The role of pattern recognition receptors in amyloid-beta-induced inflammation

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Thesis submitted to Cardiff University for the degree of Doctor of Philosophy in Medicine

September 2016

Acknowledgements

I would first like to warmly thank my supervisors, Dr Martha Triantafilou, Prof Kathy Triantafilou and Prof Paul Morgan, whose supervision, support, and advice has been invaluable throughout this project. I would also like to thank the Alzheimer's Society UK for funding this PhD project, and the patients and families who kindly donated the brain samples to the Oxford and UCL brain banks, enc5aabling part of this project to take place.

Thanks also go to my laboratory colleagues, past and present, for their assistance, the fun times spent together and for providing a welcoming working environment. Special thanks to Dr Satwik Kar, brilliant friend, and colleague, for his continuous support during this project.

Last, but not least, a big thank you to my beautiful family and friends for their continuous encouragement, love and faith in me during my studies. Along with my family, I would like to thank Janine Simpson for simply being an endless source of support and patience these last years. You have encouraged me to become the person I am today; this milestone has taken place because of you. Numerous stunning possibilities stand before us. I see that a passionate heart and a fast mind determine countless dreams and realities, all in the same timeless movement. The balance and colour of our words describe perfectly the boundless painting of the mind.

Η Ιθάκη σ' έδωσε τ' ωραίο ταξείδι.

Χωρίς αυτήν δεν θάβγαινες στον δρόμο.

Άλλα δεν έχει να σε δώσει πια.

Κι αν πτωχική την βρεις, η Ιθάκη δεν σε γέλασε.

Έτσι σοφός που έγινες, με τόση πείρα,

ήδη θα το κατάλαβες η Ιθάκες τι σημαίνουν.

– Ithaka, C.P. Cavafy

Abstract

Alzheimer's disease (AD) is the most common form of dementia among the elderly. The disease is characterized by amyloid-β (Aβ) plaques, neurofibrillary tangles, loss of synapses and neurons, and chronic neuroinflammation. The significance of neuroinflammatory processes in the on-set and progression of AD have been debated, as activated and reactive glial cells demonstrated protective and damaging properties. However, patients with AD treated with anti-inflammatory drugs demonstrated a lesser than average extent of disease development, indicating an important role of neuroinflammation in AD. Current strategies for the treatment of Alzheimer's disease are minimally effective, as we lack the methods of diagnosis at an early stage. Numerous cases present significant neuronal loss prior to diagnosis and, as restoration of function is unlikely, treatment options focus on limiting further neuronal loss. It is my hypothesis that deposition of Aβ peptide can activate the innate immune system via pattern recognition receptors (PRRs), including complement, and evoke Alzheimer's pathology. In the current study, we focused on the role of the innate immunity system of the brain in the initiation and the propagation of inflammatory process in AD and the interplay between TLRs/NLRs and the complement system.

Silencing the expression of various receptors demonstrated the involvement of NLRP3 in Aβ recognition, and use of confocal microscopy confirmed the association of Aβ interactions with this inflammasome and complement receptors. These results were confirmed in human astrocytes stimulated with Aβ and in brain tissue slides of patients with AD. Using human astrocytes and fluorescence resonance energy transfer (FRET), the associations of cell surface TLRs (such as TLR2, TLR4) with CRs (C5aR1, C3aR) were investigated; TLR2 and TLR4 were

found to interact with C5aR1. In addition, Aβ-induced responses were augmented in the presence of complement and Aβ appeared to induce the interactions of TLR4 and C5aR1 on the cell surface. The trafficking of PRRs and Aβ in human astrocytes was further investigated to determine whether internalization and trafficking of Aβ is crucial for triggering a proinflammatory response. The results demonstrated that, along with Aβ and TLR4, complement internalized in the Rab5+ endosomes, and recruited NF-kB-inducing kinase (NIK) to these endosomes in a complement-dependent manner. MAC complexes were previously demonstrated to activate an Akt+NIK+ signalosome in human endothelial cells. To the best of our knowledge, this is the first to identify such signalosomes in Rab5+ endosomes in response to complement and Aβ. These results suggest a novel possibility of a therapeutic target in the case of an Aβ-induced neuroinflammation.

C5a is the most potent anaphylatoxin, thus upon the identification of the complement-inflammasome interactions, the role of C5a in the Aβ recognition was investigated in human astrocytes. The results demonstrated that C5a augmented the Aβ-induced IL-1β production, activated pro-IL-1β and was caspase-1-dependent. In addition, a C5a-induced IL-1β release was observed following Signal 2 stimulation by Ca^{2+} and lysosomal damage. Since the signal activation resulting in inflammation in AD appears to involve multiple receptors, the therapy should be combinational and not targeting single molecules. Thus, C5aR1 blocking antibodies and PMX53 were utilized to identify a combinatory therapeutic approach in human astrocytes. The results demonstrated that the secretion of IL-1β was further reduced in the combinatory treatment of PMX53+MCC950

compared with their single treatments, suggesting a combinatory treatment to tackle the effect of Aβ in human cells.

The results of the present study add on to the current knowledge of the molecular mechanisms involved in AD, regarding Aβ association, and may lead to the design of targeted therapeutic interventions for AD.

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Abbreviations

- °C: degree Celsius
- AD: Alzheimer's Disease
- APOE: Apolipoprotein E
- APP: Amyloid precursor protein
- APS: Ammonium persulfate
- Aβ: Amyloid-beta
- AβPs: Amyloid-beta plaques
- CARD: Caspase Activation and Recruitment Domain
- CBA: Cytometric bead array
- CNS: Central Nervous System
- CpG: Cytosine-phosphate-guanosine
- Cy5: Cyanine 5
- DAMP: Damage-associated molecular pattern
- DMSO: Dimethyl sulfoxide
- dsRNA: Double-stranded ribonucleic acid
- E. Coli: Escherichia coli
- ECL: Enhanced chemiluminescence
- EDTA: Ethylenediaminetetraacetic acid
- FACS: Fluorescence activated cell sorter
- FCS: Foetal calf serum
- FITC: Fluorescein isothiocyanate
- GFP: Green fluorescent protein
- h/min: hour(s)/minute(s)

- HEK293: Human embryonic kidney 293
- HRP: Horseradish Peroxidase
- HSP: Heat shock protein
- IFN: Interferon
- IKK: IκB kinase
- IL: Interleukin
- IRAK: Interleukin-1 receptor-associated kinase
- IRF: Interferon regulatory factor
- IκB: Inhibitor of NF-κB
- LB: Luria Broth
- LPS: Lipopolysaccharide
- LRR: leucine-rich repeat
- LTA: Lipoteichoic acid
- mAb: Monoclonal antibody
- Mal: MyD88-adaptor-like
- MAVS: Mitochondrial Antiviral Signalling protein
- MHC: Major histocompatibility complex
- MyD88: Myeloid differentiation primary response gene 88
- NaAc: Sodium acetate
- NF-κB: Nuclear factor kappa-light-chain-enhancer of activated B-cells
- NLR: NOD-like receptor
- NOD: nucleotide-binding oligomerization domain
- NP: Neuritic plaque
- NT: Neurofibrillary tangle
- PAMP: Pathogen-associated molecular pattern

- PBS: Phosphate Buffered Saline
- PE: Phycoerythrin
- PFA: Paraformaldehyde
- PG: Peptidoglycan
- PG:RP: peptidoglycan recognition protein
- PGN: peptidoglycan
- PRR: Pattern recognition receptor
- RLR: RIG-I-like receptor
- RNAi: RNA interference
- RPMI: Roswell Park Memorial Institute medium
- SDS-PAGE: Sodium dodecyl sulphate polyacrylamide gel electrophoresis
- SFM: Serum free medium
- siRISC: siRNA-induced silencing complex
- SP: Senile plaque
- SR: Scavenger receptor
- ssRNA: single-stranded RNA
- TEMED: N,N,N',N'-tetra- methylethylenediamine
- TICAM: TIR domain-containing adaptor molecule
- TIR: Toll/interleukin-1 receptor
- TIRAP: TIR domain-containing adaptor protein
- TLRs: Toll-like receptors
- TNF: Tumour necrosis factor
- TRAM: TRIF related adapter molecule
- TRIF: TIR domain-containing adapter inducing IFN-β
- TRITC: Tetramethyl rhodamine iso-thiocyanate

Chapter I

Introduction

1.1 Neurodegenerative Diseases

The clinical characterization of neurodegenerative diseases is based on the pathological loss of neurons and the loss of neurological functionality. The loss of neurons in some of these diseases is often accompanied by certain histopathological findings, including Lewy bodies and Alzheimer's plaques (Arvanitakis et al. 2011; Bennet et al. 2006). On the other hand, other neurodegenerative diseases demonstrate gradual neuronal loss and atrophy without a specific pathology, or entail certain interconnected sets of neurons or anatomical systems. In the latter, the focal pathology is symmetric and bilateral or otherwise diffused and such diseases are relentlessly progressive (Crews and Masliah 2010). Neurodegenerative diseases often affect the elderly however, certain may affect children and young individuals (Bierer et al. 1995; Bussière et al. 2003). Researchers have established the biochemical basis of neurodegenerative diseases and a great number of them are inherited. Certain genes associated with these diseases were identified and DNA-based diagnosis, such as prenatal diagnosis, is available (Cozaru et al. 2016). To attain specific treatments for these diseases, an insight into their pathological mechanisms and gene products is required. Alzheimer's and Parkinson's diseases are the two most common neurodegenerative diseases.

1.1.1 Dementia

Dementia is a generic term referring to the loss of one's mental capacity. It may be the result of any pathology which can cause substantial brain damage (Uboga and Price 2010). Certain causes of dementia are neuropsychiatric disorders, effects of medication, severe organ failure or medical illness, neurodegenerative diseases (Huntington's disease, diffuse Lewy Body dementia, Parkinson's disease

or Alzheimer's Disease), multiple sclerosis (demyelinative diseases), metabolic central nervous system (CNS) disorders (peroxisomal diseases and lysosomal storage), CNS infections (HIV encephalitis), hydrocephalus, head trauma (chronic traumatic encephalopathy, diffuse axonal injury, subdural hematomas), hippocampal sclerosis, and ischemic encephalopathy and stroke (vascular or multi-infarct dementia) (Scott and Barrett 2007).

1.1.2 Alzheimer's Disease

Alzheimer's disease (AD) was first described in 1906 by Dr Alois Alzheimer, a German neuropathologist and psychiatrist. His patient (a woman over 50 years of age) named Auguste D was suffering from poor cognitive function and language skills, altered behaviour, and memory impairment (Berrios 1991). The post-mortem neuropathological assessment of the patient revealed neurofibrillary tangles, senile plaques, and histopathological lesions, which are now known as the pathological characteristics of AD (Guillozet et al. 2003; Serrano-Pozo et al. 2011; Selkoe et al. 2012). AD was recognized as the leading cause of dementia during the late 1960s and early 1970s due to the work done by Gary Blessed, Bernard Tomlinson, and Martin Roth (Blessed et al. 1968). During that time, researchers discovered that individuals with AD exhibited acute loss of cholinergic neurons that synthesize the so-called neurotransmitter acetylcholine. This discovery facilitated the efforts aimed at increasing acetylcholine levels in the patient's brain. Currently, most of the drugs for AD treatment are based on this concept. Further studies demonstrated that AD influences various neurotransmitter systems and this explained the existing drugs' modest effect. Researchers have focused their efforts on the disease's molecular mechanisms over the past two decades and they have gained further understanding of the

disease through characterization and examination of the brain lesions, based on Dr Alzheimer's initial description (Ghoshal et al. 1999; Gómez-Isla et al. 1997; Serrano-Pozo et al. 2011). The clinical characteristics of AD include delusions, depression, indifference, inability to perform calculations, loss of language function, loss of memory, a deranged view of space, and inability to learn about new things (Knudsen et al. 2001; Bird 2015). Such deficits impair the social functioning of the patients and hinder them from carrying out their daily activities. Patients with AD often die of various complications associated with chronic illness. While the disease often affects the elderly (>60 years old), it sometimes affects individuals aged between 40 and 50 years old. The prevalence of AD increases with age; ~10% of individuals >70 have significant memory loss and more than half of these individuals have AD. An estimated of 25-45% individuals >85 years old have dementia. The incidence of AD increases from 2.8 per 1,000 individuals between 65 and 69 years old, to 56.1 per 1,000 individuals over 90 years old (Kukull et al. 2002).

The two major lesions in AD are neurofibrillary tangles (NTs) and senile plaques (SPs) (Pike et al. 1995; Braak et al. 2006). The latter refers to the spherical lesions within the cerebral cortex which measures up to approximately 100 microns. The two types of senile plaques are neuritic plaques (NPs) and Aβ plaques (AβPs) (Hyman et al. 1993; Serrano-Pozo et al. 2011). The former refers to the extracellular Aβ deposits which are spherical in shape (Figure 1.1.2.1). On the other hand, NPs refers to the AβPs that consist of degenerating neuronal processes having tau-paired helical filaments (Crowther 1991). They also consist of reactive microglia and astrocytes. The Aβ found in NPs often forms small clumps or a central core, is Congo Red positive, and contains a fibrillary fine

structure (Glabe 2005; Greenberg et al. 1997; Grutzendler et al. 2007). AβPS do not cause disruptions to the neuropil. In certain cases, elderly non-demented individuals may have increased numbers of AβPs, however these are not associated with AD. A neuritic plaque scoring mechanism was developed by the Consortium to Establish a Registry for Alzheimer's disease (CERAD) which is used for ranking the neuritic plaques' density within the neocortex, as identified by the Bielschowski silver stains (Mirra et al. 1991).

NTs refer to the tau filaments found within the neuronal body. In addition, similar deposits are found within the dendrites and in the NPs' dystrophic processes (Bondareff et al. 1991; Augustinack et al. 2002). The hippocampus in advanced AD often consists of extracellular NTs that are embedded within the neuropil, like neurons' fossilized skeletons (Hoozemans et al. 2009). However, the mechanism through which the tau accumulates in the NTs is unclear. It is believed that Aβ deposition is the primary lesion in AD, while NTs are the secondary (Braak et al. 1994; Braak and Del Tredici 2011). Nevertheless, the development of NTs is independent of Aβ. Additionally, the cognitive decline has a higher association to the NTs than the SPs' number (Mandelkow and Mandelkow 1998; Guillozet-Bongaarts et al. 2005). Both the environmental and genetic risks of AD are strongly associated with the amyloid. In addition to AD, NTs are also found in several other neurodegenerative diseases, including prion diseases, chronic traumatic encephalopathy, and frontotemporal dementias. These cases are a clear indication that NTs may independently result in neurodegeneration without an Aβ deposition (Iqbal and Grundke-Iqbal 2002; Giannakopoulos et al. 2003; Ingelsson et al. 2004). However, SPs are demonstrated in AD only.

While certain cases of AD have a predominance of either NTs or SPs, most of them demonstrate a combination of both. The analysis of NTs deposition's pattern indicates that such changes initially appear within the temporal lobes' transentorhinal area prior to spreading to other regions, including the neocortex, hippocampus, and the entorhinal cortex (see Figure 1.1.2.2) (Gómez-Isla et al. 1996; Guillozet-Bongaarts et al. 2005; Harris et al. 2010). Therefore, there are three stages involved in the development of the AD's neurofibrillary pathology: isocortical, limbic and transentorhinal. These stages form the foundation for the AD Braak and Braak staging system (Braak and Braak 1991). This system's initial stages (stages I and II) correlate to the so-called transentorhinal involvement, the next two (stages III and IV) correlate to the limbic, and the last two (stages V and VI) correlate to the isocortical involvement. Neocortical NTs are associated with cognitive decline, whereas NTs in the entorhinal cortex and hippocampus are associated with memory impairment (Braak and Braak 1991).

SPs are commonly present in the association cortex. However, in very acute cases of patients, NTs and SPs are additionally observed in the brainstem and deep cerebellar nuclei, whereas the white matter does not contain such lesions. Each SP is a representation of the neuropil's focus of damage, including numerous neurons' dendrites and axon terminals, and possibly thousands of synapses (Geddes et al. 1997). Therefore, formation of SPs results in acute disconnection. The clinical pathology is associated with the lesions' distribution. The hippocampal damage justifies the memory impairment, while the association cortex's involvement is correlated with the patient's higher-level intellectual functions. Both NTs and SPs correlate with brain atrophy, the loss of synapses and neurons, as

well as the lateral ventricles' dilation because of brain tissue loss (DeKosky and Scheff 1990; DeKosky et al. 1996; Erten-Lyons et al. 2009).

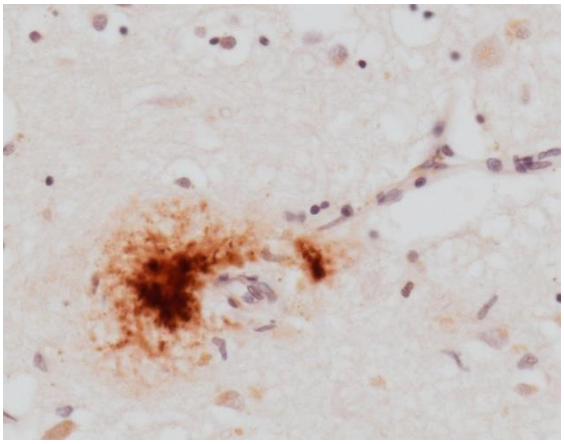

Figure 1.1.2.1 Histopathological lesions in Alzheimer's Disease. Amyloid plaque in the hippocampus area of an Alzheimer's Disease patient (image obtained during staining analysis of a tissue slide provided for this project).

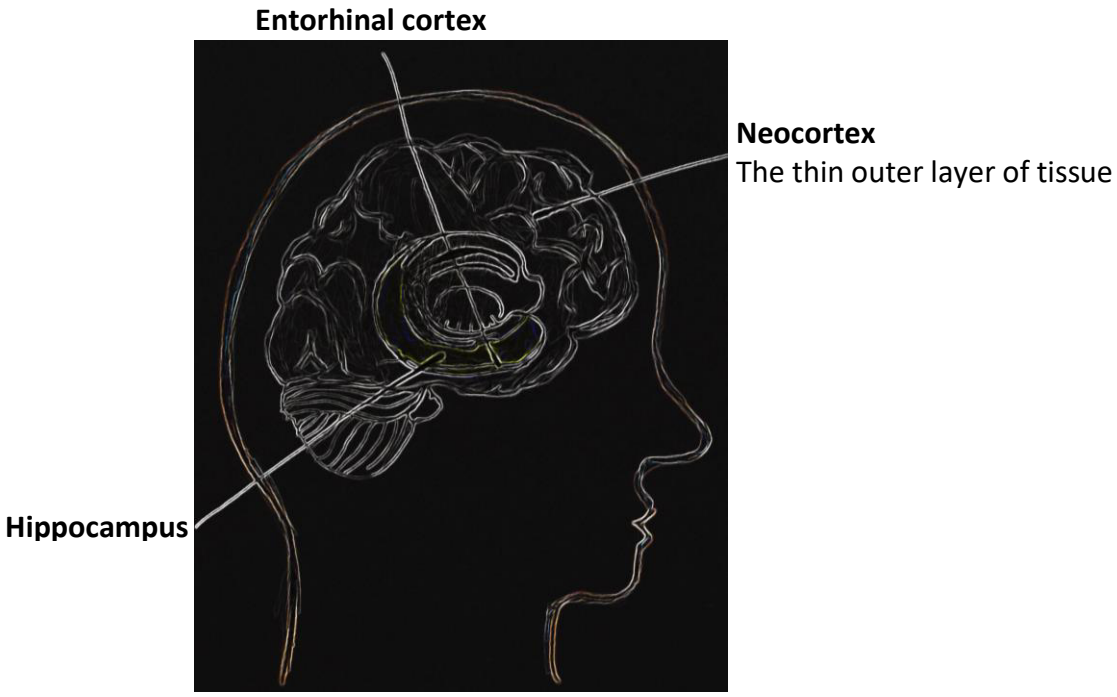

Figure 1.1.2.2 Diagram of the brain.

1.2 APP processing

Amyloid precursor protein (APP) refers to a transmembrane protein that is largely availed in various tissues. There are three varying secretases that mediate the proteolytic processing of APP: α-, β- and γ-secretases. The site of α-secretase cleavage is found between residue 16 and 17 in the centre of the Aβ sequence, sending out α-APPs from the cell membrane. The C-terminal fragment C83 is in the left membrane, and the β-secretase cleaves APP into β-APPs and the C-terminal fragment C99. γ-secretase cleaves C83 and C99, releasing p3 and Aβ, respectively (Figure 1.2). Therefore, Aβ is not produced by cleavage with γ-secretase and α-secretase and this pathway is called the non-amyloidogenic pathway. It thus follows that the amyloidogenic pathway creating the Aβ is mediated by β-secretase and γ-secretase (Esler and Wolfe 2001).

β-secretase cleavage in the brain has been observed to be interceded by a single protein, the aspartyl protease β-site APP cleaving enzyme-1 (BACE-1) (Vassar et al. 1999). This transmembrane protein is carried through the secretory pathway to the plasma membrane and clustered with APP in lipid rafts (membrane microdomains containing cholesterol). The primary sites of β-secretase activity are the lipid rafts and endosomes (Vassar et al. 1999; Ehehalt et al. 2003). The ideal pH for BACE-1 activity is 5.5 (Sinha et al. 1999), hence, the acidic environment of endosomes supports the β-secretase action. The biological significance of BACE-1 has been indistinct until recently; BACE-1 action was observed to be vital for the myelination of axons (Willem et al. 2006).

γ-secretase cleavage is performed by a protein complex comprising of 4 proteins: nicastrin, presenilin, APH-1 (frontal pharynx-deficient phenotype 1) and PEN-2 (presenilin-enhancer 2) (Edbauer et al. 2003). The γ-secretase complex cleaves

inside the transmembrane space of APP, and γ-secretase has been demonstrated to be present and active in numerous cell compartments, such as the endoplasmic reticulum, trans-Golgi system, endosomes, and the plasma membrane (Vetrivel and Thinakaran 2006; Kaether et al. 2006). Additionally, similarly to β-secretase, γ-secretase has been observed to be limited to lipid rafts (Vetrivel et al. 2004). Furthermore, the γ-secretase complex cleaves APP at ε-site (~10 residues downstream of the γ-site) to release the APP intracellular domain (AICD) (Weidemann et al. 2002).

The secretases required in APP processing are essential therapeutic targets due to their activities and scientists are researching for β- and γ-secretase inhibitors. Nevertheless, BACE-1 is difficult to target due to the considerable great active site and localization to endosomes. In addition, BACE-1 may be involved in myelination. As mentioned above, the γ-secretase complexes cleave numerous substrates, such as notch, thus inhibiting γ-secretase could result in potential side effects.

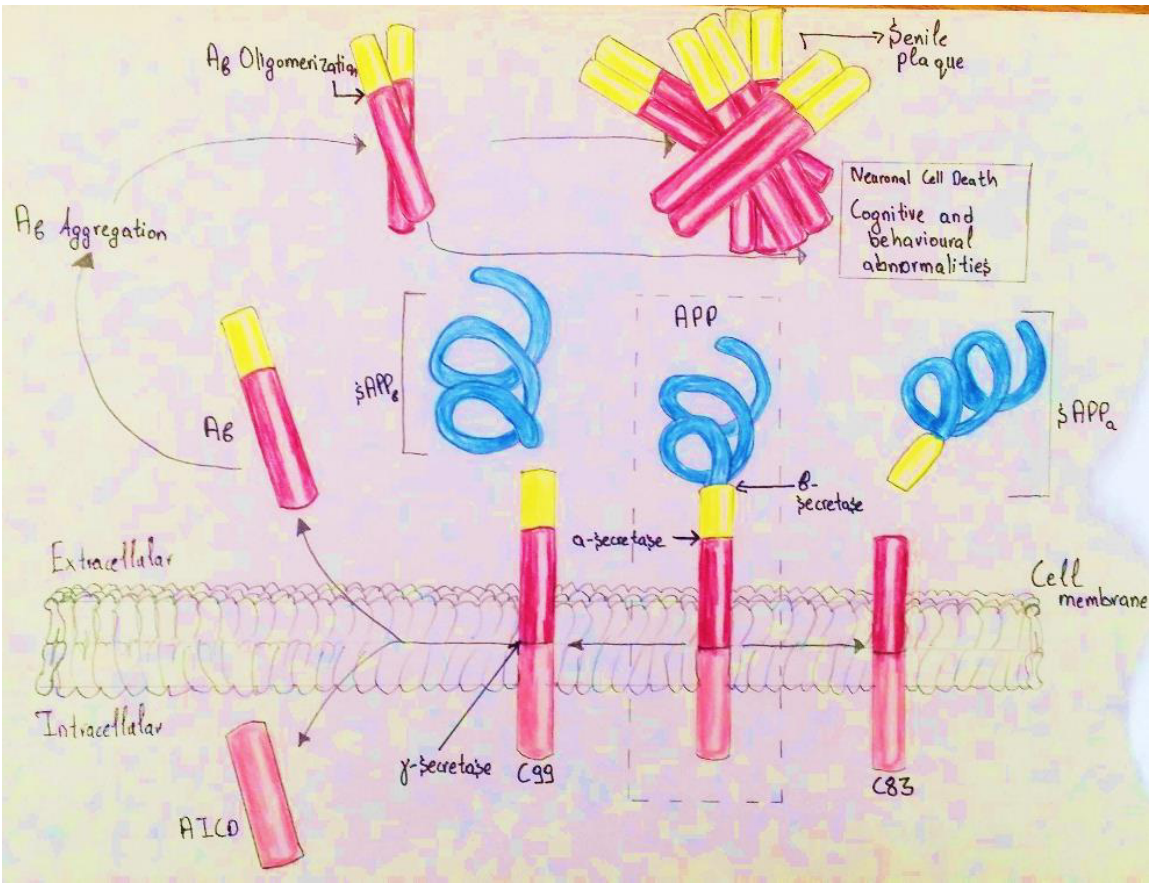

Figure 1.2 APP processing pathways. APP is cleaved by either β- or α-secretase, leaving C99 and C83 in the plasma membrane. Cleavage by the α-secretase (non-amyloidogenic pathway) results in the generation of sAPP-α and C83 (left arrow). Cleavage by the β-secretase (amyloidogenic pathway) leads to the generation of sAPP-β and C99 (right arrow). C83 is finally cleaved by the γ-secretase to generating AICD and Aβ.

1.3 The Aβ peptide

The properties of the Aβ peptide were investigated by researchers following the distinguishing proof that the peptide was the fundamental protein component of the amyloid plaques in AD. At the time, Aβ demonstrated no homology to known proteins and the amyloid cores of senile plaques were difficult to dissolve to further investigate, thus initially Aβ was believed to exist in senile plaques only. Further studies demonstrated that Aβ was detected in the plasma of healthy individuals (Seubert et al. 1992) and that numerous types of cells, not only neurons, were producing the peptide (Hartmann et al. 1997). Similarly to the fibre structures present in plaques *in vivo*, small segments of the Aβ amino-acid sequence were identified forming amyloid fibres in vitro (Kirschner et al. 1987).

Even though $A\beta_{40}$ is the major Aβ species produced by neuronal cells (Suzuki et al. 1994), the amyloid plaques primarily contain $A\beta_{42}$. Thus, the elongated peptide appeared to serve a role a great role in AD. A previous study demonstrated that the longer carboxyl terminus of Aβ influenced the amyloid formation and the subsequent aggregation of soluble Aβ peptides. For example, the rate of peptide formation was accelerated due to the additional Aβ including the C-terminal residues (Jarrett et al. 1993).

The precise number of molecules existing in the smallest nuclei of Aβ has not been identified yet, however a previous study hypothesized that a pentamer/hexamer may be the smallest unit utilized to result in fibril formation (Klein et al. 2004). The wild type of $A\beta_{1\text{-}42}$ may create this unit during the initial stages of aggregation, whereas the wild type $A\beta_{1\text{-}40}$ is formed by monomers to tetramers (Bitan et al. 2003). To investigate the effects of Aβ fibrils, the concentration of Aβ needed for fibrillization should be established. In general, previous studies suggest ~10 μM

as the optimal concentration for synthetic Aβ peptide *in vitro*. However, this is not the case for *in vivo* applications, as the concentrations of Aβ in plasma and cerebrospinal fluid are in the nM or pM range. In *in vivo* cases, the concentration of Aβ may be higher in certain cells or other factors may allow fibril formation with decreased Aβ concentration (Fezoui et al. 2002).

A previous study proposed that Aβ may create an unfolded helix with intermediate that assists in the acceleration of fibrillization. Nonetheless, the fully unfolded peptide may create fibrils at a slower rate. In *in vitro* studies, the procedure of fibril formation entails the formation of soluble oligomers. These studies identified numerous formed oligomeric Aβ species, such as protofibrils and ADDLs (Aβ derived diffusible ligands) (Walsh et al. 1997; Harper et al. 1997). The protofibrils of Aβ were characterized as curved structures of a diameter between 4 and 10 nm, and approximately 200 nm long (Walsh et al. 1997; Harper et al. 1997). In addition, fibrils were identified as insoluble structures of more than 200 nm long and a diameter of 4-10 nm, alike the protofibrils (Walsh et al. 1997). A previous study demonstrated that the fibrils consist of 6 protofilaments with β-strands, following a perpendicular to the fiber axis direction and held together by hydrogen bonding (Serpell 2000). Upon centrifugation at an average speed, fibrils form pellets and may bind β-sheet binding-dyes, such as Congo-Red and Thioflavin (Serpell 2000).

1.3.1 Aβ mediated neurotoxicity

Numerous studies have demonstrated that aggregated Aβ serves an important role as a pathogen in AD, thus further research was conducted to identify the Aβ aggregate with the most pathogenic ability. Previous studies utilizing synthetic Aβ demonstrated that protofibrils and fibrils were toxic to the cultured neurons (Hartley et al. 1999; Walsh et al. 1999; Ward et al. 2000) and influenced the electrophysiological parameters (Hartley et al. 1999; Walsh et al. 1999). The same effects were not detected for the monomeric Aβ (Hartley et al. 1999; Walsh et al. 1999; Ward et al. 2000). Despite these observations, other studies demonstrated that fibrillar Aβ was either non-toxic (Heinitz et al. 2006; Hoshi et al. 2003) or had a less effective result (Deshpande et al. 2006) in comparison to the oligomeric form. A possible explanation for these contradictory observations may be that the Aβ oligomers and fibrils provoke neurotoxicity via various mechanisms (Deshpande et al. 2006).

Furthermore, Aβ-mediated cell death was hypothesized to be dependent on continuous polymerization and not on only one specific aggregate (Wogulis et al. 2005). In addition, numerous toxic incidents, including oxidative stress, calcium overload, mitochondrial dysfunction, and pore/channel formation, have been demonstrated to result in necrotic or apoptotic events (Canevari et al. 2004). The various molecular mechanisms leading to these events may be interconnected and take place at the same time, and certain toxic incidents may be minor in comparison to the original toxic attack. For example, a previous study demonstrated that mitochondrial dysfunction is an early Aβ-mediated toxic incident preceding cell death (Deshpande et al. 2006).

1.3.2 Aβ plaques

Amyloid or Aβ plaques are extracellular accumulations of protein, consisting of the hydrophobic Aβ peptide, and various proteins and cell components, such as apolipoprotein E (ApoE), clusterin and numerous constituents of the complement system (Liao et al. 2004). An association and activation of the immune system in Aβ plaques was proposed due to the activated astrocytes and microglia cells surrounding the plaques (Eikelenboom et al. 2006). The formation of the Aβ peptide occurs following the processing of APP, via either the amyloidogenic or non-amyloidogenic pathway (Figure 1.3.2), as determined by the secretase enzyme implicated in the sequential cleavage stages.

The two APP processing pathways generate the amyloid precursor protein intracellular domain (AICD). AICD was hypothesized to serve a role in transcriptional control, however the genes affected by this process are not yet identified (Chang and Suh, 2010). An initial cleavage by β-secretase, followed by γ-secretase in the amyloidogenic pathway produces the aggregated Aβ peptide. The number of amino acid residues in the Aβ peptide species depends on the γ-secretase cleavage. $A\beta_{40}$ and $A\beta_{42}$ are frequently produced, ~90% and <10%, respectively, however species of less residues were identified (Selkoe and Wolfe, 2007). Aβ42 is particularly hydrophobic and prone to aggregation.

1.3.3 Neurofibrillary tangles

Tau is a microtubule-related protein and the principle segment of NTs. It serves a role in the adjustment and control of microtubule bundles required for the cytoskeletal integrity and axonal transport (Roy et al. 2005). The regular activity of tau is managed by a fine balance amongst phosphorylation and dephosphorylation. Disruption in this balance may result in hyperphosphorylation of tau, which further leads to the interruption of regular functioning, inclining the molecule to form paired helical filaments. These filaments are insoluble and aggregate in blocks, forming the NTs inside the cytoplasm of neuronal cells during AD (Goedert et al. 1995). The tau protein is involved in numerous neurodegenerative diseases apart from AD, including frontal temporal dementia (Neumann et al. 2009). Frontal temporal dementia may be a result of a series of mutations in the microtubule associated protein tau (MAPT) gene which encodes tau (Goedert and Jakes 2005), leading to neuronal cell death and further cognitive decline.

In the case of AD, tau tangles are believed to form after the Aβ plaques. Furthermore, APP mutations result in amyloid plaques and NTs, however tau mutations lead to NTs only (Lovestone 2000).

1.3.4 Aβ and tau in AD

Deposits of aggregated Aβ are present in the neuritic plaques and cerebral vessels (cerebrovascular amyloid angiopathy) of patients with AD (Akiyama et al. 2000). An innate immune response, including activated glial cells and augmented levels of cytokines, is associated with the Aβ deposits present in the brain of patients with AD (Akiyama et al. 2000). Previous studies demonstrated that Aβ deposits in the AD brain may activate glial cells, commencing a pro-inflammatory cascade that

leads to the production and release of cytotoxic molecules, cytokines, proteases and complement compartments, thus triggering neurodegeneration (Akiyama et al. 2000; Heneka and O'Banion 2007). Based on these observations, nonsteroidal anti-inflammatory drugs (NSAIDs) were suggested to be utilized to reduce the risk and possibly postpone the onset of AD (Breitner et al. 1995; Breitner et al 1994; McGeer et al. 1990; Stewart et al. 1997; Szekely et al. 2004; Veld et al. 2000).

A previous study demonstrated that amyloid plaques were generated by an overexpression of the APP in its mutant form in the brains of transgenic mice [9]. Reactive astrocytes and activated microglia were identified near those plaques, and the levels of interleukin (IL)-1, IL-6 and tumour necrosis factor (TNF) -α were upregulated compared with the mice lacking overexpressed APP (Morgan et al. 2005). These observations are similar to the ones demonstrated in patients with AD. Previous studies demonstrated that treatment of Tg2576 mice (AD mouse model) overexpressing APP with Ibuprofen (an NSAID), led to decreased levels of reactive astrocytes, Aβ deposition and IL-1β production (Lim et al. 2000; Lim et al. 2001). Furthermore, the process of gene deletion in transgenic mice is utilized in research to understand AD and prevent the pathology. A previous study demonstrated that removal of the TNF Type I receptor gene in a different mouse model of AD, APP23, resulted in reduced plaque formation, inhibition of Aβ and normal learning and memory activity (He et al. 2007). Another study revealed that the deletion of interferon (IFN) –γ receptor Type I gene in Tg2576 mice resulted in reduced gliosis and amyloid plaques (Yamamoto et al. 2007). In the wild type mice of the study, INF-γ elicited TNF-α production leading to increased levels of the β-site APP-cleaving enzyme (BACE1) in astrocytes, which further resulted in Aβ formation (Yamamoto et al. 2007).

The data of these studies suggest that upregulation of proinflammatory cytokines and activation of glial cells may promote AD progression.

1.3.5 The amyloid cascade hypothesis

The amyloid cascade hypothesis was proposed more than 20 years ago. The hypothesis proposes that Aβ deposition results in NT formation and numerous toxic events, which ultimately leads to AD (Mudher et al. 2002). The hypothesis, which is considered to be the foremost one in the field of AD, was initially proposed based on genetic evidence and has been contested over the years. Primarily, concerns were raised regarding the association of the plaque load and AD (Haass and Selkoe 2007). Further concerns were that the neurological deficits, which were similar to AD, were present prior to the deposition of the plaques (Williams 2006). The amyloid hypothesis states that increased $A\beta_{42}$ production as a result of a mutation or other factors, including faulty Aβ clearance, leads to $A\beta_{42}$ oligomerization and deposition. This process results in the pathogenic events correlated with AD, such as neuronal dysfunction, thus leading to dementia. Based on previous concerns and novel data coming forward over the last decade, certain amendments of the hypothesis were recommended. For example, evidence emerged on the pathological role of the soluble oligomeric Aβ species, which precede amyloid plaque formation, and on the role of intracellular Aβ (Francis et al. 1999). Aβ in its soluble form associates with synapse loss Terry et al. 1999) and memory impairment (Haass and Selkoe 2007).

The role of amyloid in neuronal dysfunction has been extended by the discovery of small, soluble, oligomers of the Aβ peptide, some forms of which have been termed ADDLs (Aβ-derived diffusible ligands) (Lambert et al. 1998). These Aβ oligomers are not only potential intermediates in the formation of amyloid

filaments, but they also have been shown to be neurotoxic themselves and to inhibit long-term potentiation (LTP), a cellular model of memory, in hippocampal slices (Klein 2002). Thus, the essential role of Aβ oligomers in the neurodegeneration process is now taken into account in the Amyloid Cascade Hypothesis. Despite its strength, the Amyloid Cascade Hypothesis is incomplete without including the essential role of amyloid- associated inflammatory proteins.

1.4 Diagnosis of AD

As mentioned above, the two hallmark pathologies required for a diagnosis of AD are the extracellular plaque deposits of the β-amyloid peptide and the NTs of the microtubule binding protein tau. Progressive dementia that has evolved over a period of time, lacking focal neurological deficits, and presents no abnormal imaging findings may be AD. Helpful biomarkers, such as the increase in tau protein or decrease of Aβ in the CSF, are tested however, a definitive diagnosis of AD occurs with a histopathological examination of the brain. Previous autopsy studies demonstrated that the brains of individuals over 65 years old without clinical dementia were positive for SPs and NTs within the hippocampus and the entorhinal cortex (Reitz et al. 2009). This observation implies that SPs and NTs are partly formed due to normal ageing. Nevertheless, in the brains of AD individuals, increased levels of SPs and NTs were demonstrated in the limbic cortex, the neocortex and other brain regions, compared with the non-demented individuals (Lacor et al. 2007).

The brains of patients with AD demonstrate reduced levels of acetylcholine (ACh), loss of cholinergic neurons in the basal forebrain and decreased acetylcholine synthesizing enzyme choline acetyltransferase in the cerebral cortex (Kar et al. 2004). In addition to the human individuals, animal models reveal that ACh serves an important role in memory and thought processing. Besides ACh, neurotransmitters, including noradrenalin, serotonin, and somatostatin, have been demonstrated in reduced levels in AD. In the case of neurotransmitter deficiency, the cognitive impairment was correlated with the loss of the cholinergic input (Martorana and Koch (2014).

Based on the above observations, acetylcholinesterase inhibitors, such as tacrine, and ACh receptor agonists, such as nicotine, were utilized as treatment for AD. However, the results indicated a minor success of this treatment, suggesting that other factors combined with ACh deficiency have an effect on cognitive function.

Identification of AD-like pathology in individuals without dementia raised concerns, as cases were characterized to be demented due to the presence of few SPs and NTs, whereas clinical dementia was not present. The CERAD criteria regarding the neuropathological assessment of AD gave emphasis to the senile or neuritic plaques, age, and clinical history of the patient (Mirra et al. 1991). An additional system has been suggested by the National Institute on Aging (NIA)-Reagan Institute to assess the topographic staging of neurofibrillary changes, as well as neuritic plaques (Newell et al. 1999). Following the above criteria for AD, a patient may be diagnosed considering the number of SPs and NTs, age, and cognitive function.

The increased formation of SPs and NTs in individuals reveals that the biochemical abnormalities occurring in AD have been initialized. For certain patients with AD, the progression of the disease is rapid and severe, whereas in others the disease progresses in a slow-rate leading to memory impairment and deterioration of mental power.

Further research is required to understand the association of neurotransmitter deficiencies and neuronal plasticity. In addition, the effect of the environment on brain interactions should be investigated. This would allow for the design of future therapies in AD.

1.5 Neuroinflammation in the Central Nervous System

1.5.1 Neuroinflammation

Inflammation is associated with all neurodegenerative diseases. During the last decade, scientists have started to understand the effect of the immune system on these various neurodegenerative disorders. Based on better technology, there is a strong research focus on investigations of the impact of microglia. However, there is specific information regarding the impact of astrocytes in neuroinflammation.

AD-related neuroinflammation is considered to be the resulting reaction of Aβ deposit formation in the brain and the subsequent neuronal cell death. It is not fully understood whether this neuroinflammatory response is the inducer itself and not a secondary response to Aβ deposition. Inflammation in general has been suggested to serve a vital role in the progression of late-onset AD. Neuroinflammation has been proposed to have both a beneficial and detrimental role in AD. For example, reactive astrocytes and activated microglia have a positive effect in the clearance of Aβ (Simard et al. 2006; Koistinaho et al. 2004), and an undesirable production of cytotoxic elements resulting in the development of AD (Akiyame et al. 2000). Microglia phagocytosis is required for the clearance of Aβ (Richard et al. 2008), however, an overstimulation of the process might result in neuronal cell death. In addition, increased production of inflammatory molecules by the glial cells has an effect on the development of AD.

The amyloid plaques consist not only of Aβ, but of acute phase proteins (Abraham et al. 1989), such as α1-antichymotrypsin and α2-macroglobulin (Abraham et al. 1990; Bauer et al. 1991), and complement components, such as C1q, C3, C5, C6, C7, C8 and C9 (Eikelenboom and Stam 1982). These Aβ-associated proteins

(AAPs) were demonstrated to have an effect on the fibrillization, deposition and transport of Aβ (Veerhuis et al. 2005; Eikelenboom et al. 2011), thus disrupting the clearance of Aβ. For instance, AAPs, such as apolipoproteins, heat-shock proteins and heparan sulfate proteoglycans co-deposit with Aβ (Veerhuis et al. 2005; Wilhelmus et al. 2007). Furthermore, Aβ may form soluble complexes with several AAPs, such as the Aβ-ApoE complex in the cerebrospinal fluid (Tai et al. 2013). In addition, alpha1-antichymotrypsin and certain heat-shock proteins affect the aggregation and fibril formation of Aβ (Janciauskiene and Wright 1998).

IL-1 is an immunoregulatory cytokine that is overexpressed within the cerebral cortical regions of the AD-affected brain. The early overexpression of IL-1 in AD, coupled with the increased number and staining intensity of IL-1-immunoreactive microglia indicates that IL-1 serves an important role in the development of the plaques. Previous *in vitro* studies have additionally demonstrated that the IL-1 cytokine had an effect on the increased production and deposit formation of Aβ via processing (Buxbaum et al. 1992) and promoting the synthesis of APP (Goldgaber et al. 1989). In the case of unsuccessful Aβ clearance by glial cells, the cells remain chronically activated, as observed in late-onset AD (Koenigsknecht-Talboo and Landreth 2005; Edison et al. 2008).

Approximately 60% of variation in cognitive decline in late-onset AD was hypothesized to be inherited, however previous studies investigated various inflammatory markers associated with the late stage of AD (Gatz et al. 2006). One of these studies investigated middle-age individuals whose parents had a history of late-stage AD. The data of the study demonstrated that augmented levels of inflammatory markers were a risk factor for the individuals to develop AD later in life (van Exel et al. 2009). The data were further compared with age-matched

individuals whose parents had no history of AD. For the experimental analysis, whole blood samples were collected and stimulated with lipopolysaccharide (LPS) *ex vivo*, and the data demonstrated that individuals with parents suffering from AD had increased levels of IL-1β, IL-6 and TNF-α. Another study suggested that the inflammatory response observed in the blood may be analogous across the blood brain barrier (BBB) (Ek et al. 2001). Based on these results, it may be that inflammation serves a critical role and could be considered a risk factor in developing AD.

1.5.2 White matter

White matter is present within the CNS of vertebrates. The volume of white matter in the human body accounts for more than 50% of the entire brain volume (Fields 2008). This is a significant proportion in comparison to other animals, such as mice, in which the proportion is as small as 25% of the whole brain volume (Fields 2008).

The role of white matter was believed to be insignificant and its function was neglected by scientists in the past. White matter was initially studied by Rudolf Virchow (Peters et al. 1991) and was presumed to be a type of connective tissue. This presumption continued for decades, until the pathologists Ramon y Cajal and Rio Hortega illustrated the 3 primary cells types white matted consisted of: microglia, astrocytes, and oligodendrocytes (Pérez-Cerdá et al. 2015). In addition to these cell types, scientists demonstrated that white matter contains numerous blood vessels and axons (connections among nerve cells). Axons function as energy transporters (cell signalling) among cells; they stretch from neurons of the grey and white matters and assist with information transport (Clark et al. 2009).

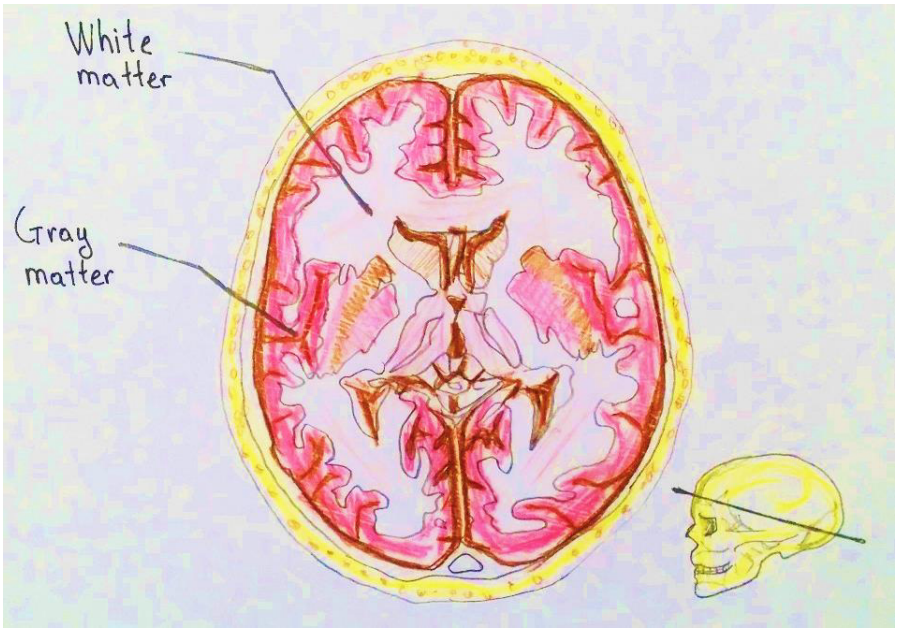

Figure 1.5.2a Location of white and grey matter in the brain. Schematic of a horizontal section of a human brain showing location of grey and white matter [adapted from American Accreditation HealthCare Commission (www.urac.org)].

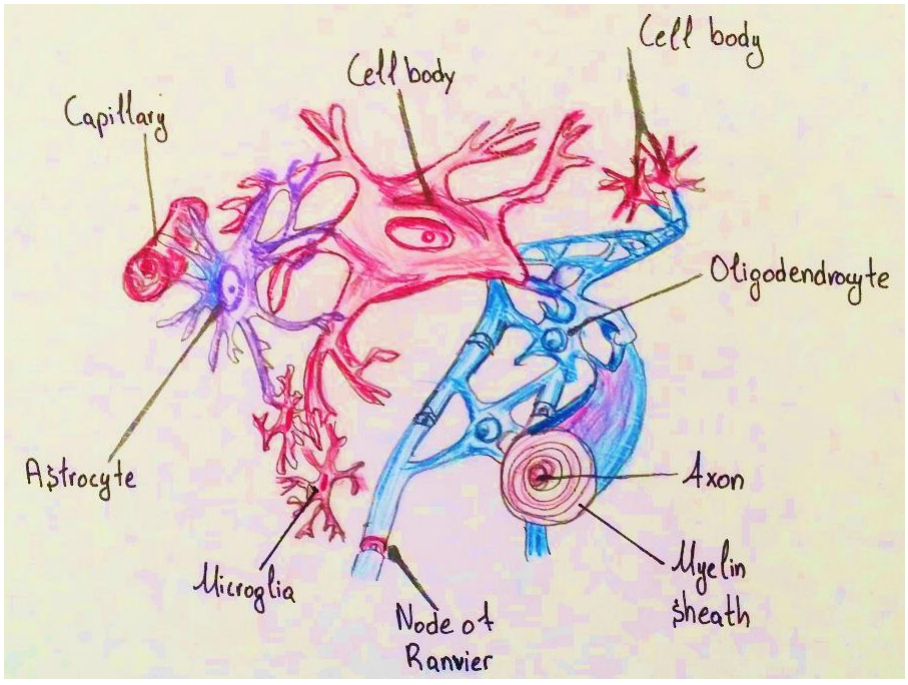

Figure 1.5.2b Components of white and grey matter. Components of grey matter (cell bodies) and white matter (oligodendrocytes, astrocytes, microglia, and axons). [adapted from The McGraw-Hill Company, Inc. (www.mcgraw-hill.co.uk)].

1.5.3 Astrocytes

Chemical interactions result in the activation of astrocytes, which may lead to phagocytosis and production of cytotoxic molecules. Astrocytes have not been researched in depth, compared with the microglia cells, as initially they were hypothesized to only connect with other astrocytes and act in an organizational matter. Nevertheless, this perception has changed, as irregular astrocytic activity has been demonstrated to associate with various neuropathologies, such as focal brain ischemia, perinatal asphyxia, retinal ischemia, diabetic retinopathy, AD, Parkinson's disease, and multiple sclerosis (Allaman et al. 2011). Astrocytes account for approximately 50% of the cells of the CNS, thus being the most common type of cell within this system. They originate in the neuroectoderm, have the shape of a star and great plasticity (Volterra and Meldolesi 2005). Astrocytes utilize gap junctions as their communication system and are structured in domains to have the least overlap, while being aware of their surroundings and swiftly respond to any alterations in their environment (Liu et al. 2011). Due to their arrangement in domains, any astrocyte may act on its own within the domain as a link with other cells of the CNS (Liu et al. 2011).

Astrocytes serve a critical role in the brain homeostasis, specifically in neuronal survival and function. For example, astrocytes offer energy to neurons via the lactate shuttle structure, they maintain ion, glutamate, and water homeostasis, are an important element of the defense system, and assist to form and maintain the BBB (Allaman et al. 2011; Sofroniew et al. 2010). Upon activation of astrocytes, the levels of intracellular Ca^{2+} rise creating a Ca^{2+} signal which proliferates to the surrounding astrocytes. This response leads to the activation of signalling

pathways within the astrocytes, which further results in morphological alterations and cell motility (Akiyama et al. 2000).

Activation of astrocytes does not necessarily correlate with proliferation, thus the term “reactive astrocytes”, instead of “active astrocytes” was proposed to describe their activation process. Following activation, astrocytes may express numerous inflammatory elements, such as complement components, iNOS, IL-1 and IL-6 (Akiyama et al. 2000).

1.5.4 Astrocytes in AD

The term astrogliosis refers to the abnormal increase in the number of astrocytes due to the destruction of surrounding neurons and, in AD, astrogliosis is observed in areas of the brain consisting of amyloid plaques (Akiyama et al. 2000). In addition, deposition of Aβ leads to increased intercellular calcium expression and, simultaneously, overactivity of astrocytes *in vitro* and *in vivo* (Kuchibhotla et al. 2009; Abramov et al. 2003).

As a response to Aβ, astrocytes produce cytotoxic compounds, such as IL-1β, IL-6 and IFN-γ, which have been suggested to activate microglia and lead to chronic inflammation, thus serving a role in the pathogenesis of AD (Garwood et al. 2011). Furthermore, a previous study demonstrated that in a culture consisting of rat primary neurons and astrocytes, following Aβ induction, the astrocytes were a factor in the resulting neurotoxicity and tau phosphorylation (Garwood et al. 2011). Following activation by Aβ, astrocytes have an effect on neuronal cultures; astrocytes accelerate Aβ-induced neurotoxicity. The so called ‘neuro-neglect hypothesis’ implies that astrocytes activated by Aβ neglect the support they would normally provide to neurons, thus leading to the vulnerability of neurons towards toxic responses (Fuller et al. 2010).

Previous studies demonstrated that, in Tg2576 mice, astrocytes surrounding the Aβ plaques produce and express BACE1. BACE1 expressed by astrocytes is additionally demonstrated in the neocortex of patients with AD (Hartlage-Rubsamen et al. 2003; Rossner et al. 2001). The above findings and the fact that astrocytes account for more than half of the brain parenchyma suggest that the contribution of astrocytes to the Aβ plaque formation requires further research.

1.5.5 Effect of chronic neuroinflammation on microglia and astrocytes

Chronic neurodegenerative diseases, such as AD or Parkinson's disease, are often age-related and primarily associated with extensive neuroinflammation (Hauss-Wegrzyniak et al. 2002; Wersinger and Sidhu 2002; Blasko et al. 2004). Various studies have shown that in chronic brain inflammation, cells of the innate immune system modify their phenotype and specifically change their receptor expression (Streit et al. 2004; Liu et al. 2005; Letiembre et al. 2007). Thus, it can be presumed that microglia and astrocytes may change to a more reactive state. In most diseases, associated with chronic brain inflammation, the expression of PRRs in microglia and astrocytes is up-regulated. PRRs and cell types of the CNS involved in the innate immune system and sensing of fibrillar Aβ need to be identified, and the correlation between PRRs and Aβ needs in depth research.

In a healthy brain, microglia and astrocytes release molecules that keep the brain running normally. In AD, microglia and astrocytes can become deregulated, causing neuroinflammation that can promote the development and progression of neurodegeneration.

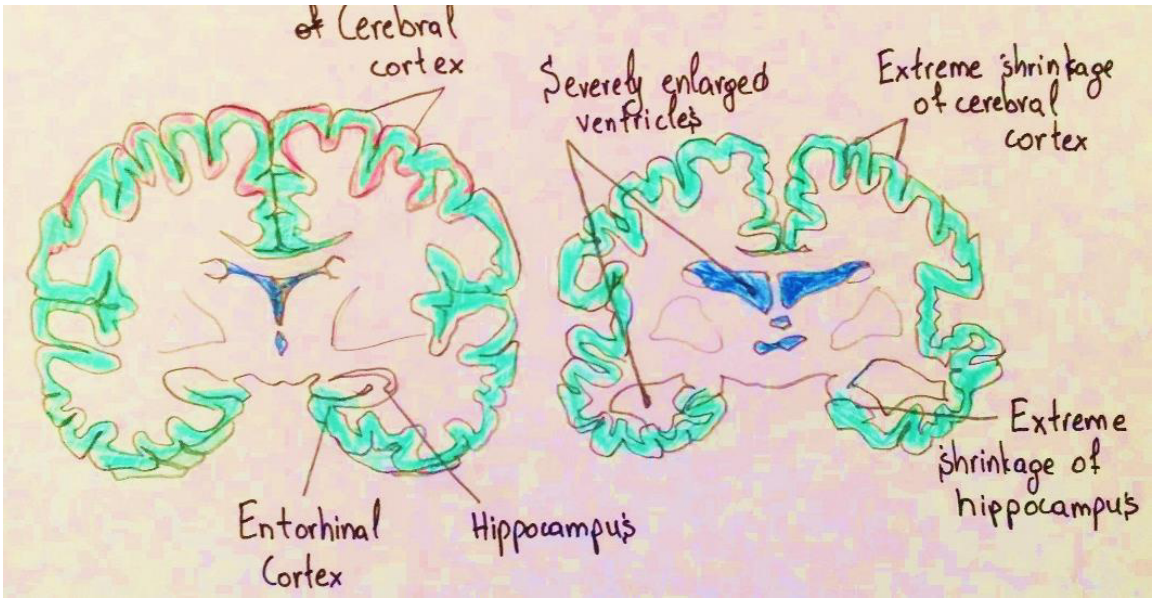

Figure 1.5.5 The effect of chronic neuroinflammation on a human brain with Alzheimer Disease. Combination of two brain diagrams comparison. On the left a normal brain without AD, and on the right an AD brain showing clear shrinkage of the cerebral cortex, hippocampus, and severely enlarged ventricles due to cerebral and hippocampal atrophy. This was caused by the multiple primary lesions which lead to the death of motor cells, overtime leading to shrinkage (adapted from ADEAR: "Alzheimer's Disease Education and Referral Center, a service of the National Institute on Aging, www.patient.info/education/alzheimers-disease).

1.6 Innate Immunity and Pattern Recognition Receptors

1.6.1 Overview

The immune system is composed of two subdivisions: the innate (non-specific) and adaptive (acquired) immune systems. The two subdivisions can identify foreign invaders, such as microorganisms, triggering an immune response to remove them from the body. The innate immune system is the first line of defence against invading organisms and the adaptive immune system operates as a second line of defence, providing protection against re-exposure to the same pathogen due to immunological memory (Janeway et al. 2001).

The primary function of the immune system is to differentiate between itself and non-self components. This function is required for protection of the body against invading pathogens and to eradicate malignant cells (altered cells). Numerous pathogens, such as viruses, replicate intracellularly and others, such as fungi and bacteria, replicate extracellularly. Therefore, the various parts of the immune system have developed in a manner of protection against these pathogens (Janeway et al. 2001).

An infection may be cleared by the immune system prior to becoming a disease. A disease may arise following un-prevented infection, increased virulence of a pathogen or a serious compromise within the immune system. The immune system may be harmful or beneficial, depending on the scenario. For example, after inflammation (immune response), local irritation and impairment to healthy tissue occur due to the toxic components being released. In addition, the immune system may attack itself in the case of autoimmune diseases, as it does not differentiate between the healthy tissue and antigens (Edwards and Cambridge 2006).

Pattern recognition receptors (PRRs) serve an important role in the innate immune response by detecting pathogen-associated molecular pattern motifs (PAMP), such as LPS, bacterial lipoteichoic acid (LTA), peptidoglycans, bacterial flagellin and bacterial DNA. PAMPs are unique to each pathogen, essential molecular structures required for the pathogens survival, and exist on Gram-positive and Gram-negative bacteria, fungi, and viruses (Akira et al. 2006). PRRs are receptors on lymphocytes which recognise antigens. Extracellular PRRs detect mainly PAMPs in bacteria and fungi, compared with the intracellular PRRs which primarily detect viral components. PRRs are separated into endocytic, such as the mannose and scavenger receptors, and signalling receptors, such as the TLRs and NOD proteins. Endocytic PRRs promote the attachment of microorganisms to phagocytes and their subsequent engulfment and destruction.

In addition to PAMPs, PRRs recognize damage-associated molecular pattern motifs (DAMP), which are either cytosolic or nuclear proteins with diverse intracellular activities (Rubartelli and Lotze 2007). Upon recognition of various bacterial components by the innate immune system, a pro-inflammatory response occurs. Pro-inflammatory mediators, including TNF-α, IL-6 and IL-1, are released, leading to uncontrollable cytokine production and subsequent cardiovascular collapse, hemodynamic instability and eventually sepsis.

1.6.2 Toll-like receptors

Toll-like receptors (TLRs) were named for their similarity to Toll, a receptor recognised in Drosophila melanogaster. Following the identification of TLR4, the first mammalian TLR, by Charles Janeway and Ruslah Medzhitov in 1997, numerous proteins were further recognized which were structurally associated with it. These proteins are now known as TLRs (Rock et al. 1988). Janeway and

Medzhitov demonstrated that TLR4 was capable of inducing the activation of the adaptive immune system and further investigated the involvement of TLRs in the innate immune system.

The family of TLRs in mammals is composed of at least 11 TLRs, and individual TLRs may detect specific microbial elements originating in pathogens, such as fungi, protozoa, viruses and bacteria (Poltorak et al. 1998). For example, TLR2 recognises Gram-positive bacteria products, such as LTA, peptidoglycan, spirochetes, mycobacteria, and yeast. TLR3 recognises viral double-stranded RNA (dsRNA) (Poltorak et al. 1998), TLR4 serves an important role in the detection of LPS (Poltorak et al. 1998), TLR5 recognises bacterial flagellin (Hayashi et al. 2001), TLRs 7 and 8 recognise single stranded RNA and viral ssRNA (Krieg and Vollmer 2007) and TLR9 is responsible for mediated immune responses to CpG DNA (Ashkar and Rosenthal 2002).

TLRs have leucine-rich repeats (LRR) in their extracellular domains, and are spread within the cell. For example, TLRs 1, 2 and 4 are located on the cell surface, compared to TLRs 3, 7, 8 and 9 which are located in intracellular compartments, including the endosome (Heil et al. 2003; Matsumoto et al. 2003; Ahmad-Nejad et al. 2002; Latz et al. 2004).

The Toll/interleukin-1 receptor (TIR) homology domain is an intracellular signalling domain observed in the IL-1 receptor and TLRs. Regardless of this resemblance between the two types of receptors, IL-1 has an immunoglobulin-like domain instead of LRRs, and the extracellular domains of the receptors do not share the same structure (Poltorak et al. 1998).

Toll-like receptor	Ligand	Cellular distribution
TLR-1:TLR-2 heterodimer	Lipomannans (mycobacteria), lipoproteins, lipoteichoic acids, cell-wall β-glucans, zymosan	Monocytes, dendritic cells, mast cells, eosinophils, basophils
TLR-2:TLR-6 heterodimer		
TLR-3	Double-stranded RNA (viruses)	NK cells
TLR-4 (plus MD-2 and CD-14)	LPS, Lipoteichoic acids	macrophages, dendritic cells, mast cells, eosinophils
TLR-5	Flagellin	Intestinal epithelium
TLR-7	Single-stranded RNA (viruses)	plasmacytoid dendritic cells, NK cells, eosinophils, B cells
TLR-8	Single-stranded RNA (viruses)	NK cells
TLR-9	DNA with unmethylated CpG (bacteria and herpesviruses)	plasmacytoid dendritic cells, NK cells, eosinophils, B cells
TLR-10	Unknown	plasmacytoid dendritic cells, NK cells, eosinophils, B cells
TLR-11 (mouse only)	Profilin and profilin-like proteins	Macrophages, dendritic cells, liver, kidney and bladder epithelial cells

Table 1.6.2 Innate immune recognition by mammalian Toll-like receptors. (Janeway's Immunobiology, 8th edition, Garland Science, 2012).

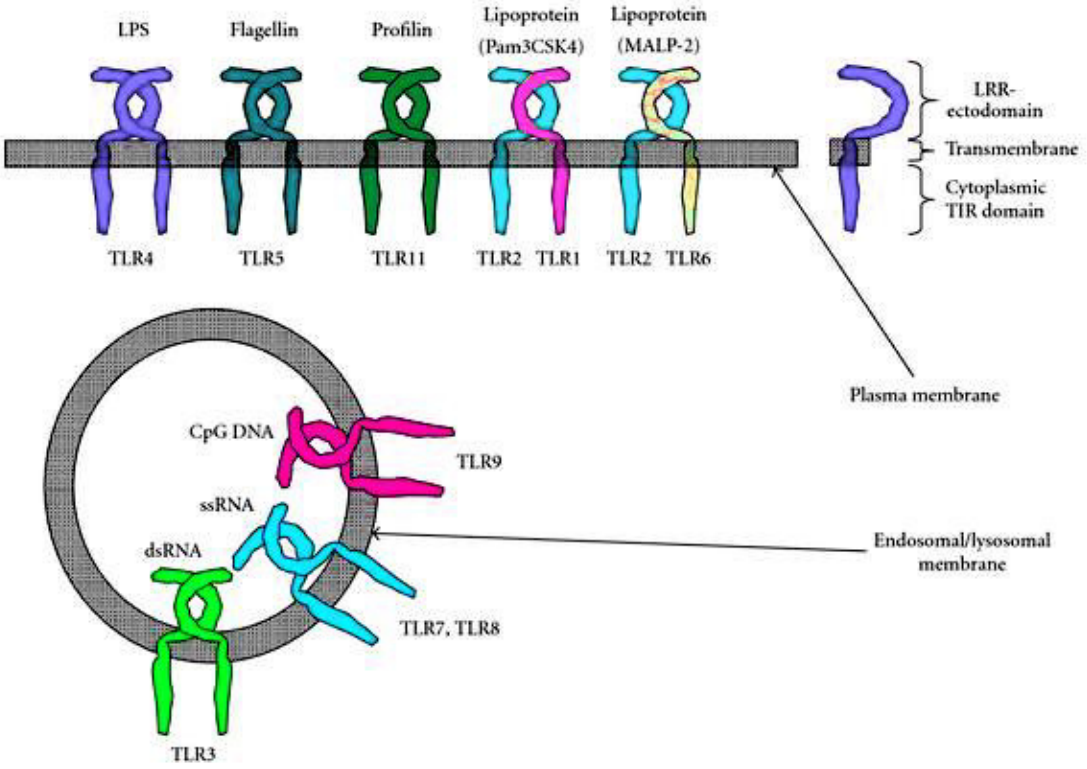

Figure 1.6.2.1 TLR localisation. TLR1, TLR2, TLR4, TLR5, TLR6, and TLR11 recognise their ligands on the cell surface, whilst TLR3, TLR7, TLR8, and TLR9 are located intracellularly, in endosomes and lysosomes (adapted from Yamamoto and Takeda 2010).

1.6.3 TLR signalling pathways

Microbial parts or damage-associated molecular pattern motifs (DAMP) are able to stimulate various TLRs and this leads to the expression of numerous genes playing a role in the immune response. The signalling pathways mediated by TLRs are investigated to reveal the molecular mechanisms utilised in TLR-induced gene expression (Akira and Takeda 2014). Upon activation, the dimerization of TLRs may take place. For example, a previous study demonstrated that TLR2 forms a heterodimer with TLR1 or 6, whereas other TLRs are hypothesized to create homodimers (Saitoh et al. 2004). The formation of dimers can initiate numerous signalling pathways that include a cytoplasmic TIR domain and MyD88 (myeloid

differentiation primary-response protein 88; TIR-domain-containing adaptor) located downstream of these pathways was demonstrated to serve a key role in the production of inflammatory cytokines, including TNF-α and IL-12 (Hayashi et al. 2001; Hemmi et al. 2002; Takeuchi et al. 2000; Kawai et al. 1999; Schnare et al. 2000; Hacker et al. 2000).

Following the investigation of the TLR-mediated pathways, two distinct pathways emerged: the MyD88-independent and -dependent pathways (Figure 1.6.3).

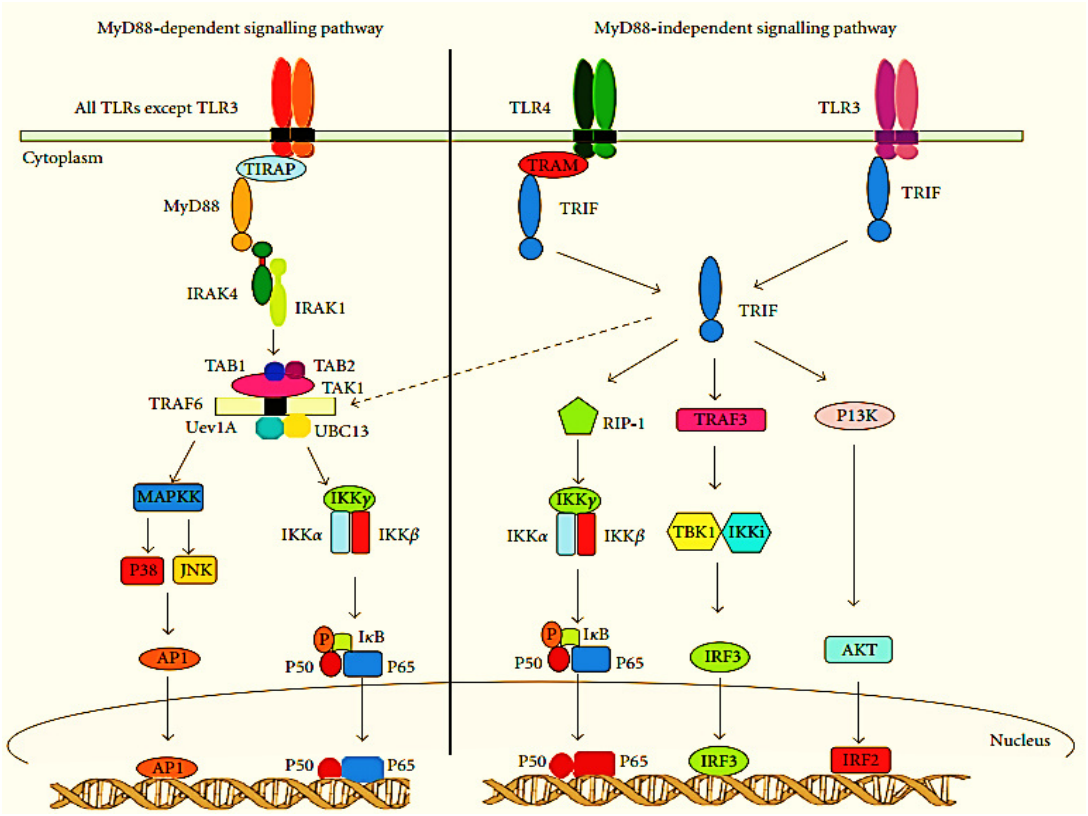

Figure 1.6.3 Visualization of the MyD88 dependent and independent TLR signalling pathways. TIR containing adaptor molecule MyD88 is essential for downstream signalling for the secretion of inflammatory cytokines through TLR signalling and the MyD88 independent pathway leads to IRF3 activation via IKKε/IKKí. Signalling through the MyD88-dependent pathway leads to the activation of MAPKK and IKK complex resulting in activation and nuclear translocation of AP-1 and NF-κB, respectively. TRIF is the main adaptor protein in the MyD88-independent pathway and can associate with TRAF6 to activate AP-1 and NF-κB (adapted from Patel et al. 2012).

1.6.4 Future prospects of TLRs

As mentioned above, microbial components or DAMPs can stimulate TLRs and initiate the production of various inflammatory cytokines, including TNF-α, and interleukins 6 and 12. Upon increased production of the proinflammatory cytokines, systemic disorders are initiated, accompanied with increased mortality rates. Thus, the human body and other organisms have developed the molecular mechanisms required for the adaptation of the immune responses provoked by TLRs (Beeson 1947). Previous studies have demonstrated an association of TLRs and inflammatory and immune diseases. An example is the continuous production of IL-10 due to flawed TLR signalling leading to chronic enterocolitis. Another study demonstrated that mutating TLR4 in mice with flawed IL-10 production has improved the intestinal inflammation, therefore suggesting that a specific TLR is mediating the microbial identification resulting in chronic enterocolitis (Kobayashi et al. 2003).

Further to enterocolitis, mice with an APOE-deficiency demonstrated less pathology of atherosclerosis following the induction of a MyD88 deficiency, thus suggesting that TLRs play an important role in the progression of the disease (Bjorkbacka et al. 2004; Michelsen et al. 2004). These studies indicate that TLRs are capable of mediating certain pathways initiating an immune response, regardless of the presence or not of an infection. Therefore, identifying the mechanisms to balance the TLR-mediated immune response may reduce the effect of the response in various disorders.

1.6.5 PRRs in AD

PRRs recognise a restricted collection of microbial signatures (PAMPs), and permit sensing of different types of microbial pathogens ranging from bacteria and viruses to fungi and spirochetes as well as danger-associated molecular patterns (DAMPs). Families of PRRs include the TLR family, the RIG-like receptor family (RLR), the NOD-like receptor family (NLR) as well as the complement system.

The precise roles of PRRs in detecting and mounting responses against Aβ, and their roles in AD are not clear. TLR expression is upregulated in the AD brain, and in murine models of AD, TLR2 and TLR7 were found to be upregulated compared to controls (Letiembre et al. 2007). Furthermore, multiple TLR genes (1-8) (Weiner and Frenkel 2006; Meyer-Luehmann et al. 2008; Akiyama et al. 2000; McGeer et al. 2006; Letiembre et al. 2009; Bsibsi et al. 2002) were found to be present in microglia in post-mortem tissue from AD patients, with varying levels of expression (Bsibsi et al. 2002). The increased expression of TLRs in AD has positioned them as potential key players in neurodegenerative mechanisms and disease progression.

In particular, the TLR4 gene has emerged as a candidate for susceptibility for AD. A common miss-sense polymorphism resulting in an adenine to guanine substitution 896 nucleotides downstream of the transcription start site causes the replacement of glycine for aspartic acid at amino acid 299 (Asp299Gly) and alters the extracellular structural domain of TLR4. This mutation attenuates TLR4 signalling in response to LPS (Arbour et al. 2000). Since its discovery, this polymorphism has been associated with decreased cardiovascular disease risk (Balistreri et al. 2004), aging (Balistreri et al. 2009) as well as decreased risk of late-onset AD in an Italian cohort (Minoretti et al. 2006; Balistreri et al. 2008),

suggesting that pro-inflammatory responses triggered via TLR4 are crucial for disease progression. Recently, Tang et al. (2014) using primary neuronal cultures from TLR4 mutant mice have shown that neurons expressing TLR4 have an increased sensitivity to Aβ and are vulnerable to degeneration in AD.

In addition to the implication of TLR4 in susceptibility to neurodegeneration, recent studies have suggested that TLR4 activation is required for clearance of Aβ in AD. Mice carrying a point mutation in TLR4 exhibit augmented deposition of Aβ in both the neocortex and hippocampus (Tahara et al. 2006). The mechanism by which TLR activation aids Aβ clearance is unclear.

1.7 Inflammasomes

Inflammasomes are intracellular macromolecular complexes which represent a major line of innate defence. They form upon recognition of PAMPs or DAMPs. The exact structure of an inflammasome depends on the activator/receptor that will cause the inflammasome to assemble (Davis et al. 2011). PAMPs are detected by PRRs and are separated to non-viral (bacterial) and viral PAMPs. Non-viral ones include bacterial LPS and peptidoglycan, and viral PAMPs include endosomal dsRNA (double-stranded RNA), uncapped ssRNA (single-stranded RNA), cytosolic DNA and several viral fusion glycoproteins. The PRRs family detecting these PAMPs is separated into four main classes; Toll-like receptors (TLRs), {NOD}-like receptors (NLRs), the IFI200 family member absent in melanoma 2 (AIM2) and {RIG-I}-like receptors (RLRs). TLRs sense PAMPs in the extracellular space and endosomes and NLRs, RLRs and AIM2 sense pathogens in intracellular compartments.

Inflammasomes are caspase-1 and caspase-5 activators. Caspase-1 controls the activation of the proforms (precursors) of IL-1β and IL-18 cytokines, by cleaving the proforms to release the cytokines (Latz et al. 2013). These cytokines lack a leader sequence and are expressed as biologically inactive proforms in the cytoplasm of the cells. Caspase-1 is expressed in an inactive proform in the cytoplasm too and it is activated by proteolytic self-processing (Latz 2010). Caspase-1 appears to be uniquely involved in participating in the inflammatory response by cleaving the precursors of IL-1 beta and IL-18. Inactive pro-caspase-1 is converted to an active enzyme via dimerization, followed by an autocatalytic reaction that generates an active molecule composed of two large and two small subunits (termed p20 and p10) (Lamkanfi et al. 2007). The precise molecular

mechanisms for the activation of the inflammasome are still unclear, however it is certain that for the maturation and release of IL-1β and IL-18 at least two signals are required; Signal 1 and Signal 2 (Latz 2010). Mechanisms triggering signal 2 for inflammasome activation are induction by reactive oxygen species (ROS), lysosomal damage (possibly coming from ROS), pore formation and K^+ efflux and possibly PAMP influx (Davis et al. 2011). A fourth mechanism was identified recently which is the calcium mobilization regarding the activation of the NLRP3 inflammasome. Findings support NLRP3 inflammasome activation by calcium-mediated mitochondrial damage (Murakami et al. 2012).

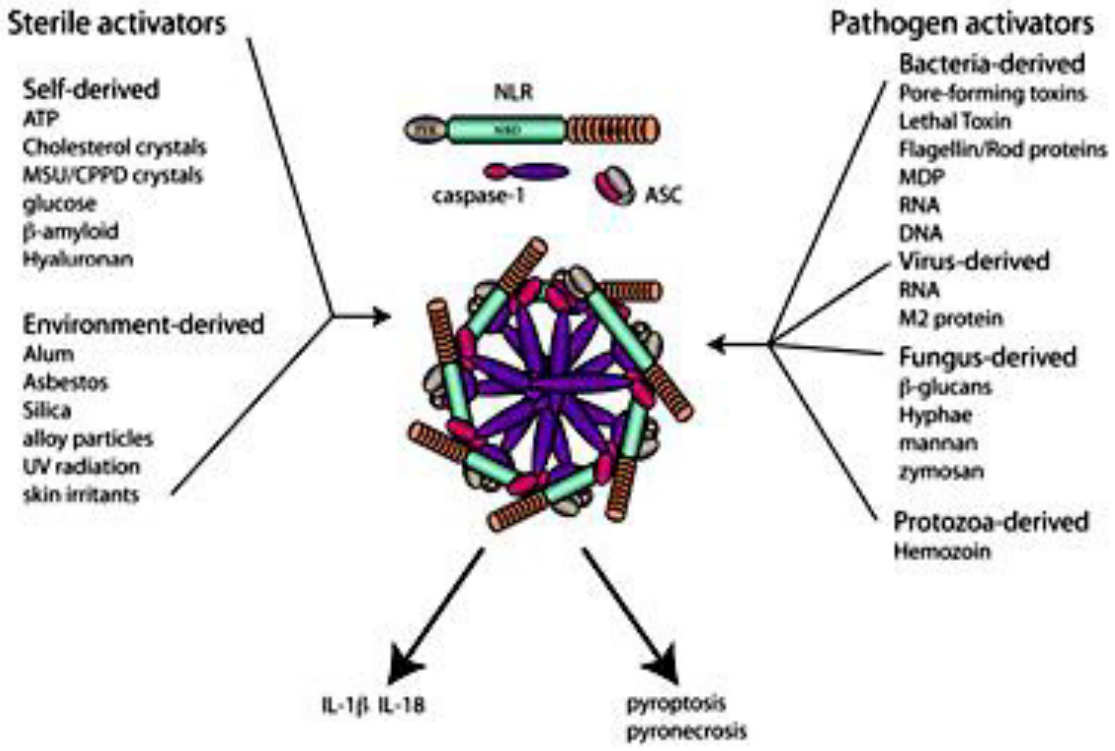

Figure 1.7 Schematic Diagram of an Inflammasome. The NLRs, ASC and Caspase-1 assembles to form the penta- or heptameric structure called the inflammasome resulting in the maturation of IL-1β and IL-18, as well as inflammatory cell death, by either pyroptosis or pyronecrosis (adapted from Davis et al. 2011).

1.7.1 NOD-like-receptors (NLRs)

The NLR family consists of three subfamilies; NODs, NLRPs and IPAF. NLRP1, NLRP3, NLRC4 (IPAF) family members assemble the inflammasomes which are responsible for the activation of caspase-1, hence cleavage of pro-IL-1β and pro-IL-18 for secretion of IL-1β and IL-18 cytokines. On the other hand, NLRX1, NLRC5 and NODs family members in general, inhibit NF-κB and type I IFN-mediated signaling pathways attenuating an inflammatory response. NLRs have a conserved NOD motif characterization and their domain organization consists of an amino-terminal effector binding region (CARD, PYD, and BIR domains), the intermediary NOD and an array of carboxy-terminal LRR (Leucine-rich repeat) motifs. The intermediary NOD is needed for nucleotide binding and self-oligomerization and the array of LRR motifs is presumed to detect conserved microbial patterns and modulate NLR activity (Kanneganti 2010).

1.7.1.1 NLRP1 (or NALP1) Inflammasome

The NLRP1 inflammasome consists of NALP1, ASC, caspase-1 and caspase-5. Human NALP1 is a multi-domain scaffold protein, containing an N-terminal PYRIN (PYD) domain, a centrally located NACHT domain, five tandem LRR domains, a FIIND domain and a C-terminal CARD (Chu et al. 2001; Hlaing et al. 2001). The mechanisms of activation of NLRP1 inflammasome are not yet clear, although it is proposed that it is a two-step activation; bacterial cofactor muramyl dipeptide (MDP) induces a conformational change in NLRP1, which then allows the protein to bind NTPs and oligomerize, thus creating a platform for caspase activation (Faustin et al. 2007). The mechanism by which MDP primes NLRP1 for subsequent NTP-dependent oligomerization is a matter for speculation (Faustin et al. 2007). NLRP1 activity is regulated by anti-apoptotic proteins Bcl-2 and Bcl-x(L)

which can inhibit NLRP1 activity, by inhibiting ATP binding to NLRP1 (Bruey et al. 2007). Cell death and IL-1β secretion induced by NLRP1 depend on K+ efflux and the lysosomal protein cathepsin B (Davis et al. 2011).

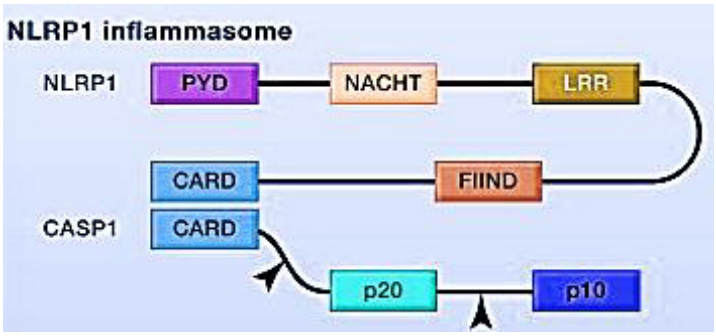

Figure 1.7.1.1 Minimal model for NLRP1 inflammasome. Depicted above in an unoligomerized inflammasome complex. CARD, caspase recruitment domain; LRR, leucin rich repeats; NACHT, nucleotide-binding and oligomerization domain; PYD, pyrin domain, FIIND, domain with function to find; HIN, HIN-200/IF120x domain (adapted from Tschopp and Schroder 2010).

1.7.1.2 NLRP3 (or NALP3) Inflammasome

The NLRP3 inflammasome is activated by a wide range of danger signals that derive not only from microorganisms, but also from metabolic dysregulation. Thanks to its wide distribution in different tissues and organs, the NLRP3 inflammasome protein complex may represent a crucial signalling pathway that facilitates organ crosstalk and local injury in tissues target of metabolic damage. The NLRP3 inflammasome is activated in response to the widest array of stimuli, leading to the theory that the dissimilar agonists induce similar downstream events that are sensed by NLRP3 (Schroder et al. 2010). In most cell types, NLRP3 must be primed and priming has long been known to increase cellular expression of NLRP3 through NF-κB signalling (Bauernfeind et al. 2009). Priming is initiated with an NF-κB–activating stimulus, such as LPS binding to TLR4 (Kolb et al. 2014), which induces elevated expression of NLRP3 (as well as IL-1B), resulting in the increased expression of the NLRP3 protein (Murakami et al. 2012; Hirota et al.

2011). Furthermore, the adaptor protein ASC becomes linearly ubiquitinated and phosphorylated resulting in inflammasome assembly. Following priming, a second signal activates NRP3 during the canonical NLRP3 inflammasome activation, which results to the formation of the NLRP3 inflammasome complex.

The mechanisms for NLRP3 activation are the extracellular ATP binding to its receptor P2X7, triggering K^+ efflux and pannexin-1 membrane pore formation, leading activators in (Tschopp and Schroder 2010). Another mechanism is through phagocytosis causing lysosomal damage, releasing the lysosomal content into the cytosol. The third mechanism associates with ROS generation causing activation to NLRP3, while the fourth mechanism is the Calcium mobilization as calcium signalling promotes mitochondrial damage which triggers NLRP3 inflammasome (Murakami et al. 2012; Tschopp and Schroder 2010).

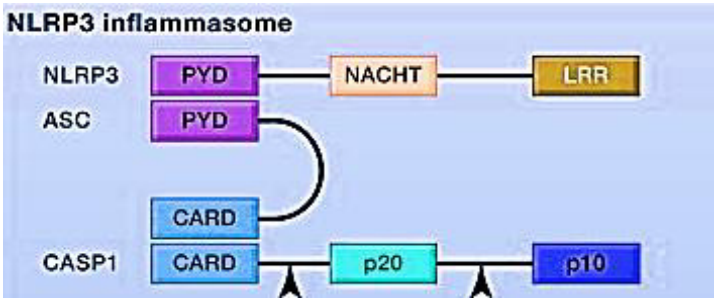

Figure 1.7.1.2a Minimal model for NLRP3 inflammasome. Depicted above in an unoligomerized inflammasome complex. CARD, caspase recruitment domain; LRR, leucin rich repeats; NACHT, nucleotide-binding and oligomerization domain; PYD, pyrin domain (adapted from Tschopp and Schroder 2010).

Activator	Proposed signalling pathway
Microorganisms (PAMPs)	
Sendai virus	ND
Influenza virus	ND
Adenovirus	ROS
Candida albicans	ROS
Saccharomyces cerevisiae	ROS
Staphylococcus aureus	ROS
Listeria monocytogenes	ND
Bacterial pore-forming toxins	ROS
Endogenous danger signals (DAMPs)	
Extracellular ATP	ROS and channel formation
Hyaluronan	ND
Glucose	ROS
MSU	ROS
Amyloid-β	Lysosome rupture
Environmental irritants	
Skin irritants	ROS
Imidazoquinolinone compounds	ROS
Silica	ROS and lysosome rupture
Asbestos	ROS
Alum	ROS and lysosome rupture

Table 1.7.1.2 Proposed signalling pathways for NLRP3 inflammasome activators (adapted from Tschopp and Schroder 2010).

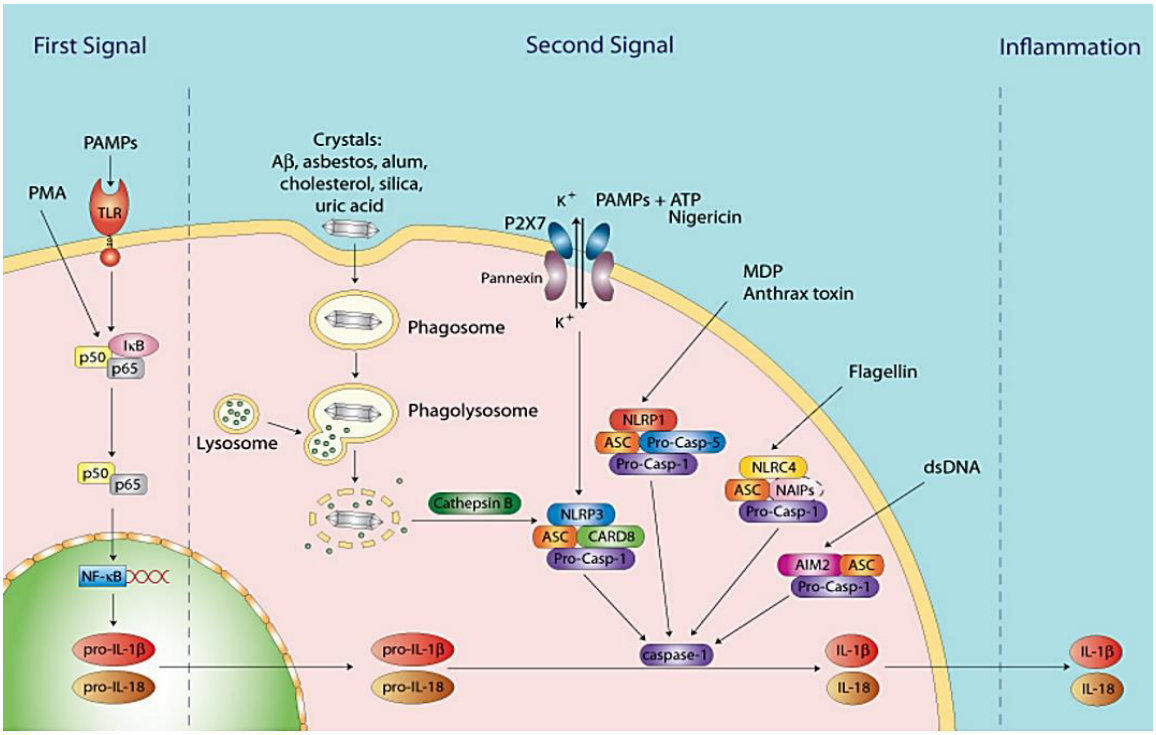

Figure 1.7.1.2b Schematic Diagram of NLRP1, NLRP3 and NLRC4 inflammasomes and their activators. The NLRs, ASC and Caspase-1 assemble to form the inflammasome resulting in the maturation of IL-1β and IL-18. For the activation and release of IL-1β, two distinct signals are

required. The nature of these signals during infection or inflammation is not completely defined. Signal 1 is triggered by various PAMPs following Toll-like receptor (TLR) activation which induces the synthesis of pro-IL-1β. Signal 2 is provided by the activation of the inflammasome and caspase-1, resulting in IL-1β processing (www.invivogen.com/review-inflammasome).

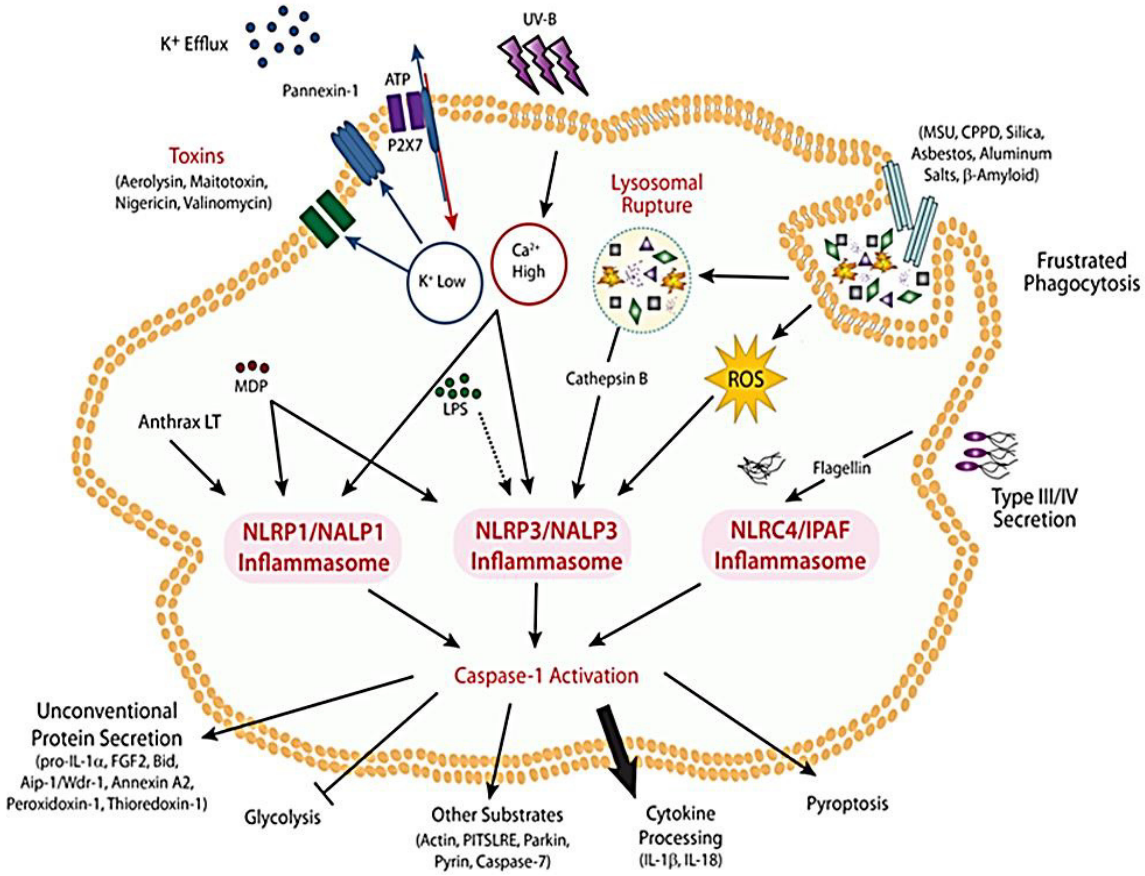

Figure 1.7.1.2c Schematic Diagram of NLRP1, NLRP3 and NLRC4 inflammasome signalling upon activation (http://www.adipogen.com/inflammasomes/?___store=gb).

1.7.1.3 NLRC4 (or IPAF) Inflammasome

NLRC4 is activated by bacteria. The inflammasome assembled by the NLR protein NLRC4 responds to bacterial flagellin and a conserved type III secretion system rod component. How the NLRC4 inflammasome detects the two bacterial products and the molecular mechanism of NLRC4 inflammasome activation are not fully understood. When bacterial flagellin enters through the cell membrane it is detected by NRLC4 and activates it causing caspase-1 activation (Franchi et al. 2006).

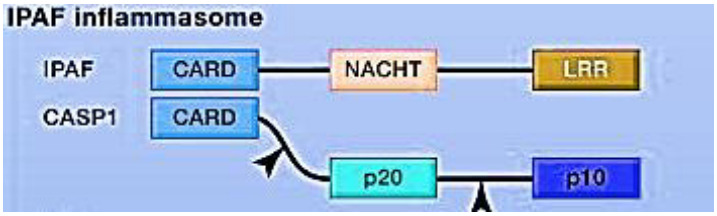

Figure 1.7.1.3 Minimal model for IIPAF (NLRC4) inflammasome. Depicted above in an unoligomerized inflammasome complex. CARD, caspase recruitment domain; LRR, leucin rich repeats; NACHT, nucleotide-binding and oligomerization domain; PYD, pyrin domain (adapted from Tschopp and Schroder 2010).

1.7.1.4 NLRC5 Inflammasome

NLRC5 contains an N-terminal CARD-like domain and it is commonly expressed in numerous tissues and cell types in human and mouse. It is considered to be negative and positive regulator in both NF-κB and type I IFN signaling pathways (Lamkanfi et al. 2011) and regulates MHC Class 1 (Major Histocompatibility Class I) (Meissner et al. 2010). It is still not clarified what is the exact role of NLRC5 in regulating immune signalling as the literature appears to be controversial (Lamkanfi et al. 2011).

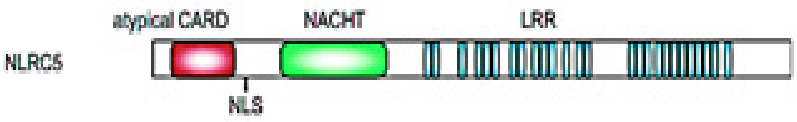

Figure 1.7.1.4 Structural Domain of NLRC5. NLRC5 has typical tripartite domains including the N-terminal atypical caspase activation and recruitment domain (CARD), the centrally located NACHT (named after NAIP, CIITA, HET-E, and TP-1 proteins) and multiple leucine rich repeats (LRRs) at the C-terminal (adapted from Yao and Qian 2013).

1.7.1.5 NLRP6 Inflammasome

NLRP6 regulates caspase-1 and NF-κB activity and it correlates with ASC to activate caspase-1 and release IL-1β. It is expressed in non-hematopoietic cells, maintaining intestinal homeostasis through regulation of the intestinal flora (Anand et al. 2012). Deficiency in NLRP6 in mouse colonic epithelial cells resulted in altered intestinal microbiota. This is because NLRP6 inflammasome regulates IL-18 and not IL-1β and deficiency in IL-18 or NLRP6 distorts the composition of the microbiome toward a colitogenic type (Anand et al. 2012; Chen et al. 2011; Elinav et al. 2011; Chen and Nunez 2011). This distortion, along with CCL5 chemokine production and recruitment of inflammatory cells stimulates a spontaneous inflammation, causing colitis. Studies support that this process and inflammation could be the initiating event in some cases of human inflammatory bowel disease (Rathinam et al. 2012). Therefore, therapeutic inhibition of NLRP6 activation could benefit in treating infectious diseases (Anand et al. 2012).

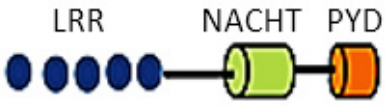

Figure 1.7.1.5 Minimal model for NLRP6 inflammasome. Depicted above in an unoligomerized inflammasome complex. LRR, leucin rich repeats; NACHT, nucleotide-binding and oligomerization domain; PYD, pyrin domain.

1.7.1.6 NLRP7 Inflammasome

NLRP7 has an N-terminal pyrin (MEFV) domain (PYD), followed by a NACHT domain, a NACHT-associated domain (NAD), and a C-terminal LRR region (Messaed et al. 2011a). NLRP7 functions as a PRR in primary human macrophages and the NLRP7 inflammasome senses microbial infection via recognition of acLP (acylated lipopeptides) (Messaed et al. 2011b). The cytosolic microbial acLP activates NLRP7, causing the assembly of the inflammasome, caspase-1 activation via ASC recruitment to the inflammasome and the release of IL-1β and IL-18. In macrophages both NLRP7 and TLR2 are required for the acLP-mediated release of IL-1β and they both play a role in the host defence against intracellular Gram-positive bacteria (Khare et al. 2012).

1.7.2 AIM2 Inflammasome

AIM2 belongs to the same family as IFI200 or HIN200 proteins which are IFN-inducible proteins. They have a 200-amino-acid repeat at the C-terminus, known as HIN domain, and an N-terminal PYD. Same as NLRP3, AIM2 N-terminal PYD recruits ASC to the inflammasome, thus causing the activation of pro-caspase-1 into caspase-1. The AIM2 inflammasome can be activated by cytosolic dsDNA from viruses or bacteria, therefore having a crucial role in the recognition of cytosolic DNA and of infection with vaccinia virus and MCMV (Kanneganti 2010).

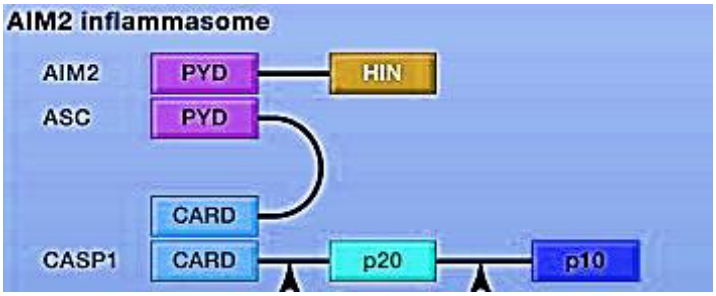

Figure 1.7.2 Minimal model for AIM2 inflammasome. Depicted above in an unoligomerized inflammasome complex. CARD, caspase recruitment domain; PYD, pyrin domain; HIN, HIN-200/IF120x domain (adapted from Tschopp and Schroder 2010).

1.7.3 RIG-I Inflammasome

RIG-I induces type I IFN production through MAVS (mitochondrial antiviral signalling protein) and activates IRF3 and IRF7 (IFN response factor 3 and 7 respectively). MDA5 and RIG-I are both members of the RLR family. MDA5 recognizes RNA virus infection that is specific dsRNA intermediates produced during infection with positive-strand RNA viruses (Kanneganti 2010; Poeck et al. 2010). MDA5 cannot interact with ASC which is needed for activation of caspase-1, thus cannot activate the inflammasome. RIG-I might be responsible to mediate both Signals 1 and 2 needed for inflammasome activation (Kanneganti 2010; Yu and Levine 2011). RIG-I triggers IL-1β and/or IL-18 production through caspase-1 and caspase-3 activation. The transfected 5'-triphosphate RNA causes RIG-I activation, leading to MAVS-CARD9-NF-κB signaling giving pro-IL-1β. ASC recruitment activates caspase-1 which causes the inflammasome pathway activation (Kanneganti 2010).

1.7.4 Therapeutic potential

A high number of viruses activate caspace-1 inducing IL-1β and IL-18 cytokines secretion, thus the inflammasomes have a potential role in the immune response to viruses (Kanneganti 2010). Even though the mechanisms regulating the inflammasomes activity are not clear, various proteins were identified that may interfere with inflammasome activation. Inflammasome activation and chronic inflammation are responsible for metabolic disorders progression, such as obesity, type 2 diabetes mellitus and atherosclerosis, and are a factor in carcinogenesis and control the adaptive immune response (Davis et al. 2011). For these reasons the molecular mechanisms leading to the activation of inflammasomes need to be further investigated to enable for specific treatments preventing sterile or microbial attacks. Some human hereditary or acquired diseases are correlated with elevated IL-1β or NLRP3 mutations, like Gout, Type 2 Diabetes Mellitus, and Alzheimer Disease (Leemans et al. 2011).

1.8 Complement System

1.8.1 Overview of complement activation and function

An important component of the immune system is the complement system. This system boosts or 'complements' the bactericidal activity of antibodies in serum (Janeway et al. 2001). The complement system acts as an element of the innate immune system, nevertheless, the adaptive immune system may adapt it to function if required. The term 'complement' was coined by Paul Ehrlich in 1899 to describe the required supplementary molecule with the function of inducing bacterial lysis (Chaplin 2005).

Originally, in 1891, the term 'alexin' (means 'to ward off' in Greek) was used by Hans Ernst August Buchner to describe the heat labile part in the blood that was capable of killing bacteria (as reviewed by Nesargikar et al. 2012). To eliminate the killing activity, the serum was heated, however, upon injection of heat-inactivated serum into animals, the serum sustained its killing properties and protected the animal from diseases. In addition to the alexin factor, further studies by Jules Bordet demonstrated that that a 'sensitizer' was another required factor to have cell lysis (Morgan 1990; Nesargikar et al. 2012). The 'sensitizer' is now known as the antibody and, following a century from the initial description of the complement, its abilities and function demonstrate something more than just a supplementary molecule.

Following penetration of a pathogen in the epithelial barrier, the defense mechanisms of the innate immune system are activated. The complement system is one of the initial factors activated, and serves an important role in the search for pathogens and antigens, as well as, acting in response to PAMPs and DAMPs (Janeway et al. 2001). The complement system is a complex of pre-cursors and

proteins that assist antibodies and phagocytic cells to eliminate bacteria and other foreign invaders from an organism. The complement proteins are in the blood and lymph, thus a molecular defense mechanism, including the complement activation, could be used immediately following infection. The activation of the complement leads to a series of enzymatic reactions, in which proteolytic cleavage and activation of successive complement proteins occur. Following activation of the proteins, complement fragments are fixed on the pathogen surface. This leads to the uptake and destruction of complement-coated microbes by phagocytes carrying the complement receptors (Wagner and Frank 2010). Following inflammation, the target cells are opsonized by the complement components C1q, C3b (iC3b) and C4b. Opsonisation occurs via deposition of antibodies, known as the classical pathway, carbohydrate-binding proteins, known as the lectin pathway or the alternative pathway (Hoffman et al 2000). The membrane attack complex (MAC) is responsible for the lysis of the targeted cell and, due to the vast functions of the complement system, it is required to maintain homeostasis in the body. To sustain this role, the complement system eradicates macromolecular aggregates, apoptotic and necrotic cells, and assists with the healing of the body by attraction and activation of cells (Janeway et al. 2001).

1.8.2. Complement activation pathways

C1q binds to the antigen-antibody complex resulting in the activation of the C1-complex, thus leading to the activation of the classical pathway. This pathway may additionally be activated by other molecules, such as Aβ, C-reactive protein and various apoptotic fragments. The lectin pathway has a rather similar activation to the classical pathway, with the opsonin mannose-binding lectin (MBL) and ficolins 1, 2 and 3, as an alternative to C1q. The above-mentioned lectin molecules are capable of binding to various sugars, including mannose and fucose, which are uncovered by the pathogens. Activation of the lectin pathway occurs following the MBL binding to mannose deposits on the pathogen surface. This binding leads to the activation of MBL-associated serine proteases (MASP) 1 and 2, which cleavage C4 into C4a and b, and additionally cleavage C2 into C2a and b (Figure 1.8.2) (Orsini et al. 2014). The binding of C2a with C4b creates the C3-convertase. The alternative pathway is activated following the spontaneous hydrolysis of circulating C3 into C3(H_2O) on the surface of the cells. A fourth pathway was proposed, the extrinsic one, which is determined by the serine protease components of the coagulation cascade. A previous study suggested there is interplay between the complement and coagulation systems in cerebrovascular diseases (Huber-Lang et al. 2006).

The complement pathways converge on the C3-convertase, except for the extrinsic one. All four pathways, however, converge on the C5-convertase and activate the final pathway, known as the terminal pathway, by cleaving C5 into C5 a and b (Figure 1.8.2). C5a and C3a are potent anaphylatoxins and are capable of provoking inflammatory responses. On the other hand, C5b is binding on the targeted cell which forms a pore, named the Membrane Attack Complex of

complement (MAC), by assembling C6, 7, 8 and 9. If small amounts of MAC are generated, then signalling events take place in host cells instead of cell lysis. However, lytic amount of MAC results in cell lysis and clearance of the pathogen or altered cell.

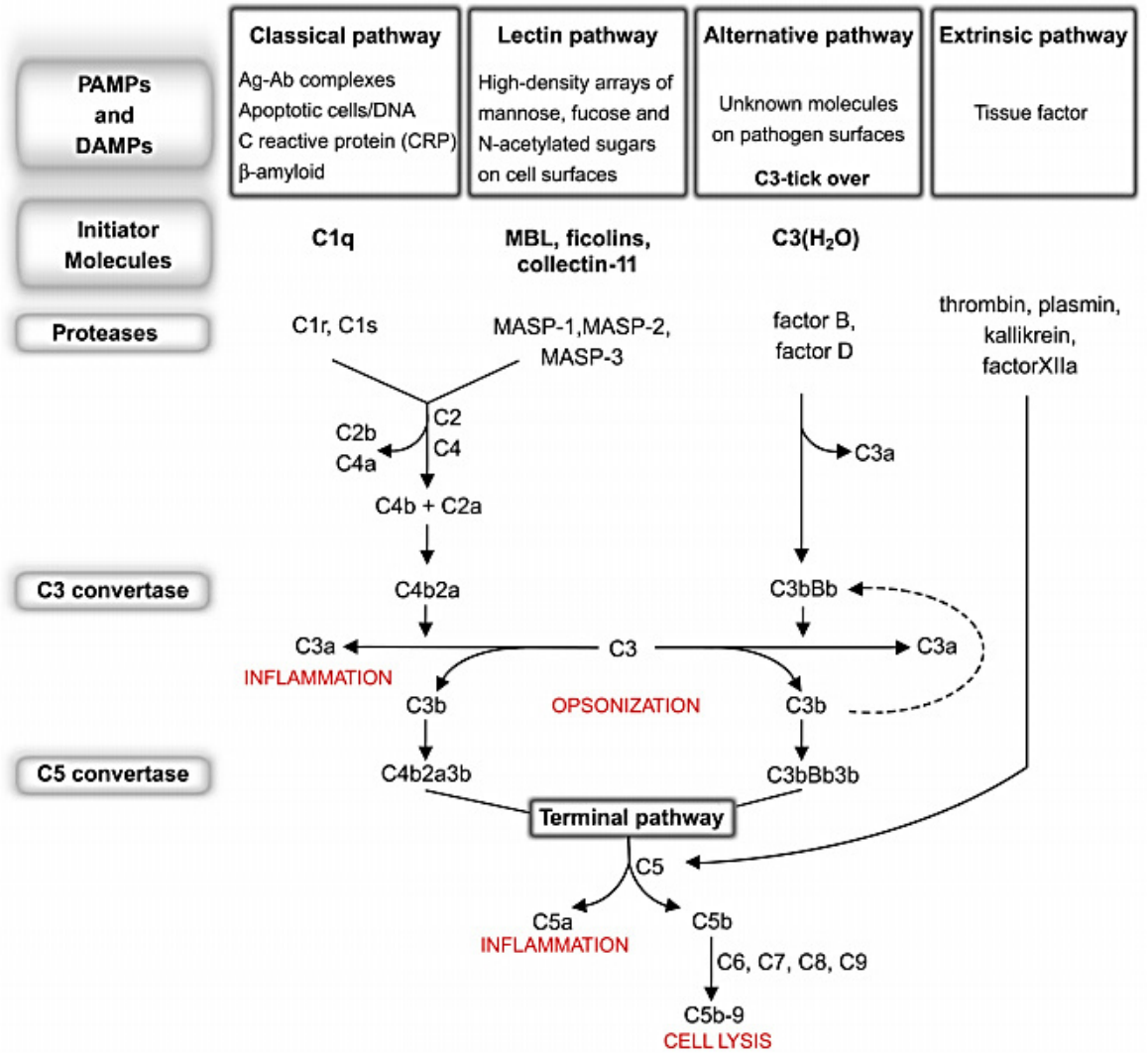

Figure 1.8.2 Overview of the complement system (Orsini et al. 2014).

Complement activation produces numerous cleavage fragments, including C3b and C4b, which act as opsonins and initiate a phagocytic response. Furthermore, complement molecules are capable of initiating an immune response via signalling to various receptors on immune cells (Ricklin and Lambris 2007). Due to the

importance of maintaining the complement balance, regulatory molecules, such as C1 inhibitor (C1-INH), exist. These molecules are separated into membrane-bound and fluid phase regulators (Tandon et al. 1994; Zipfel and Sherka 2009). Complement regulators include factor H and properdin (alternative pathway), and C1q and C1INH (classical and lectin pathways). Soluble inhibitors include clusterin and vitronectin (terminal pathway), and membrane-bound regulators include CR1, CD46, CD55 and CD59 (Zipfel and Sherka 2009).

C1-INH is a well-characterized complement inhibitor, and is a serine-protease inhibitor capable of deactivating the C1q-C1r-C1s and MBL-MASP1-MASP2 complexes (Wagner and Frank 2010). In addition, increased quantity of CI-INH deactivates the C3b fragment produced in the alternative pathway (Wagner and Frank 2010), and various molecules of the coagulation cascades (Ehrnthaller et al. 2011). The numerous roles of the complement receptors are yet to be determined, as further research is required to investigate their potential.

1.8.3 Regulation of complement

The proteins involved in the complement system make ~4% of all blood proteins and the majority of these proteins derive from the liver, normally circulating as inactive precursors (pro-proteins). Upon stimulation of the Complement system by e.g. binding of C1q to IgG or IgM, or MBL to mannose residues, changes in conformation of C1q and MBL results in activation of associated enzymes and a cascade of enzymatic activation reactions (resulting in the release of C3a and C5a), conformational changes and complex formations of the various complement components results in the formation of the membrane attack complex of complement (MAC). Hepatocytes were demonstrated to produce various types of these proteins (Morgan and Gasque 1997; Brennan et al. 2012), and epithelial, endothelial, glial cells and neurons can produce various complement factors.

The human brain has the ability to protect itself from numerous threats due to an advanced immune system specialized for the brain cells, unlike other organs in the body. Glial cells, oligodendrocytes and neurons are competent enough to produce locally receptors, regulators, and factors for the complement components (Woodruff et al. 2010). Specifically, glial, and neuronal cells are capable to synthesize C3, C1q, C1-INH and CD59, and additionally certain brain cells produce other complement components (Stevens et al. 2007).

The complement system serves an important role in neurodegenerative diseases. In a healthy brain, the production of complement proteins is minimal and is hypothesized to play a role in brain maturity and homeostasis (Woodruff et al. 2010). On the other hand, impairment or injuries of cells results to a rise in the levels of the complement components. An amplification in the production of complement proteins leads to inflammation and tissue destruction (Woodruff et al.

2010). Previous studies demonstrated that in chronic neurodegenerative and acute CNS diseases, the complement system was activated, with neuronal cells specifically showing complement-mediated damage. This specific damage may be because neurons have a low expression of membrane-bound complement regulators, such as CD46 and CD55 (Singhrao et al. 2000).

Initially, the complement system was hypothesized to be activated only upon injury/penetration of the BBB. Over the last decades, studies have demonstrated that complement proteins are widely expressed within the CNS and are involved in innate immune responses and signalling cascades (Stephan et al. 2012). For example, Shinjyo *et al.* demonstrated that interplay between C3a and C3aR has an effect on the adult neuronal development, by controlling the migration and differentiation of neuronal stem cells (Shinjyo et al. 2009). Furthermore, previous studies demonstrated that astrocyte-induced expression of complement proteins in neurons was necessary for the clearance of redundant synapses in the CNS while expanding (Schafer et al. 2012).

Complement regulators and factors are located in glial cells, and the complement system appears to carry out major inflammatory responses within the brain (Veerhuis et al. 2011). In addition, astrocytes and microglia are capable of secreting oxidative products, pro-inflammatory cytokines or have phagocytotic functions following activation of the complement system (Fu et al. 2012). Finally, brains of mice and patients with AD demonstrated an increase in the levels of complement proteins, further supporting the association of AD and complement (Stoltzner et al. 2000; Loeffler et al. 2008). More specifically, complement proteins of both the classical and alternative pathways, such as C1q, C4, C3, and Factor

B, have been co-localized with fibrillar amyloid plaques and cerebral vascular amyloid in the cerebral cortex and hippocampus of AD patients.

	Complement Components					
Cell type	**Classical**	**Alternative**	**Terminal**	**C-receptors**	**C-regulators Soluble**	**Membrane-bound**
Astrocytes	C1q, C1r, C1s, C2, C3, C4	C3, fB, fD	C5, C6, C7, C8	C1qR, CR2, C3aR, C5aR	C1-inh, fH, fI, clusterin	CD59, DAF, MCP, CR1
Microglia	C1q, C1r, C1s, C2, C3, C5	C3, fB, fD	-	C1qR, CR3, C3aR, CR4, C5aR	C1-inh	CD59, CR1

Table 1.8.3 Expression of C proteins by human astrocytes and microglia (adapted from Veerhuis et al. 2011).

Regarding genetic factors, clusterin (complement regulator) (Harold et al. 2009) and CR1 (Lambert et al. 2009) were demonstrated to be genetic risk factors of AD, and play a role in the activation of complement during AD.

Lian et al. revealed that, in agreement with previous studies, Aβ provokes the activation of complement in astrocytes, microglia, and neurons (Lian et al. 2015; Haga et al. 1993; Fu et al. 2012), nevertheless the consequence of complement activation *in vivo* continues is not fully understood. A previous study demonstrated that deletion of C1q led to the development of amyloid plaques and loss of synapses in an AD-mouse model (Fonseca et al. 2004). Furthermore, C1q was additionally demonstrated to protect from amyloid toxicity (Pisalyaput and Tenner 2008).

Complement system has a therapeutic potential and various antagonists and agonists available should be further investigated in *in vitro* and *in vivo* studies to understand their full potential.

1.8.4 Complement system and TLRs

TLRs and complement system play a major role in the first line host defence and various PAMPs can activate both systems. How these systems cooperate when co-activated it is not clear yet, however infections can induce simultaneous activation of both systems, indicating the systems are inter connected (Hajishengallis and Lambris 2010; Hawlisch and Kohl 2006; Mollnes et al. 2008). It is believed that TLR signalling is regulated by the complement system (Hajishengallis and Lambris 2010). Moreover, there are evidence indicating that TLRs can target the second C5a receptor, C5aR2, by reducing C5aR2 activity (Raby et al. 2011) and in general TLR activation interacts with C5a (Damman et al. 2011). C5aR1 and C5aR2 are seven transmembrane G-protein coupled receptors. As mentioned above, complement activation ultimately leads to the formation of the anaphylatoxins C3a, C4a, and C5a. Of these, C5a is one of the most phlogistic peptides involved in diverse immune and non-immune responses. Human C5a (approximately 15 kDa) is glycosylated, consists of 74 amino acids and has an N-linked glycosylation site at Asn 64 that is not essential for biological activity, although it has been suggested to regulate the C5a activity *in vivo* (Monk et al. 2007. C5a is a potent anaphylatoxin that at low nanomolar concentrations acts as a chemoattractant for myeloid cells, including neutrophils, monocytes, macrophages, basophils, and eosinophils. At higher concentrations, C5a can elicit superoxide generation and enzyme release responses (Gerard and Gerard 1994). The biological activity of C5a is greatly reduced by the removal of the carboxyl-

terminal residue (Arg) by carboxypeptidases, which is then known as C5adesArg (Manthey et al. 2009). The potent inflammatory functions of C5a (see Table 1.8.4) indicate that inhibition of this ligand, or its receptor(s), might alleviate certain inflammatory conditions. There are two receptors known to bind to C5a; C5aR1 (CD88) and C5aR2 (GPR77) (Llee et al. 2008).

Cell type	***Activity***
Neutrophils	Enhanced expression of adhesion molecules; Chemotaxis; Oxidative burst (O2 consumption); Phagocytosis; Release of granule enzymes; Delayed apoptosis
Eosinophils	Release of granule enzymes; Chemotaxis
Basophils	Histamine release
Mast cells	Histamine secretion; Chemotaxis
Plasmacytoid dendritic cells	Chemotaxis
Macrophages/monocytes	Chemotaxis; Cytokine release
Thymocytes	Enhances apoptosis
Endothelium	Vasodilation; Chemokine release
Hepatocytes	Enhanced regeneration
Microglia	Chemotaxis

Table 1.8.4 Functional activity of C5a on different cell types (as reviewed in Llee et al. 2008)**.**

Okusawa et al. (1987) demonstrated that when human monocytes were exposed to both C5a and LPS, the IL-1β induction levels were increased compared with when the monocytes were stimulated with either agonist alone. Their results suggested a synergistic interaction between C5aR1 and TLR4 receptors. It may well be that *in vivo* the two systems are activated simultaneously upon recognition

of a pathogen. Zhang et al. (2007) has also demonstrated that while LPS-mediated IL-12 production is suppressed by the C5a-C5aR1 interaction, C5a had enhanced the LPS-induced TNFα, IL-1β, and IL-6 production in mice.

C5a was additionally demonstrated to synergize with TLR2, which reacts with *Porhyromonas gingivalis*, resulting in increased TLR2-mediated cAMP production in macrophages. Due to this synergistic effect, the nitric oxidedependent killing of *P. gingivalis* is inhibited (Wang et al. 2010). TLRs appear able of enhancing C5a-mediated responses (Raby et al. 2011). Stimulation of human peripheral blood monocytes with TLR ligands, such as LPS and zymosan, and a subsequent stimulation with C5a indicated an enhanced expression of IL-8, suggesting that TLR activation can modulate C5aR1-mediated responses (Raby et al. 2011).

The same complement receptors regulating TLR signalling, like CR3, C5aR1, gC1qR and CD46, can be controlled by bacterial or viral pathogens to interfere with the hosts' protective immunity (Hajishengallis and Lambris 2010). The more associations between TLR and complement signalling pathways are discovered and researched, the more enhanced protective immunity is offered by the host as therapeutic drugs will be developed based on specific signalling cascades.

1.8.5 Complement system and Alzheimer Disease

The complement system is implicated in various diseases, among them some of the central nervous system such as AD. It is detected that an up-regulation of the expression of the complement proteins of the classical pathway occurs in AD affected brain regions (Van Beek et al. 2000; Veerhuis 2010). The levels of complement mRNAs for C1q, C1r, C1s, C2, C3, C4, C5, C6, C7, C8, and C9 were detected in the 11 regions of brain that were investigated. The mRNA levels were markedly up-regulated in affected areas of AD brain, such as the entorhinal cortex, hippocampus, and midtemporal gyrus, as C1q mRNA was increased 11- to 80-fold over control levels, and C9 mRNA 10- to 27-fold. Protein analysis of AD-affected hippocampus established the presence of all the native complement proteins as well as their activation products C4d, C3d, and the membrane attack complex. These data indicate that high levels of complement are being produced in affected areas of AD brain, that full activation of the classical complement pathway is continuously taking place, and that this activation may be contributing significantly to AD pathology (Yasojima et al. 1999).

The correlation of complement factors and amyloid deposits in AD was first described in immunohistochemical studies by Eikelenboom and Stam in 1982 (Eikelenboom and Stam 1982). Detection of complement activation via monoclonal antibodies and use of knockout mice to validate these antibodies to be used in mice with neurodegeneration, has strengthened the validity of the reports of complement component association with fibrillar amyloid containing plaques Amyloid plaques was proved to activate complement *in vivo*, and this suggested a role for complement in AD pathophysiology (Veerhuis et al. 2011; Gasque et al. 1995; Lambert et al. 2009).

Neuronal and glial cells express TLRs and complement receptors, and nearly all complement components can be locally produced in the brain, usually after injury or developmental cues. Complement proteins promote proliferation and regeneration in various tissues (Ricklin et al. 2010) and this could also be applied in similar functions in the Central Nervous System, since neuronal stem cells differentiate and migrate in response to complement. For example, it is observed that C3a-C3aR interactions are a positive regulator of adult neurogenesis (Bogestal et al. 2007; Shinjyo et al. 2009). Neurons and astrocytes express several serine protease inhibitors, including C1-Inh, nevertheless, in neurodegenerative diseases like AD, expression levels of several regulatory proteins, including C1-Inh and alternative pathway C-regulators fH and fI, remain low or are decreased (such as protease nexin 1), causing probable uncontrolled actions of the proteases (as reviewed in Veerhuis et al. 2011). The functions of some regulatory proteins, such as α2M which regulates thrombin, can be taken over by others since many proteases and protease inhibitors act in the complement, like C1-Inh, which is not only a physiological regulator of C1 activation, it is also a major inhibitor of MASP2 of the lectin pathway (Beinrohr et al. 2008).

In AD Aβ initiate the complement cascades and the Aβ-induced activation could lead to a disturbed protease - protease inhibitor balance, particularly when the synthesis of the proteases increase too, as it has been observed in AD. These imbalances could be the cause of initiation of the consequent steps in neurodegenerative processes in AD (Veerhuis et al. 2011). Moreover, in Alzheimer Disease patients, both TLR2 and CD14-positive microglia were associated with Aβ plaques, and many CD14-positive cells were found surrounding diffuse plaques compared to dense-core plaques (Letiembre et al. 2007; Landreth and Reed-

Geaghan 2009). This indicates that the increased expression of CD14 and TLRs is associated with increased inflammation, which is associated with aging. When all these are joined with various Alzheimer Disease risk factors, the disease progresses through an inflammatory response (Landreth and Reed-Geaghan, 2009), thus more research is needed into this field.

In conclusion, polymorphisms of complement factors and TLR signalling are coupled with neurodegenerative diseases. Therefore, by controlling the complement activation via use of inhibitors of the complement cascade events we could avoid the consequences of this activation and positively affect the rate of progression of these diseases (Veerhuis et al. 2011).

1.9 Aims of the project

Neuroinflammation plays a key role in neurodegeneration in AD. Complement, TLRs and NLRs are swiftly activated in response to infection or damage: there is increasing evidence for extensive bidirectional cooperation between the two systems. Aβ linked with AD can activate both types of these receptors, but whether and how these two innate immune systems interact with each other in the context of AD has not been studied before. **It is my hypothesis that deposition of Aβ peptide can activate the innate immune system via PRRs, including complement, and evoke Alzheimer's pathology.** Deposition of Aβ in the brain might activate astrocytes and microglia, initiating a pro-inflammatory cascade that results in the release of potentially cytotoxic molecules, such as cytokines, complement, proteases and other acute phase proteins, ultimately causing neurodegeneration. **In the current study, I focused on the role of the innate immunity system of the brain in the initiation and the propagation of inflammatory process in AD and the interplay between TLRs/NLRs and the complement system.**

To test my hypothesis, the following questions were examined:

1. Which PRRs are involved in the innate immune sensing of fibrillar Aβ?
2. How do PRRs - Aβ associate and how is signalling being triggered? Is interplay between complement and TLR/NLR receptors the key to the chronic inflammatory response observed in AD?
3. Is the clearance of fibrillar Aβ through its internalization and trafficking crucial for triggering a pro-inflammatory response?
4. Can we inhibit inflammatory responses by antagonizing PRRs involved in neuroinflammation?

Chapter II

Materials and methods

2.1 Materials

2.1.1 Chemicals

All fine chemicals were obtained from Sigma-Aldrich (UK).

2.1.2 Antibodies

Primary Abs	Species	Company	Catalogue no.
anti-NLRP1 (B2)	Mouse Monoclonal	Santa Cruz Biotechnology	SC-166368
anti-NLRP3 (Cryopyrin-H 66)	Rabbit Polyclonal	Santa Cruz Biotechnology	SC-66846
anti-NLRC5 (NOD4 - N-17)	Goat Polyclonal	Santa Cruz Biotechnology	SC-248091
Anti NLRP3	Goat polyclonal	Santa Cruz Biotechnology	SC-58902
anti C9 neoantigen	Mouse Monoclonal	Hycult	HM2264
anti-CR1	Rabbit Polyclonal	Santa Cruz Biotechnology	SC 20924
anti-CR3	Rabbit Polyclonal	Santa Cruz Biotechnology	SC 28664
anti-C5aR1 (S 5/1)	Human, mAb	HyCult Biotechnology (Netherlands)	HM2094
anti-C3aR (H-300)	Rabbit Polyclonal	Santa Cruz Biotechnology	SC-20138
anti-CD55 (H-319)	Rabbit Polyclonal	Santa Cruz Biotechnology	SC-9156
anti C9	Rabbit Polyclonal	Kindly donated by Prof Paul Morgan and Dr T.Hughes (Triantafilou et al. J Cell Sci 2013)	
anti-CD59	Rabbit	Kindly donated by Prof Paul Morgan and Dr T.Hughes (Triantafilou et al. J Cell Sci 2013)	N/A
anti Phospho-IKappaBAlpha	Rabbit	New England Biolabs	2859L
anti Beta amyloid	Mouse monoclonal	Santa Cruz Biotechnology	SC28365

anti TLR4	Mouse Monoclonal	Santa Cruz Biotechnology	SC293072
anti-Factor H	Rabbit	Kindly donated by Prof Paul Morgan and Dr T.Hughes	N/A
anti-TLR2 (C-18)	Goat Polyclonal	Santa Cruz Biotechnology	SC-8690
anti amyloid	Rabbit polyclonal	Thermofischer Scientific	700254
anti NIK	Mouse Monoclonal	Santa Cruz Biotechnology	SC8417
antiTLR2	Mouse Monoclonal	Abcam	TLR2.1
Anti NIK	Rabbit polyclonal	New England Biolabs	4994S
anti EEA1	Goat polyclonal	Santa Cruz Biotechnoly	SC34567
anti EEA1	Mouse Monoclonal	Santa Cruz Biotechnoly	SC11176
anti Rab5	Goat Polyclonal	Santa Cruz Biotechnoly	SC42376
anti LAMP1	Goat polyclonal	Santa Cruz Biotechnoly	SC23114
anti-TLR4 (C-19)	Goat Polyclonal	Santa Cruz Biotechnology	SC-8694

Table 2.1.2a List of primary antibodies utilized for experiments. This table summerizes the primary antibodies used in this study.

Secondary Abs Species	Company	Catalogue no.
Rabbit anti-mouse IgG- FITC	DAKO	00044532
Goat anti-rabbit IgG- FITC	JaksonImmunoresearch	111-095-045
Donkey anti-Goat IgG (H+L), Alexa Fluor 488	Thermofischer Scientific	A-11055
Donkey anti-Rabbit IgG (H+L) Alexa Fluor 488	Thermofischer Scientific	A-21206
Donkey anti-rabbit IgG (H+L) Alexa Fluor 546	Thermofischer Scientific	A-11157
Donkey anti-Goat IgG (H+L) Alexa Fluor 546	Thermofischer Scientific	A-11056
Goat anti-Rabbit IgG (H+L) Alexa Fluor 546	Thermofischer Scientific	A-11010
Donkey anti-mouse IgG (H+L), Alexa Fluor 555	Thermofischer Scientific	A-31570
Donkey anti-mouse IgG (H+L), Alexa Fluor 488	Thermofischer Scientific	A-21202
Donkey anti-mouse IgG (H+L), Alexa Fluor 647	Thermofischer Scientific	A-31571
Donkey anti-rabbit IgG (H+L), Alexa Fluor 647	Thermofischer Scientific	A-31573
Donkey anti-Goat IgG (H+L), Alexa Fluor 633	Thermofischer Scientific	A-21082
Rabbit anti-goat IgG- FITC	DAKO	00053061
Goat anti-rabbit IgG- FITC	JaksonImmunoresearch	111-095-045
Goat anti-rabbit IgG-FITC	JaksonImmunoresearch	111-095-045
Rabbit anti-mouse IgG- FITC	DAKO	00044532
Goat anti-rabbit IgG-FITC	JaksonImmunoresearch	111-095-045
Goat anti-rabbit IgG-FITC	JaksonImmunoresearch	111-095-045
Goat anti-rabbit IgG- FITC	JaksonImmunoresearch	111-095-045
Goat anti-rabbit IgG- FITC	JaksonImmunoresearch	111-095-045
Rabbit anti-goat IgG- FITC	DAKO	00053061
Goat anti mouse IgG-HRP	Thermofischer Scientific	A10551
Goat anti Rabbit IgG-HRP	Thermofischer Scientific	31460
Rabbit anti-goat IgG FITC	DAKO	00053061

Table 2.1.2b List of secondary antibodies utilized for experiments.

2.2 Tissue culture

To ensure sterile conditions during tissue culture a Microflow Advanced Biosafety Class II laminar flow hood was used. Plasticware, glassware and solutions were autoclaved for tissue culture. Hood and equipment was sterilized with 1% aqueous Virkon (Antec International). Cells were incubated at 37˚C with 5% CO_2 humidified atmosphere.

2.2.1 Cell line investigated

The cell line used for experiments is a Human brain astrocytoma cell line from ECACC (European Collection of Cell Cultures, a Public Health England Culture Collection); 1321N1. The adherent cell line, astrocyte cells (glial morphology, human species), was cultured in RPMI 1640 medium + 10% FCS (Sigma-Aldrich), 0.25% Trypsin/EDTA (Sigma-Aldrich) and maintained in an incubator at 5% CO_2 at 37°C.

2.2.2 Trypsinization

Foetal calf serum (FCS; growth factor) was added to the growth medium (10% to final volume) to provide the required nutritions and viable environment for cell growth. Upon high confluency (cells cover >90% of the flask), cells required either propagation or maintenance. Cell propagating ensures that the cells have the necessary space to grow and the cell line is maintained indefinitely.

For trypsinization, the supernatant was discarded from the flasks containing the monolayer of cells. The single layer of cells was then washed using 2 ml of 1X phosphate buffered saline (PBS) to remove the cellular debris. PBS was discarded and 2 ml of trypsin (Sigma-Aldrich) were then added to the cells ($2x10^6$ cells), and incubated for 5 min at 37ºC at 5% CO_2. Trypsin is a serine protease

that is utilized to hydrolyse the cell-adherent proteins at the bottom of the flask. After 5 min of incubation, 2 ml of growth medium (equal volume to trypsin) were added to the cells to neutralize the effect of the trypsin.

The cells were equally divided and growth medium was added bringing the flasks up to a total volume of 5 ml. The flasks were left in the incubator at 37°C at 5% CO_2.

2.2.3 Cryogenic preservation

To freeze the adherent cells for preservation, the confluent monolayer of cells ($2x10^6$ cells) was washed with 1X PBS and trypsinized as described above. The cell volume, including trypsin, was then transferred into a 15 ml Falcon tube, and centrifuged at 1200 rpm for 5min at room temperature (RT). The supernatant was aspirated and discarded, and 1 ml of freezing medium was added to the pellet and resuspended. The mixed sample was quickly transferred into appropriately labelled CryoTube vials (Nunc; Sigma-Aldrich) and stored at -80°C.

The CryoTube vials were transferred into liquid nitrogen (-196°C) after 24 h for long-term storage. Depending on the cells (transfected or wild type), the freezing medium consists of 10% dimethyl sulfoxide (DMSO) in FCS. In the case of transfected cells, the freezing medium consists of 10% DMSO, 30% FCS and 60% selection medium (with antibiotics; see transfection subchapter). The freezing medium is necessary for the preservation of cells to avoid cell damage and is acting by forming ice crystals around the cells. The DMSO/FCS-resuspended pellet of cells was transferred into CryoTube vials and stored in the freezer in less than 10 min, as DMSO is a cryo-protectant, however toxic to the cells in room temperature following exposures longer than 15 min.

2.2.4 Thawing Cells

CryoTube vials stored in liquid nitrogen may be carefully removed and utilize as required in experiments. To use the frozen cells, they require thawing first. The DMSO/FCS/cell mixture from the vials was transferred into 15 ml Falcon tubes as soon as it was defrosted and 10 ml of appropriate growth medium were quickly added. The sample was centrifuged at 1200 rpm for 5 min at RT and the supernatant was discarded. Growth medium (5 ml) was added to the pellet of cells and resuspended carefully. The cell mixture was then transferred into a flask and left in a 37ºC at 5% CO_2 incubator.

2.2.5 Amyloid

A peptide of amino acids 1–42 of Aβ (*DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIA)*, as well as revAβ (AIAEGDSHVLKEGAYMEIFDVQGHVFGGKIFRVVDLGSHNVA) (control nonfibrillary peptide with identical sequence in reverse order), were obtained from Anaspec. To induce fibril formation, $A\beta_{1-42}$ was resuspended in H_2O at 1 mg/ml and incubated for 1 week at 37°C (Moore et al 2002). Fibril formation from our preparation was confirmed by atomic force microscopy staining performed by Professor Louise Serpell (University of Sussex) who is an expert in amyloid structure, as previously described (Gosal et al 2005). Aβ preparations were tested for endotoxin with a limulus amebocyte lysate assay. Aliquots of Aβ were frozen and then they were used as needed to prevent microbial growth and degradation of fibrils. $A\beta_{1-42}$ and revAβ were used at 10uM in all studies unless otherwise stated.

2.2.6 Brain tissue samples

Brain tissue samples used in this study were obtained from The Oxford Brain Bank as well as the University College London Brain Bank by a Material transfer agreement. Tissue was supplied as paraffin-embedded slides. Alzheimer's Disease subjects and non-demented controls were recruited through the Oxford Brain Tissue Research Center or the UCL Brain Bank. Each subject was diagnosed at intake and each brain underwent an extensive neuropathology examination. A table indicating the tissue samples is listed below.

	Sample number	Gender	Age	Pathological diagnosis	Limbic region section	Neuropathological record
Controls	Cs00017	Female	57	Normal	hippocampus	
Controls	Cs22348	Female	76	Normal	hippocampus	
Controls	Cs0099	Female	61	Normal	hippocampus	
Controls	Cs00567	Female	67	Normal	hippocampus	
Controls	Cs11123	Male	54	Normal	hippocampus	
Controls	Cs00765	Male	37	Normal	hippocampus	
Controls	Cs11123	Male	54	Normal	hippocampus	
Controls	Cs341	Female	48	Normal	hippocampus	
Controls	Cs00978	Male	57	Normal	hippocampus	
Controls	20111016	Male	60		hippocampus	
Controls	19960207	Female	49	Normal	hippocampus	
Controls	20000059	Female	55	Normal	hippocampus	
Controls	20020062	Male	70	Normal	hippocampus	
Controls	20090017	Male	18	Normal	hippocampus	
Controls	20100098	Female	63	Normal	hippocampus	
Controls	20100320	Male	33	Normal	hippocampus	
Controls	20100987	Male	62	Normal	hippocampus	
Controls	20110561	Male	64	Normal	hippocampus	
Controls	20100486	Female	27	Normal	hippocampus	

Controls	20121029	Male	52	Normal	hippocampus	
Controls	20131902	Male		Normal	hippocampus	
Controls	19820196	Female		Normal	hippocampus	
Controls	19891029	Male		Normal	hippocampus	
Controls	19910252	Female	67	Normal	hippocampus	
Controls	19920202	Female			hippocampus	
Dementia	Cs05479	Female	89	Alzheimer	hippocampus	Stage IV
Dementia	Cs34578	Male	80	Alzheimer	hippocampus	Stage III
Dementia	Cs1198	Male	77	Alzheimer	hippocampus	Stage IV
Dementia	Cs12345	Female	78	Alzheimer	hippocampus	Stage V
Dementia	CS111	Male	81	Alzheimer	hippocampus	Stage V
Dementia	CS1118	Female	77	Alzheimer		
Dementia	Cs0087	Female	83	Alzheimer	hippocampus	Stage VI
Dementia	Cs0056	Male	55	Normal	hippocampus	Stage IV
Dementia	Cs1234	Male	70	Alzheimer	hippocampus	Stage IV
Dementia	Cs0096	Female	65	Normal	hippocampus	Stage III
Dementia	Cs5809	Female	74	Alzheimer	hippocampus	Stage IV
Dementia	Cs2456	Male	86	Alzheimer	hippocampus	Stage VI
Dementia	Cs3478	Male	75	Alzheimer	hippocampus	Stage V
Dementia	Cs3477	Male	70	Alzheimer	hippocampus	Stage IV
Dementia	Cs0091	Female	68	Alzheimer	hippocampus	Stage IV
Dementia	Cs34567	Female	71	Alzheimer	hippocampus	Stage IV
Dementia	CS1239	Male		Alzheimer	hippocampus	Stage III
Dementia	Cs11888	Male	89	Alzheimer	hippocampus	Stage VI
Dementia	19940092	Male		Alzheimer	hippocampus	
Dementia	19940144	Female	92	Alzheimer	hippocampus	Stage IV
Dementia	20040091	Female	87	Alzheimer	hippocampus	Stage IV
Dementia	20050087	Male	73	Alzheimer	hippocampus	Stage III
Dementia	20050102					
Dementia	20070049	Female	69	Alzheimer	hippocampus	Stage IV

Dementia	20090067	Male	72	Alzheimer	hippocampus	Stage IV
Dementia	20100729	Male	70	Alzheimer	hippocampus	Stage III
Dementia	20110891	Female	88	Alzheimer	hippocampus	Stage IV
Dementia	20040091		77	Alzheimer	hippocampus	Stage VI
Dementia	20050087	Male	83	Alzheimer	hippocampus	Stage V
Dementia	20050102	Female	87	Alzheimer	hippocampus	Stage VI
Dementia	20070049	Female	66	Alzheimer	hippocampus	Stage IV
Dementia	20090067	Male	81	Alzheimer	hippocampus	
Dementia	20100729	Female	84	Alzheimer	hippocampus	
Dementia	20110891	Female	86	Alzheimer	hippocampus	Stage V
Dementia	20111016	Male	90	Alzheimer	hippocampus	Stage V
Dementia	19960207	Male	76	Alzheimer	hippocampus	Stage VI
Dementia	20000059	Female	70	Alzheimer	hippocampus	Stage V
Dementia	20020062	Male	69	Alzheimer	hippocampus	Stage VI
Dementia	20090017	Female	82	Alzheimer	hippocampus	Stage V
Dementia	20100098	Female	75	Alzheimer	hippocampus	Stage VI
Dementia	20100320	Female		Alzheimer	hippocampus	Stage VI
Dementia	20100987	Female	78	Alzheimer	hippocampus	Stage IV
Dementia	20110561	Female		Alzheimer	hippocampus	Stage V
Dementia	20100486	Male	69	Alzheimer	hippocampus	Stage III
Dementia	20121029	Female	72	Alzheimer	hippocampus	Stage IV
Dementia	20131902	Male	72	Alzheimer	hippocampus	Stage V
Dementia	19820196	Female		Alzheimer	hippocampus	Stage VI
Dementia	19891029	Male	85	Alzheimer	hippocampus	Stage IV
Dementia	19910252	Female	78	Alzheimer	hippocampus	Stage V
Dementia	19920202	Male	71	Alzheimer	hippocampus	Stage VI

Table 2.2.6 A table indicating the samples from patients with Alzheimer's Disease and healthy controls used for this study.

2.3 Immunofluorescence

2.3.1 Underlying principles

Immunofluorescence is a technique that utilizes light microscopy with a fluorescence microscope to detect and quantify an antigen present on the cell membrane or intracellularly. Antibodies have specificity towards certain antigens, thus a fluorophore (fluorescent dye) is attached to an antibody to bind the antigen of interest. The fluorophore assists in the visualization of the distribution of the targeted antigen and the fluorescence detected corresponds to the quantity of the antigen of interest. Immunofluorescence may be used on cell lines or tissue sections to investigate the distribution of molecules (Bauer and Jaccoberger 1994).

Fluorophores are fluorescent chemical compounds which absorb light energy of a certain wavelength and emit energy at a different wavelength. Depending on the fluorophores' structure and environment, the energy transfer efficiency, absorbed wavelengths and time prior to emission change accordingly. Two of the most commonly used fluorophores are the derivatives of fluorescein, FITC (fluorescein isothiocyanate), and rhodamine, TRITC (tetramethylrhodamine isothiocyanate). FITC has an excitation wavelength of 495nm (cyan) and an emission wavelength of 519nm (green), whereas TRITC has an excitation wavelength of 547nm (green) and an emission wavelength of 572nm (yellow) (Haugland 1995; Liu et al. 2013). Alexa 488 (excitation wavelength of 495nm and an emission wavelength of 519nm), Alexa 594 (excitation wavelength of 590nm and an emission wavelength of 617nm) and Alexa 405(excitation wavelength of 401nm and an emission wavelength of 421nm) are additional fluorescent dyes chosen for this project.

Additional fluorescent dyes, such as TOPRO (excitation at 642 nm and emission at 661 nm), or DAPI (excitation at 350 nm and emission at 470 nm), are nuclear and chromosomal counterstains that infiltrate compromised membranes (characteristic of dead cells), thus used as an indicator of cell death within a cell population. The fluorescence of TOPRO, a long-wavelength light-absorbing dye, is not normally concealed due to tissue auto-fluorescence, thus it is an ideal dye for tissue counterstaining. In addition, TOPRO stain has very strong binding affinity for dsDNA, with dissociation constants in the μM range, therefore offers a robust and selective nuclear staining in tissue sections or cultured cells (Tavecchio et al. 2008).

The two main methods of immunofluorescence are direct (primary) and indirect (secondary).

2.3.2 Direct immunofluorescence

In direct immunofluorescence (IF) a single antibody is directed against the antigen of interest and it is chemically conjugated to a fluorescent dye (Figure 2.3.3). The experimental procedure of direct IF is simpler and shorter compared with the indirect IF, as only one labelling step is required. However, the signal detected in direct IF may appear weak compared with indirect IF. This is because signal amplification provided using secondary antibodies does not occur in direct IF. Nevertheless, non-specific binding is reduced in direct IF via the use of conjugated primary antibodies. Finally, direct IF allows for the investigation of ligand binding properties, in addition to labelling specific antigens (Coons et al. 1941; Bacallao et al. 2006).

2.3.3 Indirect immunofluorescence

Indirect IF uses two antibodies: a primary antibody that is un-conjugated and specific for the antigen of interest, and a fluorophore-conjugated secondary antibody directed against the primary antibody to detect it (Figure 2.3.3). This method is highly specific, as the positive and negative samples create very different signals, thus are easily differentiated. Each bound antibody demonstrates a typical fluorescence pattern depending on the location of the individual antigens. The technique of IF allows for simultaneous detection of antibodies against numerous biochemically different antigens on one single biological substrate. Therefore, indirect IF is chosen in cases for which the test antigens would be difficult to be individually prepared for enzyme immunoassays. The selection of primary antibodies is a crucial step in IF. The secondary detection of two different targets in the same sample depends on the primary antibody for each target being raised in different host species to avoid cross-reactivity between the secondary antibodies.

To detect different targets in the same sample, the structure of antibodies serves an important role. An antibody consists of two regions: An Fc region (fragment crystallisable region) and a Fab region (fragment antigen-binding region). The Fab region contains variable sections that determine which antigen is bound and the Fc region is constant in a class of the same species. Antibodies may be designed to contain the same Fc region and different Fab regions. Therefore, primary antibodies with the same Fc region may be used to detect various antigens (due to different Fab regions), and still be detected by a single fluorescently-conjugated secondary antibody specific to the Fc region of the primary antibody.

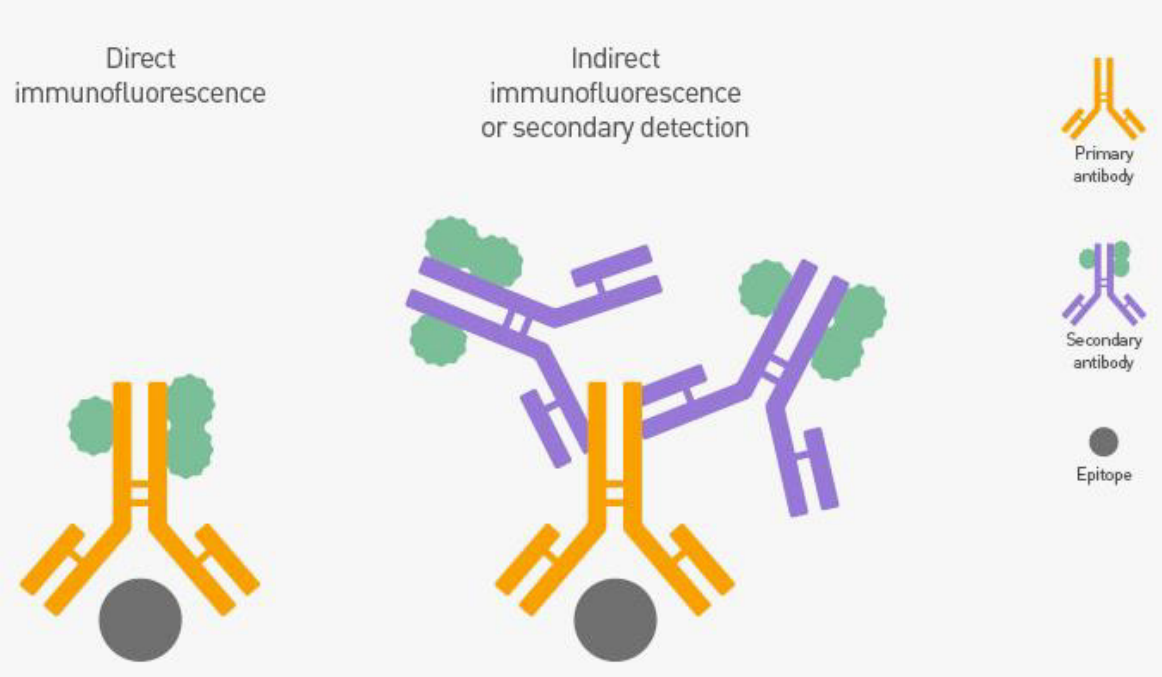

Figure 2.3.3 Two types of immunofluorescence (IF). In direct IF, the antibody (orange) is labelled with fluorophores (green) and binds the epitope (grey). In indirect IF, the primary antibody (orange) binds the epitope (grey) and a fluorophore-labelled secondary antibody (purple) binds specifically to the primary antibody (Thermofisher Scientific, Inc., URL).

2.4 Flow Cytometry

2.4.1 Underlying principles

Flow cytometry is utilized in cell counting, cell sorting, biomarker detection and protein engineering. A flow cytometer defines and counts cells passing in a stream through a laser beam, and it can identify and isolate different populations of cells within a sample. To identify the different cell populations, it is detecting the fluorescence from tagged primary or secondary antibodies (Janeway et al. 2001). The cells are forced though a narrow nozzle with a much larger volume of saline solution, to release cells individually, hence the laser beam will excite them singularly. The laser beam excites the fluorescent tags and then sensitive photomultiplier tubes detect the fluorescence. The size and granularity of each cell and the fluorescence of bound labelled monoclonal antibodies are calculated, providing information on the expression of the cell-surface proteins of each cell.

This method is used to investigate numerous processes, including cell cycle, apoptosis, and detection of biomarkers. The method assesses the physical and chemical characteristics of cells (between 0.2 and 150μm in size), which are fluorescently labeled. As the fluorescing cell passes through the laser beam, it creates a peak or pulse of photon emission over time. These are detected by the photomultiplying tube sensors and converted to a voltage pulse, known as an event. The flow cytometer measures the total pulse height and area. The measured voltage pulse area will correlate directly to the intensity of fluorescence for that event.

A. B.

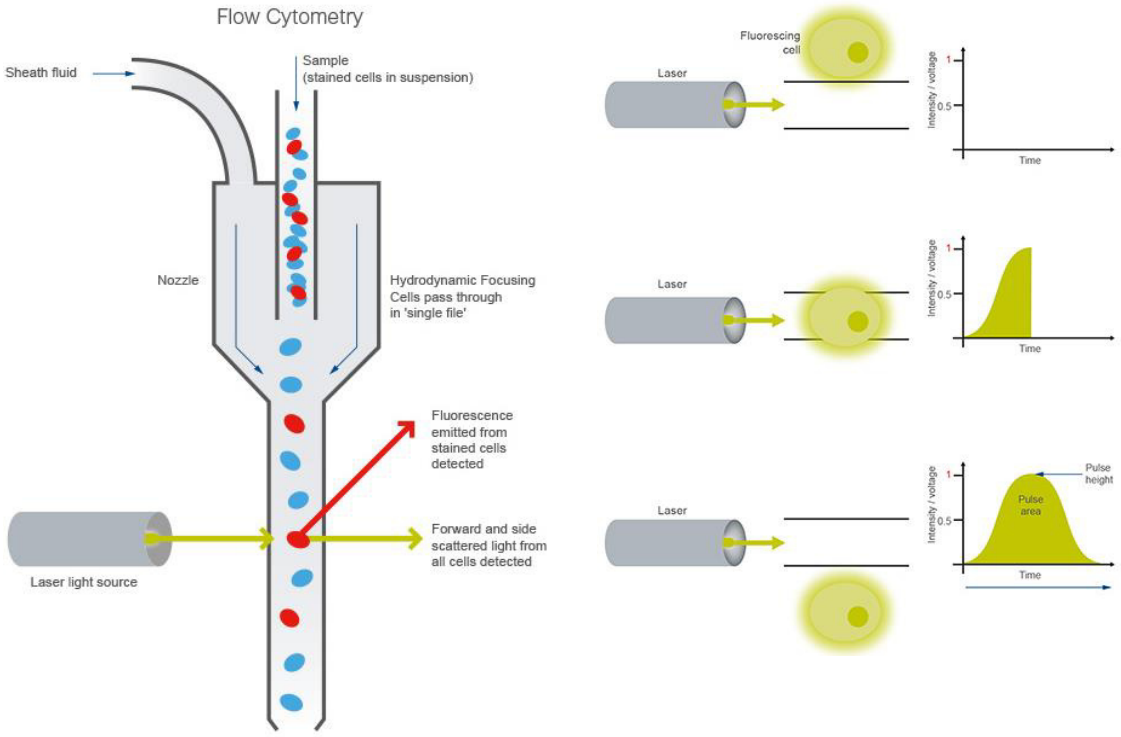

Figure 2.4.1 (A) Overview of a flow cytometer. (B) Photomultiplying tube sensors measurement. Measurement of the pulse area of the voltage created each time by a fluorescing cell releasing photons (Abcam PLC, URL).

The flow cytometer, also known as a Fluorescence Activated Cell Sorter (FACS), can quantify and distinguish between molecules according to their fluorescence and physiological structure. In this study, fluorescently labelled cells/cytokines were analysed using a Becton Dickinson Fluorescent Activated Cell Sorter (FACSCalibur) with the software supplied by Cell Quest.

2.5 Determining PRR expression levels

2.5.1 Trypan blue viability test

As the quality of the cell sample was vital for potential downstream experiments, viability measurements were routinely performed. The Trypan blue exclusion test is used to determine the number of viable cells present in a cell suspension. It is based on the principle that live cells possess intact cell membranes that exclude certain dyes, such as Trypan blue, Eosin, or propidium, whereas dead cells do not. In this test, a cell suspension is simply mixed with 1% trypan blue (ThermoFisher Scientific) and then visually examined under a microscope to determine whether cells take up or exclude the dye. In the protocol used, a viable cell had a clear cytoplasm whereas a nonviable cell had a blue cytoplasm.

2.5.2 Cell stimulation and assays analyses

Indirect immunofluorescence and flow cytometry were utilised to elucidate NLRs, TLRs and complement receptors expression in astrocytes. To perform the indirect immunofluorescence assay, confluent cells ($1x10^6$) were either unstimulated, or stimulated with 10μM of Aβ or scrambled peptide (identical sequence to Aβ, but scrambled) for 2, 4 or 6 hours.

At each time point, the supernatants were collected from the flasks and transferred to appropriately labelled eppendorf tubes. The tubes were stored in the -20°C to be used at a later point for the cytometric bead array assay. The monolayer of cells in the flasks was washed with 2 ml 1X PBS and 1 ml of 1X PBS was added to each flask to scrape of the cells and collect them into eppendorf tubes. The samples were then centrifuged at 13000 rpm for 5 minutes at RT and the PBS was aspirated without disturbing the pellet of cells. Cells were fixed using 300 μl of 4%

paraformaldehyde (PFA; Sigma-Aldrich) for 15 minutes at RT and 500 µL of 1X PBS were added to wash the cells by centrifugation at 13000 rpm for 5 minutes at RT. The PFA and PBS were aspirated and 100 µL of 1X PBS/0.02%BSA/0.02%NaN_3/0.02%Saponin were added to the cells and left to incubate for 5 minutes at RT. BSA is used as a carrier protein to antibodies and as a general protein blocking agent. The amphipathic nature of Saponin makes it act as a surfactant, enhancing the penetration of proteins through the cell membrane. NaN_3 prevents the internalization of surface antigens, which could produce a loss of fluorescent intensity. The appropriate primary antibodies (1 ug) were added and samples were incubated at room temperature for 1 hour. Samples were washed twice with PBS/BSA/NaN_3/Saponin and the appropriate secondary antibody conjugated to FITC was added. The cells were incubated with the secondary antibody (0.5 ug) for 45-60 min in the dark (to avoid photobleaching), washed twice and resuspended in 500µl PBS/BSA/NaN_3/Saponin. The samples were transferred to flow tubes and analyzed using the Becton Dickinson Fluorescent Activated Cell Sorter (FACSCalibur) and the Cell Quest software. 10,000 not gated cells were analyzed for each sample.

2.6 SDS-PAGE

2.6.1 Underlying principles

Polyacrylamide gel electrophoresis (PAGE) is a method used in combination with western blotting to separate proteins according to their size, shape, and net charge. Sodium dodecyl sulphate (SDS)-PAGE separates the proteins according to their size only, as proteins migrate in the gel in response to the electric field applied. This is because SDS, as a denaturing detergent, dissolves the hydrophobic molecules to become linear. SDS makes the protein negatively charged for the proteins to migrate through the gel from negative to positive charge. Therefore, depending on their molecular weight (MW), small proteins will move faster in the gel than the ones with higher MW.

After the separation of the proteins on the gel, the proteins are transferred to nitrocellulose membranes and probed with antibodies along with enzymatic chemiluminescent to detect the proteins. The advantage of using SDS-PAGE instead of ELISA is the denaturing condition where under proteins are quantified, which is of high relevance when analysing highly fibrillogenic proteins, such as Aβ.

2.6.2 Continuous SDS-PAGE

For continuous SDS-PAGE, the same buffer ions are present at constant pH in the gel and electrode reservoirs. Therefore, it is much quicker and easier to prepare, compared with the discontinuous SDS-PAGE. For this method, the DNA (or RNA) samples should be sufficient to run for enough resolutions of electrophoresis, where as to simply observe whether a protein is present in a sample the discontinuous method is preferred.

2.6.3 Discontinuous SDS-PAGE

For discontinuous SDS-PAGE, different buffer ions are present in the gel and electrode reservoirs. By using different buffers in the gel and in the electrode solutions and adding a stacking gel to the resolving gel, samples are compressed into a thin starting band and individual proteins are finely resolved and separated. The gel consists of two parts: the upper stacking gel and the lower resolving gel. The 4% stacking gel has larger pores (low percentage) and a low pH of 6.8, whereas the lower 10% resolving gel has smaller pores with a higher pH of 8.8. The protein samples are loaded at the interface between the two gel layers, in very thin and sharp wells (made with a 1 mm comb). Following the application of an electrical field, the proteins travel through the resolving gel and are separated according to their MW, as described above.

2.6.4 Experimental procedure

The gels were made up in liquid form to be poured into appropriate casts to set. The polymerisation of the gel occurs upon addition of ammonium persulfate (APS) and N,N,N',N'-tetra-methylethylenediamine (TEMED), as the gels form cross-links creating the sieve-like matrix required. For the SDS-PAGE analysis in the present study the Mini-PROTEAN Tetra Handcast System (Bio-Rad Laboratories, Inc.) was utilised (Figure 2.6.4).

To prepare the gel cast to load the proteins, the resolving gel mixture (see Table 2.6.4) was prepared and immediately poured into the cast. Isobutanol (~1 ml) was added to the top to make the gel surface smooth and flat. The gel was left to set at RT for 45 min and then isobutanol was rinsed with dH_2O and removed. The 4% stacking gel mixture (see Table 2.6.4) was poured on top of the resolving gel and a 1mm comb was placed in it to form the wells. The stacking gel was left to set at

RT for 45 min and then the comb was carefully removed. The gels were placed into the electrophoresis tank and 1X running buffer [25Mm Tris, 192mM glycine and 0.1% (w/v) SDS, pH 8.3] was poured on top to cover it.

Figure 2.6.4 The Mini-PROTEAN Tetra handcast system (Bio-Rad Laboratories, URL).

2 gels	10% Resolving gel	4% Stacking gel
Distilled water	4.02 mL	6.1 mL
1.5M Tris-HCl pH 6.8	2.5 mL	2.5 mL
10% (w/v) SDS	100 µL	100 µL
Acrylamide/Bis	3.33 mL	1.3 mL
10% APS	50 µL	50 µL
TEMED	5 µL	10 µL
Total Monomer	10.0 mL	10 mL

Table 2.6.4 Composition of resolving and stacking gels used in SDS-PAGE.

To prepare the samples for running on the gel, X2 SDS-PAGE Reducing Sample Buffer (100ml; 10.0g Glycerol, 20ml 0.5M Tris pH 6.8, 16ml 10% SDS, 4ml 14.3M β-mercaptoethanol, few mg of Bromophenol Blue) was added to cells that had been either unstimulated, or stimulated with Aβ or scrambled for 2, 4, or 6 hours. The SDS in the reducing sample buffer is an anionic surfactant which both denatures secondary and non-disulphide-linked tertiary structures in proteins, and places a negative charge onto each protein in proportion to its mass. This ensures

that each protein is linear and separated only by its molecular weight. The β-mercaptoethanol present further denatures proteins by cleaving their disulphide bonds, thereby disrupting the tertiary and quaternary structure of them. The glycerol helps preserve the proteins at low temperatures, and weighs down the samples when loading them into the wells, and the Tris present acts as a buffer. The addition of Bromophenol Blue allows the samples to be visualised as they pass through the gel. Each sample (100µl) was added to separate eppendorfs and boiled for 10 minutes, along with biotinylated SDS-PAGE standards (2µl + 40µl X2 SDS-PAGE Reducing sample buffer), to help denature the proteins further. The standards are a mixture of biotinylated proteins with consistent molecular weights, allowing for accurate molecular weight determination of immune detected proteins. 40µl of the samples (10µl of standards) were then loaded into the wells, and the apparatus was run at a constant voltage of 200V for approximately 45 minutes.

2.7 Western blot analysis

2.7.1 Underlying principles

Western blotting refers to the transfer of biological samples from a gel to a membrane and their subsequent detection on the surface of the membrane. Towbin et al. (1979) was the first to demonstrate the method that it is now widely used to analyze proteins. The specificity of the antibody-antigen interaction allows for a target protein to be identified within a complex protein sample.

The SDS-PAGE is used to electrophoretically separate the proteins, which are then transferred to a nitrocellulose membrane via electroblotting. The proteins are transferred into a portion of nitrocellulose membrane in the same formation as they were in the gel. Electroblotting was used to pull proteins from the poly-acrylamide gel onto the nitrocellulose membrane using the Mini Trans-Blot Cell system (Bio-Rad Laboratories, Inc.; Figure 2.7.1).

Figure 2.7.1 The Mini Trans-Blot cell system. The Mini Trans-Blot cell transfers up to two gels in an hour and the self-contained Bio-Ice cooling unit absorbs the heat generated during transfer (Bio-Rad Laboratories, URL).

Following SDS-PAGE, the resolving gel was placed in a transfer cassette on top of a porous pad, two pieces of blotting paper and a nitrocellulose membrane, all soaked in transfer buffer (20mM Tris acetate, 0.1% SDS, 20% isopropanol, pH 8.3). Two pieces of blotting paper and a porous pad were then placed on top of the gel, and the cassette was closed and placed in the transfer tank. A blue cooling unit was placed inside the tank along with the cassette and then it was filled to the top with transfer buffer. Transferring was performed at a constant current of 220mA for 60 minutes.

2.7.2 Probing the membranes with antibodies

Subsequent to protein transfer, the membrane was placed in blocking buffer (20g milk powder dissolved in 100ml 1X PBS) on rotary for 1 h. The nitrocellulose membrane has a high binding affinity and allows all proteins to bind strongly, in addition to antibodies. The blocking mixture has high quantities of proteins to prevent the unspecific binding of antibodies, thus blocking the membrane ensures that these proteins will bind to the rest of the membrane to block the antibodies from binding (Abcam, URL).

Subsequent to blocking, the membrane was washed for 2x15 min in 1X PBS with 0.1% Tween 20 on a rotary and then incubated with the appropriate primary antibody diluted in 1X PBS-Tween for the specific protein of interest. Primary antibody incubation was performed on rotary at RT for 1 h and then the membrane was left in the fridge overnight. The following day, the membrane was washed for 2x15 min in 1X PBS-Tween on a rotary. The membrane was then incubated with a secondary antibody specific to the primary antibody and conjugated to horseradish peroxidise (HRP). Secondary antibody incubation was performed on

rotary at RT for 45 min and the membrane was then washed for 2 h in 1X PBS-Tween whilst changing the washing buffer every 15 min.

Subsequent to the washes, the membrane was placed in a developing cassette, treated with an enhanced-chemiluminescence (ECL) Prime reagent (Amersham, UK) in a dark room and high performance chemiluminescence film (Hyperfilm; GE Healthcare) was placed onto the membrane. The ECL reagent enables the HRP to emit light only where the protein bands are present. The film was finally exposed for a short time, depending on the intensity of the luminescence.

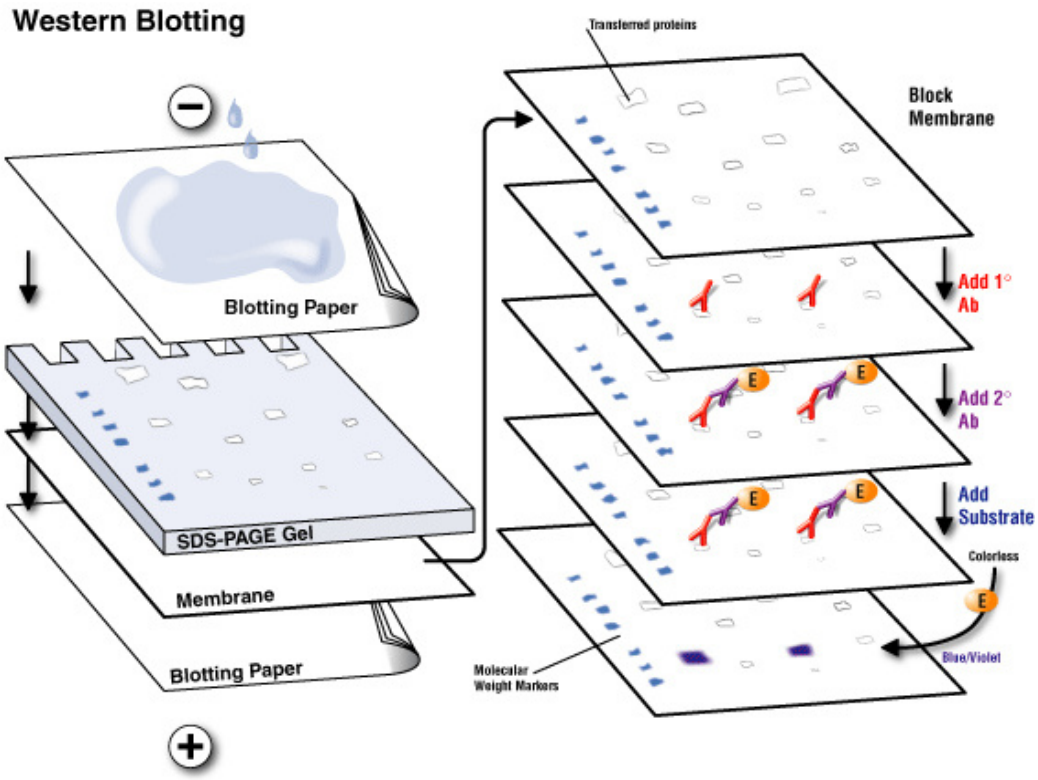

Figure 2.7.2 Principles of western blotting. The proteins are electrophoretically transferred from the sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel onto the nitrocellulose membrane. The membrane is then blocked, and primary and secondary antibody incubations take place. Subsequent to the incubations, a substrate is added for the proteins to be visualised utilizing enhanced chemiluminescence (Komabiotech, URL).

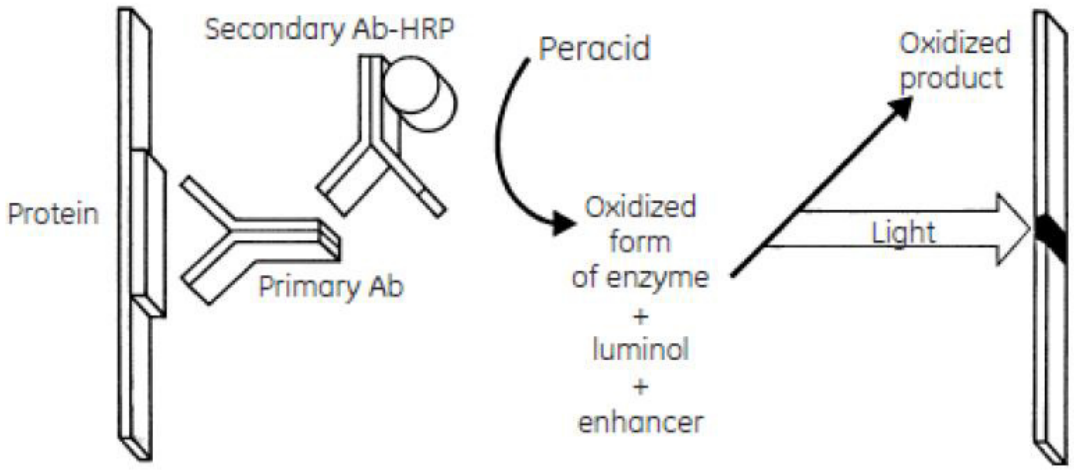

Figure 2.7.3 Principle of electro-chemiluminescence in western blotting. The nitrocellulose membranes are treated with an electro-chemiluminescence (ECL) reagent, causing the HRP to emit light only where the protein bands are present. HRP catalyses the oxidation of luminol to its excited state and decays back to the ground state via a light emitting pathway (GE Healthcare Life Sciences, URL).

2.7.3 Stripping and re-probing membranes

Following the initial use of nitrocellulose membranes with bound proteins and antibodies, the membrane may be stripped and re-probed with different antibodies. To strip the membrane of previous bound antibodies and keep only the proteins bound, the membrane is incubated in stripping buffer at 50°C, on a shaker incubator for 5-7 min. Following incubation, the membrane was washed with 1X PBS-Tween for 20 min on a shaker and then blocked for 1 h. Subsequent to blocking the membrane may be re-probed with the same or different antibodies, as described above (see 2.7.2 Probing membranes with antibodies section).

2.8 Plasmid DNA

2.8.1 Preparation of plasmid DNA

2.8.1.1 Transformation

All procedures were carried out using sterile tips near a Bunsen burner to ensure sterility. Agar plates were previously prepared with 25 ug/ml Zeocin. psiRNA-plasmids (Invivogen) were transformed into a competent E. Coli strain (E. Coli GT116). 1 ug of the plasmid was added to 100 µl of E. Coli competent cells and incubated on ice for 30 min. This was followed by heat shocking the tubes at 42ºC for 45 sec, then placing them back on ice for 2 min. Luria Broth (500 µl; Bactopeptone, Yeast extract, NaCl, dH_20) was added to the transformed cells and the tubes were incubated at 225 rpm at 37ºC for 1 h. The transformed cells (100 µl) were then placed on Zeocin (25µg/ml) incorporated agar plates. Plates were left to grow overnight in an incubator shaker at 37ºC at 125 rpm. The plasmids contained a Zeocin-resistance gene, so that only the E. Coli that have taken up the plasmid would survive on the Zeocin agar plates. Following the growth of the E. Coli colonies, separate colonies were selected with sterile toothpicks and placed in different 25 ml vials of Zeocin (25µg/ml) incorporated luria broth, and incubated overnight at 225 rpm at 37ºC.

2.8.1.2 DNA Isolation

The luria broth mixture containing the E. Coli culture was centrifuged at 4000rpm in 50ml Falcon tubes for 30 min at RT and the supernatant was then swiftly discarded without agitating the pellet of cells. STET buffer (400 µl) was added to the pellet and was vortexed to lyse the E. Coli cell walls. The mixture was then transferred to sterile eppendorf tubes and 10 µl lysozyme (50mg/ml) (Sigma

Aldrich, UK) was added to each eppendorf. The eppendorfs were placed in a 100°C waterbath for 1 min and subsequent on ice for 5 min. The samples were then centrifuged for 30 min at 13000 rpm at RT and the pellet was removed using a sterile toothpick. RNAse A (5 µl; 20µg/ml; degrades RNA) (Sigma Aldrich, UK) was added to each sample and left to incubate at 42°C for 30 min. Following incubation, 400 µl phenol/chloroform/isoamyl alcohol (P3803; Sigma-Aldrich UK) were added to the samples, vortexed carefully and centrifuged for 15 min at 13000 rpm at RT. The supernatant was carefully collected and transferred to a new set of sterile eppendorfs, while the rest was discarded. Chloroform/isoamyl alcohol (400 µl) (25666; Sigma-Aldrich UK) was added to the samples, vortexed and centrifuged as before for 30 min at 13000 rpm at RT. The supernatants were again carefully collected and transferred to another set of sterile eppendorfs. A mixture consisting of: 20 µl (1/20th of total volume) sodium acetate 2M pH 6.5 and 1 ml (2.5 times the total volume) 100% ethanol, was added to each sample, vortexed and placed in the -80°C freezer for at least 1 h.

After 1 h, the samples were immediately centrifuged from frozen at 13000 rpm for 20 min at RT. The supernatants were carefully discarded, keeping the tiny pellets of purified DNA in the eppendorfs. Samples were centrifuged at 13000 rpm for 1 min at RT to remove any excess supernatant and sterile water (60 µl) was then added to each sample. DNA concentration was measured using the NanoDrop 2000 UV-Vis Spectrophotometer. Samples were stored in -80°C for future experiments.

2.8.1.3 Agarose gel electrophoresis for purity control

Agarose gel electrophoresis was used to separate a mixed population of DNA through a matrix made of agarose. The DNA samples were separated according

to the size of the sample fragments; smaller fragments translocated further than the longer fragments, through the agarose gel. An electrical gradient was applied on both ends of the gel, for the negatively charged molecules to translocate and separate the fragments. To verify the purity of the prepared plasmid (see 2.8.1.2 section), the samples were run on a 1% w/v agarose gel with 1% GelRed DNA stain. The samples were mixed with the ELFO loading buffer (10 µl sample + 5 µl buffer) and run at 100V constant voltage for 45 min. Once run, the bands were observed using the Stratagene Eagle Eye II still video system (Stratagene; Agilent Technologies Inc.) to detect whether the DNA was purified, thus could be used in future experiments.

2.8.2 Silencing (RNA interference)

RNA interference (RNAi)i involves the control of genes at the level of RNA. In this study astrocytes were transformed with NLRP1, NLRP3, TLR2, TLR4 and NLRC5 psiRNAs to knock down their expression.

The psiRNA is transcribed producing shRNA this is processed by the Dicer class of RNase III enzymes into siRNA, removing the hairpin and leaving a two nucleotide long 3' overhang. The siRNA is then bound by siRNA-induced silencing complex (siRISC) which has an RNase component (argonaute) which is activated and sets about degrading one of the siRNA strands. The strand left intact, with RISC attached, is complimentary to and binds endogenous mRNA. This complex not only prevents transcription of the mRNA, but also when bound the RNAse component of RISC leads to cleavage and degradation of the mRNA (Figure 2.8.2).

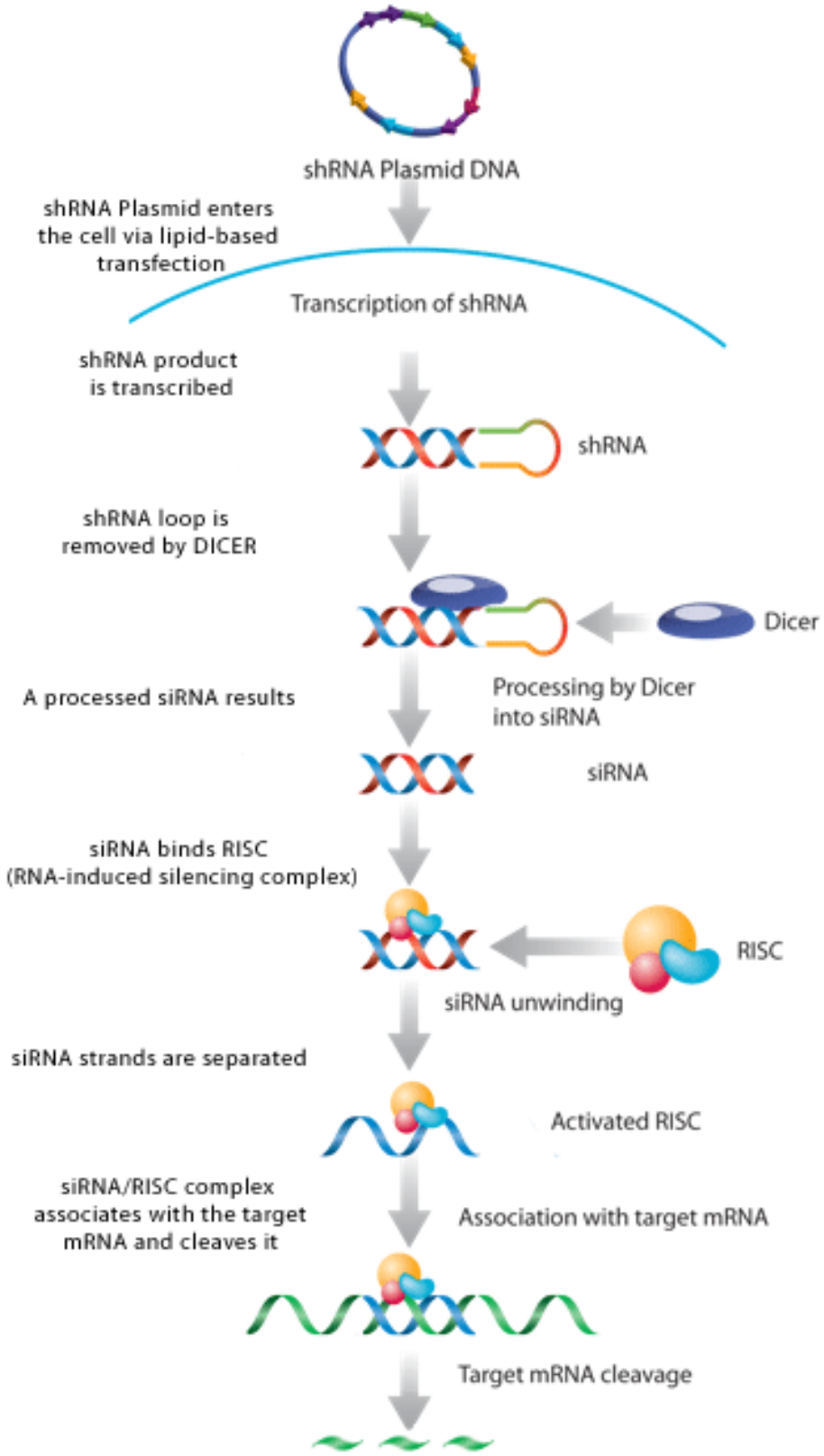

Figure 2.8.2.1 Gene silencing by shRNA. Transfection of a psiRNA plasmid into a mammalian cell leads to the cleavage and degradation of the target mRNA. This results in the knock down of gene expression (Santa Cruz Biotechnology, Inc., URL).

The TLR2 and TLR4 shRNAs were produced by the psiRNA-h7SKGFP::Zeo plasmid from the human 7SK RNA pol III promoter (Figure 2.8.2.2; InvivoGen). The plasmids feature a GFP::Zeo fusion gene that allows simple monitoring of transfection efficiency and selection in both E. Coli and mammalian cells.

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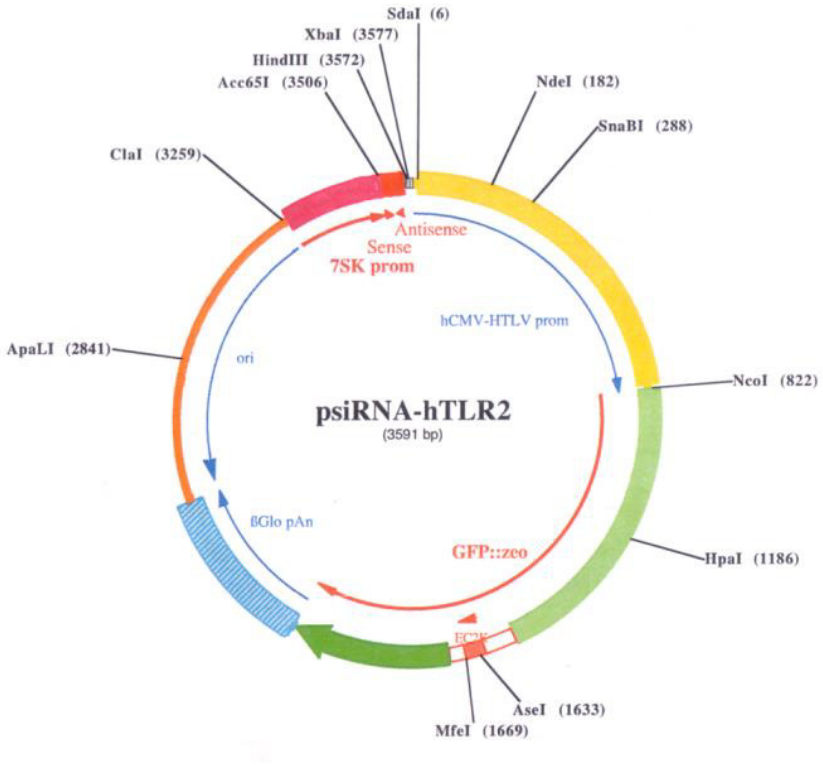


Figure 2.8.1.2 TLR2 psiRNA plasmid. This is a schematic representation of the psiRNA-h7SKGFP::Zeo plasmids from the human 7SK RNA pol III promoter. The siRNA region (red) encodes TLR2 interfering RNA (Invivogen psiRNA 2009 manual).

Different psiRNA clones were generated using the psh7SK vector from Invitrogen the most efficient was against the sequence: for NLRP3 GGAAGTGGACTGCGAGAAGTT, for NLRC5, GAACCTGTGGAGCTGTCTTGT and GCAACAGCATCTGCGTGTCAA, for NLRC4, GGATGCTGCTAGAGGGATCAT and GACAACTGGGCTCCTCTGTAA for NLRP1, GAAGGAGGAGCTGAAGGAGTT and GGCCTGATTATGTGGAGGAGA. For TLR4 GCCAGGAGAACTACGTGTGAA and for TLR2 GTCAATTCAGAACGTAAGTCA.

2.8.3 Transfection

During the experimental process of transfection, foreign DNA enters the cells and the protein expression is examined and investigated. In the present study, the method of transient transfection was chosen. In transient transfection, the cells temporarily express the gene in the introduced plasmid, as it is not integrated into the host chromosomes and would be lost upon a certain number of cycles of cell division. This type of transfection has the advantage of being a quick and easy technique to express a gene of interest into a cell population. In the other type of transfection, the stable one, the plasmid is integrated into the hosts' DNA and becomes a stable part of the transfected cells' genetic material, however, the procedure takes between 6 and 8 weeks. For both types of transfection, the transfected cells are detected and selected using a selection growth medium with antibiotics. In this study lipofectamine was used as a transfection agent. Liposomes are synthetic analogues of the phospholipid bilayer, the building block of the cellular membrane. These transfection compounds share several characteristics with their natural counterparts, including the presence of hydrophobic and hydrophilic regions of each molecule which allow for the formation of spheroid liposomes under aqueous conditions. In the presence of free DNA or RNA, liposomes encapsulate the nucleic acids to create an efficient delivery system. The charge, composition and structure of the liposome defines the affinity of the complex for the cellular membrane. Under specific conditions, the liposome-nucleic acid complex can interact with the cell membrane to gain access into the cell by endocytosis and subsequently release the nucleic acids into the cytoplasm.

Briefly, in the experimental process, plates, or flasks of astrocytes ($1x10^6$ cells) were prepared for transfection using 4ug of plasmid shRNA and Lipofectamine 2000 (following the manufacturer's instructions).

4ug of plasmid siRNA were re-suspended in 30µl GIBCO Opti-MEM (31985-062, Life Technologies Ltd. UK), complexed with 10µl Lipofectamine 2000 (11668027, Life Technologies Ltd. UK) + 40µl Opti-MEM. $1x10^6$ cells were washed with 6ml GIBCO Opti-MEM, the supernatant was aspirated and then 800µl of Opti MEM was added per plate. After the addition of Lipofectamine 2000-complexed DNA, cells were incubated overnight. The following day the medium was removed and cells were, re-suspended in GIBCO RPMI 1640 GlutaMAX™-I + 100ug/ml Zeocin to apply antibiotic selection pressure. Control plates with untransfected cells, cells with just the psh7SK vector and psh7SK with scrambled sequence were also used. Flow cytometry determined efficiency of transfection, since the plasmids expressed GFP, as well as western blotting for the receptor of interest. The procedure was repeated until 85% knockdown efficiency was achieved and then the cells were used for stimulations.

2.9 Cytometric Bead Array

2.9.1 Underlying principles

The cytometric bead array (CBA) system allows for the simultaneous measurement of multiple analytes in sample volumes too small for traditional immunoassays. In addition, the assay enables the evaluation of multiple analytes in a single sample and the results are reproducible and comparative with previous experiments. In the present study, the Human Inflammation BD Cytometric Bead Array (BD Biosciences) kit was used to measure the concentrations of the released cytokines. The BD CBA kit was utilized to quantitatively measure interleukin-8 (IL-8), IL-1β, IL-6, IL-10, tumour necrosis factor (TNF)-α and IL-12p70 protein levels in a single sample. The release of cytokines was measured to quantify the inflammatory response of the cells in response to various stimuli, as cytokines are the messengers that co-ordinate inflammation and give a direct representation of cellular response (Morgan et al. 2004).

To detect the analytes under the flow cytometer, beads of known size and fluorescence from the CBA kit were utilized to capture the singular soluble or the sets of analytes. The capture beads are each unique and conjugated with a specific antibody and a combination of phycoerythrin (PE)–conjugated antibodies (BD CBA kit) was used as the detection mixture. This reagent of PE-conjugated antibodies gives a fluorescent signal in proportion to the quantity of the bound analyte(s). Following the incubation of the capture beads and PE-reagent with an unknown sample, the beads, the analytes in the sample and the PE-conjugated antibodies form a complex. These complexes can be measured using flow cytometry to identify particles with fluorescence characteristics of both the bead and the detector.

The FCAP Array analysis software gates on each individual bead population and determines the median fluorescence intensity (MFI) for each analyte in the array. It generates a standard curve and performs interpolation of sample concentrations compared to the standard curve and generates an analysis report in tabular format.

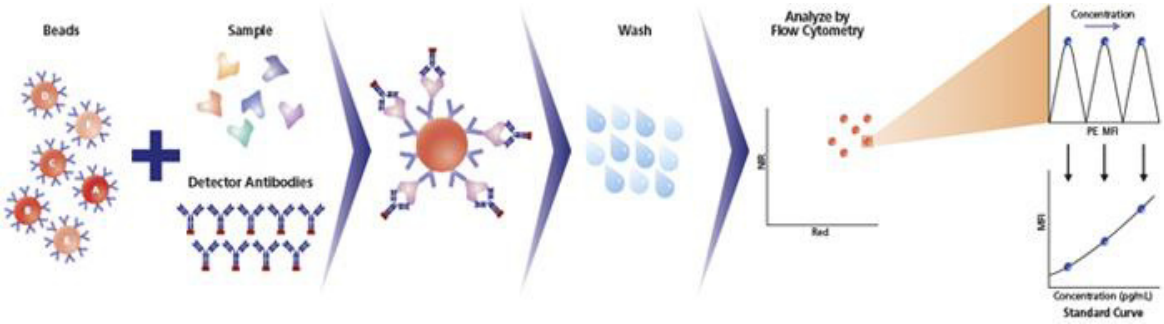


Figure 2.9 Cytometric bead array (CBA) system. CBA beads bind to cytokines in the sample. Each capture bead in the array has unique fluorescence intensity and is coated with a capture antibody specific for a single analyte. A combination of different beads is mixed with a sample or standard and a mixture of detection antibodies that are conjugated to a reporter molecule (PE). Following incubation and subsequent washing, the samples are acquired on a flow cytometer and analyzed (BDbiosciences, URL).

2.9.2 Experimental procedure

The BD cytometric bead array Human Inflammatory Cytokine kit (551811 BD, Biosciences, USA) was used according to the manufacturer's instructions. Briefly, for the experimental procedure, the mixed capture beads (50 µl) were added to each of the samples (50 µl) in flow tubes and incubated for 1 h in RT. Then 50 µl of the phycoerythrin detection reagent was added to the samples and mixed, followed by further 3 h incubation in the dark at RT. Following incubation, washing buffer (1 ml) was added to the samples and were then centrifuged at 200xg for 5 min. The supernatant was carefully discarded, and 300 µl wash buffer was added and the sample was resuspended. The samples were analysed using flow cytometry, with the data acquired being analysed using the FCAP Array software (Becton Dickinson).

2.10 HEK-IFN-α/β and HEK IL-1β reporter cells

HEK IFNα/β ((Invivogen) were generated by stable transfection of HEK293 cells (human embryonic kidney 293) with the human STAT2 and IRF9 genes to obtain a fully active type I IFN signalling pathway. The other genes of the pathway (IFNAR1, IFNAR2, JAK1, TyK2 and STAT1) are naturally expressed in sufficient amounts. The cells were further transfected with a SEAP reporter gene under the control of the IFN-α/β inducible ISG54 promoter. Stimulation of HEK-Blue™ IFN-α/β cells with human IFN-α or IFN-β activates the JAK/STAT/ISGF3 pathway and subsequently induces the production of serum embryonic alkaline phosphatase (SEAP).

HEK-Blue IL1β cells (Invivogen) enable the detection of IL1β by monitoring the activation of the ISGF3 pathway. HEK 293 cells are modified immortal cell lines engineered to express (SEAP) in response to the activation of downstream signalling cascades induced by IL1-β binding to its cognate receptor IL1R as is shown in figure 2.9.1 below depicting IL1-β as a worked example.

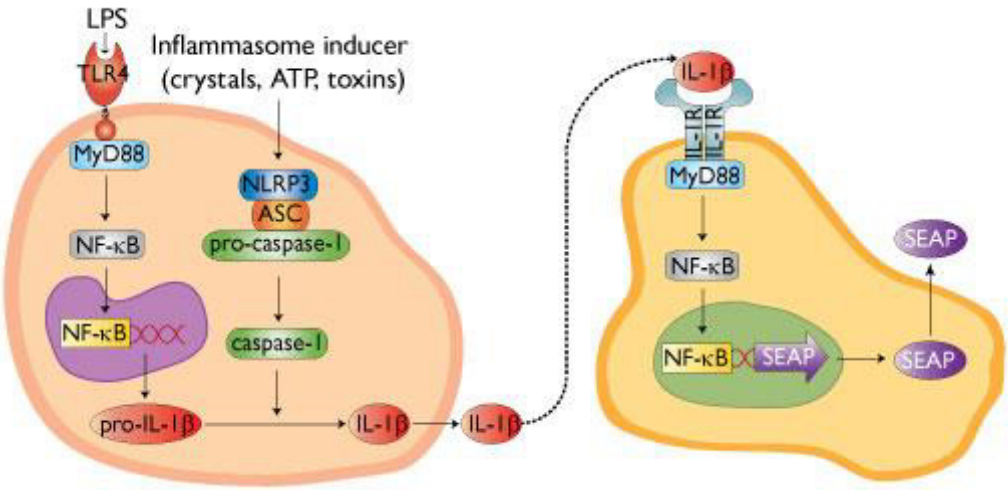


Figure 2.10 Mechanism of cytokine detection with reporter cells.

Initial incubation of an appropriate population of reporter cells in the presence of supernatant from samples of interest will result in the generation of SEAP proportional to the concentration of cytokine in the assay. If the particular cytokine is absent in the supernatant of interest, no SEAP will be produced by the reporter cells. Incubation of the SEAP-containing supernatant with alkaline detection reagent results in a light absorbance shift in the supernatant which may be quantified by photospectrometry analysis(; Yang et al. 1997).

Reporter cells and detection reagent were obtained from Invivogen USA. Reporter cells specific for IL1-β (HEK) or IFNα/β were incubated for 24 hr with triplicate supernatants reflecting each experiment and associated time-points. The following day supernatant was transferred to Eppendorfs containing 150µl QuantiBlue alkaline phosphatase detection reagent (catalogue number: rep-qb1), incubated for 60 minutes prior to analysis using a Shimadzu Biospec-1601 DNA/Protein/Enzyme Analyzer (Tokyo, JP) photospectrometer to determine cytokine expression at different time points compared to un-stimulated supernatants.

2.11 Histology

2.11.1 Underlying principles

This is a simple method for the staining of tissues/cells to view their structure. The most commonly used stains are haematoxylin and eosin (H&E). Haematoxylin stains acidic structures, such as DNA, and eosin stains basic structures, such as proteins in the cytoplasm. When used together, the haematoxylin indicates nuclei in blue and eosin indicates the cytoplasm in pink. It is commonly used to investigate the overall structure of tissue, such as the thickness of vessel walls, or the presence of tumour tissue. It may also be used to count cells. Prior to H&E staining, tissue must have been fixed, embedded and sectioned.

In the present study, immunohistochemistry (IHC) was utilized to stain the tissue sections obtained from the Oxford and UCL Brain Banks. The method of IHC is utilized to detect certain antigens in a tissue section and it is widely used by laboratories for diagnostic reasons or to identify the expression patterns of various proteins. IHC is based on the property of antibodies to bind specific antigens. For example, antibodies recognizing specific antigens of interest, such as primary antibodies, are detected using secondary antibodies conjugated to an enzyme or a fluorochrome (immunohistofluorescence). The secondary antibody is directed against the Fc portion of the primary antibody and is, therefore, specific to the species in which the primary antibody was raised in (Duraiyan et al. 2012).

In the present study, the ImmPRESS Polymerized Reporter Enzyme Staining System (Vector Laboratories Ltd, Cambridgeshire, UK) was utilized to detect the proteins of interest. This is an enzymatic, non-biotin, one-step detection kit that provides high sensitivity with low background staining in immunohistochemical and immunocytochemical applications. The ImmPRESS Reagent utilizes a novel

approach to conjugate horseradish peroxidase micropolymers to affinity-purified secondary antibodies. This conjugation technology avoids the use of large dextrans or other macromolecules as a backbone and enables a higher density of enzymes per antibody to bind to the target with minimal steric interference. Successfully labelling multiple antigens in the same tissue section entails the use of detection systems with high sensitivity and low background, and substrate choices that produce an ideal colour contrast (ImmPRESS REAGENT manual; Vector Laboratories Ltd.). The peroxidase and alkaline phosphatase detection systems offered by Vector Laboratories are widely acknowledged for having the above qualities, thus they were chosen the applications of this project.

2.11.2 Immunohistochemical procedure – paraffin fixed tissue

Sections were heated in an oven maintained at 60^{o}C for 30 min and then emerged in xylene for 2 x 20 min, followed by submersion in graded alcohol series (100% ethanol: 5 min, 95% ethanol: 5 min, 80% ethanol: 5min, 70 % ethanol: 5 min). Slides were washed for 5 minutes in distilled water and then immersed in 1X antigen retrieval solution (DAKO UK Ltd.) for 30 min in a 100^{o}C waterbath. Most antigens require an antigen retrieval step before antibody binding can occur on fixed tissue. This is due to cross-linking of proteins during fixation which masks epitopes. The two methods of antigen retrieval are heat-induced and enzymatic. Both methods act by breaking the methylene bridges and exposing the epitopes to allow binding of the primary antibody. Heat-induced epitope retrieval is most often performed using a pressure cooker or a microwave at maximum power (850W). The antigen retrieval was collected and the slides were placed for 5 minutes in a microwave. Then they were left for 30 minutes at RT. Slides were rinsed twice with 1X PBS and appropriate primary antibodies (1ug/ml; diluted in

1X PBS/BSA/NaN_3/Saponin) were added. Slides were left to incubate in RT for 1-2 hours, depending on the primary antibody used, and then washed with 1X PBS/BSA/NaN_3/Saponin. Following washing, the appropriate IMM-PRESS secondary antibody (Vector VIP kit) was added and slides were incubated at RT for 30 minutes. Slides were washed with1X PBS for 5min at RT and then incubated with the appropriate Chromogen buffer (peroxidase substrate solution from the kit) for 1-2 minutes. Depending on the enzyme and substrate used, the specific antigen on the tissue was coloured either red, blue, or brown.

Enzyme	Substrate	Colour
Horseradish Peroxidase (HRP)	3,3'-Diaminobenzidine (DAB)	Brown
	3-Amino-9-ethylcarbazole (AEC)	Red
Alkaline phosphatase (AP)	5-Bromo-4-chloro-3-indolyl phosphate:Nitroblue tetrazolium (BCIP/NBT)	Blue
	Vector Blue	Blue
Glucose oxidase	Nitroblue tetrazolium (NBT)	Blue

Table 2.11.2 Enzyme and substrate used to produce certain colours.

Following incubation, slides were washed with distilled water, counterstained for the nucleus with either haematoxylin (blue), nuclear fast red, or methyl green and mounted using Vectashield (Vectorlabs, UK) (schematic representation in Figure 2.11.2).

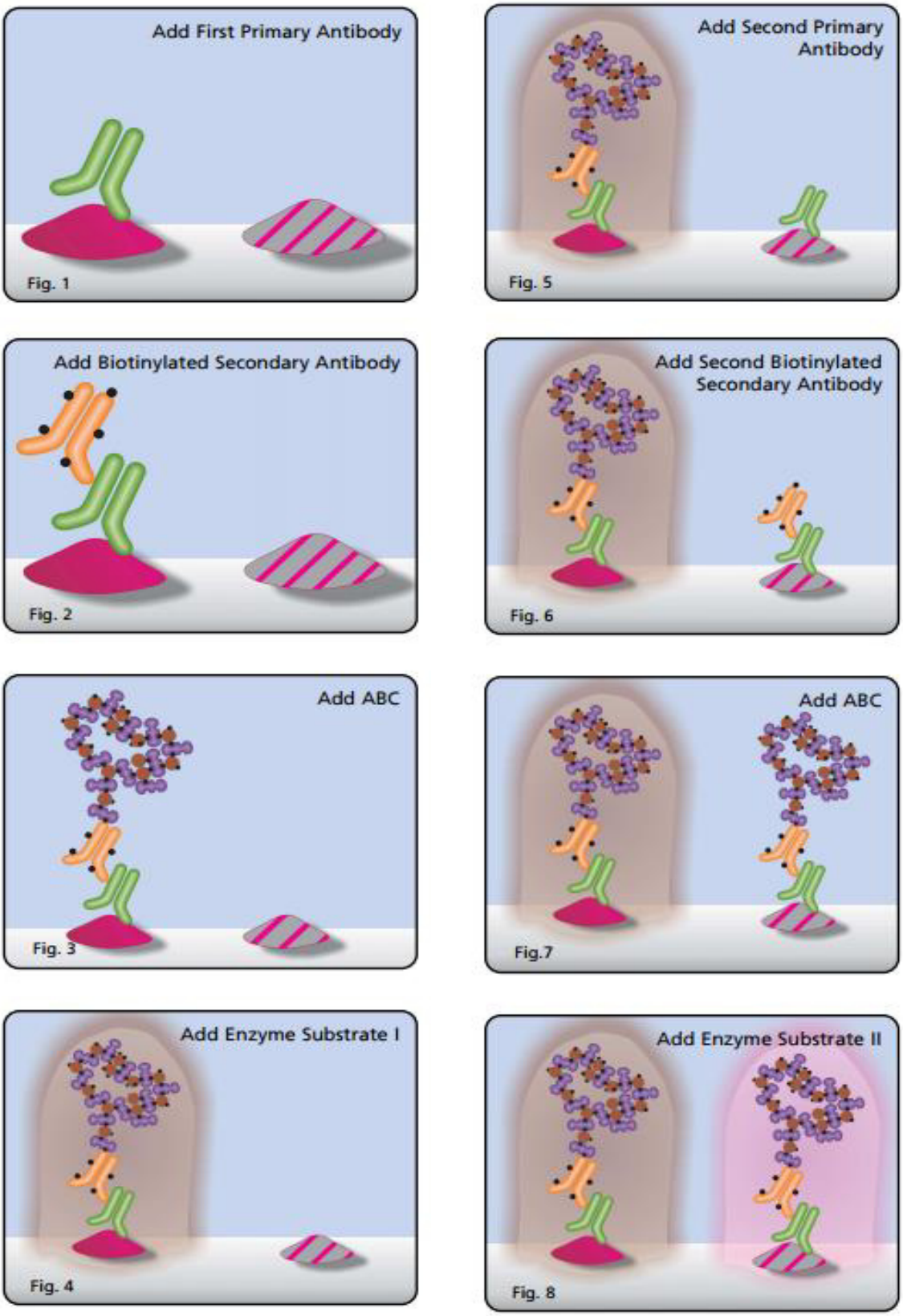


Figure 2.11.2 Multiple antigen labelling using the VECTASTAIN Systems. (Vectorlabs, URL)

2.12 Confocal Microscopy

2.12.1 Underlying principles

Confocal microscopy is an optical imaging technique utilized to increase the optical resolution and contrast of a micrograph by adding a spatial pinhole positioned at the confocal plane of the lens to eliminate out-of-focus light. Confocal microscopy generates clear images of large specimens or complete cells and was developed to enhance immunofluorescence microscopy. This method allows for optical sectioning (sets of images at different depths within a specimen) and the reconstruction of 3D structures from the obtained images (Hotton et al. 1997; Pawley 2006).

Following the initial concept of the confocal microscope by Marvin Minsky in 1955 (Minsky 1957; Minsky 1988), confocal microscopy has evolved drastically due to technological advances. At present, there are various types of confocal scanning techniques, such as single beam scanning [confocal stage scanning microscopy (CSSM) and confocal laser scanning microscopy (CLSM)] and multiple-beam scanning. CLSM utilizes a pulsed laser beam of an appropriate wavelength that is powerful enough to excite the fluorochrome of interest when it is focused at the focal plane of the layer in the specimen. The light emitted by other layers is out-of-focus and is ignored by the detector due to the confocal pinhole. Molecules and/or structures in a cell may be labelled with varied fluorochrome conjugated antibodies that emit light of different wavelengths. Simultaneous observation of various fluorochromes on different parts of the cells is allowed due to the variety of wavelengths of the light utilized (Masters 2006).

Present day confocal microscopes rely on a dedicated computer for control and image storage. The images obtained from the microscope are analysed by a

computer that assigns different colours to the various wavelengths, thus allowing for image overlap and detection of co-localization of molecules and/or structures in a cell. Therefore, confocal microscopy may be used to investigate and assess the effect of various stimuli on different components of a cell. In addition, multiple labelling of cells with various fluorochrome-conjugated antibodies for antigens of interest provides a great insight into the biological processes of cells.

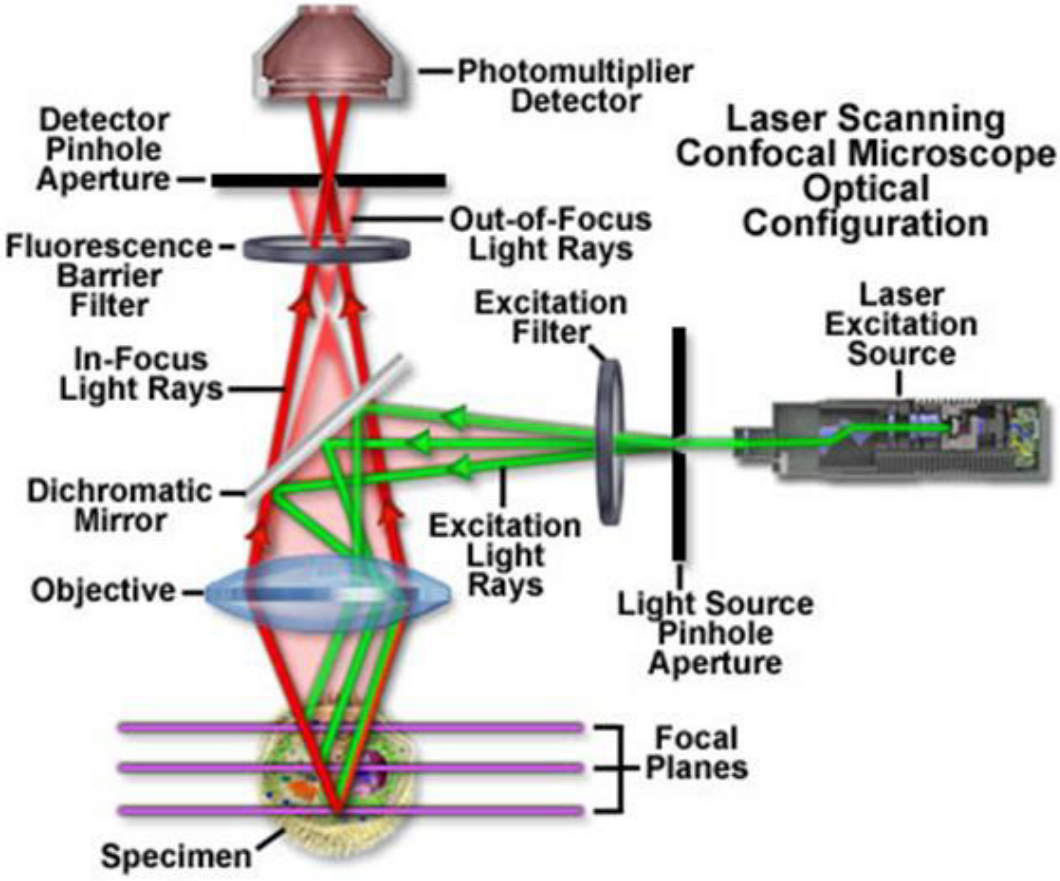


Figure 2.12.1 Principles of a confocal microscope. Coherent light emitted by the laser system (excitation source) passes through a pinhole aperture positioned in a conjugate plane (confocal) with a scanning point on the specimen and a second pinhole aperture located in front of the detector (a photomultiplier tube). As the laser is reflected by a dichromatic mirror and scanned across the specimen in a specific focal plane, secondary fluorescence emitted from points on the specimen (in the same focal plane) pass back through the dichromatic mirror and are focused as a confocal point at the detector pinhole aperture (Nikon MicroscopyU, URL).

The distribution of signals in fluorescence microscopy images may be used to determine whether two probes co-distribute with one another or co-localize. A confocal microscope is widely used to assess co-localization, as it excites certain fluorophores and gate a specific wavelength of emitted light to produce an image of colour-labelled antigens, such as green, red, or blue. The location of specific antigens on a cell is revealed due to the fluorochrome-conjugated antibodies, thus merging the images of numerous fluorochrome-labelled antigens in the same cell location could suggest a possible co-localization upon colour change. Fluorescence co-localization analysis is more appropriately used to determine whether two molecules associate with the same structures. For example, to determine whether a particular protein associates with endosomes (Babey et al. 2006), mitochondria (Lynch et al. 1996) or microtubules (Nicolas et al. 2008) or whether two proteins associate with the same sub-nuclear structures (van Steensel et al. 1996). Evaluating co-localization at this scale is still susceptible to the limits of resolution. For example, an overlap in fluorescence does not necessarily indicate co-localization of two probes in the same cellular structure. However, the observation of repeated coincidence of two probes in multiple structures throughout a cell increases the confidence that the two occupy the same structures.

Quantitative assessment of the images is required to verify co-localization. A number of factors, such as pixels of overlaid images, may indicate to be in close proximity and appear to the human eye as overlapping when they are not. To eliminate user bias and provide a quantitative value of the extent of co-localization, the images obtained were automatically thresholded and the interdependency of the red and green channels was measured. A correlation coefficient was used to

calculate the interdependence of the two variables, which equates to the extent of co-localization that may be occurring.

2.12.2 Dual labelling via indirect immunofluorescence

To prepare the slides, 10,000 astrocytes cells were seeded on coverslips or labtek slides (10,000 cells per well). The slides were then incubated until they reached desired confluency. And then stimulated as required at certain time points. The cells were fixed to prevent potential re-organisation of the `proteins during the experiment. Cells were permeabilised using PBS/0.02% BSA/0.02% Saponin and labelled with antibodies for NLRP3, C9, Amyloid, or TLR4 (2μg) (listed in Table 2.1.2a). Followed by an appropriate species specific secondary antibody (2μg) conjugated to Alexa 488, Alexa546 or Alexa 647 (listed in Table 2.1.2b). We have validated our confocal experiments with isotype control antibodies as well as with only secondary antibody.

In the Instances of dual labelling, secondary antibodies conjugated to Alexa488 and Alexa546 were used and Topro-3 (1/10,000 dilution) was the selected nuclear stain since it has far-red fluorescence (Excitation/Emission: 642/661nm) similar to Alexa 647.

To reduce photobleaching slides were mounted using Slowfade antifade reagent (Fischer Scientific, UK) or Vectashield (Vector labs).

Cells were imaged on a Carl Zeiss, Inc. LSM510 META confocal microscope (with an Axiovert 200 fluorescent microscope) using a 1.4 NA 63x Zeiss objective. The images were analysed using LSM 2.5 image analysis software (Carl Zeiss, Inc.).

To quantify the degree of co-localization, we used ImageJ software (MacBiophotonics). The analysis uses Costes' approach. This allows for the calculation of Pearson's correlation coefficient *R*(obs), which also accounts for any

random overlay of pixels by generating the mean correlation coefficient *R*(rand) between *n* images that have identical average pixel intensity to the original images, but a random distribution of pixels.

2.12.3 ImageJ and JACoP analysis

Statistical software programmes have been developed to quantify co-localization. By considering pixels individually in separately gated images, these programs accurately quantify the amount of co-localization between two fluorescent labels. In the current study, ImageJ (version 1.43) analysis software and the JACoP plugin were utilized to assess co-localization. ImageJ allows for the analysis of the co-localization of two images using a number of standard analysis methods simultaneously. For the purposes of the current study, the Costes` randomization method (Pearson`s correlation coefficient) was utilized to quantify the co-localization of fluorochrome-labelled antigens.

The images obtained were converted to TIF files to be accessed by ImageJ and JACoP. ImageJ (ImageJ, URL) is an open source image processing and analysis software designed for scientific multidimensional images. The JACoP (Just Another Colocalisation Plugin) (available from ImageJ Plugins, URL) plug-in is a compilation of co-localization tools (Bolte and Cordelieres, 2006), that calculates a set of commonly used co-localization indicators, including the Pearson's coefficient.

2.12.4 Pearson`s correlation coefficient

The Pearson`s correlation coefficient (Rr) calculates the covariance between the intensities of the corresponding pixels in each gated image. The images are then analysed as grey for consistency and the statistical analysis is independent of the

image background. Furthermore, Rr is independent of pixel intensities due to the thresholds being automatically created for each gated image (Manders et al. 1993). The maximum intensity of an image was used to set the image threshold, with the resulting Rr ranging from -1 to 1. Rr is equal to 1 for 100% co-localization (i.e., the 2 images compared are the same), 0 for random overlap of proteins and -1 indicates full anti-correlation (i.e. no red where there is green). Negative values of Rr were not used for co-localization, as they indicate an anti-co-localized situation where a pixel is bright in one channel and dim in the other. The fact that that Rr is invariant to background or intensity scales makes the correlation coefficient a robust estimator for co-localization. Furthermore, a more biologically meaningful set of coefficients are the proportion of each protein co-localized with the other (Manders et al. 1993).

2.12.5 Mander's overlap coefficient

Mander's overlap coefficient is measured from the Pearson's coefficient. It is sensitive to background intensity, however, it is an easier method to interpret than Pearson's coefficient. The data obtained range from 0 (total negative correlation) to 1 (total positive correlation). Mander's co-localization coefficients (M1 and M2) demonstrated the extent of green pixels in one channel overlapping red pixels in the other channel, and vice versa. The data obtained indicate the percentage of the total pixels in each channel that overlap with pixels in the other channel (Manders et al. 1993).

2.12.6 Costes' randomisation method

The Costes` method evaluates the statistical significance of the Rr obtained. The Rr coefficient measures the amount or degree of co-localization, however, initially

there was no comparison to understand the meaning of this coefficient. Therefore, Sylvain Costes designed a statistical significance test to assess the probability that the measured value of Pearson's correlation Rr between the two-colour channels is significantly greater than values of Rr that would be calculated if there was only random overlap of the same information. The result of these tests indicates whether the Pearsons' Rr and Manders' coefficients measured are better than pure chance or not.

The Costes' method compares the Rr observed from the original two images, r{obs}, with an average Rr obtained from randomized images of the two original images, r{rand}, which have been created by shuffling pixels' blocks (Figure 2.12.6). The Costes' randomisation method then compares r{obs} and r{rand} to obtain a significance (p-value) expressed as a percentage, that is inversely correlated to the probability of getting r(obs) by chance. Results obtained from this analysis range from 0 (total negative correlation) to 1 (total positive correlation) (Costes et al. 2004).

Figure 2.12.6 Randomized image of two original images, r{rand}, created by shuffling pixels' blocks. r{rand} is the average of a comparison of 1000 pairs of scrambled original images and represents the events occurring due to chance.

2.13 Fluorescence resonance energy transfer (FRET)

2.13.1 Underlying principles

Fluorescence (or Förster) resonance energy transfer (FRET) is a technique that enables the quantification of the association among fluorescently labelled molecules between 1 and 10 nm apart. It is a biophysical method probing the proximity of molecules on the cell surface, under conditions very close to the physiological state of the cells, thus it is a useful tool for investigating molecular associations. FRET may be used to reveal interactions between proteins or structural changes within a molecule. This method measures the non-radiative transfer of photon energy from an excited fluorophore (the donor) to another fluorophore (the acceptor), and it is one of few tools available for measuring nanometre scale distances and the changes in distances, both *in vitro* and *in vivo* (Wu and Brand 1994; Ankerhold 2001).

FRET is utilized to investigate molecular level interactions, due to its sensitivity to distance changes, thus uncovering cellular localizations which were previously undetectable. The present technological advances assisted in developing qualitative and quantitative enhancements for the method, such as distance range, increased spatial resolution and sensitivity. However, FRET requires the donor emission spectrum to overlap the excitation spectrum of the acceptor to work correctly and only specific pairs of fluorophores are eligible. Fluorescence microscopy techniques rely upon the absorption by a fluorophore of light at one wavelength (excitation), followed by the emission of secondary fluorescence at a longer wavelength. The mechanism of FRET includes a donor fluorophore in an excited electronic state, which may transfer its excitation energy to a neighbouring (1-10 nm) acceptor, that subsequently emits light (Kenworthy and Edidin 1999).

Fluorescent proteins (FPs), such as the green-fluorescent-protein (GFP), are usually used for FRET experiments. FPs may be genetically fused to proteins of interest and expressed in cells making them an excellent reporter system for gene expression and protein localization. Numerous enhanced FP variants with different spectral properties are commercially available, and CFP (cyan-coloured) is used as the donor, whereas YFP (yellow-coloured) is used as the acceptor.

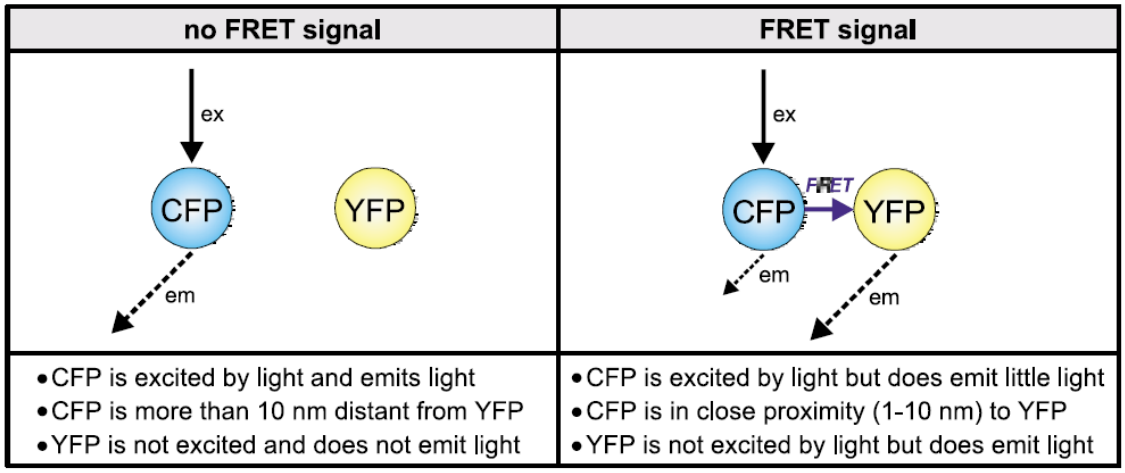


Figure 2.13.1 The principles of fluorescence resonance energy transfer (FRET). ex, excitation; CFP, cyan-coloured fluorescence protein; YFP, yellow-coloured FP; em, emission (Ankerhold 2001).

2.13.2 FRET detection methods

FRET detection methods are able to identify living cells or interactions between proteins in fixed cells, and has been utilized to detect the activation process of enzymes in apoptosis in real time (Xu et al. 2009), to analyze the function of transcriptional proteins (Camuzeaux et al. 2005) or investigate the activation of gene transcription (Liu et al. 2006). The detection methods of FRET are: sensitized emission (seFRET), acceptor bleaching (abFRET) and lifetime measurements. These methods have different properties depending on the sample tested (Diaspro 2010). The abFRET method is performed on most fluorescence microscopes by

emitting the excitation light (of a certain wavelength to excite the donor and not the acceptor) on samples with and without the acceptor fluorophore, and then monitor the donor fluorescence. abFRET is a quantitative approach that does not require sophisticated and expensive instrumentation and was initially introduced to determine transfer efficiency on a pixel-by-pixel basis in a microscope (Jovin and Arndt-Jovin 1989).

For the successfully implementation of FRET, three factors are required: 1) the emission spectrum of the donor must overlap with the excitation spectrum of the acceptor; 2) the distance between the donor and the acceptor must be sufficiently small and; 3) the dipoles of the donor and the acceptor must have a certain spatial orientation (Lam et al. 2012; Cardullo 2013). The rate of energy transfer is inversely proportional to the sixth power of the distance between the donor and acceptor (Kenworthy et al. 1998). The efficiency of energy transfer (E) is defined with respect to r and R_0, the characteristic Förster distance, using the following formula:

$$E = \frac{1}{1 + \left(\frac{r}{R_0}\right)^6}$$

where E is the FRET effect; r is the distance between fluorophores and R_0 is the distance between the donor and acceptor fluorophores when the energy transfer effect is 50% (Kenworthy et al. 1998).

2.13.3 Experimental procedure

In the present study, FRET was quantified as previously described (Kenworthy and Edidin 1999). Briefly, the change in donor (Cy3) emission following the acceptor (Cy5) bleaching was measured. Bleaching of the acceptor eliminates the light excitation of the fluorophore and FRET would not occur between donor-acceptor, therefore the donor emission will not be sequestered. If the donor/acceptor pair is ≤10nm apart the donor emission will increase after bleaching of the acceptor. Scaling factor of 10,000 was used to expand E to the scale of the 12-bit images.

Astrocytes cells were seeded on 8 well glass slides (Nunc Lab-Tek II Chamber Slide System; ThermoFisher Scientific, Inc.). Cells labelled only with the 26ic-Cy5 probe were used to determine the minimum time required to bleach Cy5. Cy5 was bleached by continuous excitation with an arc lamp using a Cy5 filter set for 10 minutes. Under these conditions, Cy3 was not bleached. FRET images were calculated from the increase in donor fluorescence subsequent to the acceptor photobleaching using the following formula:

E(%) x 100 = 10,000 x [(Cy3 postbleach – Cy3 prebleach)/Cy3 postbleach].

Fluorsecent probes against TLR4, TLR2, NLRP3, ASC, NLRP1, C9 have been generated by Dr Martha Triantafilou as Fab fragments conjugated to specific fluorophores (Triantafilou et al. 2001; Triantafilou et al. 2000). These probes were kindle provided for labelling in my FRET experiments.

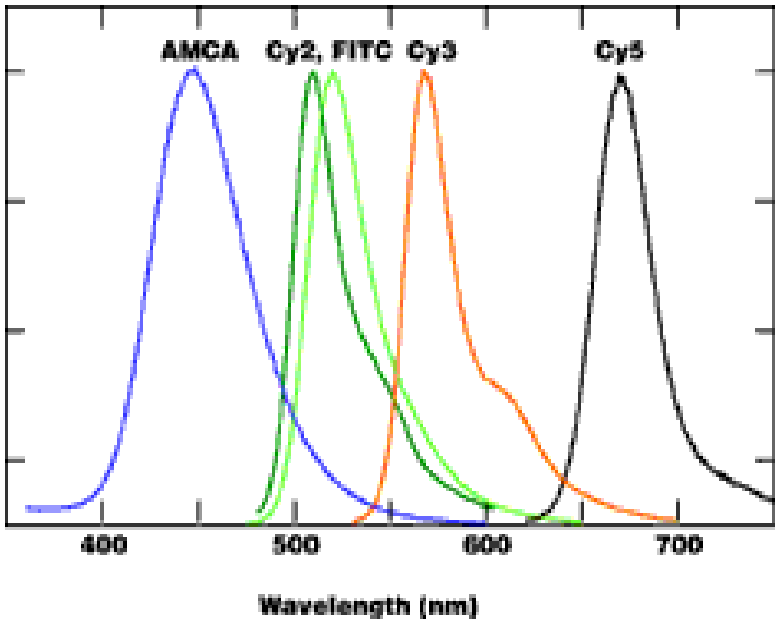


Figure 2.13.3 Indication of the excitation and emission wavelength of Cy3 and Cy5.

Astrocytes were labelled with 100 µl of a mixture of donor conjugated antibody (Cy3) and acceptor conjugated antibody (Cy5). The cells were then rinsed twice in PBS/BSA/NaN_3, prior to fixation with 4% PFA for 15 min. Cells were imaged on a Carl Zeiss LSM510 confocal microscope (with an Axiovert 200 fluorescent microscope) using a 1.4 NA 63x Zeiss objective (Carl Zeiss, Inc.). The images were analysed using the LSM 2.5 image analysis software (available from Zeiss, URL), and Cy3 (Excitation/emission: 548/561nm) and Cy5 (Excitation/emission: 647/665nm) were detected using the appropriate filter sets. Using typical exposure times for image acquisition (<5 sec), no fluorescence was observed from the Cy3-labelled specimen using the Cy5 filters, nor was Cy5 fluorescence detected using the Cy3 filter sets.

2.14 Statistical Analysis

Data were evaluated by analysis of variance and the Dunnett multiple-comparison test using the GraphPad Prism Software (San Diego, CA). Where appropriate (comparison of two groups only), two-tailed t-tests were performed. Statistical differences were considered significant at the level of $P<0.05$. Experiments were performed in triplicate samples and were performed three times or more to verify the results.

Chapter III

Involvement of PRRs in the innate immune sensing of fibrillar Aβ

3.1 Introduction

Dementia is the primary result of Alzheimer's disease (AD) and is commonly characterised by the presence of amyloid plaques (NPs) and neurofibrillary tangles (NFTs). NTs are structures deposited extracellularly containing a fibrillar amyloid-beta (Aβ) core which is highly insoluble (Iversen 1995). This is formed of fragments 39-42 amino acids which are surrounded by reactive astrocytes, dystrophic neurites and microglia produced as a biproduct of neuronal degenerative processes.

Astrocytes create the brain environment and provide for brain defence. In the hippocampus, ~60 % of all axon-dendritic synapses are surrounded by astroglial membranes (Sofroniew and Vinters, 2010). The presence of Aβ in microglia and astrocytes is a primary contributing factor in the development of AD progression, leading to deteriorating neuronal cells. An innate immune response is elicited due to the presence of these plaques and deposits; a series of cellular events resulting in a robust inflammatory response (Akiyama *et al*, 2000). These events result in the production of nitric oxide (NO), reactive oxygen species (ROS) and a plethora of pro-inflammatory cytokines. This may eventually lead to deleterious effect and neuronal cell death (Kitazawa *et al*, 2004).

During the early stages of AD, activated astrocytes are located in two regions: the inner layer of the cerebral cortex and near the amyloid deposits below the pyramidal cell layer. The pathological changes leading to the activation of astrocytes in AD are not fully understood, however it has been demonstrated that the presence of amyloid activates astrocytes. Microglia and astrocytes have been reported to play a central role as moderators of Aβ clearance and degradation. Upon activation, astrocytes may phagocytose and degrade amyloid. This suggests

that astrocytes may contribute to the removal of accumulated Aβ (Ries and Sastre, 2016). Astrocytes are activated through TLR-dependent pathways, thus causing local inflammation that eventually could intensify neuronal death (Halassa and Haydon, 2010; Henneberger *et al*., 2010). Cellular infection and stress are causative agents for activating molecular platforms called inflammasomes. Inflammasomes are responsible for the production and maturation of pro-inflammatory cytokines, such as IL 1-β and IL-18. For the release of these cytokines, that leads to potential inflammation, two signals are required: priming (signal 1), and activation and assembly of the inflammasome (signal 2) (Latz E, 2010; Bauernfeind *et al*, 2009; Bauernfeind *et al*, 2011; Embry *et al*, 2011). Several sterile inflammatory diseases are caused by the excessive secretion of interleukin (IL)-1β, such as type 2 diabetes, atherosclerosis, and AD (Sheedy *et al*, 2013). Increased production may cause or potentiate the neurodegeneration, specifically upon a prolonged exposure as in the AD brain.

In addition, the fibrillar amyloid-β (fAβ) in senile AD plaques, or amyloid-containing amylin/islet amyloid polypeptide (IAPP) on pancreatic β-cells in T2D3 is a hallmark of AD (Sheedy *et al*, 2013). Recent studies demonstrated that that these specific materials cause inflammation by recruiting cytosolic sensors, such as NLRP3 (Duewell *et al*, 2010; Halle *et al*, 2008; Masters *et al*, 2010; Rajamaki *et al*, 2010). A previous study demonstrated that injection of Aβ oligomeric forms into the retrosplenial cortex of rats lead to astrocyte activation. The results revealed activation of the transcription factor NF-κ B and demonstrated the presence of inflammatory mediators, such as tumour necrosis factor (TNF-α) and IL-1β (Carrero *et al*., 2012). NF-κB controls chemokine and adhesion molecules secretion in astrocytes, increasing the inflammatory response (Moynagh, 2005).

The continuation of this process results in a self-regulating mechanism, which leads to neurodegeneration.

In this chapter, we shed some light on how PRRs, such as the NOD proteins and TLR play a role in eliciting inflammation by recruitment of inflammasome and otherwise. In addition, we investigated how amyloid aggregates cause such a form of innate immune response resulting in neuronal inflammation and AD pathogenesis.

3.2 Aβ induced cytokine secretion in astrocytes

To test our hypothesis that Aβ triggers pattern recognition receptors in the brain leading to neuroinflammation and AD pathology, we initially investigated what cytokine response is triggered in astrocytes by Aβ. Fibrillar Aβ (amino acids 1-42), as well as control non-fibrillary peptide (identical sequence in reverse order) was obtained from AnaSpec Inc. Fremont (USA) and prepared as previously described by Gasque and colleagues (Gasque P. *et al*, 1997).

Different concentrations of Aβ (5uM, 10uM, 15uM) were utilised to determine the optimum concentration of Aβ which would be used. Cells were stimulated at different time points (6h and 8h) and cell viability was also tested. The concentrations used did not affect cell viability. The 10uM concentration were chosen for our subsequent stimulations since it triggered a similar response of IL1β seen with the higher concentration (Figure 3.2.1)

The astrocytes were stimulated with 10uM $A\beta_{1-42}$, and HEK-IFN-α/β sensor cells and HEK IL-1β sensor cells were used to identify the Human Type I IFN and IL-1β response (Figure 3.2.2). Reporter cell lines were incubated with the supernatant from unstimulated, $A\beta_{1-42}$ stimulated and scrambled peptide stimulated astrocytes

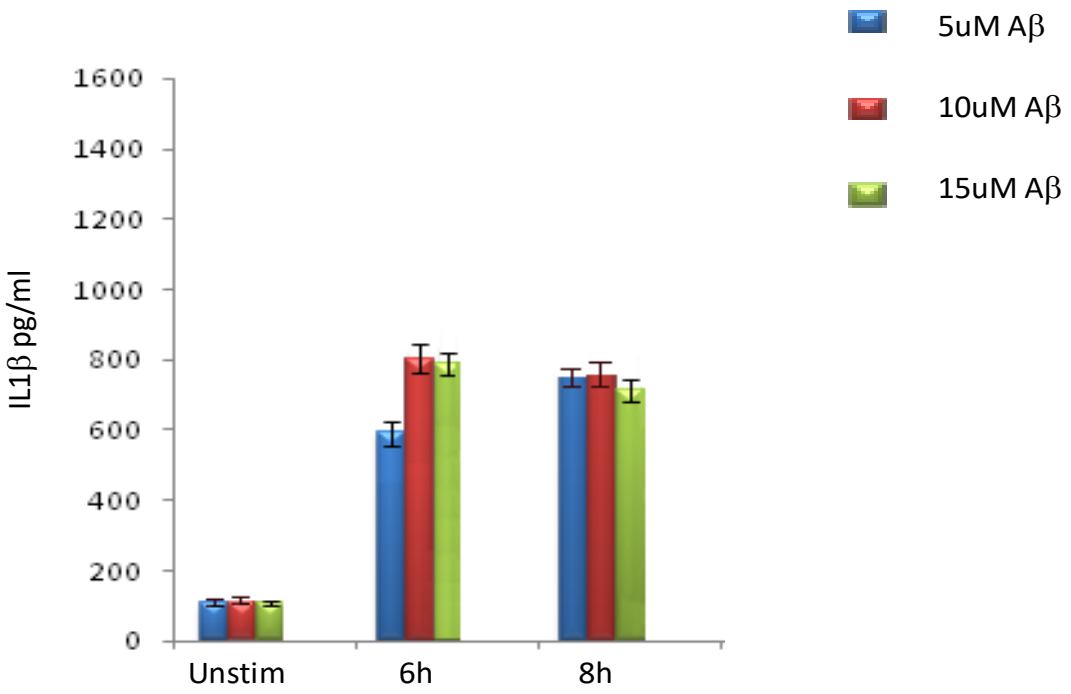


Figure 3.2.1 IL-1β secretion levels in astrocytes stimulated with different concentrations of Aβ.

for 24 hours. The next day, supernatants from reported cell lines were collected and incubated with HEK-Blue detection medium and the expression levels of IFN-α/β and IL-1β were detected using a spectrophotometer at a wavelength of 650 nm.

Results indicate that amyloid $A\beta_{1-42}$ triggers a type I interferon response which is specific since there is no IFN secretion in the unstimulated cells or cells in the presence of the scrambled peptide. IFN secretion is probably via TLRs since it is known that an alternative pathway exists to induce IFNβ downstream of TLR4 in an MyD88-independent manner (Sato et al. 2003; Noppert et al. 2007). As for IL-1β, higher levels of activation were observed indicating secretion, when cells were stimulated with amyloid compared with the unstimulated cells, thus confirming inflammasome activation in the presence of amyloid.

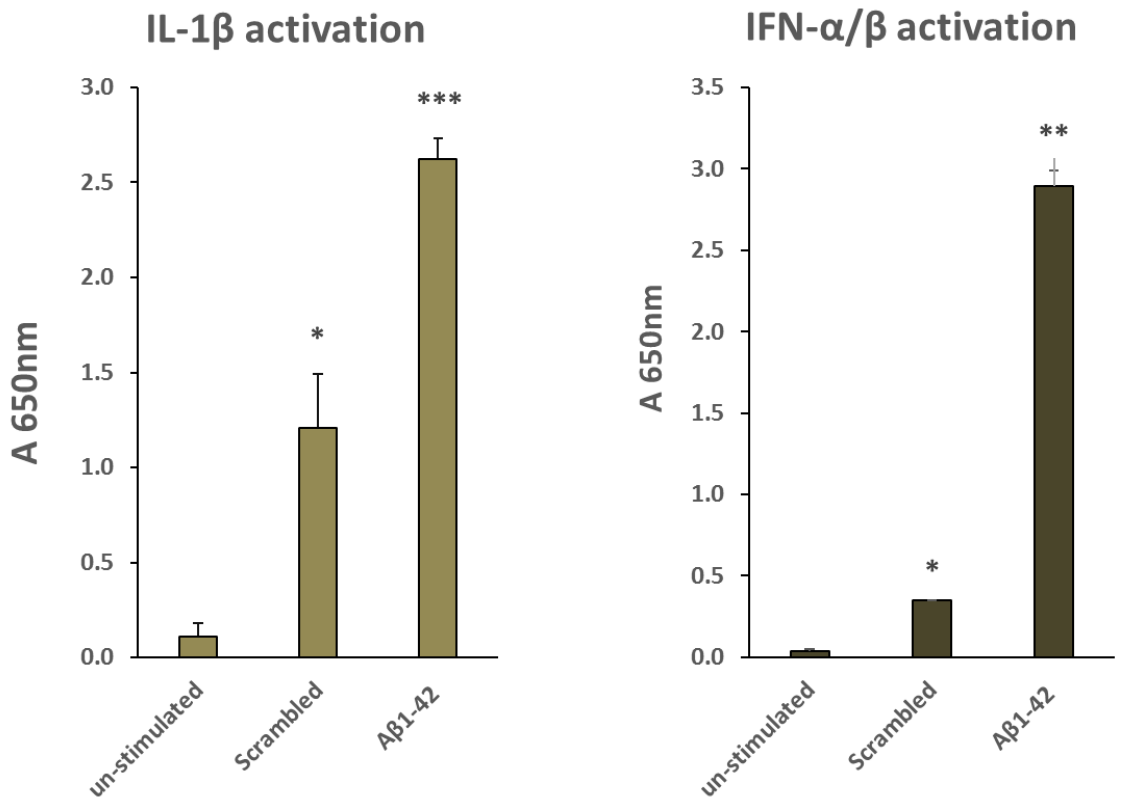


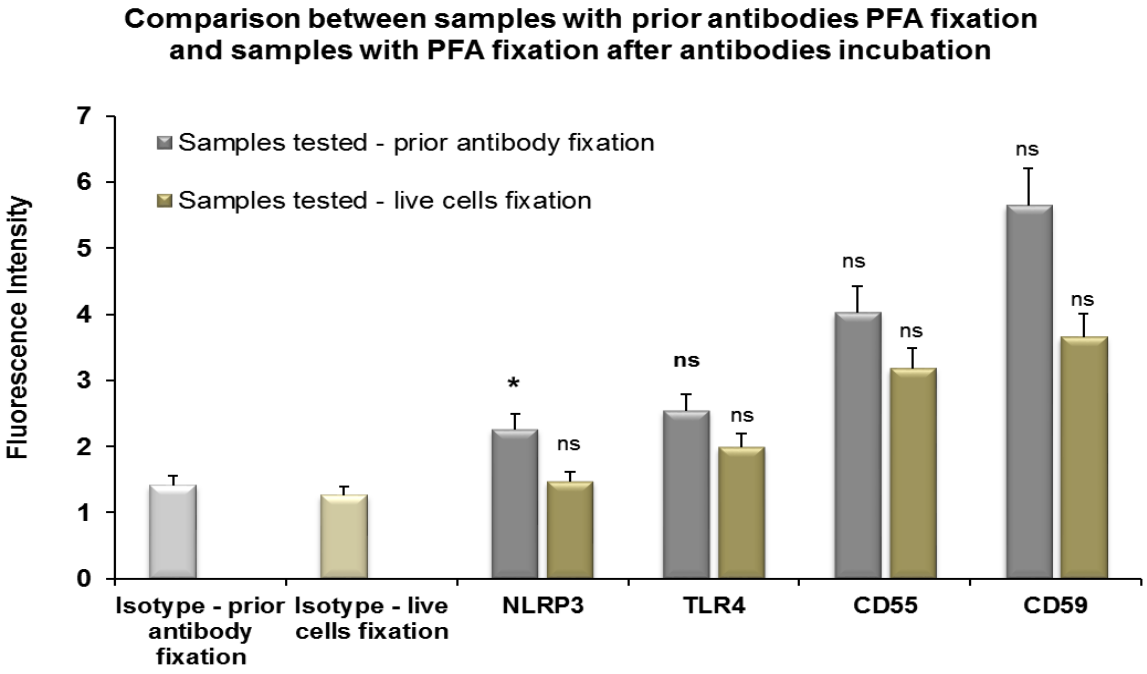
Figure 3.2.2 IFN-α/β and IL-1β activation in HEK-Blue sensor cells stimulated with supernatants from unstimulated, $A\beta_{1-42}$ stimulated and scrambled peptide (10ug) stimulated astrocytes. Supernatant was added to HEK IFNβ/ and HEK IL-1β reporter cells and incubated for 24 hours. Quanti-Blue was added and the levels of SEAP were measured using a spectrophotometer at 630 nm. The SEAP levels correspond to the expression levels of IFNβ/IL1β. *P<0.05, **P<0.01 and ***P<0.001 vs. unstimulated. The results are presented as the mean + standard deviation, and are representative of three independent experiments.

3.3 Involvement of PRRs in Aβ detection

To determine which PRRs are involved, we initially investigated changes in the expression levels of TLRs, NLRs and complement receptors in response to different concentrations of fibrillar Aβ and scrambled peptide in human astrocytes. Immunological techniques, such as immunofluorescence and flow cytometry, were employed to determine the expression levels of different TLRs (TLR2, TLR4), NLRs (NLRP1, NLRP3) and complement receptors (CD55, CD59, C5aR1, C3aR, CR1, CR3) at different time points following stimulation. The same techniques were employed to determine the expression levels of different TLRs (TLR2, TLR4), NLRs (NLRP1, NLRP3 and NLRC5) and complement receptors (CRs; C5aR1, C3aR, CR1, CR3, CD55, CD59 and Factor H) before and after Aβ stimulation at different time points following fixation with paraformaldehyde (PFA).

3.3.1 Does PFA fixation prior to antibody incubation have an adverse effect on fluorescence intensity?

PFA (4%) was used in this occasion to fix the cell monolayers at the end of each time point to avoid receptor re-organization. To determine whether our fixation method had an adverse effect on the receptor expression levels, live cells were incubated with primary antibodies and then with the specific secondary antibodies, followed by PFA fixation at the end (Fig 3.3.1-B). Cells were also fixed and then labelled with primary antibodies followed by the appropriate secondary antibodies (Fig 3.3.1-A). Rabbit IgG, polyclonal (Isotype Control) (ab27478, abcam, UK) was also used as a control. Flow cytometry was used to analyse receptor surface expression levels. Our results demonstrated no difference in the two methods.



Figures 3.3.1 Basal level of PPRs surface expression in astrocytes with PFA fixation prior to antibody staining and PFA fixation after antibody staining. Astrocytes were harvested, incubated for 30 minutes with primary antibodies against the receptors of interest, followed by a 30-minute incubation with the appropriate secondary antibody conjugated to FITC. Astrocytes were

fixed with 4% PFA either prior, or after antibody staining (experimental processes run on ice). The graph compares the two methods and the fluorescence was detected by flow cytometry using a FACSCalibur (BectonDickinson). P>0.05 (ns), *P<0.05 vs. the respective isotype control. The results are presented as the mean + standard deviation, and are representative of three independent experiments.

3.3.2 PRRs expression in response to fibrillar $A\beta_{1-42}$

Immunofluorescence and flow cytometry was employed to determine the total expression levels of different TLRs (TLR2, TLR4), NLRs (NLRP1, NLRP3 and NLRC5) and complement receptors (CRs; C5aR1, C3aR, CR1, CR3, CD55, CD59 and Factor H) before and after $A\beta_{1-42}$ stimulation at different time points in human astrocytes. At 2-hour stimulation (Figure 3.3.2), there is an upregulation of NLRP3, C5aR1 as well as Factor H (FH) receptor. At 6-hour incubation there is no significant upregulation observed in the receptors of interest (Figure 3.3.2).

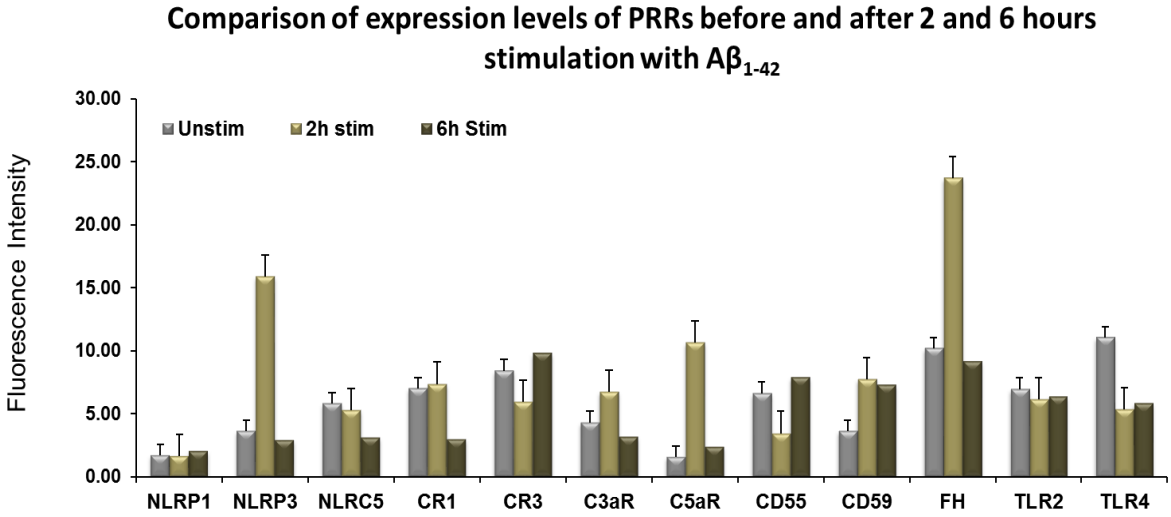


Figure 3.3.2 Comparison of expression levels of PRRs before and after 2 and 6 hours stimulation with $A\beta_{1-42}$. Basal level of PPRs expression in unstimulated and $A\beta_{1-42}$ stimulated astrocytes. Astrocytes were stimulated with 10 ug/ml of $A\beta_{1-42}$ for 2 and 6 hours and subsequently were harvested, fixed, permeabilised with 4% PFA and incubated with primary antibodies against the receptors of interest, followed by an appropriate secondary antibody conjugated to FITC.

Fluorescence was detected by flow cytometry using a FACSCalibur (BectonDickinson). P>0.05 vs. the respective unstimulated cells. The results are presented as the mean + standard deviation, and are representative of three independent experiments.

3.3.3 Signalling cascades triggered by Aβ

To determine which signalling cascades are triggered in response to Aβ, astrocytes were either not stimulated, stimulated with 10 ug/ml $A\beta_{1-42}$ or 10 ug/ml scrambled peptide for 2 and 6 hours and subsequently lysed and analysed for the presence of phospho-IkB and caspase-1 p10. Phospho-IκB is indicative of activated NF-κB which is a result of TLR activation upstream of the signalling cascade. Presence of phospho-IκB was investigated to determine whether Aβ triggers TLR activation. Similarly, caspase-1 p10 activation acts downstream of inflammasome (NLR) activation, therefore caspase-1 p10 detection was also investigated to determine whether Aβ triggers inflammasome activation. Western blot analysis of phospho-IkB and caspase-1 p10 expression in unstimulated, scrambled peptide stimulated and $A\beta_{1-42}$ stimulated astrocytes was performed.

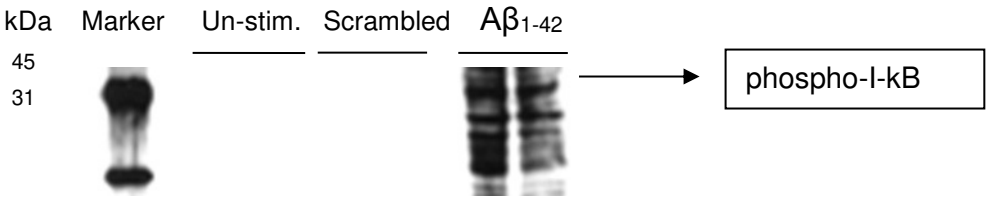


Figure 3.3.3.1 Detection of phospho-IkB expression on astrocytes by western blot analysis. Astrocytes were prepared and incubated as described in Chapter 2, Material and Methods. Cell lysates from astrocytes stimulated with $A\beta_{1-42}$ for 2h and 6h were collected and analysed for phospho-IκB using SDS-PAGE gel electrophoresis and western blot. Primary antibodies specific for phospho-IκB were used followed by the appropriate secondary antibodies conjugated to HRP. The bands were visualised using the ECL procedure. The data is a representative of four independent experiments.

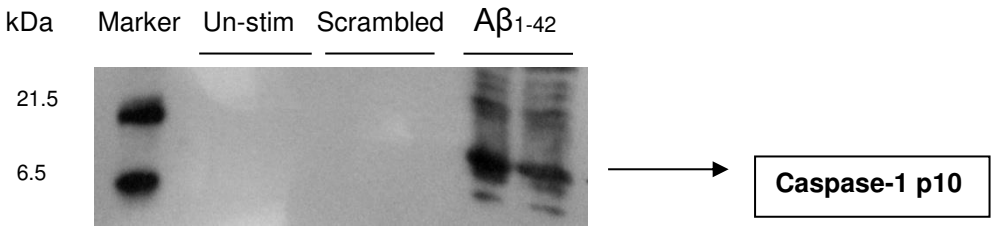


Figure 3.3.3.2 Detection of caspase-1 p10 expression on astrocytes by western blot analysis. Astrocytes were prepared and incubated as described in Chapter 2, Material and Methods. Cell lysates from astrocytes stimulated with $A\beta_{1-42}$ for 2h and 6h were collected and analysed for caspase-1 p10 using SDS-PAGE gel electrophoresis and western blot. Primary antibodies specific for caspase-1 p10 were used followed by the appropriate secondary antibodies conjugated to HRP. The bands were visualised using the ECL procedure. The data is a representative of four independent experiments.

3.4 Involvement of inflammasome in the innate immune response to Aβ

3.4.1 Aβ-induced inflammatory responses via TLRs, NLRs and CRs

To determine the involvement of TLR4, TLR2, NLRP3, NLRP1 and complement in Aβ recognition, silencing and confocal microscopy were used. RNA interference was utilised to knock down the expression of TLRs and NLRs involved in Aβ recognition during the expression experiments mentioned above.

Astrocytes were silenced for TLR4, TLR2, NLPR3 and NLRP1. I also looked at the effect of NLRP3 silencing as a control on other NLRs. Thus, the expression of NLRC5 or NLRP1 was also investigated. The results showed that NLRP3 silencing was specific and did not affect the expression of other NLRs. siRNA with scrambled sequence was also used as a control which showed that the transfection had no effect on NLRP1, NLRP3, TLR2 or TLR4 (Fig 3.4.1.1).

Once knock-down of the receptors of interest was confirmed in human astrocytes, we proceeded to stimulate the cells with different concentrations of Aβ and measure pro-inflammatory cytokines secretion using CBA.

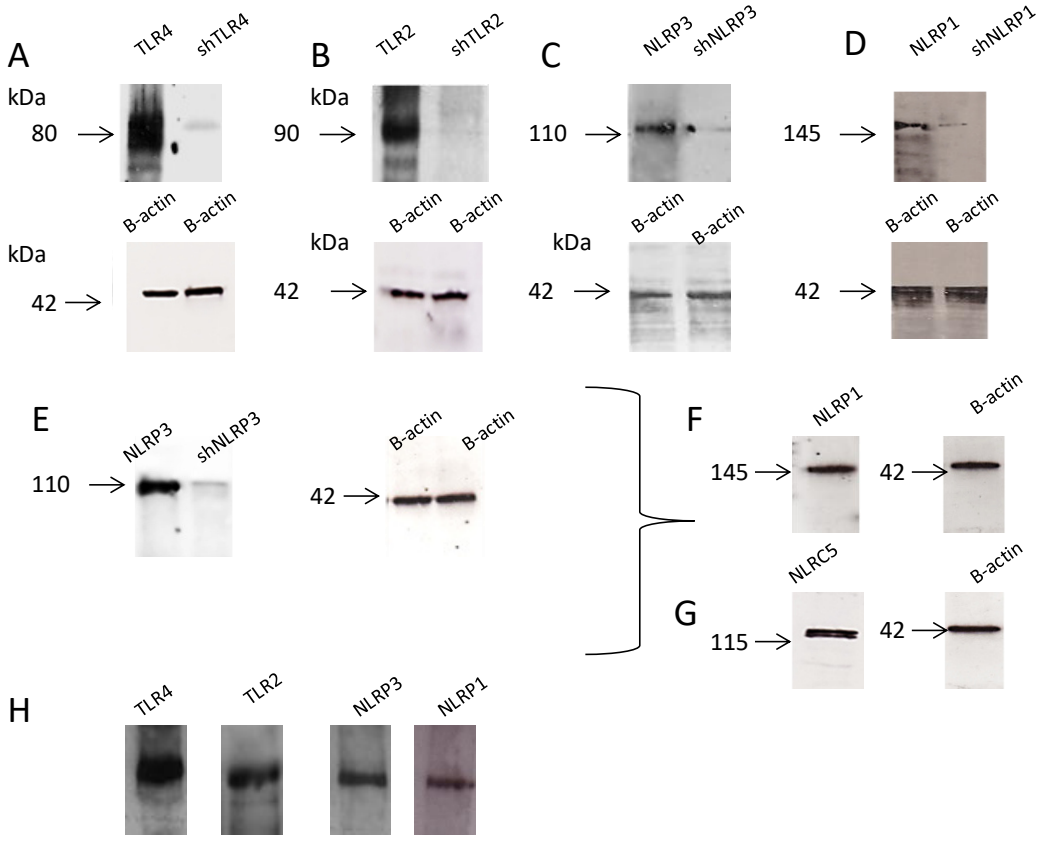


Figure 3.4.1.1 Human astrocytes silenced for TLR4, TLR2, NLRP3, NLRP1 and NLRC5. Astrocytes were silenced for (A) TLR4, (B) TLR2, (C) NLPR3, (D) NLRP1 and percentage of knockdown was compared to wild type astrocytes. The effect of NLRP3 silencing (E) as a control on NLRC5 or NLRP1 expression levels is shown on panel F and G, respectively, and β-actin expression is also depicted as a control. Panel H shows different gels showing the expression of TLR4, TLR2, NLRP3, NLRP1 when psiRNA with scrambled sequence was used. The data is a representative of four independent experiments.

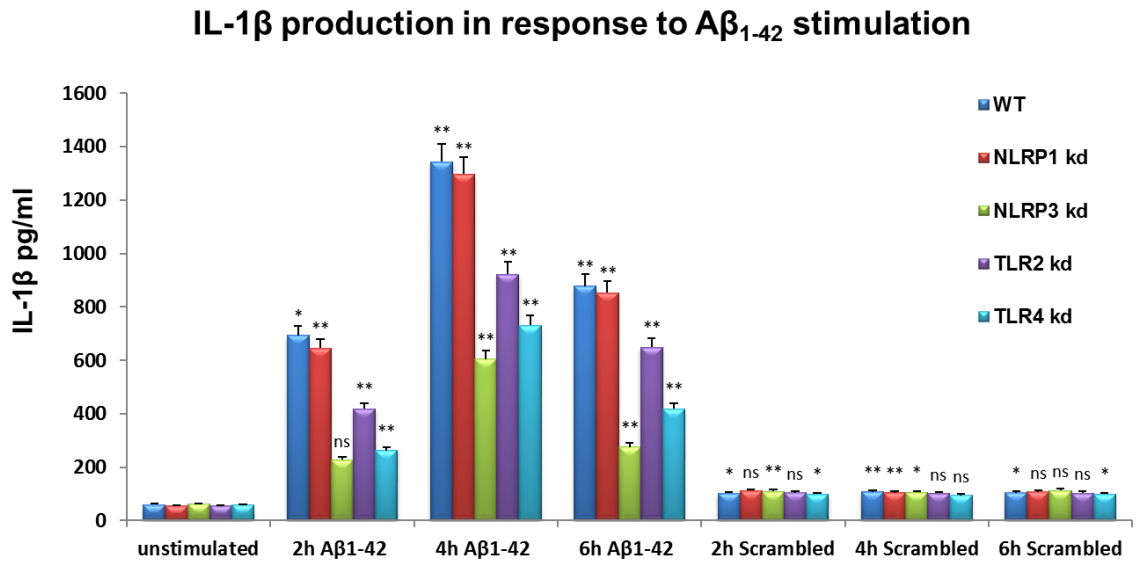
Astrocytes are a major inducible source of IL-6. Many factors have been demonstrated to induce IL-6 expression. IL6 is is a marker of inflammation with no direct role in inflammasome (McGeough et al. 2012). IL-6 is consistently detected in the brains of patients with AD, but not in the brains of non-demented elderly persons. IL-6 immunoreactivity was established in diffuse Aβ plaques from AD patients and it was demonstrated to regulate neuronal survival and function. In

addition, IL-1β is a key player associated with the development and onset of AD (Wang, et al.2015).

To determine whether Aβ triggers pro-inflammatory cytokine secretion, we measured the secretion of IL-1β (Figure 3.4.2-A) and IL-6 (Figure 3.4.2-B) using the cytometric bead array system (CBA; Becton Dickinson). Cytokine secretion was observed in response to $A\beta_{1-42}$ stimulated astrocytes at 2, 4 and 6 hours. In the presence of scrambled peptide, the secretion of cytokines was minor, indicating that cytokine production occurred only in response to $A\beta_{1-42}$ stimulation. To further determine the role of PRRs in Aβ recognition, we investigated the secretion of IL-6 and IL-1β in astrocytes silenced for NLRP1, NLRP3, TLR2 or TLR4. The data demonstrated a significant reduction in IL-1β secretion (Figure 3.4.2-A) when NLRP3, TLR2 or TLR4 activity was silenced, while there was no significant change observed in IL-1β secretion when NLRP1 activity was silenced (Figure 3.4.2-A).

Concerning IL-6 secretion, when TLR2 was silenced there was a decrease in IL-6. TLR4 silencing resulted to a more pronounced reduction of IL-6. NLRP3 and NLRP1 silencing as expected, since they do not play a role in IL-6 activation and secretion, had no effect on IL-6 production (Figure 3.4.2-B).

A.



B.

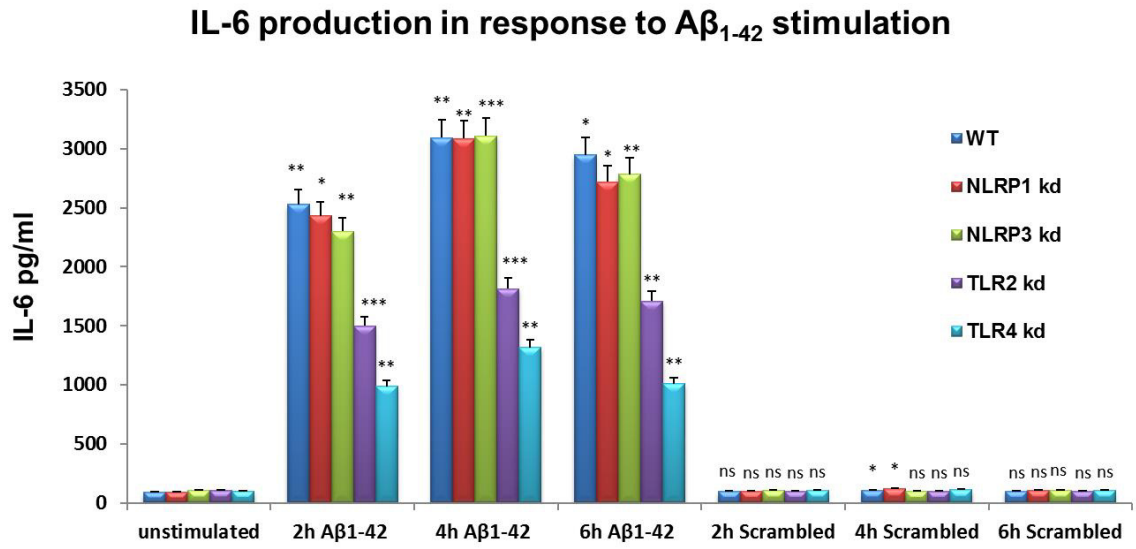


Figure 3.4.1.2 Cytokine production in response to $A\beta_{1-42}$ stimulation. Astrocytes silenced for NLPR1, NLRP3, TLR2 or TLR4 and wild type astrocytes were stimulated with $A\beta_{1-42}$ and scrambled peptide. Supernatant was collected at 2, 4 and 6 hours post incubation and analysed for IL-1β (A) and IL-6 (B) using CBA on a FACSCalibur (Becton Dickinson). *P<0.05, **P<0.01 and ***P<0.001 vs. respective unstimulated. The results are presented as the mean + standard deviation, and are representative of three independent experiments.

3.4.2 Aβ$_{1-42}$ triggers NF-kB pathway (Signal 1)

To determine which signalling cascades are triggered in response to Aβ, wild type and astrocytes silenced for TLR4 and TLR2 were either not stimulated, stimulated with 10 ug/ml Aβ$_{1-42}$ or 10 ug/ml scrambled peptide for 2, 4 and 6 hours (Figure 3.4.3 A and B). Cells were subsequently lysed and analysed for the presence of phospho-IkB by western blotting. As NF-kB activation acts downstream of TLR activation, presence of phospho-IkB was investigated to determine whether Aβ triggers TLR activation.

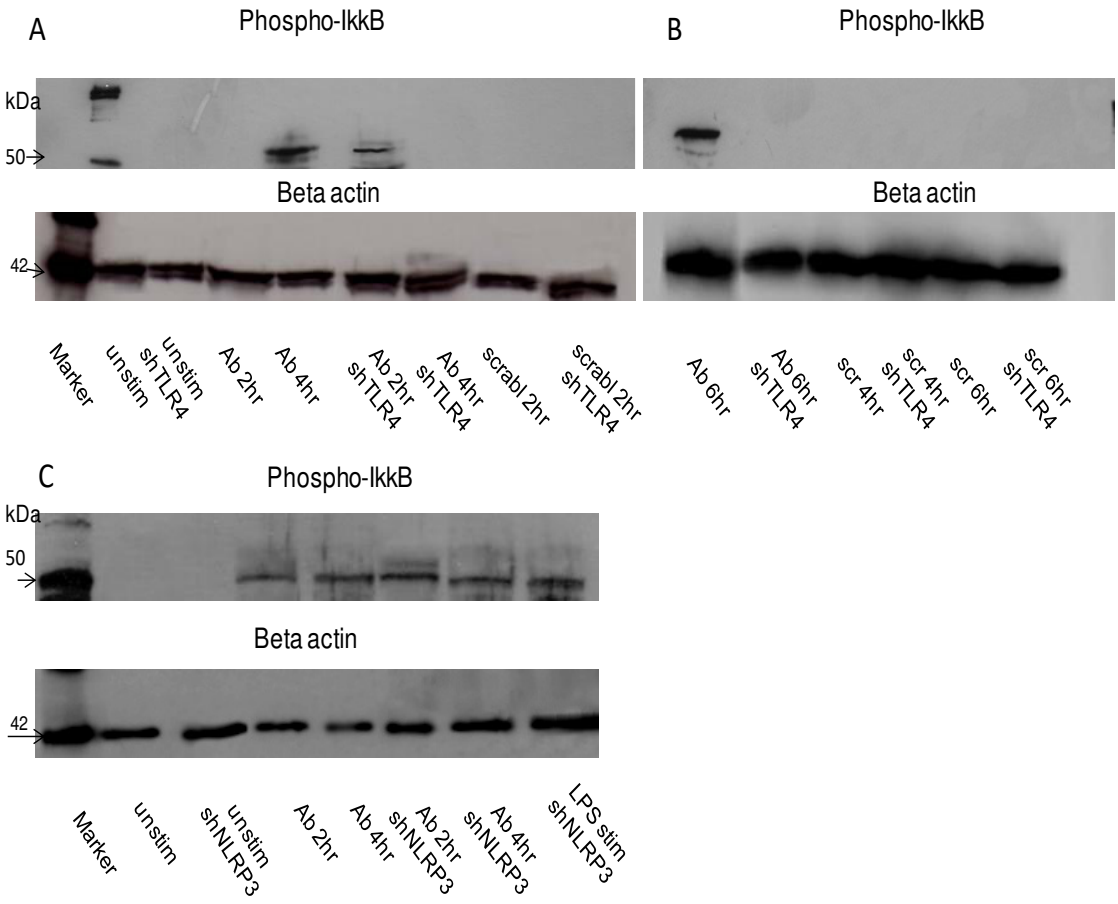


Figure 3.4.2 Western blot analysis of silenced astrocytes. Cell lysates were analysed for the presence of phospho-IkB by western blotting when TLR4 was silenced and cells were stimulated with Aβ and scrambled Aβ for 2h, 4h and 6h (A) when TLR2 was silenced and cells were stimulated

with Aβ and scrambled Aβ (B). Furthermore, the presence of phospho-IkB was investigated when NLRP3 was silenced and cells were stimulated with Aβ and scrambled Aβ as well as LPS as a positive control (C). Beta actin loading controls are also shown. The gels are representatives of three independent experiments.

The data shows that Aβ stimulation resulted in phospho-IkB production while the scrambled peptide had no effect. When TLR2 and TLR4 were silenced. there was inhibition of phospho-IkB. Probably because $A\beta_{1-42}$ is a PAMP recognized by TLR2 and TLR4 and thus resulting in NF-kB activation. When NLRP3 was silenced and cells were stimulated with $A\beta_{1-42}$ or LPS which is the PAMP for TLR4 there was phospho-IkB production which is what we expected since NLRP3 has no direct role in NF-kB activation and it is known that it cannot recognize PAMPs/ligands directly. Therefore, TLR2 and TLR4 seem to recognize Aβ and trigger NF-kB activation and cytokine production.

3.4.3 $A\beta_{1-42}$ triggers inflammasome activation (Signal 2)

To investigate whether PRRs play a role in inflammasome activation, inflammasome activation was assessed by western blotting, probing for caspase-1 p10 in astrocytes silenced for TLR4, TLR2, NLRP3 and NLRP1 (Figure 3.4.3). Caspase-1 p10 is present in $A\beta_{1-42}$ stimulated wild type astrocytes, at all time points, indicating inflammasome activation. The data demonstrated a reduced signal for caspase-1 p10 activation in $A\beta_{1-42}$ stimulated shTLR2. However, no signal was detected for caspase-1 p10 in $A\beta_{1-42}$ stimulated shTLR4 at all time points. This demonstrated that TLR2 and TLR4 play a role in the inflammasome activation through $A\beta_{1-42}$ recognition as a PAMP which is consistent with the phospho-IKB production already seen previously, thus acting as signal 1 for inflammasome. Knockdown of NLRP3 abrogates caspase-1 p10 production

indicating that NLRP3 is vital in detection of amyloid by inflammasomes triggering signal 2 which leads to the cleavage and activation of caspase1 and resulting in IL1β secretion.

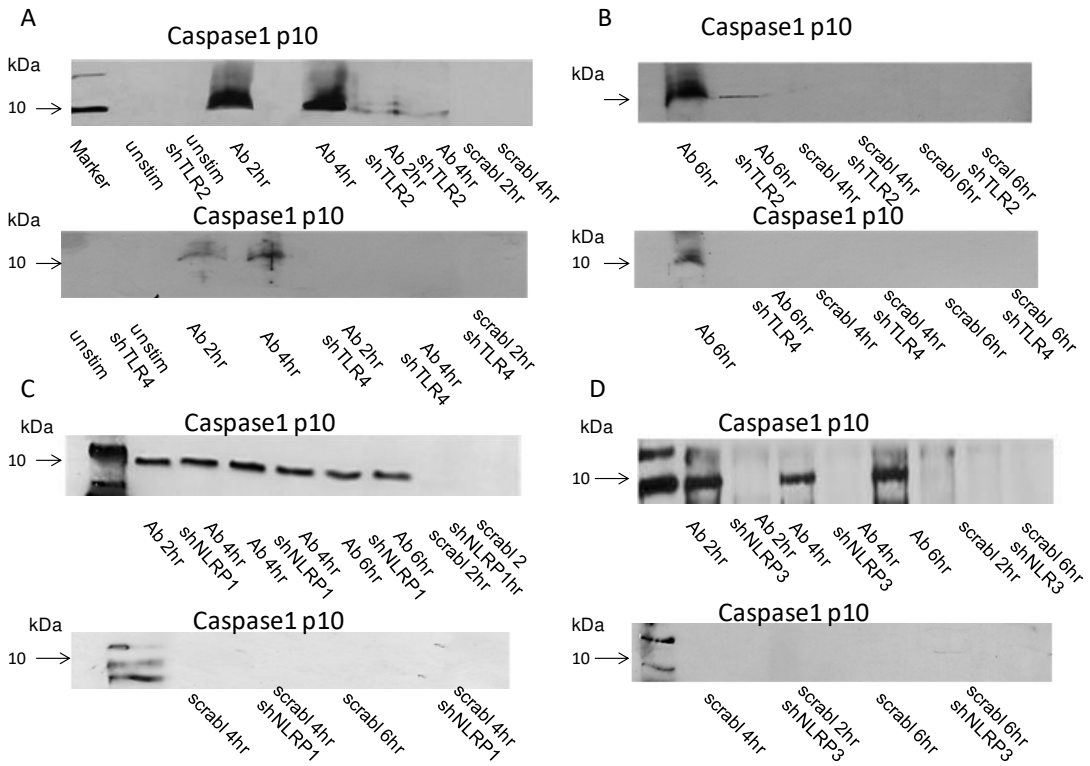


Figure 3.4.3 Western blot analysis of silenced astrocytes. Cell lysates were analysed for the presence of phospho-IkB by western blotting when TLR4 was silenced and cells were stimulated with Aβ and scrambled Aβ for 2h, 4h and 6h (A) when TLR2 was silenced and cells were stimulated with Aβ and scrambled Aβ (B). Furthermore, the presence of phospho-IkB was investigated when NLRP3 was silenced and cells were stimulated with Aβ and scrambled Aβ as well as LPS as a positive control (C). Beta actin loading controls are also shown. The gels are representatives of three independent experiments.

3.4.4 Aβ and NLRP3 presence in the AD brain

To determine if Aβ is present in the AD brain, AD patients' tissue sections were stained for Aβ using immunohistochemistry. Amyloid plaques were observed in the brain of the AD patients. To determine whether NLRP3 is present in the Aβ plagues in the AD brain, patients' tissue was stained for NLRP3 and Aβ (Figure 3.4.4). In these experiments the peroxidase substrate yields a grey/blue stain and the DAB label a brown stain enabling the two primary antibodies to be visualised on the same slide in different colours.

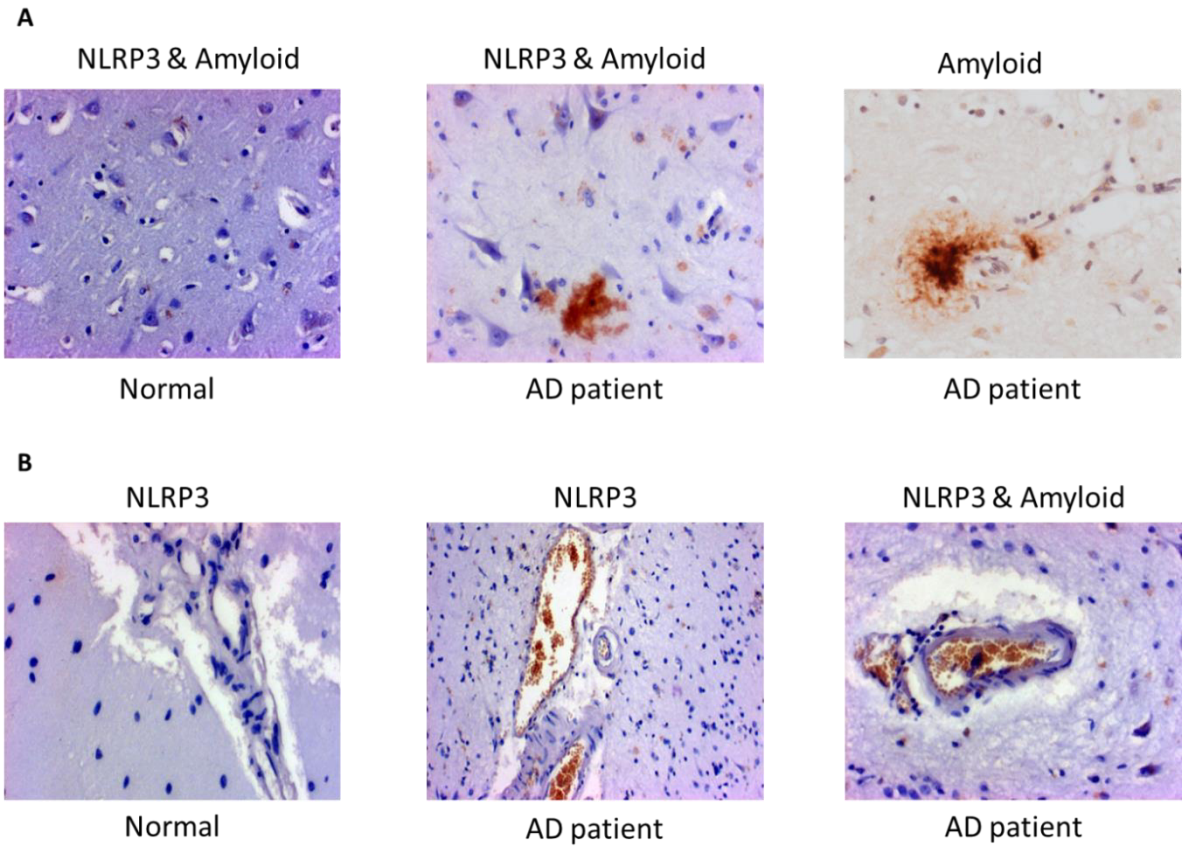


Figure 3.4.4 Tissue staining demonstrating NLRP3 activation in the AD brain. Paraffin wax sections (original magnification x400) of hippocampus of normal controls and AD patients immunolabelled with anti an Amyloid-β mAb (brown) to detect plaques and an anti NLRP3 rabbit IgG (blue), The active plaque shows the presence of amyloid and NLRP3.

3.4.5 NLRP3-MAC association in AD

To determine whether MAC triggers NLRP3 activation in the AD brain, patients' tissue was stained for NLRP3 and C9 neoepitope [signifies the presence of the membrane attack complex (MAC) in the human brain] using immunohistochemistry. Dual labelling via indirect immunofluorescence was also used to determine whether there is NLRP3 activation in the AD brain. Brain tissue from normal and AD patients was stained for NLRP3, ASC (its adaptor molecule once activated) and C9. It was shown that NLRP3 and ASC are present in the brain of AD patients and not in healthy controls (Figure 3.4.5-A).

Furthermore, results demonstrated that NLRP3 colocalizes with C9 in the amyloid plagues, suggesting that there is inflammasome-complement interaction in the AD brain (Figure 3.4.5-B).

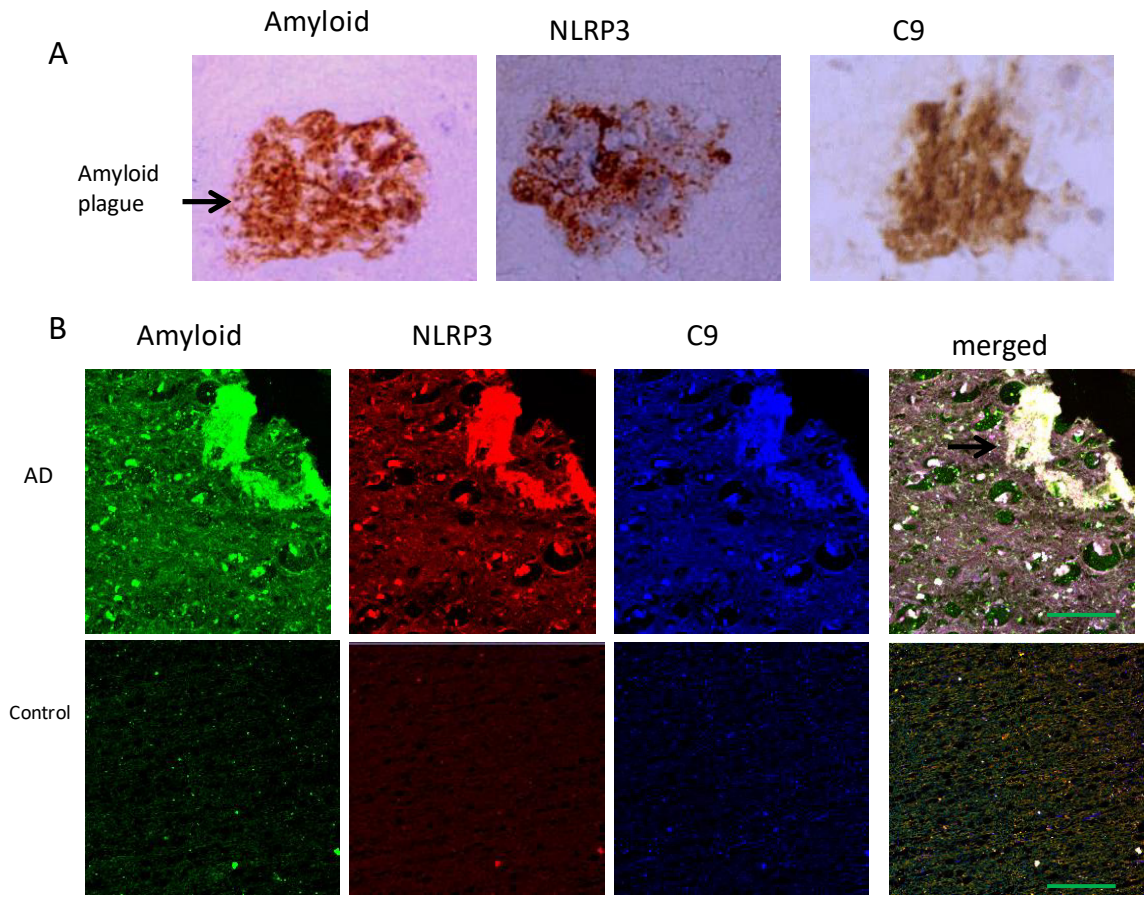
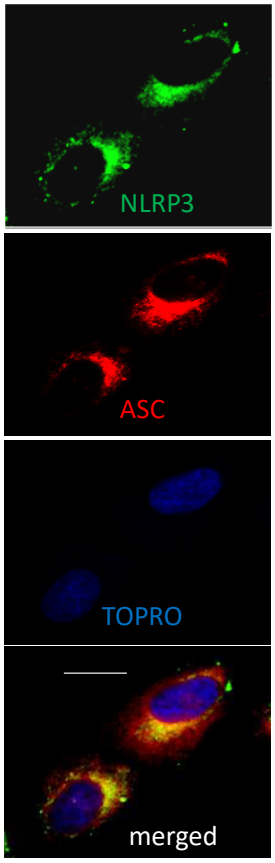


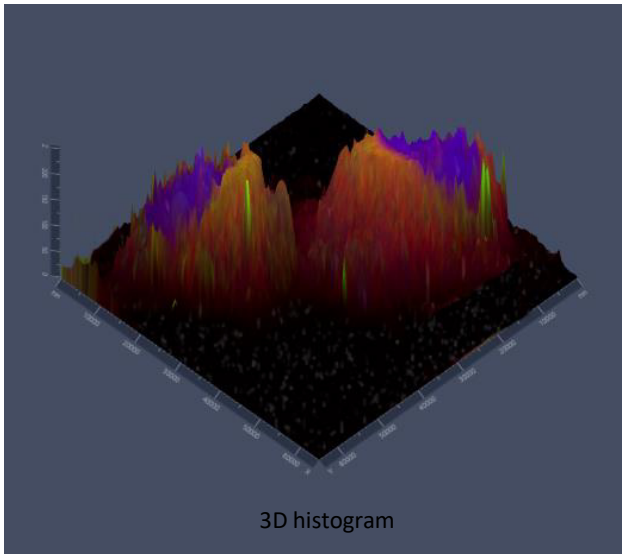
Figure 3.4.5 Tissue staining demonstrating NLRP3 and C9 activation in the AD brain. (A) Paraffin wax sections (Original magnification x400) showing specific cytoplasmic cell staining (brown, Vector DAB) on brain tissue of AD patient for amyloid using an Amyloid-β mAb, for NLRP3 using a rabbit anti-NLRP3 antibody and for C9 using a mouse monoclonal antibody anti-C9 neoantigen with Haematoxylin (blue) counterstain. The presence of NLRP3 and ASC and NLRP3 and C9 in tissue sections of AD patients and normal controls is also shown (B). Tissue was stained for NLRP3 using a primary goat anti-NLRP3 with secondary donkey anti-goat conjugated to Alexa 546 and for C9 using a mouse monoclonal antibody anti C9 neoantigen with secondary donkey anti-mouse conjugated to Alexa 647. Amyloid was labelled with a rabbit anti amyloid antibody followed by donkey anti rabbit secondary conjugated to Alexa 488. Cells were imaged using a Zeiss 510 confocal microscope. (Scale bar 100um). The arrows on figure depict the amyloid plague.

3.4.6 Aβ interacts with NLRP3

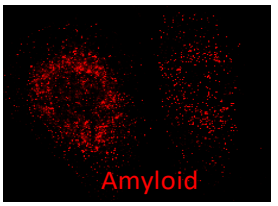
Aβ and NLRP3 were shown to be present in brain tissue of AD patients. To investigate if Aβ associates with NLRP3 in astrocytes, we stimulated wild type astrocytes with Aβ for 4h and 6h when there is the peak of IL1β secretion this confirming inflammasome activation. Cells were then labelled with primary antibodies against NLRP3, ASC and Aβ and secondary antibodies conjugated to Alexa fluorophores. (Figure 3.4.6).

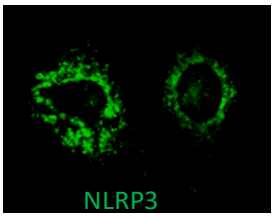


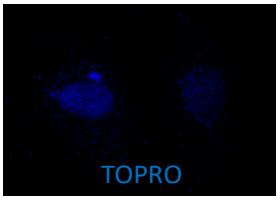
A

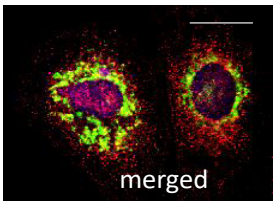


R(obs) =0.930

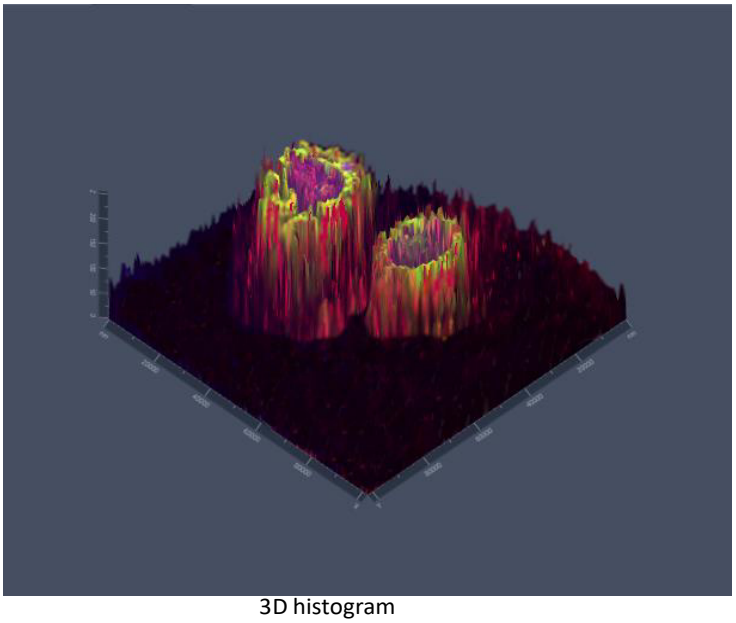






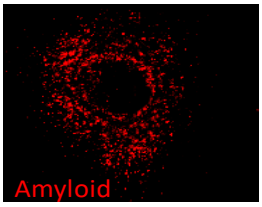


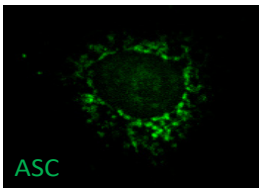
B

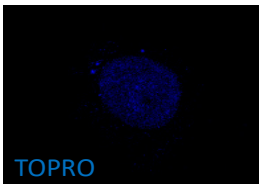


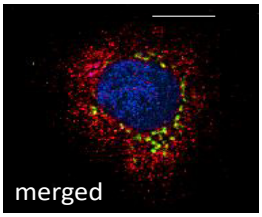
3D histogram

R(obs) =0.880

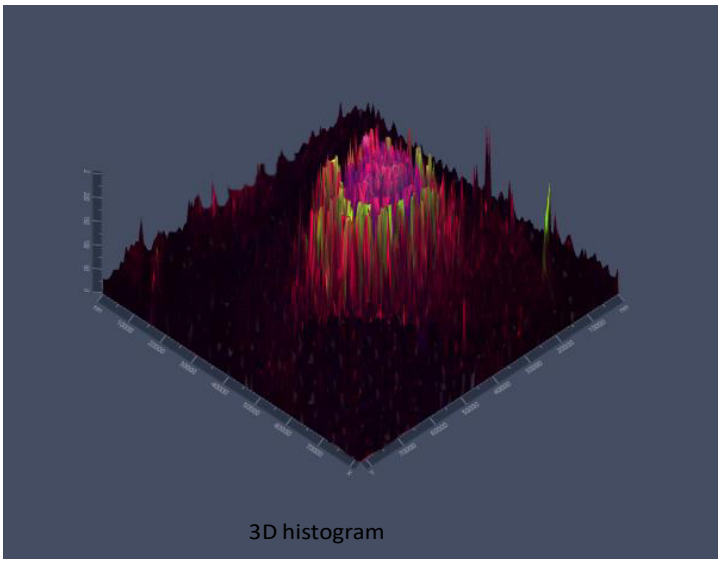








C



R(obs) =0.865

Figure 3.4.6 NLRP3 and amyloid interactions on astrocytes. Confocal images of astrocytes stimulated with Aβ for 4h are shown. NLRP3 was labelled using a rabbit anti NLRP3 antibody

followed by a donkey anti-rabbit secondary conjugated to Alexa 488 while ASC was stained using a goat anti ASC antibody followed by a secondary donkey anti-goat conjugated to Alexa 546 (A) Aβ was stained using a primary mouse monoclonal anti-amyloid-β with secondary donkey-anti mouse conjugated to Alexa 546 and NLRP3 was labelled using a rabbit anti NLRP3 antibody followed by a donkey anti-rabbit secondary conjugated to Alexa 488 (B) Aβ was stained using a primary mouse monoclonal anti-amyloid-β with secondary donkey-anti mouse conjugated to Alexa 546 and ASC was stained using a goat anti ASC antibody followed by a secondary donkey anti-goat conjugated to Alexa 488 (C). Cells were imaged using a Zeiss 510 confocal microscope. Bars shown are 10um. Histograms indicating 3D representation of the image generated by ZEN blue software are also shown. The data presented are a representation of three independent experiments. The merged images show apparent localized interaction R(obs) between NLR3 and ASC, NLRP3 and amyloid as well as ASC and amyloid, determined using ImageJ software analysis via the Costes' method.

In our imaging experiments ASC and NLRP3 were used as a positive control which returned *R*(obs) of 0.930 which are close to the theoretical maximal value confirming these co-localizations are highly significant (Fig. 3.4.6 A). The statistical significance of NRP3 and amyloid co-localisation, was calculated by Costes' approach, by using ImageJ which returned *R*(obs) of 0.880, therefore suggesting that these co-localizations are significant (Fig. 3.4.6 B).

Our data showed as expected since amyloid colocalises with NLRP3 that ASC interacts with amyloid as well *R*(obs) of 0.865 (Fig. 3.4.6 C) confirming these co-localizations are significant.

3.5 Investigating Aβ internalization

3.5.1 The endosomal pathway

Early endosomes are the initial station of the endocytic pathway and often located in the periphery of the cell. These endosomes receive most vesicles originating in the cell surface and are primarily sorting organelles where many ligands dissociate from their receptors in the acid pH of the lumen (Lundmark et al. 2008; Mellman 1996). This is the site for receptors to separate into the transcytotic pathway to late components (via vesicular component, which form multivesicular bodies, or endosomal carrier vesicles).

Internalized materials en route to the lysosomes, from early endosomes in the endocytic pathway, the trans-Golgi network in the biosynthetic pathway or the phagosomes in the phagocytic pathway, travel via the late endosomes (Mukherjee et al. 1997). The late endosomes have numerous membrane vesicles and proteins characteristic of lysosomes, such as glycoproteins, and are believed to mediate the final events prior to delivery of materials to the lysosomes.

The final part of the endocytic pathway is the lysosomes. The primary function of lysosomes is to break down cellular waste products, fats, carbohydrates, proteins, and other macromolecules into simple compounds. These compounds are then returned to the cytoplasm as new cell-building materials. To accomplish this, lysosomes use different types of hydrolytic enzymes, all of which are manufactured in the endoplasmic reticulum and modified in the Golgi apparatus (Luzio et al. 2000).

To investigate whether the internalization and trafficking of Aβ was crucial for triggering a pro-inflammatory response, we utilized confocal microscopy. Briefly, following the internalization of molecules from the plasma membrane, they fuse

with early endosomes, sent to late endosomes (degradation), and destroyed in lysosomes. Along with Aβ, we investigated TLR4 and CR3 to identify whether a co-localization occurs, hence Aβ traffics via either of these receptors.

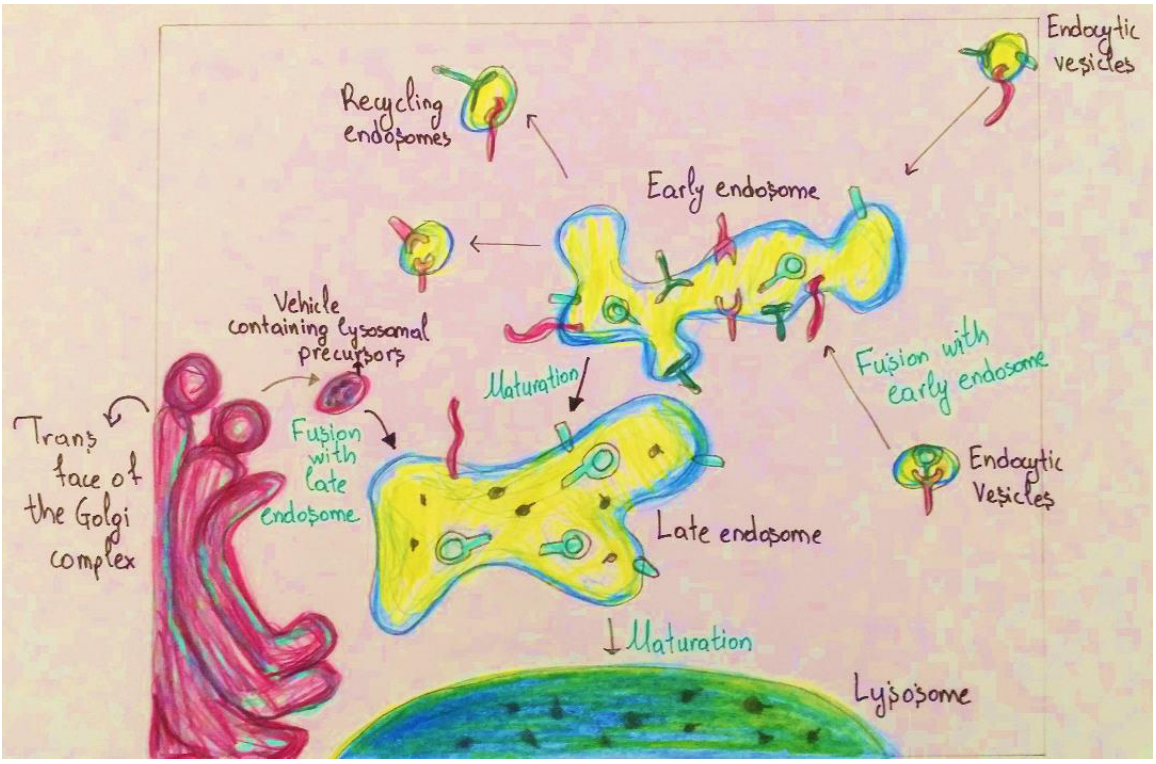


Figure 3.5.1 The endosomal pathway (adapted from www.theartofmed.tumblr.com/post/119579864277).

3.5.2 TLR4 internalization

The best characterized TLR, in terms of its key function in both innate immunity and signal transduction, is TLR4. TLR signalling consists of at least two distinct pathways: a MyD88-dependent pathway that leads to the production of inflammatory cytokines, and a MyD88-independent pathway associated with the stimulation of IFN-β and the maturation of dendritic cells. Previous studies demonstrated that TLR4 is the main sensor of LPS from Gram-negative bacteria and senses endogenous molecules causing inflammation and cell damage. TLR4

traffics to early endosomes from the plasma membrane and activates IRF3, and then traffics to late endosome, where it undergoes degradation.

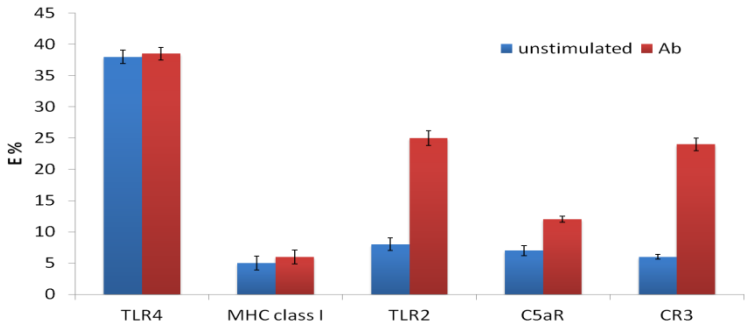
3.5.3 TLR4 interactions with cell surface PRRs in response to Aβ

To determine whether Aβ interacts with different cell surface PRRs, we employed fluorescence resonance energy transfer (FRET), a biophysical method probing the proximity of molecules on the cell surface, under conditions very close to the physiological state of the cells. FRET occurs over distances of 1-10nm and is a useful tool for investigating molecular associations. Using FRET, we investigated TLR4 heterotypic associations on the cell surface of human astrocytes in response to Aβ. The results demonstrated that subsequent to Aβ stimulation, TLR4 co-clustered with TLR2 (E = 25 ± 2.1%), to some extent with C5aR1 (E = 14 ± 1.2%) and with CR3 (E = 24 ± 2.1%). TLR4 interactions with MHC class I were used as a negative control (E = 5 ± 0.9%), while interactions between two different epitopes of TLR4 were used as our positive control (E = 38 ± 1.5%) (Figure 3.5.3 A).

Following the identification of these PRRs associated on the cell surface in response to Aβ, we investigated their interactions upon internalization. We utilised confocal microscopy to visualize in which compartment (endosomes, lysosomes, or cytoplasm) these associations take place and in what time frame. To determine the internalization route of the PRRs in response to Aβ, the PRRs of interest (TLR4 and CR3) and the endosomal compartments (early, late, recycling) were labelled using fluorescent antibodies/dyes in the presence of Aβ at 30 min, 2h and 4h. The astrocytes were imaged on an LSM710 ELYRA P1 confocal microscope using a 1.4NA 100x Zeiss objective (Carl Zeiss, Inc.) and the images were then analysed with the ZEN Black image analysis software (Carl Zeiss, Inc.).

The results demonstrated that Aβ traffics through the endosomal/lysosomal pathways (Figure 3.5.3). Furthermore, Aβ had internalised along with TLR4 and CR3 into the early endosome (EEA1: early endosome marker) within 30 min (Figure 3.5.3 B and C), and within 2h into the late endosome containing Rab7 (Figure 3.5.3 D). In addition, co-localisation of Aβ with CR3 in the lysosomal pathway (LAMP1: lysosome marker) was observed after 4h (Figure 3.5.3 E).

A.



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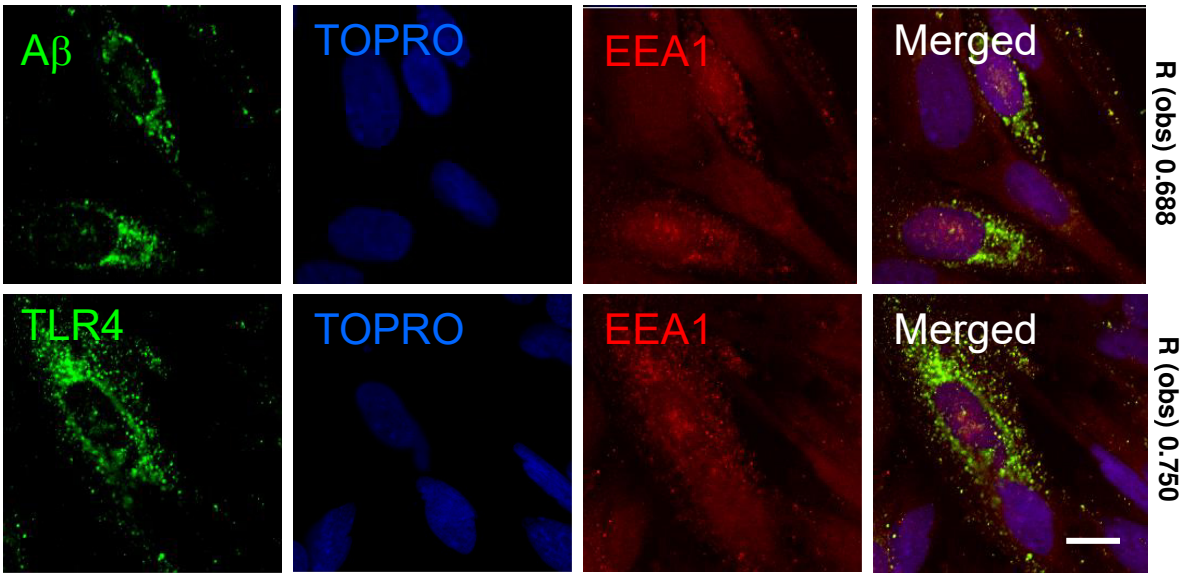
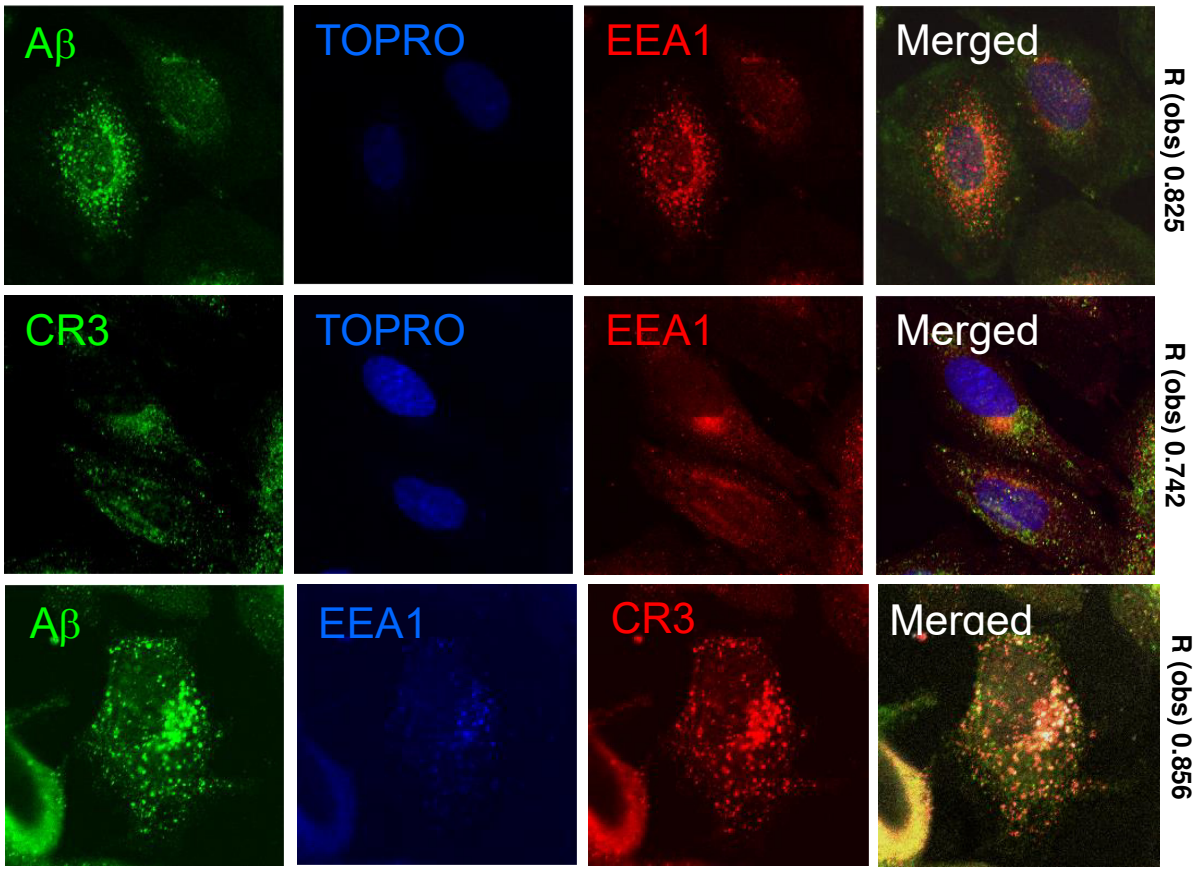
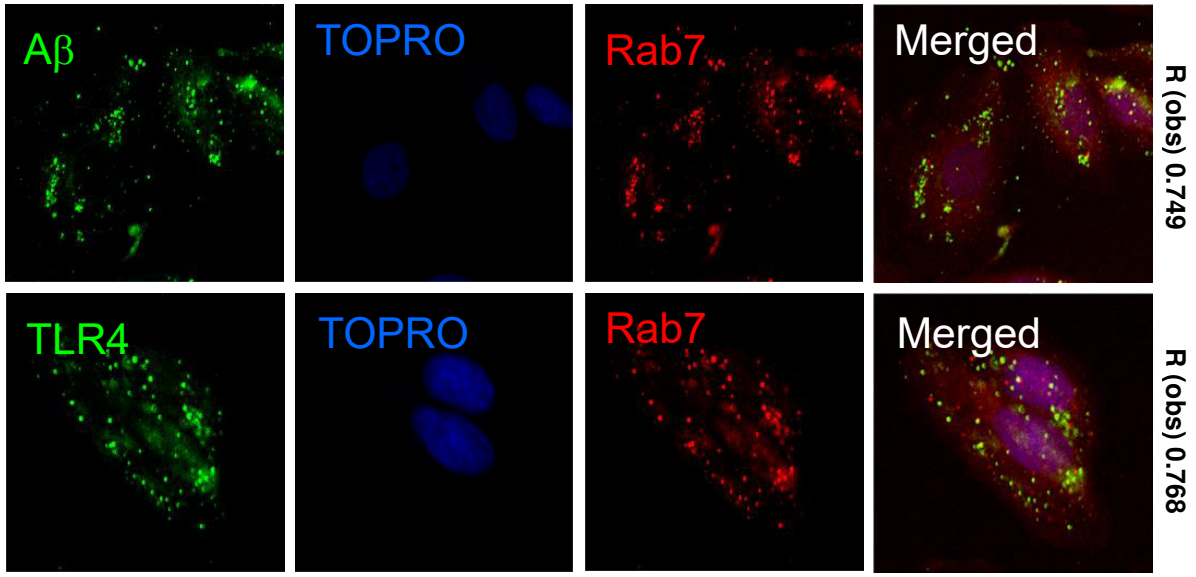


C.


Aβ
TOPRO
EEA1
Merged
R (obs) 0.825
CR3
TOPRO
EEA1
Merged
R (obs) 0.742
Aβ
EEA1
CR3
Merged
R (obs) 0.856

D.


Aβ
TOPRO
Rab7
Merged
R (obs) 0.749
TLR4
TOPRO
Rab7
Merged
R (obs) 0.768

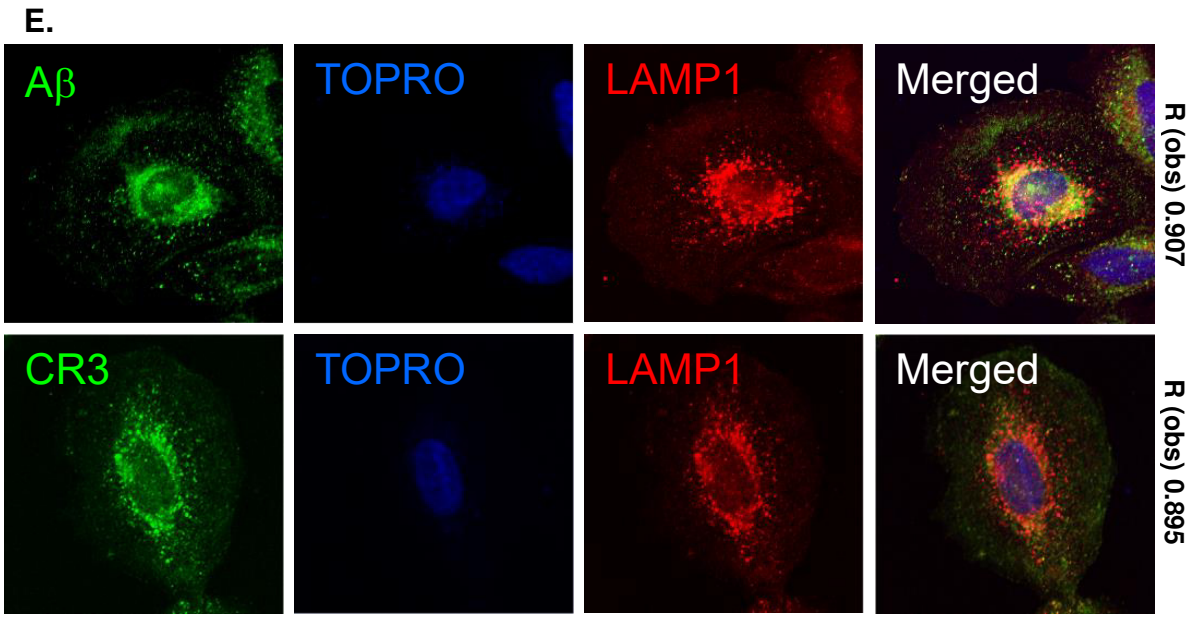


Figure 3.5.3 PRR associations induced in the presence of Aβ and Aβ internalization. (A) FRET measurements between TLR4 and different receptors in response to Aβ. Human astrocytes were stimulated with Aβ for 30 min and subsequently fixed. Energy transfer between TLR4 (Cy3) and the different receptors (Cy5), was measured from the increase in donor (Cy3) fluorescence following acceptor (Cy5) photobleaching. Fab fragments from two different antibodies for TLR4 (targeting different epitopes) one conjugated to Cy3 and one to Cy5 were used as a positive control. W6/32 (antibody specific for MHC- class-I) Fab fragments conjugated to Cy3 and anti TLR4 Fab fragments conjugated to Cy5 were used respectively as probes for MHC- class-I and TLR4 as negative control since MHC –class- I does not associate with TLR4. The percentage of energy transfer (E%) and standard deviation were calculated from three independent experiments (A). Human astrocytes were stimulated with Aβ for 30 min (panel B), 1h (panel C), 2h (panel D) or 4h (panel E). The cells were fixed and labelled with primary and secondary antibodies with the appropriate fluorescent dye, and the nucleus was stained with TOPRO-3. Images were collected using a Zeiss 710 confocal microscope and are representative of three independent experiments. Aβ was stained using a primary mouse monoclonal anti-amyloid-β with secondary donkey-anti mouse conjugated to Alexa 488 and TLR4 was labelled using a mouse monoclonal antibody followed by a donkey anti-mouse secondary conjugated to Alexa 48. EEA1 was stained using a goat anti EEA1 antibody followed by a secondary donkey anti-goat conjugated to Alexa 546 Rab7 was stained using a goat anti RAb7 followed by a secondary donkey anti-goat conjugated to Alexa 546. LAMP1 was stained using a goat anti LAMP1 followed by a secondary donkey anti-goat conjugated to Alexa 546. CR3 was stained using a mouse monoclonal with secondary donkey-anti

mouse conjugated to Alexa 488. Cells were imaged using a Zeiss 510 confocal microscope. Bars shown are 10um. The data presented are a representation of three independent experiments. The statistical significance of receptor co-localisation was calculated by Costes' approach, by using ImageJ.

3.6 Conclusion

Alzheimer's disease (AD) is a neurodegenerative disorder and the most common cause of dementia in the elderly. Patients show a gradual onset and progression of memory loss and other cognitive deficits. It is widely accepted that the extracellular accumulation of Aβ in senile plaques is a principal event in the pathogenesis of Alzheimer's disease (Weiner HL and Frenkel D 2006), but the cellular events leading to plaque-induced neuronal dysfunction are still unclear. Genetic factors have been suggested to either cause or predispose to AD including mutation in amyloid precursor protein (APP), which generates Aβ by proteolytic processing, mutation in presenilins 1 and 2 as well as polymorphism to apolipoprotein A and Complement receptor 1 (CR1) (Weiner HL and Frenkel D 2006). It is our hypothesis that deposition of Aβ peptide can activate the innate immune system via PRRs, including complement, and evoke Alzheimer's pathology initiating a pro-inflammatory cascade that results in the release of cytokines, complement, proteases and other acute phase proteins, ultimately causing neurodegeneration.

The precise roles of PRRs in detecting and mounting responses against Aβ, and their roles in AD are not clear. To determine which PRRs are involved, the expression levels of TLRs as well as NLRs and complement receptors in response to fibrillar Aβ were investigated in cultures of astrocytes. A concern over the use of fixation prior to antibody staining was raised, thus a further comparison of cells

fixed prior to antibody staining and after was performed to determine receptor surface expression. The results showed that the two methods were comparable, thus we proceeded to determine total expression of receptors at different time points using PFA fixation and permeabilization with Saponin prior to antibodies staining.

Furthermore, the results showed that in un-stimulated astrocytes all the PRRs of interest such as TLRs (TLR2, TLR4), NLRs (NLRP1, NLRP3 and NLRC5) as well as complement receptors (CRs; C5aR1, C3aR, CR1, CR3, CD55, CD59 and Factor H) were present albeit at low levels (Figure 3.3.2) suggesting that these cells are able to detect and respond to a wide range of PAMPs and DAMPs. When we examined PRRs expression following 2h-stimulation with $A\beta_{1-42}$, we observed up-regulation for some of them. From the NLRs, NLPR3 had a high expression level and from the CRs, C3aR, C5aR1, CD59 and Factor H were found to be highly expressed. The results suggested there was a difference in expression level at various time points, thus different time points of stimulation were studied as well to give us a better picture of PRR expression following Aβ stimulation.

Inflammation has been shown to be present in AD patients and IL-6 and TNF-a have been demonstrated in cerebrospinal fluid samples from AD patients, when compared to their age-matched healthy controls (Blum-Degen D et al. 1995) whereas IL-6 immunoreactivity is established in diffuse Aβ plaques from AD patients (Gadient et al. 1997). In addition, increased levels of IL-1β have been shown in the circulation of AD patients (Licastro et al 2000).

To determine which PRRs are involved in neuroinflammation, we investigated inflammasome activation since IL-1β secretion have been linked with AD (Licastro et al 2000) as well as the involvement of TLRs and complement. The data

demonstrated that Aβ can trigger cytokine secretion (IL1β, as well as non inflammasome cytokines IL6 and IFNβ), NF-kB activation (which was detected by the presence of Phospho-IKKb) as well as cleavage to caspase 1 p10 which results in inflammasome assembly.

To determine the role of TLRs and NLRs in Aβ recognition, we utilised RNA interference to knock down expression of TLRs and NLRs. Knockdown of TLR2 and TLR4 not only significantly decreased levels of TLR2 and TLR4 protein in astrocytes, but also resulted in reduced NF-kB activation (determined by the presence of phospho-Ikkb by western blotting), as well as decreased caspase-1 protein levels in response to Aβ. IL-6 secretion which is TLR dependent as well as IL-1β secretion was also inhibited. These findings suggest that TLR2 and TLR4 could be linked to the upstream' activation' of inflammasome assembly.

Since inflammasomes have been shown to be triggered by diverse ligands, it has been suggested that two or potentially more signals are required for full activation. The first, or priming signal, can by triggered from a transcriptionally active PRR or cytokine receptor, this leads to transcriptional activation of the genes encoding pro-IL1β and pro-IL18 (Martinon et al. 2009). The second signal is triggered in response to various stress signals associated with damaged self resulting in NLR assembly (Bauernfeind et al. 2009). Thus, it seems that Aβ is detected by TLR2 and TLR4 which provide the priming signal since knocking down these TLRs resulted in a reduction in expression of IL-1β and IL-6.

To determine which NLR is involved in the inflammasome assembly, NLRP3 and NLRP1 were knocked down using shRNA in astrocytes and cells were stimulated with Aβ. When IL-1β secretion and caspase 1 expression were examined, the data showed a significant reduction in IL-1β secretion and a reduction in caspase 1 p10

expression when NLRP3 was knocked down, whereas there was no significant change in IL-1b or caspase 1 when NLRP1 was knocked down.

To confirm the role of NLRP3 in amyloid induced neuroinflammation, AD brain samples from patients were used and stained for Aβ to visualize the plagues, as well as NLRP3 and MAC (C9). A study by our group has demonstrated that following sublytic MAC attack, there is an increase in intracellular Ca^{2+} concentration leading to Ca^{2+} accumulation in the mitochondrial matrix and loss of mitochondrial transmembrane potential. This triggers NLRP3 inflammasome activation and IL-1β release, demonstrating as association of NLRP3 and MAC (Triantafilou K et al. 2013). Thus, to identify NLRP3 and complement interaction in the AD brain, AD brain tissue was stained for NLRP3 and MAC (C9) (Figure 3.4.5). NLRP3 and MAC co-localize in amyloid plaques of the AD brain, further indicating an inflammasome-complement interaction. The data points that our hypothesis is right and neuroinflammation is due to the PRRs and complement.

In addition to neuroinflammation, we also looked at Aβ trafficking to determine the intracellular route that Aβ follows to enter cells. When cells were stimulated with Aβ FRET studies showed associations of TLR4 with TLR2 as well as CR3 and C5aR1. To determine whether Aβ internalizes with these receptor cluster, we labeled intracellular organelles for early and late endosome as well as lysosome at different time points since previous studies have shown that the lysosomal system can play a role in Aβ trafficking (Zheng et al. 2012). Our results showed that Aβ internalises via early endosomes (EEA1) and associates with TLR4 and CR3 and then accumulates in lysosomes (LAMP1). This is constant with other studies since Aβ has been shown to accumulate within lysosomes, apparently promoting neuronal death through lysosomal destabilization (Nixon et al. 2000;

Ditaranto 2001). It is possible that the Aβ accumulation in the lysosome could be triggering oxidative stress and since inflammasome activation is triggered in response to various stress signals that's why there is inflammasome assembly in the Aβ plagues.

Chapter IV

Amyloid internalises along with the complement membrane attack complex within Rab5+ endosomes and triggers non-canonical NF-kB signalling resulting in NLRP3 inflammasome activation

4.1 Introduction

Alzheimer's disease (AD) is a neurodegenerative disorder and the most common cause of dementia in the elderly. Amyloid β (Aβ) accumulation in senile plaques is a principal event in the pathogenesis of Alzheimer's disease (Weiner and Frenkel 2006; Meyer-Luehmann et al. 2008), but the cellular events leading to plaque-induced neuronal dysfunction are still unclear. Genetic factors have been suggested to either cause or predispose to AD including mutation in β-amyloid precursor protein (APP) (Goate et al. 1991), which is the protein that generates Aβ by proteolytic processing, mutation in presenilins 1 and 2 (Sherrington et al. 1995; Rogaev et al. 1995), as well as polymorphism to apolipoprotein A and Complement receptor 1 (CR1) (Lambert et al. 2009). In addition to genetic factors, studies have suggested that inflammatory and immunological processes are not just mere bystanders, but that microglia and invading bone marrow derived mononuclear phagocytes are central in the initiation and progression of Alzheimer's disease (Weiner and Frenkel 2006). Therefore, it is now accepted that the innate immune system plays a crucial role in the development of the disease.

The innate immune system has been primarily viewed as the first line of defence against "danger" acting quickly by mounting an inflammatory response and eliminating the intruders. It employs pattern recognition receptors (PRRs), such as Toll like receptors (TLRs), Nod-like receptors (NLRs) and complement to detect "danger". The precise roles of PRRs in detecting and mounting responses against Aβ, and their roles in AD are not clear. TLR expression is upregulated in the AD brain, and in murine models of AD, TLR2 and TLR7 were found to be upregulated compared to controls (Letiembre et al. 2009). Furthermore, multiple TLR genes (1-8) were found to be present in microglia in post-mortem tissue from AD patients,

with varying levels of expression (Bsibsi et al. 2002). The increased expression of TLRs in AD has positioned them as potential key players in neurodegenerative mechanisms and disease progression.

The TLR4 gene has emerged as a candidate for susceptibility for AD. A common miss-sense polymorphism resulting in an adenine to guanine substitution 896 nucleotides downstream of the transcription start site causes the replacement of glycine for aspartic acid at amino acid 299 (Asp299Gly) and alters the extracellular structural domain of TLR4. This mutation attenuates TLR4 signalling in response to LPS (Arbour et al. 2000). Since its discovery, this polymorphism has been associated with decreased cardiovascular disease risk (Balistreri et al. 2004), aging (Balistreri et al. 2009) as well as decreased risk of late-onset AD in an Italian cohort (Minoretti et al. 2006; Balistreri et al. 2008), suggesting that pro-inflammatory responses triggered via TLR4 are crucial for disease progression. Recently, Tang et al. (Tang et al. 2008) using primary neuronal cultures from TLR4 mutant mice have shown that neurons expressing TLR4 have an increased sensitivity to Aβ and are vulnerable to degeneration in AD.

In addition to the implication of TLR4 in susceptibility to neurodegeneration, recent studies have suggested that TLR4 activation is required for clearance of Aβ in AD. Mice carrying a point mutation in TLR4 exhibit augmented deposition of Aβ in both the neocortex and hippocampus (Tahara et al. 2006). The mechanism by which TLR activation aids Aβ clearance is unclear.

The complement system is another pattern recognition arm of the innate immune system, providing protection from infection and resolution of injury. It recognises PAMPs; it tags them and eliminates the pathogen. It is one of the innate immune mechanisms considered to play a role in the death of the brain neurons in patients

with AD. Evidence for complement mechanisms in microglial responses to Aβ has been reported, but may not been fully appreciated. Microglia and other brain cells express the genes and/or proteins for virtually all classical and alternative pathway components (Walker et al. 1995; Walker and McGeer 1992). Both the classical and alternative pathways are directly activated by Aβ in an antibody-independent fashion (Daly and Kotwal 1998). The classical pathway has been shown to be directly activated in AD by both fibrillar Aβ deposits and neurofibrillary tangles (Shen et al. 2001; Rogers et al. 1992). All components of the classical pathway (Shen et al. 1997) and the alternative pathway (Strohmeyer et al. 2000) have been identified in neurons of the AD brain. The end product of the terminal pathway, the membrane attack complex (MAC), was also found in neuronal membranes of the AD brain (Webster et al. 1997). The presence of MAC in the AD brain suggests that C5a has been released since the formation of MAC is initiated by C5b, which is generated when C5a is cleaved from C5 by C5 convertase from either the classical or alternative pathways. These studies suggest the involvement of C5a as well as its receptor, C5aR1 in the neuroinflammation observed in AD. The expression of the C5aR1 in microglia cells of human and rodents supports this hypothesis (Gasque et al. 1997). A recent study by Fonseca et al. (Fonseca et al. 2009) has demonstrated that treatment with a C5aR1 antagonist decreases pathology and enhances behavioural performance in murine models of AD, suggesting that C5aR1 is a novel therapeutic target for AD.

Long before TLRs and complement had been implicated in autoimmune diseases, studies had demonstrated that neuroinflammation plays a key role in neurodegeneration in AD. Both complement and TLRs are swiftly activated in response to infection or danger and there is increasing evidence for extensive

bidirectional cooperation between the two systems, shaping the immune response through both synergistic and antagonistic crosstalk (Hajishengallis et al. 2010; Hajishengallis and Lambris 2016). TLRs and complement have been individually linked to AD. Aβ linked with AD can activate both types of pattern recognition sensors, but whether and how these two innate immune systems interact with each other in the context of AD has not been studied before. In this study, I aimed to answer some of the key questions that arise: 1) whether interplay between complement and TLR receptors is the key to the chronic inflammatory response observed in AD; 2) how are PRRs involved in Aβ clearance; and 3) whether the internalization and trafficking of Aβ is crucial for triggering a pro-inflammatory response.

4.2 Aβ-induced inflammatory responses in the presence of complement

To address the question whether the interplay between complement and TLR/NLR receptors is the key to the chronic inflammatory response observed in AD, we initially investigated what cytokine response is triggered in astrocytes by Aβ in the presence and absence of complement. Fibrillar Aβ (amino acids 1-42), as well as control non-fibrillary peptide (identical sequence but in reverse order) was obtained from American Peptide (USA) and prepared as previously described (Moore et al. 2002). Utilising an astrocytic cell line for these experiments, we stimulated astrocytes by incubating the cells with different concentrations of Aβ. Following incubation, supernatants were collected and frozen until they were analysed for their cytokine content. Pro-inflammatory cytokines were analysed using cytometric bead arrays (CBAs) from BectonDickinson. As demonstrated in the previous chapter (Figure 3.4.1.2), it was shown that IL-1β as well as IL-6 were triggered in response to Aβ (Figure 4.2 A and B). This response was greatly enhanced in the presence of normal human serum (NHS) as a source of complement, especially in the case of IL-1β, suggesting that complement augments Aβ-induced IL-1β secretion. An NHS titration was performed and 10% NHS was found to be the optimum concentration to be used. To verify complement activation, flow cytometry was used in order to confirm the presence of the MAC through positive staining for C9 neoepitope after activation of complement **(**Figure 4.2 C, open histogram), whereas cells treated with the equivalent concentration of heat-inactivated NHS (HI-NHS) were negative for MAC staining (Figure 4.3 C, grey histogram).

Furthermore, to confirm complement-involvement in the Aβ-induced IL-1β secretion, we stimulated the cells with Aβ in the presence of NHS depleted for complement terminal components. It was shown that when HI-NHS or C5-depleted NHS was used, the augmented IL-1β response was not observed, suggesting a role for the terminal pathway of complement components. In contrast IL-6, which is not dependent on inflammasome activation, was marginally enhanced by the addition of NHS, and there was not a significance difference when incubated with either heat inactivated NHS or C5-depleted NHS, suggesting that the terminal pathway of complement components does not play a role in IL-6 responses.

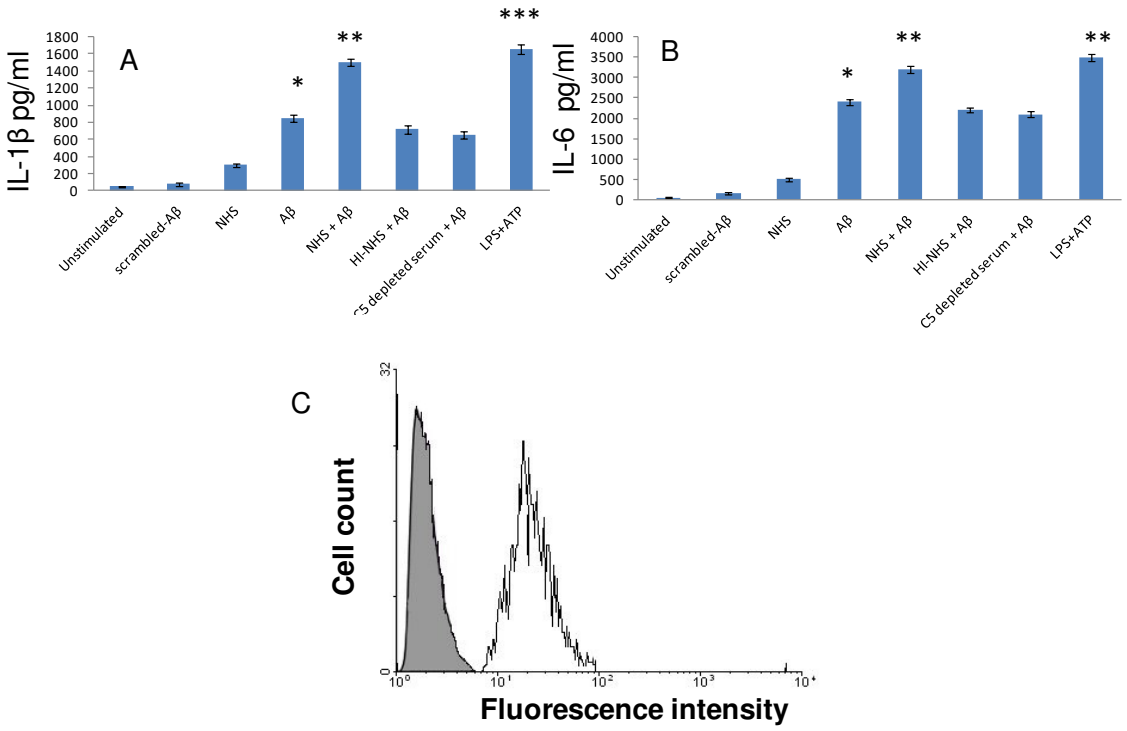


Figure 4.2 Aβ-induced inflammatory responses in the presence of complement. Human astrocytes were incubated with fibrillar Aβ in the presence and absence of normal human serum (NHS) for 6 h. The supernatants were harvested and assayed for cytokine contents using the Cytometric Bead Array (CBA) system (Becton Dickinson) (A, B). The level of MAC deposition at different doses of serum was revealed by mean fluorescence intensity upon staining for the anti-C9 neoepitope (white histogram) using flow cytometry (C). Fluorescence was detected using a

FACSCalibur (BectonDickinson). The data presented are the mean ± standard deviation (n=3) from three independent experiments. *P<0,05, **P<0,01, ***P<0,001 vs respective unstimulated.

4.3 Aβ triggers inflammatory responses via TLR2, TLR4 and NLRP3

To determine which PRRs are involved in triggering the inflammatory responses observed, we utilised RNA interference to knock down expression of TLRs and NLRs. Once we had confirmed knock-down of the receptors of interest in astrocytes cells, we proceeded to stimulate the cells with different concentrations of Aβ and measure pro-inflammatory cytokine using CBAs and compared the cytokine profile of normal and knocked down cells (Figure 4.3). TLR2, TLR4 and NLRP3 knock down reduced the Aβ induced IL-1β while NLRP1 knock down had no effect (as previously shown in 3.4.1.2. at times between 2-6 h). When cells were co-incubated with Aβ and NHS, knockdown of NLRP1 had no effect on IL-1β secretion, while knockdown of NLPP3 completely abrogated the induced IL-1β secretion. Knockdown of TLR2 and TLR4 only partially reduced the IL-1 secretion. Co-incubation of heat inactivated serum or C5-depleted serum with Aβ, resulted in a similar pattern of IL-1β secretion as incubation with Aβ on its own.

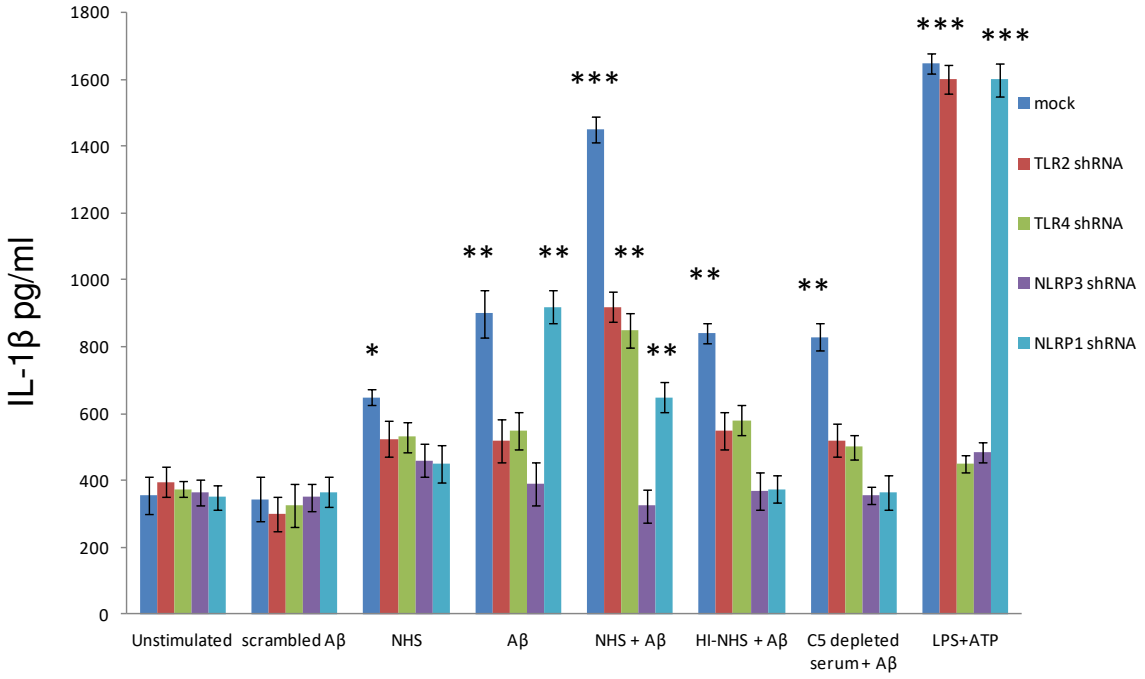


Figure 4.3 RNA interference reveals PRR involved in Aβ-induced IL-1β secretion. RNA interference was used to knock down expression of TLRs and NLRs, and subsequently the cells were stimulated with Aβ in the presence and absence of complement. The supernatants were harvested and assayed for cytokine contents using the CBA. The data presented are the mean ± standard deviation (n=3) from three independent experiments carried out in triplicate. *P<0,05, **P<0,01, ***P<0,001 vs respective unstimulated.

4.4 PRR associations induced in the presence of Aβ and complement

To shed light into PRR and Aβ interactions we utilised fluorescence resonance energy transfer (FRET). FRET is a non-invasive imaging technique used to determine molecular proximity. FRET can occur over 1-10 nm distances, and effectively increases the resolution of light microscopy to the molecular level (Stryer 1978). It involves non-radiative transfer of energy from the excited state of a donor molecule to an appropriate acceptor. FRET was measured in terms of dequenching of donor fluorescence following complete photo-bleaching of the acceptor fluorophore.

Using FRET, we investigated TLR4 heterotypic associations on the cell surface of human astrocytes in response to Aβ. We found that following Aβ stimulation, TLR4 co-clustered with TLR2 (E = 25 ± 1.1%) and to some extent C5aR1 (E = 16 ± 1.2%), which was enhanced in the presence of complement (NHS) (E = 32 ± 0.5 and 35 ± 0.9%, respectively) (Figure 4.5). C5 depleted serum did not enhance the effect of Aβ, suggesting the involvement of activation of the terminal pathway of Complement.

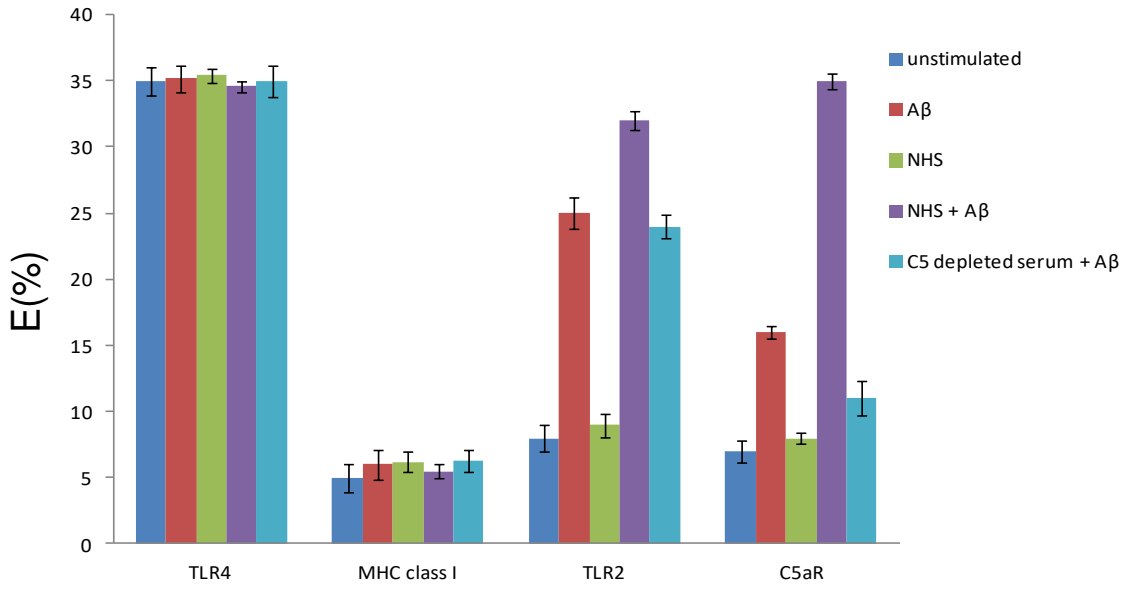


Figure 4.4 PRR associations induced in the presence of Aβ and complement. FRET measurements between TLR4 and different receptors in response to Aβ. Human astrocytes were stimulated with Aβ for 30 min and subsequently fixed. Energy transfer between TLR4 (Cy3) and the different receptors (Cy5), was measured from the increase in donor (Cy3) fluorescence following acceptor (Cy5) photobleaching. The percentage of energy transfer (E%) and standard deviation were calculated from three independent experiments performed in triplicate.

4.5 Aβ in the presence of complement triggers non-canonical NF-kB signalling

In Chapter 3, the internalization of Aβ was investigated. It was shown that Aβ internalizes in endosomal compartments. In order to determine whether signalling is triggered from these locations, Rab5+ containing endosomes were labelled using fluorescent antibodies/dyes as well as signalling molecules in the presence of Aβ. Interestingly, it was shown that NF-kB-inducing kinase (NIK) which leads to the activation of the non-canonical NF-kB pathway, was recruited to Rab5+ containing endosomes in response to Aβ and complement (10% NHS) (Figure 4.5 A, top row), but not when cells were stimulated with Aβ and 10% HI-NHS (Figure 4.5A, bottom row). To verify these findings, astrocytes were stimulated with Aβ in the presence of complement and lysed to perform western blotting. Western blotting confirmed that following Aβ stimulation in the presence of 10% NHS as a source of complement, non-canonical NF-kB signalling is triggered (Figure 4.5 B). NIK could only be detected in the presence of NHS, whereas when the complement terminal components were removed from the serum, NIK activation was inhibited (Figure 4.5 B), suggesting that it is only recruited in the presence of complement.

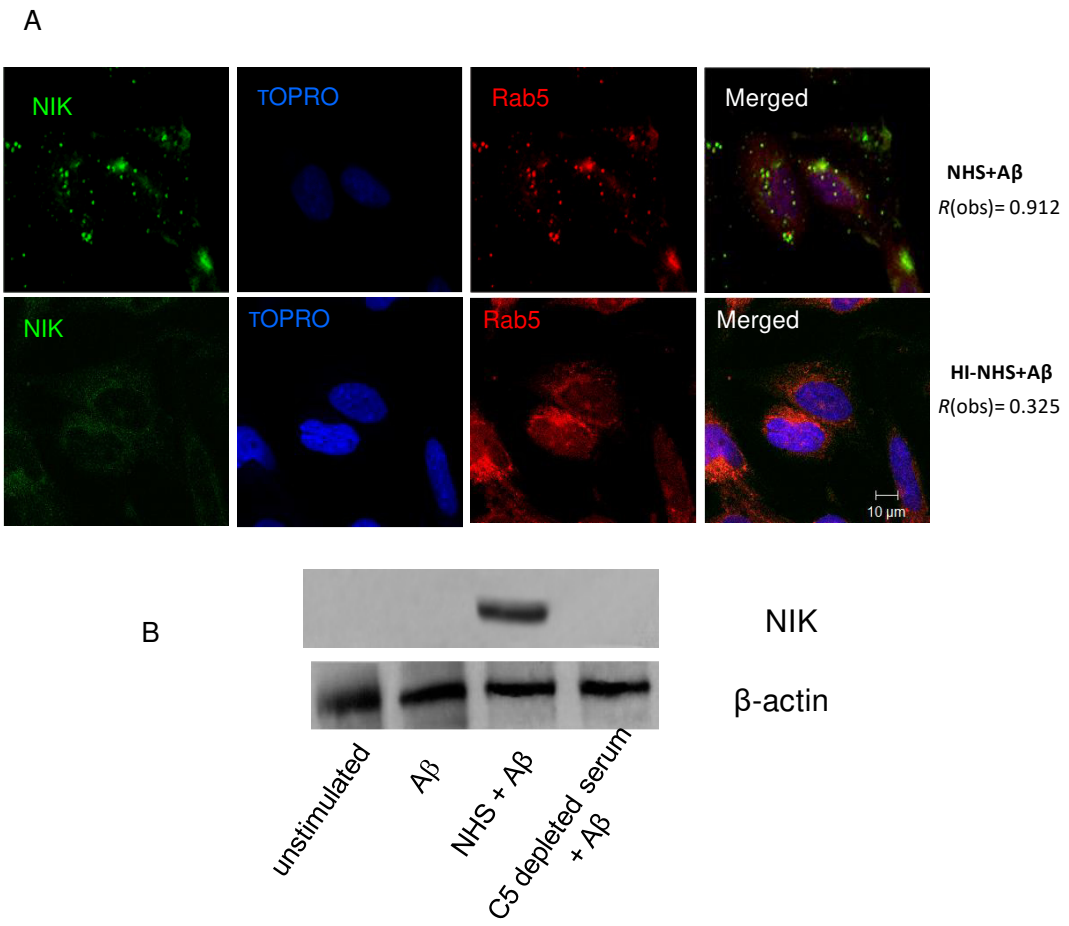


Figure 4.5 Aβ in the presence of complement triggers non-canonical NF-kB signalling. (A) Human astrocytes were stimulated with Aβ, in the presence of 10% NHS or HI-NHS for 30 min. The cells were fixed and labelled with primary and secondary antibodies with the appropriate fluorescent dye (A) or lysed and analysed using western blotting (B). Fluorescent images were collected using a Zeiss 710 Confocal microscope. The data are representative of three independent experiments.

4.6 Clearance of fibrillar Aβ through its internalization and trafficking, is crucial for triggering a non-canonical NF-kB signalling via Rab5+ containing endosomes

To investigate the recruitment of NIK to Rab5+ containing endosomes, we labelled human astrocytes that had been stimulated with Aβ in the presence of NHS for PRRs, NIK and Rab5+. It was shown that within 30 min following Aβ stimulation, C9, a component of the membrane attack complex (MAC), was found to co-localise with NIK within the Rab5+ containing endosomes (Figure 4.6, top row). In addition, TLR4 and Aβ were found to localised in these endosomes at the same time (Figure 4.6, middle and bottom row). In the presence of HI-NHS (Figure 4.6, second row), there was no MAC deposition/internalization and neither was co-localisation of NIK in endosomal vesicles.

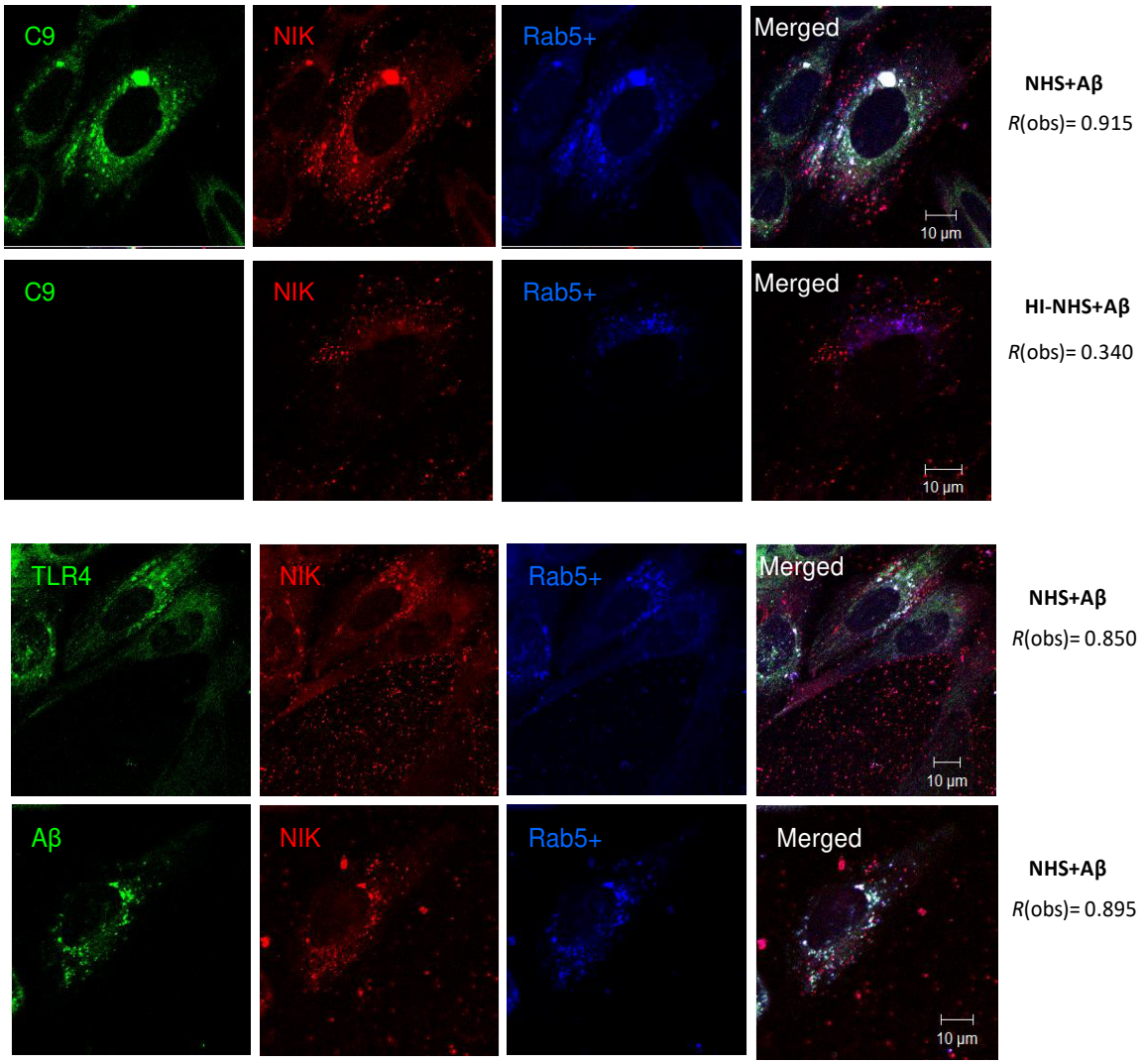


Figure 4.6 Internalization of Aβ in the presence of complement triggers non-canonical NF-kB signalling. Human astrocytes were stimulated with Aβ, either in the presence of 10% NHS or 10% HI-NHS for 30 min. The cells were fixed and labelled with primary and secondary antibodies with the appropriate fluorescent dye. Fluorescent images were collected using a Zeiss 710 Confocal microscope. The data are representative of three independent experiments. Bars 10 µm. The data represent the means of three independent experiments. Co-localization coefficients [*R*(obs)] were calculated using Costes' approach.

4.7 Conclusion

Aβ linked with AD can activate TLRs/NLRs and complement, but whether and how these two parts of the innate immune systems interact with each other in the context of AD has not been studied before.

In this study, we set out to investigate the effect of the complement system in Aβ-induced inflammasome activation. Initially we investigated whether Aβ could trigger IL-1β responses and it was shown that Aβ could trigger some inflammasome activation. Interestingly, this activation was enhanced in the presence of human serum as a source of complement, which seemed to be dependent of the terminal pathway activation, since serum depleted for C5 did not enhance the Aβ-induced IL-1β response. This is in agreement with recent publications, demonstrating how crystal aggregates (An et al. 2014; Samstad et al. 2014; Pilely et al. 2016), such as MSU or cholesterol crystals, can trigger inflammasome activation in the presence of complement and in particular C5a.

Since inflammasomes seem to require two signals for activation, one signal via a PRR that leads to the production of pro-IL-1β and pro-IL-18 in the cytosol and a second signal that leads to the assembly of the oligomeric inflammasome structure that activates caspase-1 leading to the processing of pro-IL-1β/18 into IL-1βand IL-18 (Stutz et al. 2009); we proceeded to investigate which PRRs are involved in triggered Signal 1 and Signal 2 of inflammasome activation in the presence of Aβ and complement.

Using RNA interference, we demonstrated that both TLR4 and TLR2 are able to contribute to Signal 1 of inflammasome activation, whereas NLRP3 seems to be the NLR that forms the inflammasome in response to Aβ and complement. This is in agreement, with recent literature where complement components have been

shown to be able to enhance innate immune responses and induced inflammasome activation and in particular NLRP3 activation (An et al. 2014; Samstad et al. 2014; Pilely et al. 2016; Laudisi et al. 2013; Triantafilou et al. 2013). Thus, NLRP3 seems to be the NLR that is involved in most complement-induced inflammasome responses.

Since RNA interference had demonstrated the involvement of TLR2 and TLR4 on the cell surface, we proceeded to investigate the molecular interactions of these PRR on the cell surface following stimulation with Aβ in the presence of complement. For these experiments, we utilised fluorescence resonance energy transfer (FRET), to study molecular associations. Interestingly, Aβ in the presence of NHS induced heterotypic association of TLR2 and C5aR1 with TLR4, NHS on its own had no effect, but when co-incubated with Aβ, it enhanced the effect of Aβ. C5 depleted serum did not enhance the effect of Aβ, suggesting the involvement of activation of the terminal pathway of the complement system.

We proceeded to investigate using confocal microscopy, what happens to the oligomeric structures that are formed in response to Aβ+NHS on the cell surface. It was shown that TLR4/ C5aR1/Aβ complexes internalized within 30 min and are targeted to Rab5+ containing endosomes, stabilising NF-kB-inducing kinase (NIK) and leading to the activation of the non-canonical NF-kB pathway, suggesting that internalization and signalling are interlinked in the case of Aβ. Surprisingly, complement was found to internalize along with Aβ and TLR4 in Rab5+ endosomes, and recruited NIK to these endosomes. This recruitment of the non-canonical NF-kB pathway appeared to be complement-dependent as it was inhibited in the absence of terminal complement components. This is in agreement with a recent paper that has demonstrated that MAC complexes activate an

Akt+NIK+ signalosome (Jane-Wit et al. 2015) in human endothelial cells. Our study, is the first study to identify such signalosomes in Rab5+ endosomes in response to complement and Aβ, suggesting new possible therapeutic targets for Aβ-induced neuroinflammation.

Chapter V

Inhibition of inflammatory responses by antagonising PRRs involved in neuroinflammation

5.1 Introduction

Astrocytes and microglia express various immune receptors, including Toll-like receptors (TLRs) and complement receptors, to identify pathogen-associated molecular patterns (PAMPs) on invading organisms and mediate the immune response of the host. The activation of TLRs leads to the production of cytokines, which may either assist in the protection of the central nervous system (CNS) from pathogens (Pan et al. 2011), or have a damaging effect on neurons. For example, HIV1-associated dementia is characterized by neuronal apoptosis which is believed to be the result of cytokine secretion, such as interleukin (IL)-1β, IL-6 and tumour necrosis factor (TNF)-α, by astrocytes and microglia (Kaul 2009). Furthermore, an immune response in the CNS was demonstrated by a previous study to be involved in the neurodegeneration of certain neurological diseases, such as Alzheimer's disease (AD) (Whitney et al. 2009). The accumulation of extracellular fibrils of amyloid-β (fAβ) and intracellular neurofibrillary tangles in neurons lead to the formation of senile plaques. These events result in the neurodegeneration observed in AD (Weiner and Frenkel 2006).

For certain cytokines, it may be beneficial to inhibit their activity. For example, IL-1β may induce excito-neurotoxicity by activating the tyrosine phosphorylation of N-Methyl-D-aspartate (NMDA) receptors, leading to an increase in the levels of intracellular Ca^{2+} and loss of cultured hippocampal neurons (Viviani et al. 2006). On the other hand, the IL-6 cytokine was demonstrated to have a neuroprotective role against an NMDA-induced death of the cerebellar neurons (Wang et al. 2009a). These data indicate that pro-inflammatory cytokines may be either neuroprotective or neurotoxic via the modulation of neurotransmission. fAβ activates the classical and alternative pathways of complement with consequent

C3 activation, C5a production and membrane attack complex (MAC) formation (Bradt et al. 1998). The role of the complement system in the removal of the infectious pathogen occurs through the activation of a variety of receptors including CR1, CR3 and C5aR1, and certain of these receptors serve a prominent role in the inflammatory response induced in AD (Crehan et al. 2012); not to mention the pro-inflammatory effects that complement might be contributing to neuroinflammation.

As already mentioned, IL-1β seems to be playing a critical role in neuroinflammation but how it is triggered in AD is unclear. Aβ could be triggering inflammasome activation (mainly Signal 1), but the questions that remain are what is the crosstalk of complement with inflammasome in AD and in particular what could be triggering Signal 2 for inflammasome activation? Several ions have been suggested to be able to trigger Signal 2, Reactive oxygen species (ROS) and Ca^{2+} are at least two of those (Dostert et al. 2008; Segovia et al. 2012). Both ions can be linked to AD, but the question remains whether they are involved in Aβ-induced responses in the presence of complement.

ROS is produced in numerous normal and abnormal processes in humans, including asthma, cancer, and aging. In addition, the basal levels of ROS production in cells may be associated with other physiological functions, such as cell proliferation, apoptosis, and homeostasis (Lambeth 2004). Excessive ROS production (above basal levels) impairs and oxidizes DNA, lipids, sugars, and proteins, thus leading to the dysfunction of these molecules and cell apoptosis. A leading theory of the cause of aging indicates that free radical damage and oxidative stress serve an important role in the pathogenesis of AD. This is because the brain utilizes 20% more oxygen than other tissues that also undergo

mitochondrial respiration. Cellular oxidative stress, including augmentation of protein oxidation, was demonstrated in AD in response to Aβ, and it is believed that treatment with anti-oxidant compounds may provide protection against oxidative stress and Aβ toxicity (Park et al. 2005).

Furthermore, Ca^{2+} serves an important role in the normal functioning of cells. Deregulation of Ca^{2+}-mediated signalling has been implicated in numerous neurodegenerative diseases, including AD. The amyloid precursor protein (APP) was demonstrated to alter the Ca^{2+} homeostasis, and lead to synaptic dysfunction and dendritic spine loss in neurons (Carafoli 1991). Aβ and the presenilins identified in AD have an effect on Ca^{2+} homeostasis during the early stages of the disease development. Thus, the synaptic transmission and function is affected prior to the neurotic plaque development. An alteration in the Ca^{2+} may provide Signal 2 for inflammasome activation and induce inflammatory responses, protein modifications and neuronal degeneration. As functional synapses and synaptic transmission are fundamental processes in memory formation, alterations in these processes may result in neuronal dysfunction and memory deficit, such as observed in AD (LaFerla 2002). It is thus important to identify whether a Ca^{2+} inhibition, in combination with other inhibitors has a beneficial effect in astrocytes in response to Aβ.

At present, there are no drug treatments that can cure AD or any other common type of dementia. To be able to develop AD-specific therapeutic interventions, we need to decipher the exact molecular mechanisms that are involved in Aβ-induced inflammatory responses. So far, our study has demonstrated that complement and in particular the terminal pathway can augment Aβ-induced responses, but which

part of the terminal pathway plays a role in triggering Signal 1 or 2 of inflammasome activation is something that we have to address.

In this chapter, we aim 1) to decipher which part of the terminal pathway of the complement system contributes to the enhanced Aβ-induced inflammasome responses; 2) how is Signal 2 being triggered in the presence of NHS+Aβ; and 3) how can we inhibit the NHS+Aβ-induced inflammasome response as a potential therapeutic intervention.

5.2 C5a augments Aβ-induced IL-1β production

Our data so far suggested complement-inflammasome interactions both *in vitro* (astrocytes) and *in vivo* (AD brain tissue). In the AD brain, we demonstrated NLRP3, C9 and amyloid co-localization (see Chapter III). We have shown that Aβ is capable of delivering signal 1 (NF-kB activation) and non-canonical NF-kB in the presence of complement (see Chapter IV), but which complement components are involved in triggering Signal 1? Since they co-localize, could complement component(s) released upon complement activation also be potential triggers? We therefore focused on the effect of C5a, the most potent anaphylatoxin, on inflammasome activation in astrocytes. To determine the role of C5a in Aβ recognition, we investigated the secretion of IL-1β in astrocytes upon a 6-h stimulation with C5a, Aβ and combinatory treatment of C5a+Aβ (Figure 5.2). The results demonstrated that C5a stimulation resulted in increased IL-1β secretion compared with the unstimulated astrocytes. Furthermore, an augmented effect was observed in the combinatory treatment of C5a+Aβ compared with the individual treatments of C5a or Aβ (Figure 5.2). This was even more pronounced if cells were “primed” (or pre-treated) with C5a 30 min prior to exposure to Aβ (Figure 5.2); thus, demonstrating that the effect is synergistic, rather than additive.

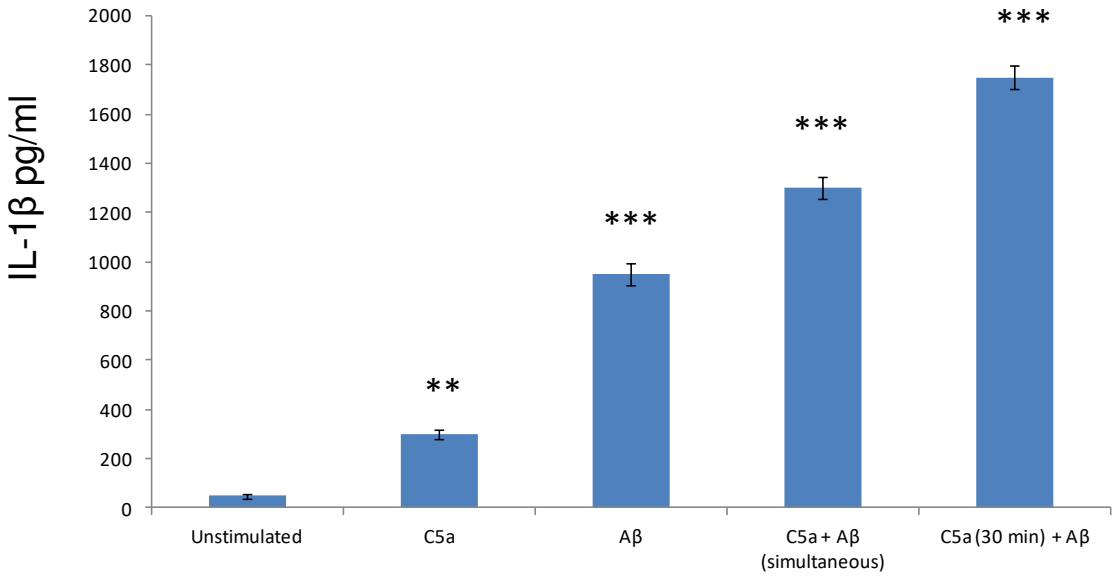


Figure 5.2 Aβ-induced production of IL-1β. To determine the role of C5a in Aβ recognition, the secretion of IL-1β in human astrocytes was investigated upon stimulation with C5a, Aβ and a combination of C5a+Aβ. Human astrocytes were stimulated with 50ng/ml C5a and 10mg/ml Aβ for 6h. Cells were either pretreated with C5a for 30 min and subsequently Aβ was added, or both C5a and Aβ were added simultaneously. The supernatants were harvested and assayed for cytokine contents using the Cytometric Bead Array (CBA) system (Becton Dickinson). The data presented are the mean ± standard deviation (n=3) from three independent experiments carried out in triplicate. *P<0,05, **P<0,01, ***P<0,001 vs respective unstimulated.

5.3 C5a triggers Signal 1

To identify whether C5a triggers signal 1, we run western blots probing for pro-IL-1β. Human astrocytes were stimulated with C5a, Aβ and a combinatory treatment of C5a and Aβ for 6 hours. Membranes were probed with Pro-IL-1β to identify whether Signal 1 was activated.

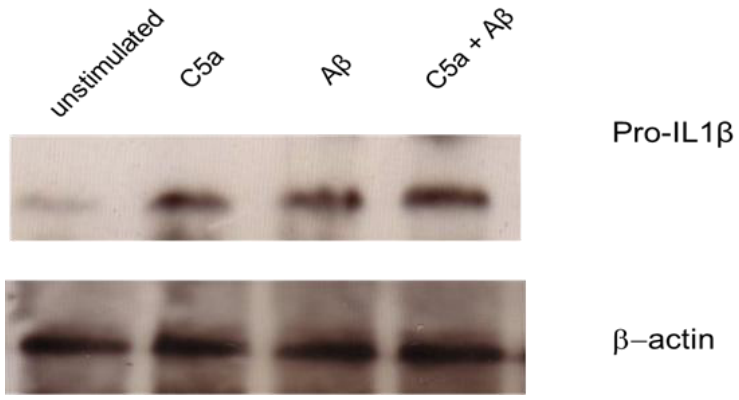


Figure 5.3 C5a triggers Signal 1. To identify whether C5a triggers Signal 1, western blotting was utilized, probing for pro-IL-1β. Human astrocytes were stimulated with 50ng/ml C5a and 10mg/ml Aβ for 6 hours. Pro-IL-1β was present in C5a-, Aβ- and C5a + Aβ-stimulated astrocytes, and not in unstimulated astrocytes. This is a representative from three independent experiments.

The results demonstrated that Pro-IL-1β was present in the C5a-, Aβ- and C5a+Aβ-stimulated astrocytes, and not in unstimulated astrocytes. Therefore, C5a was able to trigger Signal 1.

5.4 C5a-induced IL-1β release is C5aR1 and caspase-1-dependent

To determine whether the effect of C5a is through the C5aR1 and not the C5aR2, anti-C5aR1 (10μg/ml; Cambridge Biosciences), as well as control mouse IgG (7ug; Cambridge Biosciences; relevant IgG added as control) was used. Cells were pre-treated for 30 min with the blocking antibody prior to challenge with C5a as well as C5a+Aβ (Figure 5.4). The data clearly demonstrates that the effects observed are C5aR1 dependent, as pre-treatment with the blocking antibody inhibited C5a-induced responses, whereas the control IgG had no effect.

In addition, we proceeded to investigate whether the C5a effects observed are caspase-1 dependent. Z-YVAD-FMK (10μM; Abcam; irreversible caspase-1 inhibitor, cell-permeable) was utilized to determine whether the secretion of IL-1β, following C5a and Aβ stimulation, was caspase-1-dependent. The inhibitor was added (at the concentrations mentioned above) to the human astrocytes for 30 minutes prior to the addition of C5a and/or Aβ for 6 hours. A viability test was performed using Trypan blue (see Chapter II) to verify that the selected concentrations of the inhibitors did not kill off the cells. Cells remained unaffected when the inhibitors were used in the concentrations mentioned above.

It was shown that Z-YVAD-FMK was able to inhibit IL-1β responses triggered by either C5a, Aβ, or a combination of both, demonstrating that the inflammasome activation observed is caspase-1-dependent.

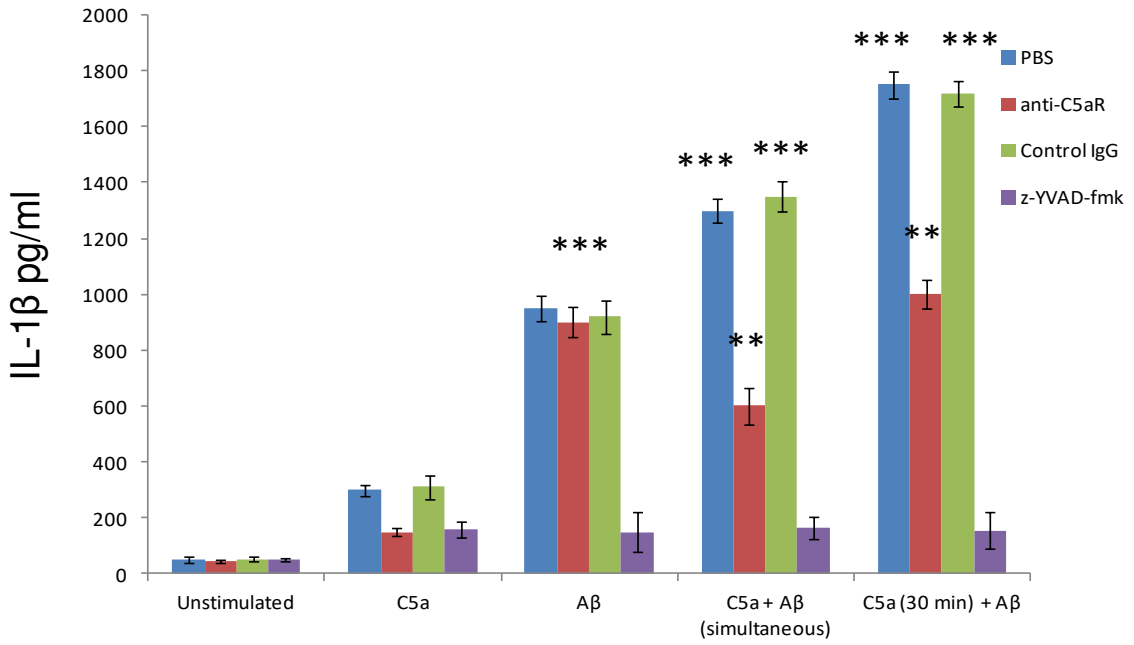


Figure 5.4 C5a-induced IL-1β release. Inhibitors (anti-C5aR1, control hIgG and Z-YVAD-fmk) and PBS were added for 30 min to the human astrocytes and then accordingly C5a, Aβ and C5a+Aβ were added for a total of 6 h. The supernatants were harvested and assayed for cytokine contents using the Cytometric Bead Array (CBA) system (Becton Dickinson). The data presented are the mean ± standard deviation (n=3) from three independent experiments carried out in triplicate. *P<0,05, **P<0,01, ***P<0,001 vs respective unstimulated.

The results demonstrated that following C5a stimulation, the Ca^{2+} inhibition using BAPTA as well as the ROS inhibition with DPI reduced IL-1β secretion significantly compared with the PBS treatment (Figure 5.4). In contrast, the CA-074 Me treatment had no effect on the IL-1β secretion compared with the PBS treatment. In contrast in Aβ (alone) stimulation, BAPTA and DPI treatments had no effect on the IL-1β secretion, compared with the PBS treatment. However, treatment with CA-074 Me inhibiting Cathepsin B resulted in decreased levels of IL-1β compared with the other treatments (Figure 5.4), suggesting that Aβ must be triggering Signal 2 via lysosomal damage and Cathepsin B release.

As expected, in the combinational treatments with both C5a+Aβ stimulation, ROS, Ca2+ as well as Cathepsin B inhibition seemed to be effective in reducing IL-1β secretion compared with the PBS treatment (Figure 5.4). Cathepsin B had the highest effect, with ROS inhibition being the least effective of the three inhibitors, suggesting that Cathepsin B and Ca2+ are the main triggers of Signal 2 for inflammasome activation.

5.5 C5a-induced IL-1β release following Signal 2 stimulation: Ca^{2+} and lysosomal damage

Thus far, C5a was demonstrated to augment Aβ-induced IL-1β production, trigger Signal 1 (pro-IL-1β activation) and be caspase-1-dependent (caspase inhibitor blocked IL-1β production). To further investigate whether C5a triggers Signal 2, we utilized certain inhibitors, as follows: (i) BAPTA-AM, membrane-impermeable Ca^{2+} chelator, (ii) DPI (NADPH oxidase inhibitor diphenylene iodonium chloride), reactive oxygen species (ROS) inhibitor, and (iii) CA-074 Me, cathepsin B inhibitor (lysosomal damage). Previous studies utilized inhibitors that reportedly discriminate between cathepsin B and related lysosomal cysteine proteinases, and have implicated the enzyme in a wide range of physiological and pathological processes. The most popular substance to selectively inhibit cathepsin B *in vivo* is CA-074 Me, the methyl ester of the E-64 derivative CA-074.

Similar to the above experiments, the inhibitors Cathepsin B (CA-074; 100 μM), DPI (20 μM) and BAPTA (30 μM; all from Sigma-Aldrich) were added to the human astrocytes for 30 minutes, and then added accordingly C5a and/or Aβ for a total of 6 hours.

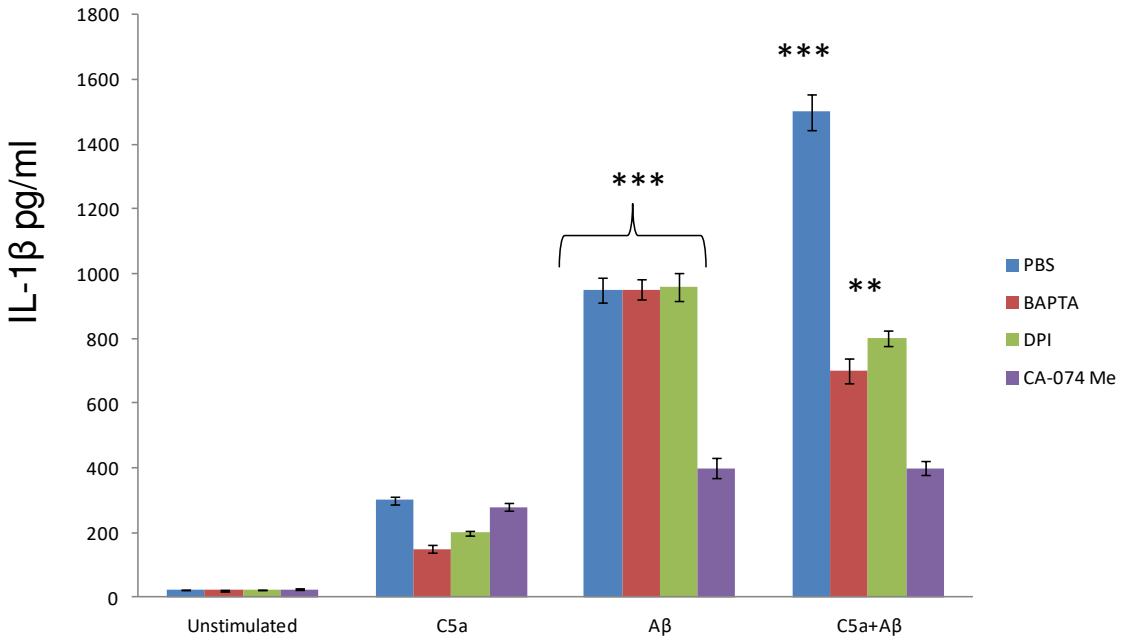


Figure 5.5 C5a-induced IL-1β release. Inhibitors (BAPTA, DPI and CA-074 Me) and PBS were added for 30 min to the human astrocytes, and then accordingly C5a, Aβ and C5a+Aβ were added for a total of 6 h. The supernatants were harvested and assayed for cytokine contents using the Cytometric Bead Array (CBA) system (Becton Dickinson). The data presented are the mean ± standard deviation (n=3) from three independent experiments carried out in triplicate. *P<0,05, **P<0,01, ***P<0,001 vs respective unstimulated.

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5.6 Therapeutic interventions for AD: the MCC950 inhibitor and PMX53 antagonist

Following the identification of the PRRs involved and the signalling cascades, the next step was to target them therapeutically. We decided to test inhibitors for inflammasome, complement and combinations of both *in vitro*. A previous study demonstrated the development of MCC950, an inhibitor of NLRP3, and the possibility of being used as a potential therapeutic for inflammatory diseases (Coll et al. 2015). This inhibitor blocks canonical and non-canonical NLPR3 activation at nanomolecular concentrations, possibly by interacting directly with NLRP3.

PMX53 (complement inhibitor), which has already been shown to be beneficial for AD (Fonseca et al. 2009), was also utilized for the experiments. PMX53 belongs to a class of selective small cyclic peptides, which potently inhibit C5a effects. For example, PMX53 inhibits C5a-induced myeloperoxidase release from human neutrophils (March et al. 2004). This antagonist of C5aR1 is also effective in numerous inflammatory disease models in mice and rats in which the physiopathology depends on complement system activation, such as rheumatoid arthritis (Woodruff et al. 2002), inflammatory bowel disease (Woodruff et al. 2003) and neurodegeneration (Woodruff et al. 2006). It is possible that if PMX53 is used in combination with TLR/NLR antagonists, such as MCC950, it will be more beneficial. Since the activation appears to involve multiple receptors, the therapy should be combinational and not targeting single molecules. MCC950 was not demonstrated by Coll et al. (2015) to block K^+ efflux or $Ca2^+$ flux and targeting the NLRP3 may present certain advantages over the use of biologic inhibitors of IL-1β.

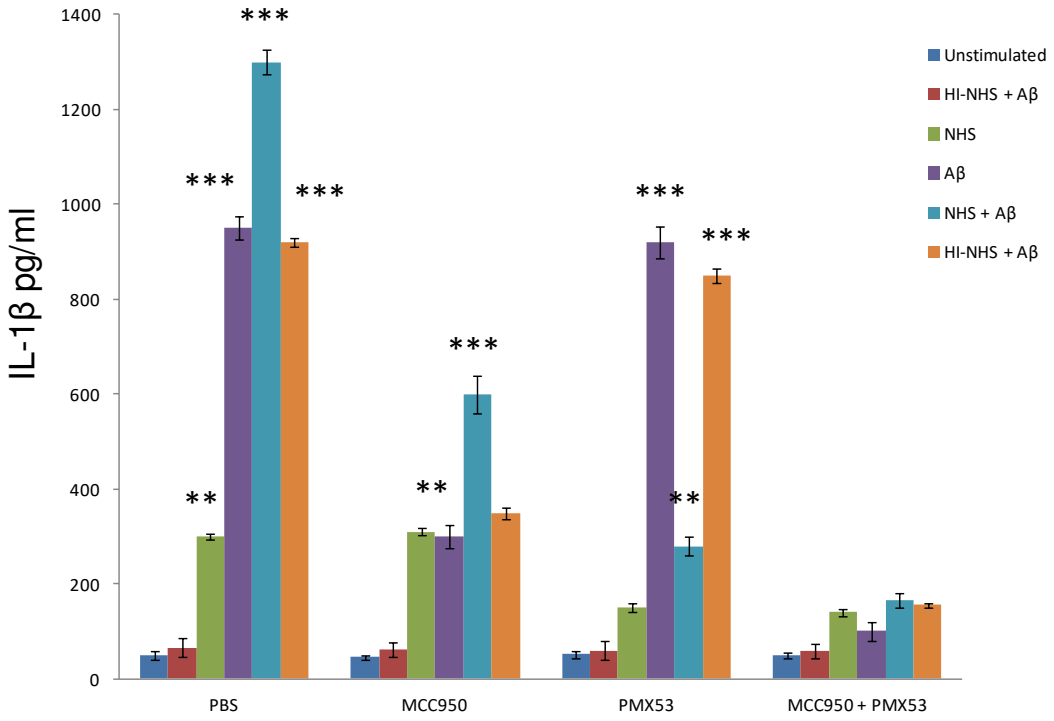

Figure 5.6 Complement-inflammasome inhibitors. Human astrocytes were incubated with 10% normal human serum (NHS), fibrillar Aβ and a combinatory treatment of NHS+Aβ, and heat-inactivated (HI)-NHS+Aβ for 6 h. PBS**,** 10 µM MCC950 (NLRP3 inflammasome inhibitor, Sigma, UK), 10 nM PMX53 (complement inhibitor, TOCRIS, UK) or a combinatory treatment of MCC950+PMX53 were added to the cells for 6 h. The supernatants were harvested and assayed for cytokine contents using the Cytometric Bead Array (CBA) system (Becton Dickinson). The data presented are the mean ± standard deviation (n=3) from three independent experiments carried out in triplicate. *P<0,05, **P<0,01, ***P<0,001 vs respective unstimulated.

MCC950 inhibition of NLRP3 had no effect on the NHS induced IL-1β secretion, while it largely prevented the Aβ induced IL-β secretion in all treatments containing Aβ, however the NHS+Aβ induced IL-1β secretion was least affected (Figure 5.5). In contrast PMX53, the C5aR1 antagonist, prevented the NHS and NHS+Aβ induced IL-1β secretion but not the Aβ induced secretion, demonstrating that C5a generated in the NHS was responsible. PMX53 had a stronger effect on the NHS+Aβ than expected. We were expecting that only the NHS component would have been affected, but it seemed to have a very strong inhibition, possibly

because the response is synergistic and not additive, it is possible that the complement component is contributing more to the inflammatory response.

A combination of both MCC950 and PMX53 was able to inhibit IL-1β secretion in response to any of the stimulations (Aβ alone or in combination with complement) (Figure 5.5), suggesting that a combinational therapeutic intervention targeting both the inflammasome and the complement system might be the most beneficial.

5.7 Conclusion

The pathogenesis of AD and other neurodegenerative diseases is characterised by inflammatory processes, which may be triggered by molecules of the NOD-like receptor (NLR) family, such as NLRP3. NLRP3 may form the inflammasome complex and upon activation leads to a cascade of events resulting in the secretion of cytokines, such as IL-1β. The irregular activation of the inflammasome is observed in cases of AD patients, hence targeting the specific molecule is of great interest.

Previous studies identified NLRP3 inhibitors, however the MCC950 inhibitor is more potent and specific than other NLRP3 inhibitors and has several advantages which are listed below. Coll et al. (2015) demonstrated that the MCC950 inhibitor overcomes the side effects of other anti-inflammatory drugs commonly used and observed that it blocks the production of inflammatory factors in blood samples from patients with a severe inflammatory disorder. In addition, MCC950 may be orally administered, hence has pharmaceutical potential as a therapeutic drug.

IL-1β maturation may be mediated by a number of different enzymes, such as serine proteases (Latz et al. 2013). MCC950 was demonstrated to not block the major antimicrobial inflammasomes NLRC4 and NLRP1, therefore a specific targeting of NLRP3 will not result in the complete blockade of IL-1β *in vivo* during infection (Coll et al. 2015). MCC950 may have less immunosuppressive effects compared with biologics, such as canakinumab, and a shorter half-life (Dinarello and van der Meer 2013). Thus, it may be withdrawn should unwanted effects, such as infections, occur. Furthermore, MCC950 is a small molecule and may be more cost effective compared with other biologic agents (Fautrel 2012). In conclusion,

the clinical progression of MCC950 or other derivatives may lead to the development of novel anti-inflammatory therapies for Alzheimer's disease.

In the case of complement, over the last decades, its role in the genesis of the inflammatory process and inflammatory diseases, including AD, has been gaining increasing attention (Sarma et al. 2006). One of the main effector components of the complement system is the anaphylatoxin C5a: one of the most potent inflammatory peptide mediators binding to its G-protein-coupled receptor (C5aR1) present in inflammatory cells (Werfel et al. 1992). C5a is a potent neutrophil chemoattractant and induces an increase in oxidative burst, phagocytosis, and release of granule enzymes (Hetland et al. 1998).

In the current study, the data demonstrated the importance of C5a in Aβ-induced inflammasome responses, supporting the hypothesis that the inhibition of C5a-induced inflammation via its receptor, C5aR1, reduces the secreted IL-1β levels. C5a was found to be able to "prime" the cells, especially when added prior to the addition of Aβ, and subsequently augment the inflammasome response. Therefore, the interaction of C5a+Aβ seems to be synergistic and not additive. Single 1 (priming) of inflammasome activation is delivered via C5a- C5aR1 axis, whereas Signal 2 seems to be calcium and Cathepsin-B dependent. Calcium must most likely be triggered by C5a, since it has been shown that C5a stimulates calcium influx (Monk and Partridge 1993). In the case of Cathepsin-B release, we believe that it must be attributed to the Aβ. Internalization of Aβ must eventually result in lysosomal damage and Cathepsin B release into the cytosol (Halle et al. 2008), thus both calcium and Cathepsin B release must be delivering Signal 2 of inflammasome activation.

Since both complement and Aβ contribute to the inflammasome activation observed, it would be beneficial for therapeutic interventions to be targeting both pathways. Thus, in this study, we tested inhibiting the complement system, the inflammasome and both. PMX53, which is a C5aR1 antagonist, was able to potently inhibit NHS+Aβ responses. It had a stronger effect on the NHS+Aβ than expected, as we were expecting that only the NHS component would have been affected, but it seemed to also affect the Aβ; possibly suggesting there is a synergistic effect of NHS+Aβ when added together (and the effect is not simply additive), that once you remove the complement component, you reduce most of the inflammation. This is in agreement with recent literature, that demonstrates that C5a in particular primes the cells for inflammasome activation (An et al. 2014; Samstad et al. 2014) and subsequently addition of crystals or aggregates (as it is in this case with the Aβ), further trigger the inflammasome response.

The secretion of IL-1β was further reduced in the combinatory treatment of PMX53+MCC950, indicating an interplay between complement and inflammasome, which agrees with the current literature as mentioned above. Therefore, suggesting that combinatory treatments to tackle the effect of Aβ in cells would be beneficial. This combinatory therapeutic approach is attractive due to the specificity of the pathways being inhibited, the potential for oral delivery for both drugs, the already successful Phase 1 testing of PMX53 in humans, and the fact that these drugs could be prescribed following clinical diagnosis of early stage AD. In conclusion, further investigation is required to identify the potential combinatory use of a C5a receptor antagonist and an NLRP3 specific inhibitor as a therapeutic for AD in humans.

Chapter VI

Discussion

Alzheimer's disease is a neurodegenerative disorder and the most common cause of dementia in the elderly. Patients show a gradual onset and progression of memory loss and other cognitive deficits. It is widely accepted that the extracellular accumulation of Aβ in senile plaques is a principal event in the pathogenesis of Alzheimer's disease (Meyer-Luehmann et al. 2008), but the cellular events leading to plaque-induced neuronal dysfunction are still unclear.

The innate immune system has been primarily viewed as the first line of defence against "danger" acting quickly by mounting an inflammatory response and eliminating the intruders, thus buying the adaptive immune system time to pick up momentum. The innate immune system employs PRRs, such as Toll like receptors (TLRs) and complement to detect "danger". Both complement and TLRs are swiftly activated in response to infection or danger and have both been linked to neuroinflammation in the AD brain. Neuroinflammation is proposed to play a key major role in AD pathogenesis. Neuroinflammation is concentrated at sites of Aβ plaques, which exhibit increased levels of pro-inflammatory cytokines, complement components and proteases (Akiyama et al. 2000; McGeer et al. 2006). Aβ plaques are surrounded and infiltrated by activated astrocytes and microglia, which are believed to be the major source of local inflammatory components (Akiyama et al. 2000). TLRs and complement have been individual linked to AD, but currently, there is increasing evidence for extensive bidirectional cooperation between the two systems, shaping the immune response through both synergistic and antagonistic crosstalk.

The complement system is a well-known powerful effector mechanism of the innate immune system, providing protection from infection and resolution of injury. It recognises PAMPs, quickly tags them and eliminates the pathogen. It is one of

the innate immune mechanisms considered to play a role in the death of the brain neurons in patients with AD. Evidence for complement mechanisms in microglial responses to Aβ has been reported, but may not been fully appreciated. Microglia and other brain cells express the genes and/or proteins for virtually all classical and alternative pathway components (Walker et al. 1992; Walker et al. 1995). As demonstrated by Linton and Morgan (1999) and Hawlisch et al. (2004), the complement system is involved in numerous inflammatory conditions. The effectors of the complement were originally believed to be mediators due to their capability of enhancing phagocytosis, hence protecting the host from pathogens (van Beek et al. 2003). As demonstrated later, the complement served a role in the physiopathology of numerous acute and chronic inflammatory diseases. For example, the complement can recruit and activating leukocytes in various diseases, including rheumatoid arthritis, primarily via C5a (Weissmann 2006).

The classical and alternative pathways are directly activated by Aβ in an antibody-independent fashion (Daly and Kotwal 1998). In particular, the classical pathway has been shown to be directly activated in AD by both fibrillar Aβ deposits and neurofibrillary tangles (Shen et al. 2001; Rogers et al. 1992). All components of the classical pathway (Shen et al. 1997) and the alternative pathway (Strohmeyer et al. 2000) have been identified in neurons of the AD brain. The end product of the terminal pathway, the membrane attack complex (MAC), was also found in neuronal membranes of the AD brain (Webster et al. 1997).

Long before TLRs and complement had been implicated in autoimmune diseases, studies had demonstrated that neuroinflammation plays a key role in neurodegeneration in AD. Both complement and TLRs are swiftly activated in response to infection or danger and there is increasing evidence for extensive

bidirectional cooperation between the two systems, shaping the immune response through both synergistic and antagonistic crosstalk. TLRs and complement have been individually linked to AD. Aβ linked with AD can activate both types of these receptors, but whether and how these two innate immune systems interact with each other in the context of AD has not been studied before. It is our hypothesis that deposition of Aβ peptide can activate both the TLR and complement systems and evoke Alzheimer's pathology.

The cells of the innate immune system can recognize molecules shared by groups of related microbes, PAMPs, which are essential for the survival of the invading organisms. Similarly to microglia, astrocytes have an essential role in the immune response against CNS pathogens. They are a major source of inflammatory chemokines, and TLRs have been proved to exist in astrocytes (Bsibsi et al. 2002; Bowman et al. 2003; Carpentier et al. 2005; Farina et al. 2005). It has been previously demonstrated that primary astrocytes express low levels of TLR2, TLR3, TLR5 and TLR9, while the expression of each TLR homolog was rapidly up-regulated, following exposure to its corresponding ligand (Kielian 2006). In addition, certain microbial components have been shown to up-regulate the expression of other TLR homologs instead of their corresponding TLR homologs (Jack et al. 2005; Konat et al. 2006), whereas the exposure of astrocytes to inflammatory cytokines may also augment TLR expression. The results of the above studies imply that cell activation by microbial components and/or inflammatory cytokines could possibly sensitize astrocytes by increasing the expression of PRRs. Therefore, subsequent to CNS damage or infection, this could increase the immune responses of astrocytes (Konat et al. 2006).

Furthermore, IL-1β is a key player associated with the development and onset of AD. It can be released by glial cells and activates the NF-kB pathway to up-regulate cytokine production. Increased levels of cytokines such as IL-1β, IL-6 and TNF-a have been previously demonstrated in cerebrospinal fluid samples of patients with AD, when compared to their age-matched healthy controls (Blum-Degen et al. 1995). In addition, increased levels of IL-1β have been observed in the circulation of AD patients (Licastro et al. 2000). IL-6 immunoreactivity was established in diffuse Aβ plaques of patients with AD by a previous study, and it was demonstrated to regulate neuronal survival and function (Gadient et al. 1997). Increased production of inflammatory compounds may lead to the neurodegeneration observed in AD. In the present study, IL-1β production was investigated in response to inflammasome activation induced by Aβ in astrocytes. Silencing different NLRs and TLRs allowed us to discover their involvement in inflammasome activation.

Initially we demonstrated that all PRRs of interest were present albeit at low levels in wt astrocytes, therefore astrocytes were able to detect and respond to a wide range of PAMPs and DAMPs. Our data demonstrated an increase of IL-1β in response to Aβ stimulation in wt astrocytes. Upon silencing the activity of NLRP3, TLR2 or TLR4, a decrease in IL-1β secretion was observed at 2, 4 and 6 h time points, compared to the level of secretion of either Aβ-stimulated wt or Aβ-stimulated shNLRP1 astrocytes. This further indicated the key role of NLRP3, TLR2 and TLR4 activity in the activation of the inflammasome and the recognition of Aβ.

One of the signalling cascades that can be triggered by Aβ is the NF-kB pathway. NF-kB resides in the cytoplasm and is associated with its inhibitory protein IkB.

Once stimulated, IkB is phosphorylated leading to proteasomal degradation. NF-kB then becomes free and is translocated into the nucleus leading to the transcription of cytokines. This is an essential step in inflammasome activation, as a Signal 1 (priming signal) is needed to produce pro-IL-1β and pro-IL-18 in the cytosol. TLRs act upstream of NF-kB activation and to identify whether the pathway was activated we probed for phospho-IkkB. In our study, it was shown that activation of TLR2 and TLR4 was essential to trigger Signal 1 of Aβ-induced inflammasome activation. This agrees with previous studies that have shown that TLR4 is linked to Aβ recognition.

The other signalling cascade that can be triggered by Aβ is Signal 2, leading to the activation of caspase-1 and the secretion of IL-1β and IL-18. To investigate whether inflammasome activation) we probed for caspase-1 p10. It was shown that NLRP3 was essential in activating the inflammasome in response to Aβ. The experiments confirmed that NLRP3, TLR2 and TLR4 play a major role in the recognition of Aβ, which agrees with recent studies that have shown that Aβ is linked to the NLRP3 inflammasome (Halle et al. 2008).

AD brain samples from previous studies have demonstrated increased expression of caspase-1 and activated NLRP3 inflammasome. In the present study, we observed that subsequent to stimulations with Aβ, NLRP3 was activated and neuroinflammation was present in the AD brain, as demonstrated by confocal microscopy. To identify NLRP3 and complement interaction in the AD brain, AD brain tissue from patients with AD and healthy controls was stained for NLRP3 and MAC (C9), demonstrating that NLRP3 and MAC co-localize in amyloid plaques of the AD brain, further indicating an inflammasome-complement interaction. The data indicated that our hypothesis was right and neuroinflammation is due to the

PRRs and complement. These findings are extremely novel, as the interaction of NLRP3 and C9 in the brain of individuals with AD has never been demonstrated before. This finding is significant as the link of inflammasome-complement interactions in the amyloid plaque demonstrates that both parts of the innate immune system are linked with AD, but questions remain as to which parts of the complement system are responsible for this interaction, how is Signal 1 and Signal 2 triggered and how can we inhibit it. Therefore, we proceeded to analyse further the exact molecular mechanisms that are involved.

When we started to investigate the innate immune responses in the presence of both Aβ and complement, it was demonstrated that Aβ-induced responses were augmented in the presence of complement. Aβ in the presence of complement appeared to induce the heterotypic interactions of TLR4 and C5aR1 on the cell surface, triggering Signal 1 of inflammasome activation.

These heterotypic complexes (TLR4/ C5aR1/Aβ) complexes internalized within 30 min and were found to be targeted to the Rab5+ containing endosomes. Interestingly, this internalization step was crucial for triggering signalling, as signalling molecules were found to be recruited to the endosome. In particular, the NF-kB-inducing kinase (NIK), which results in the activation of the non-canonical NF-kB pathway, was recruited to the endosomes, and was found to co-localise with Aβ, TLR4 and C5aR1, suggesting that internalization and signalling are interlinked in the case of Aβ – especially in the presence of complement. In addition, we demonstrated that complement internalized along with Aβ and TLR4 in Rab5+ endosomes, and recruited NIK to these endosomes, which is a finding that is extremely novel.

Although we had demonstrated a synergistic interaction between NHS as a source of complement and Aβ we had to decipher which part of the complement system was responsible for triggering the response. When we utilised C5-depleted serum, the IL-1b response was reduced, suggesting the terminal pathway of complement is the one responsible for augmenting the response. More specifically, when we utilised C5a purified complement components to stimulate the cells in the presence of Aβ, the results demonstrated that C5a stimulation resulted in increased IL-1β secretion compared with the unstimulated astrocytes. Furthermore, an augmented effect was observed in the combinatory treatment of C5a+Aβ compared with the individual treatments of C5a or Aβ. This was even more pronounced if cells were “primed” (or pre-treated) with C5a 30 min prior to exposure to Aβ; thus, demonstrating that the effect is synergistic, rather than additive.

C5a is the most potent anaphylatoxin and chemoattract and it has been implicated in many inflammatory conditions, thus it is not surprisingly that it seems to contribute to Aβ-induced inflammation. C5a triggers its functional effects by binding to two receptors, C5aR1 and C5aR2. It is thought that most functional effects of C5a occur via C5aR1 and the role of C5aR2 is a bit unclear (Llee et al. 2008). In our study, we wanted to identify which C5a receptor are the functional effects triggered by. Using function-blocking mAbs, it was demonstrated that the effects that we observed are triggered via the C5a- C5aR1 axis. Thus C5aR1, along with TLR2 and TLR4 is one of the receptors involved in triggering Signal 1 of inflammasome activation.

As already mentioned, the inflammasome requires two signals for activation, a priming signal (Signal 1) coming from an activated PRR (in this case TLR2/TLR4,

C5aR1) and an activating signal (Signal 2), which activates caspase-1 and catalyses the processing of pro-IL-1β into IL-1β. Although it is easier to determine Signal 1, Signal 2 seems to be rather diverse. It has been demonstrated to be triggered by K^+/Na^+ fluxes, as well as Ca^{2+} imbalances and the generation of reactive oxygen species (ROS). To determine the mechanism of inflammasome activation by Aβ in the presence of complement in our study we investigated various inhibitors that affect ion homeostasis in the cell. The inhibitors that we utilized are as follows: (i) BAPTA-AM, membrane-impermeable Ca^{2+} chelator, (ii) DPI (NADPH oxidase inhibitor diphenylene iodonium chloride), reactive oxygen species (ROS) inhibitor, and (iii) CA-074 Me, cathepsin B inhibitor (lysosomal damage).

The results demonstrated that following C5a stimulation, the Ca^{2+} inhibition using BAPTA as well as the ROS inhibition with DPI reduced IL-1β secretion significantly compared with the PBS treatment. In contrast, the CA-074 Me treatment had no effect on the IL-1β secretion compared with the PBS treatment.

In Aβ (alone) stimulation, BAPTA and DPI treatments had no effect on the IL-1β secretion, compared with the PBS treatment. However, treatment with CA-074 Me inhibiting Cathepsin B resulted in decreased levels of IL-1β compared with the other treatments, suggesting that Aβ must be triggering Signal 2 via lysosomal damage and Cathepsin B release.

As expected, in the combinational treatments with both C5a+Aβ stimulation, ROS, Ca2+ as well as Cathepsin B inhibition seemed to be effective in reducing IL-1β secretion compared with the PBS treatment. Cathepsin B had the highest effect, with ROS inhibition being the least effective of the three inhibitors, suggesting that

Cathepsin B and Ca2+ are the main triggers of Signal 2 for inflammasome activation.

Following the identification of the PRRs involved and the signalling cascades, the next step was to target them therapeutically. Since the irregular activation of the inflammasome was observed in cases of patients with AD, we decided to target specific molecules of interest. The presence of MAC in the AD brain suggests that C5a has been released since the formation of MAC is initiated by C5b, which is generated when C5a is cleaved from C5 by C5 convertase from either the classical or alternative pathways. Previous studies suggested the involvement of C5a as well as its receptor, C5aR1 in the neuroinflammation observed in AD. The expression of the C5aR1 in microglia cells of human and rodents supports this hypothesis (Gasque et al. 1997). A recent study by Fonseca et al. (Fonseca et al. 2009) has demonstrated that treatment with a C5aR1 antagonist decreases pathology and enhances behavioural performance in murine models of AD, suggesting that C5aR1 is a novel therapeutic target for AD.

Since both complement and Aβ contribute to the inflammasome activation observed, it would be beneficial for therapeutic interventions to be targeting both pathways. Thus, in this study, we tested inhibiting the complement system, the inflammasome and both. PMX53, which is a C5aR1 antagonist, was able to potently inhibit NHS+Aβ responses. It had a stronger effect on the NHS+Aβ than expected, as we were expecting that only the NHS component would have been affected, but it seemed to also affect the Aβ; possibly suggesting there is a synergistic effect of NHS+Aβ when added together (and the effect is not simply additive), that once you remove the complement component, you reduce most of the inflammation. This is in agreement with recent literature, that demonstrates

that C5a in particular primes the cells for inflammasome activation (An et al. 2014; Samstad et al. 2014) and subsequently addition of crystals or aggregates (as it is in this case with the Aβ), further trigger the inflammasome response.

The secretion of IL-1β was further reduced in the combinatory treatment of PMX53+MCC950, indicating an interplay between complement and inflammasome, which is in agreement with the current literature as mentioned above. Therefore, suggesting that combinatory treatments to tackle the effect of Aβ in cells would be beneficial. This combinatory therapeutic approach is attractive due to the specificity of the pathways being inhibited, the potential for oral delivery for both drugs, the already successful Phase 1 testing of PMX53 in humans, and the fact that these drugs could be prescribed following clinical diagnosis of early stage AD.

The last decade has witnessed an impressive growth of knowledge regarding the signaling pathways regulating glial biology and pathophysiology. In the present study we focused on some of the important examples, such as complement and inflammasome pathways, calcium efflux, reactive oxygen species (ROS) presence and lysosomal damage leading to inflammasome activation. These factors serve essential and multifaceted roles in maintaining homeostasis in brain cells. In addition, they have an impact on neurodegenerative processes in a complex and context-dependent manner, depending on their unique neuron–glial signalling profile or the cross-talk among TLRs and complement pathways.

Based on the results of the present study, we proposed a model of activation, as detailed in Figure 6.1.

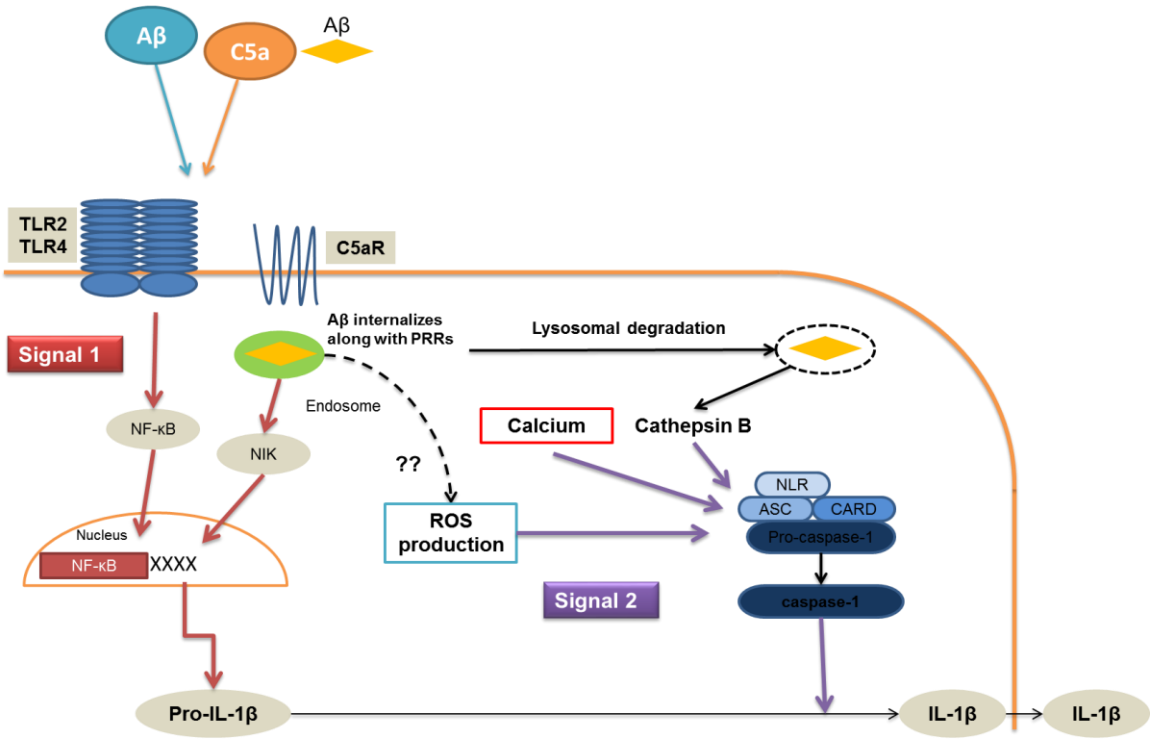

Figure 6.1 Proposed model of activation. TLR2 and TLR4 were demonstrated to be involved in Aβ recognition (see Chapter III). Data suggests that a synergistic interaction between complement receptors and the TLR pathway may be an important mechanism by which complement promotes inflammation. Preliminary data from FRET indicated that C5aR1 clusters with TLRs 2 and 4 (see Chapter IV). The canonical pathway is initiated by receptors on cell surface and the non-canonical pathway by the endosomes (see Chapter IV).

Astrocytes were the glial cells of interest in the current study, as being a specialized type of glial cells they outnumber neurons by over fivefold. They contiguously tile the entire central nervous system (CNS) and exert many essential complex functions in the healthy CNS (Sofroniew and Vinters 2009). A vital question arises, however, as to how many types of astrocytes exist in the CNS. Furthermore, the morphological and molecular signatures of astrocytes have not been fully elucidated, particularly the way these signatures may alter during aging and in pathological conditions. There is a possibility that different types of astrocytes exist in the hippocampus and frontal lobe (areas primarily affected in

AD) and have different responses to brain stimuli/insults. In addition to astrocytes, future work should focus on the use of microglia compared to astrocytes in order to see the importance of microglial cells in complement-inflammasome interactions. It has not been possible to use such cells for comparison in this study, but in the future with the generation of *iPSC (induced pluripotent stem cells)*-derived *microglia*-like macrophage microglial cells, there would be an opportunity to compare the results.

Future work should also include testing the inhibitors that we have used in vitro in an *in vivo* model of Alzheimer's (such as J20 mouse, or the 3Tg mouse strains). It would be very interesting to try to inhibit the inflammasome (using MCC950), the complement system (with PMX53 or even Compstatin) and a combination of both in a mouse model of Alzheimer's. In addition to testing the pro-inflammatory profile of the mice throughout the time of the experiment, as well as, performing immunohistochemistry of the brain tissue, it would be beneficial to image the mice using MRI and PET (using antibodies against NLRP3 and complement components) to assess any changes in their brain. Behavioural studies in order to establish whether there is cognitive impairment should also be conducted.

Our studies and others have confirmed the inflammatory nature of Aβ. We have moved the field a step further forward in deciphering the molecular mechanisms involved, by demonstrating that there is complement-inflammasome crosstalk. Only by targeting these pathways can we hope to be able to develop a therapeutic intervention for Alzheimer's for the future.

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