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SOLUBLE AMYLOID-BETA 42 AGGREGATES STIMULATE CELLULAR INFLAMMATION INDEPENDENT OF CD47

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Advisory Committee

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For my Family

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

Aβ	Amyloid-beta	aCSF	Artificial cerebrospinal fluid
AD	Alzheimer's disease	ADDLs	Aβ-derived diffusible ligands
AFM	Atomic force microscope	AICD	APP intracellular domain
APLP	APP-like protein	ASC	Apoptosis-associated speck-
ApoE	Apoliporotein E	APP	Amyloid precursor protein
BACE	β-site APP-cleaving enzyme	CARD	Caspase recruitment domain
BBB	Blood brain barrier	BSA	Bovine serum albumin
CD	Circular dichroism	HFIP	Hexafluoroisopropanol
CNS	Central nervous system	CSF	Cerebrospinal fluid
DAMPs	Danger-associated molecular	DLS	Dynamic light scattering
DMSO	Dimethyl sulfoxide	DMEM	Dulbecco's modified Eagle's
ECL	Enhanced chemiluminescence	EDS	Energy dispersive X-ray
ELISA	Enzyme-linked immunosorbent	EOAD	Early-onset AD
EM	Electron microscopy	ER	Endoplasmic reticulum
FPLC	Fast protein liquid chromatography	FBS	Fetal bovine serum
GM-CSF	Granulocyte macrophage colony-stimulating factor	GuHCl	Guandium hydrochloride
IgG	Immunoglobulin G	HRP	Horseradish peroxidase
IL	Interleukins	IFN	Interferon
KO	Knock out	ROS	Reactive oxygen species
LDH	Lactate dehydrogenase	LOAD	Late-onset AD
LPS	Lipopolysaccharide	LTP	Long term potentiation

MyD88	Myeloid differentiation protein 88	$MyD88^{-/-}$	MyD88 Knock out
NLR	Nod-like receptor	NFT	Neurofibrillary tangles
PAMPs	Pathogen associated molecular patterns	PHF	Paired helical filaments
PBS	Phosphate buffered saline	NMR	Nuclear magnetic resonance
PRR	Pattern recognition receptor	PMS	Phenazine methosulfate
R_H	Hydrodynamic radius	PVDF	Polyvinylidene difluoride

ABSTRACT

Karki, Sanjib, Ph.D., University of Missouri-St. Louis, August 2015. Soluble amyloid-beta 42 aggregates stimulate cellular inflammation independent of CD47, Major Professor: Michael. R. Nichols

Alzheimer's disease (AD) is a neurodegenerative disease characterized by the accumulation and deposition of aggregated amyloid-β protein (Aβ). Senile plaques found in brains of Alzheimer's patients mainly have 39 to 42 amino-acid residue Aβ peptides. These Aβ peptides are formed by proteolytic cleavage of amyloid precursor protein. Biochemical studies have shown Aβ(1-40) and Aβ(1-42) peptide residues play a vital role in neurodegenerative pathology of the disease and therefore most of the studies are focused into these two peptides. AD-related inflammation results from the activation of glial cells, of which microglia are the most well-studied. However, the mechanism of Aβ-microglial interaction for proinflammatory response is not completely understood. For this interaction several cellular components have been purposed to be involved including receptor CD47. Therefore we sought to determine the role of CD47 receptor in Aβ(1-42) protofibril induced cytokine response. My research using CD47 antagonist peptide 4N1K showed inhibition of Aβ42 protofibril-triggered microglial production of cytokines tumor necrosis factor-α (TNFα) and interleukin-1β (IL-1β). However, in our study 4N1K peptide also blocked the lipopolysaccharide (LPS)-mediated TNFα production in microglial cells showing non-specificity of this peptide. Further assessment using anti-CD47 neutralizing antibodies didn't block Aβ(1-42) induced cytokine production. Additionally no difference between $CD47^{-/-}$ and wild type microglial cytokine production was observed. These results together demonstrated that Aβ42 protofibril-mediated cytokine production in microglial cells is not dependent on CD47 receptor and 4N1K inhibition may be independent of CD47.

Stability studies between Aβ(1-42) and Aβ(1-40) peptides were done in order to understand the early events that occurs in Aβ aggregation. In our study, use of conformation specific antibodies showed the presence of elements of fibrillar structure between Aβ(1-42) and Aβ(1-42) aggregates. However, for Aβ(1-40) aggregates loss of immunoreactivity on treatment with chaotropic agents indicated them to be less stable compared to Aβ(1-42) aggregates and protofibrils. Additionally our study showed early changes in freshly SEC-purified Aβ(1-42) monomer through OC positive immunoreactivity that was SDS-sensitive initially but became resistant later. These changes occurred much before the significant ThT fluorescence increase therefore indicating importance of early events that occurs in Aβ aggregation.

Another project was to explore possible Aβ structure dependent microglial activation. Initially, time dependent study of Aβ(1-42) protofibril and monomer induced

microglial TNFα production was carried out. The protofibril species induced the cytokine production earlier compared to the monomer showing the significance of preassembled species for proinflammatory response. Majority of this TNFα production occurred through MyD88 dependent pathway. Our in situ aggregation studies between Aβ(1-40) and Aβ(1-42) monomers in presence of microglial cells showed a correlation of proinflammatory response with aggregation. Additionally this aggregation study was also monitored by using fluorophores ThT and Bis-ANS. Fluorescence increase from these fluorophores, ThT and Bis-ANS, showed the changes related to β-sheet structural content and surface hydrophobicity respectively which might have been important for significant microglial inflammatory response.

CHAPTER 1. GENERAL INTRODUCTION

1.1 History of amyloid disease and its pathology

In 1906, Bavarian psychiatrist Alois Alzheimer described the neurological disorder that later was termed as Alzheimer Disease (AD) based on the detail observation he made for five years on fifty-year-old female patient Auguste D., who was admitted to the Frankfurt Psychiatric Hospital in 1901. This patient was observed having a continuous decline in memory, progressive confusion, and a sleeping disorder with other worsening psychological changes (Hippius & Neundörfer, 2003; Selkoe & Podlisny, 2002).

Alzheimer was also able to do morphological and histopathological studies on brain samples after the death of his patient. Though his precise study and results were presented later in scientific meetings and were also published, still contemporary scientific and non-scientific audiences were reluctant to accept the disease and instead considered dementia as a natural process of ageing (Reger, 2002). It was only later with advancement in scientific research techniques and neurological research in 1970s that the disease was accepted. From histopathological observation, Alzheimer was also successful in characterizing two pathological lesions called as neurofibrillary tangles (NFTs) and senile plaques (Figure 1.1). Additionally, on performing detailed histological studies of brain samples from another patient named Josef F., only senile plaques were observed, unlike brain samples from Auguste D. that had both NFTs and plaques. Alzheimer concluded that these differences represented different stages in the progression of illness,

hence became first person to describe different stages in this newly discovered AD (Hippius & Neundörfer, 2003; Reger, 2002; Schachter & Davis, 2000).

Still NFTs and senile plaques remained as basis of postmortem AD diagnosis (Selkoe, 1994). It was only in 1984 that amyloid beta (Aβ) protein was found to be the major constituent of senile plaque, and two years later hyperphosphorylated microtubule associated tau protein was found to be the main component in NFTs (Glenner & Wong, 1984; Grundke-Iqbal et al., 1986). The deposition of Aβ in senile plaques has been found not to be restricted to the brain, but also occurs in walls of capillaries, arteries and meninges, resulting in more diverse pathological complications (Selkoe, 1994; Vinters, 2006).

1.1.1 Neurofibrillary tangles

Neurofibrillary tangles are intra-neuronal bundles of paired, helically wounded filaments (PHFs) of approximately 10 nm diameter fibrils mainly found in different parts of AD brain including hippocampus, entorhinal cortex, amygdala and cortices of frontal, temporal and parietal lobes. The subunit protein of PHF is the microtubule associated tau protein and PHFs area found to be resistant against heat and acid treatments (Selkoe, 2011; "Subunit structure of paired helical filaments in Alzheimer's disease," 1985). Native tau protein is typically unfolded protein, without fixed conformation suitable for structural analysis by crystallography. Sequence analysis reveals an acidic N-terminal domain, proline rich domain and C-terminal basic region, lending the macromolecule a dipolar character. The normal function of tau protein is to stabilize microtubules via its tubulin, binding domain and its expression is higher in neuronal axons (Kolarova et al., 2012). The phosphorylation of tau protein is responsible for regulating axonal transport

coordinated by kinesin and dynein proteins as shown in figure 1.2. Loss of functional tau has been attributed to post-translational modifications such as hyperphosphorylation, acetylation, glycation, nitration and conformational changes (Carrell & Gooptu, 1998; Grundke-Iqbal et al., 1986; Kolarova et al., 2012; Kuhla et al., 2007). In NFTs found in AD brain, hyperphosphorylated tau protein is found to be the main component of oligomers which may progress into fibrils (Maeda et al., 2007).

Hyperphosphorylation of tau may reduce the protein's microtubule binding affinity, resulting in microtubule destabilization and and increased degeneration of neurons (Mietelska-Porowska et al., 2014; Wolfe, 2012). Further observations of tau aggregates within NFTs in complex with ubiquitin have been considered as evidence that the abnormal tau protein is resistant to degradation by the proteasome (Selkoe, 2011).

1.1.2 Senile plaques

Senile plaques or neuritic plaques (Figure 1.1) obtained from AD brains are primarily composed of an insoluble fibrillar form of amyloid-β protein (Masters et al., 1985). Later, studies utilizing carboxy-terminal specific monoclonal antibodies identified two isoforms, Aβ(1-42) and Aβ(1-40), with Aβ(1-42) being primary component. Aβ(1-42) possesses two additional hydrophobic amino acids at its carboxy-terminus (Iwatsubo et al., 1994; Kang et al., 1987). Apart from Aβ, these plaques were also found to be associated with activated microglia and astrocytes, as well as other components such as proteoglycans, inflammatory molecules and metal ions with immune cells playing a role in plaque homeostasis (Atwood et al., 2002).

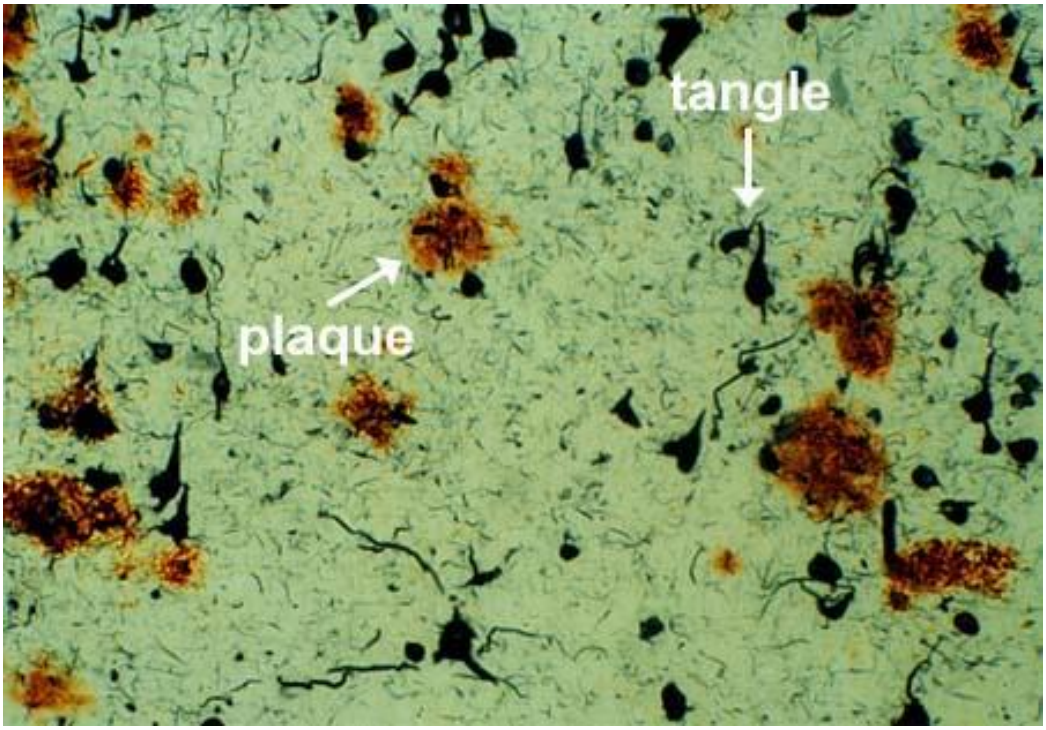


Figure 1.1 Histopathological hallmarks of AD brain.

AD brain senile plaque and NFTs that are made up of Aβ and tau protein respectively http://ladulab.anat.uic.edu/

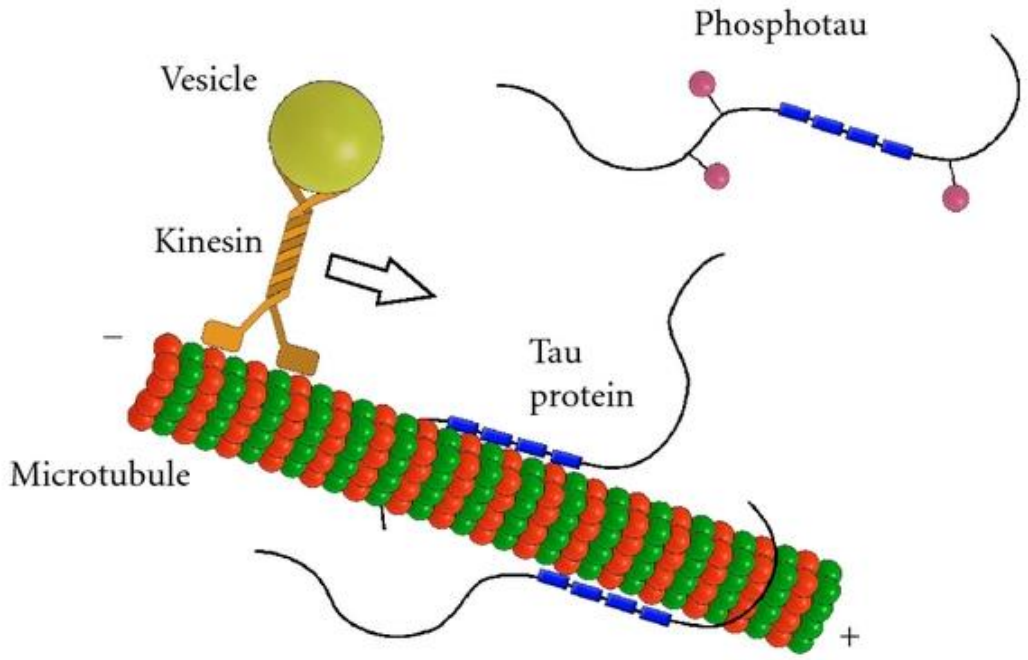


Figure 1.2 Functioning of tau protein in association with microtubule.

Microtubule represented by cylindrical arrangement of green and red balls is stabilized by tubulin binding domains (blue boxes) of tau protein. The phosphorylation of tau (pink balls) protein regulates its ability to bind to microtubule hence may modulate plus-end directed transport of vesicles along microtubules by kinesin (Kolarova et al., 2012)

Studies using antibodies against Aβ showed that senile plaques were also associated with a diverse mixture of non-fibrillar forms of the protein surrounding the plaques with various degrees of immune cell activation. The Aβ forms present in diffuse plaques are not well characterized, and are primarily amorphous, though it is believed that these species later give rise to senile plaques (Selkoe, 1994, 2011).

1.2 Aβ peptide production

Aβ peptide generation occurs via sequential proteolysis of a larger precursor protein called amyloid precursor protein (APP). APP belongs to family of mammalian proteins which includes amyloid precursors like protein-1 (APLP1) and protein-2 (APLP2) (O'Brien & Wong, 2011). The human APP gene, approximately 240 kilobases in length, is located in chromosome 21 (Yoshikai et al., 1990). Out of the three most alternate splicing-derived APP isoforms, isoform APP_{695}, 695 amino-acids in length, is the most dominant form of APP glycoprotein in brain. The other common isoforms, 750 (APP_{750}) and 751 (APP_{751}) amino acids in length, are expressed ubiquitously (Bayer et al., 1999; Selkoe, 1994; Tanaka et al., 1989).

Despite a long history of APP study, its exact function and mechanism still remains unknown. However, certain studies have shown some potential biological roles of APP such as cell growth and survival in cell lines, regulation of synaptic function, and kinesin mediated axonal transport, as well as development of the peripheral nervous system (Kamal et al., 2001; Karaulanov et al., 1992; Merdes et al., 2004; Terry et al., 1991). Studies with APP knock out mice showed increased rates of cerebral ischemic mortality, defects in locomotor function and long-term memory, reactive gliosis, and defects in long-term potentiation (Dawson et al., 1999; Koike et al., 2012; Zheng et al., 1995).

The first cDNA sequence of APP was obtained in 1987 showing the presence of cysteine rich region, which was possibly involved to stabilize N-terminal domain folding, glycosylation sites, and a patch of hydrophobic amino acids at a transmembrane domain (Kang et al., 1987). The three-dimensional structure of APP (Figure 1.1) shows APP having a cysteine rich ectodomain (E1), containing heparin as well as a copper metal binding domains; an α- helix rich domain (E2); and Aβ and intracellular domains (Dawkins & Small, 2014) (Figure 1.4 A). The Aβ domain is composed of 28 amino-acid residues from the extracellular domains, and extends 14 to 15 amino-acid residues into the transmembrane domain (TMB)—i.e., amino acids 597–613 of APP_{695} transcript.—This sequence is a highly conserved sequence (Dawkins & Small, 2014; O'Brien & Wong, 2011; Suh & Checler, 2002). The intracellular domain composed of conserved YENPTY residue is linked to a transmembrane domain by three consecutive lysine residues, and is proposed to regulate endosomal-lysosomal intracellular targeting (Bonifacino & Traub, 2003).

Heavy production of APP in neurons is followed by prompt processing. APP processing involves several post-translational modifications, such as O- and N-linked glycosylation, phosphorylation, sulfation and palmitoylation, in addition to its proteolytic cleavages by enzymes called secretases, producing subsequent numbers of smaller APP fragments (Bhattacharyya et al., 2013; Dawkins & Small, 2014; Lee et al., 2008; Selkoe, 2001). APP proteolytic cleavage follows one of two pathways: amyloidogenic and non-amyloidogenic (Figure 1.2). APP, after passing through the trans-golgi apparatus, is transported by clatherin- coated vesicles into endosomes or to the cell surface (O'Brien & Wong, 2011). APP on the cell surface which is directly cleaved by α-secretase followed

by γ-secretase, does not generate full length Aβ—hence this route is called a non-amyloidogenic pathway (Figure 1.3). This is attributed to the initial APP cleavage within the APP Aβ domain (between Lys16 and Leu 17) by α-secretase producing soluble ectodomain (APPsα) and membrane-bound α-carboxy terminal fragment of 83 amino-acid sequence length(α-CTF or C83).

The α-CTF form of APP is subsequently cleaved by γ-secretase, producing a truncated Aβ peptide called p3, which is devoid of pathological significance (Haass et al., 1993; Haass et al., 2012). APP sorted to endosomal compartment, either from the trans-golgi apparatus or from the cell membrane through re-internalization into vesicles, is acted upon by β-secretase also known as β-site APP-cleaving enzyme 1 (BACE-1) (O'Brien & Wong, 2011). This sheds the comparatively shorter ectodomain part of APP (APPsβ) leaving a membrane-bound APP carboxy-terminal fragment of 99 amino acids (βCTF or C99). βCTF is then processed by γ-secretase enzyme forming not only intact Aβ, but also APP intracellular domain (AICD) in the cytosol in the amyloidogenic pathway (Dawkins & Small, 2014; Haass et al., 2012) (Figure 1.3). γ-secretase is a multi-protein complex composed of four subunits, including presenilin 1 (PS-1) or PS-2, having a catalytic aspartyl protease domain, nicastrin anterior pharynx defective (APH-1), and PS enhancer (Steiner et al., 2008). The cleavage by γ- secretase is promiscuous, not limited to one site, but occurs several times forming Aβ

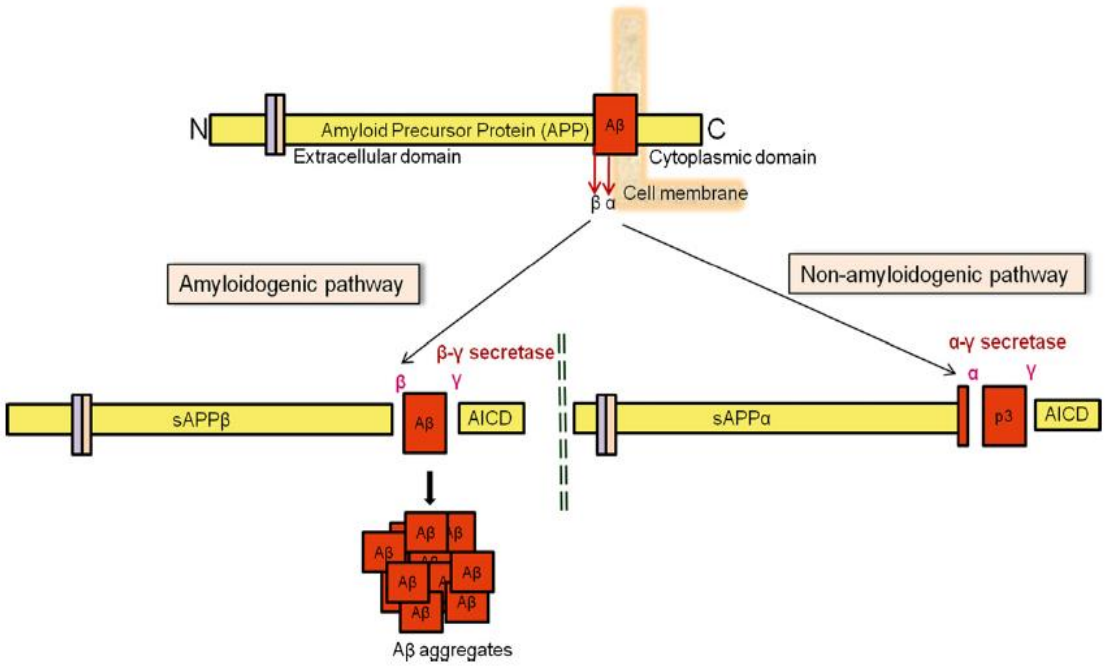


Figure 1.3 APP proteolytic processing and Aβ fragment generation.

Enzymatic processing of APP requires α, β-(BACE-1) and γ secretases. Amyloidogenic pathway is carried out through APP cleavage by β-secretase producing a C-terminal fragment of 99 residues (C99) containing intact amyloid-peptide domain. Then this C99 fragment is cleaved by γ-secretase producing 39 to 42 amino-acid length fragments that are prone to aggregation. Another non-amyloidogenic pathway involved sequential cleavage of APP by α-secretase within the amyloid peptide domain and by γ-secretase acting on membrane bound carboxy-terminal fragment, producing soluble p3 much shorter in length than Aβ (3, 16, 17), which does not undergo aggregation (Mathew et al., 2011)

fragments of 39-42 amino-acids in length (Selkoe, 2001). The dominant Aβ fragments Aβ(1-40) and Aβ(1-42) are both found in cerebrospinal fluid at nanomolar in concentrations. But, Aβ(1-42) is more prone to aggregation-forming fibrillar species compared to Aβ(1-40), hence could possibly be the reason for it being a major component of Aβ senile plaques in the AD brain(Tamaoka, 1998). This leads to speculation over the imbalance between production and clearance of Aβ as a major cause for disease progression, and can provide the therapeutic window for targeting the components involved in APP processing (Haass et al., 1994; Seubert et al., 1993; Shoji et al., 1992; Walsh et al., 2000).

1.3 Genes behind Alzheimer's disease:

The increase in life expectancy has made us more vulnerable to Alzheimer's disease. Most of the AD cases are seen in people beyond the age of 65, and such occurrences are classified as Late Onset AD (LOAD). However, relatively more aggressive in nature is the Early Onset AD (EOAD) occurring before age of 65, which constitutes a small portion of AD cases. The culprit for this familial or hereditary EOAD has been attributed to four genes, namely: APP, PS1, PS2 and ApoE. Mutations in each of these genes has been shown to cause excessive Aβ deposition in the brain, followed by neuronal dysfunction in regions important for cognitive function (Panegyres & Chen, 2013; Selkoe & Podlisny, 2002). APP, PS1 and PS2 are directly implicated in Aβ production as previously discussed, hence mutation in any of these genes would result in a gain in amyloidogenic toxic effect.

Most of the APP missense mutation occurs within or near α-, β- or γ-secretase cleavage sites with the overall effect being increased amyloid β production. Studies have

shown the enhancement in amyloidosis favors greater productions of Aβ42 peptide, and therefore increases tendency for fibrillation and cerebral deposition (Citron et al., 1992; Hardy, 1997; Scheuner et al., 1996; Selkoe & Podlisny, 2002; Suzuki et al., 1994). The familial AD characteristics such as cerebral amyloid angiopathy, cerebral parenchymal amyloidosis, and neurodegeneration have also been associated with single codon mutations within the Aβ protein region. These mutations are E22K Italian mutation, A21G Flemish mutation E22G Artic mutation, E22Q Dutch mutation, and D23N Iowa mutation (Figure 1.4 B). Though several studies point out the effect of these mutations at Aβ production level, recent studies show their dominant effect on rates of protofibril and fibril formation, as well as mutant Aβ resistant to degradation with naturally occurring enzymes like neprilysin (Betts et al., 2008; Nilsberth et al., 2001; Oakley et al., 2006; Tsubuki et al., 2003). Surprisingly, the recent discovery of single-point mutation A673T in APP displayed protective effect against AD and cognitive decline in normal elderly people. This was probably due to the mutation being near to the β-secretase cleavage site, hence hindering APP β-cleavage and amyloidogenic peptide production (Jonsson et al., 2012).

Studies on FAD (Familial Alzheimer's Disease) lead to the development of several transgenic mice models displaying AD-like pathology (Janus et al., 2001). Mutations in PS-1 or PS-2 genes, which are expressed to form part of the γ-secretase enzyme complex, have been shown to alter the Aβ42: Aβ40 ratio, increasing Aβ42 concentration while keeping Aβ40 constant (De Strooper, 2003; Jankowsky et al., 2004; Takeda et al., 2004). APP transgenic mice with BACE-1 genes knocked out displayed failures in Aβ generation and amyloid deposition, as well as improvement in memory, suggesting

BACE-1 as therapeutic target gene (Cai et al., 2001; Ohno et al., 2007; Vassar et al., 2014). Recent studies in a mouse model with five familial mutations (5XFAD) with heterozygous BACE-$1^{+/-}$ suggested greater than 50% BACE-1 inhibition is important in lowering Aβ level and likely progression of AD (Sadleir et al., 2015). Double knock in mice APP/PS1 displayed oxidative stress and lipid peroxidation, suggesting a probable role for oxidative stress in AD (Matsuoka et al., 2001; Mohmmad Abdul et al., 2006).

Another gene found to be associated with late onset AD is apolipoprotein E (ApoE), the study was initiated with the observation of substantial ApoE-Aβ binding in the cerebrospinal fluid (Strittmatter et al., 1993). ApoE is an approximately 34 kDa protein involved in cholesterol transport in plasma and the central nervous system (CNS). It is expressed dominantly in the brain and liver. In the CNS, it is synthesized primarily by astrocytes and microglia (Mahley, 1988; Pitas et al., 1987; Uchihara et al., 1995). The three common isoforms for this protein are ε2, ε3 and ε4. Although ε3 accounts for around 77% expression, in the general population, ε4 in AD has been shown to have increased from 15% to 40%. The least expressed allele, ε2, has been linked to being AD protective (Bertram & Tanzi, 2008; Corder et al., 1993; Holtzman et al., 2000; LaDu et al., 1994; Mahley, 1988; Schmechel et al., 1993).

Several mechanisms have been suggested for role of ApoE in AD predisposition. Some of these are modulation on Aβ concentration, aggregation and clearance, fibrillation, hyper-phosphorylation of tau tangles, neurotoxicity and synaptic impairment

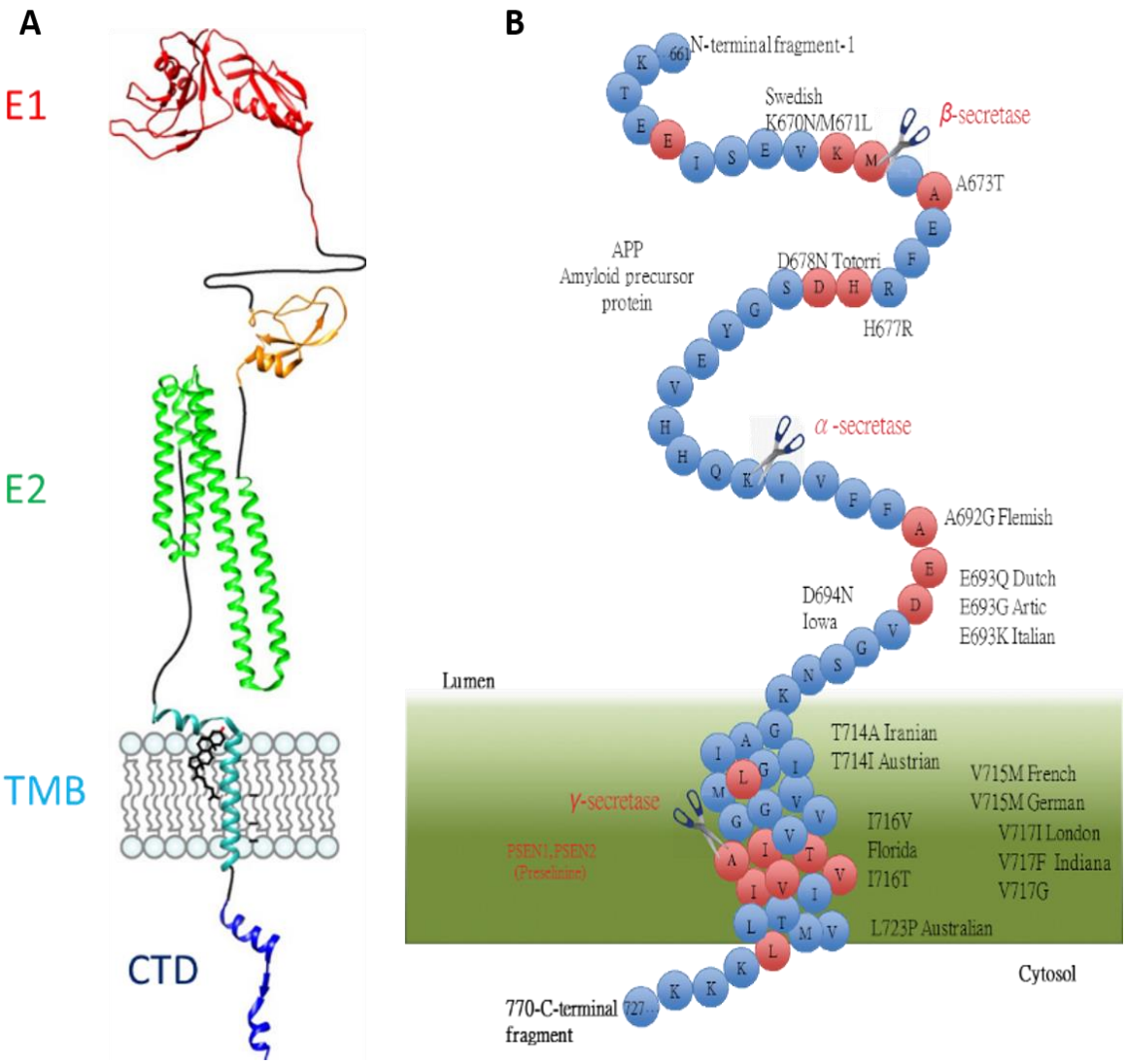


Figure 1.4 Structure of APP and its genetic mutations.

Panel A shows hypothetical three dimensional structure of APP based on protein data bank files showing the four main domains: ectodomain (E1), α-helix rich domain (E2) transmembrane domain (TMB), and C-terminal domain (CTD) (Dawkins & Small, 2014). Panel B represents APP, with the three principal cleavage sites (β-secretase or BACE, α-secretase, and γ-secretase) and several known mutations (Modarresi et al., 2011)

(Beffert & Poirier, 1996; Beffert & Poirier, 1998; Buttini et al., 2002; Holtzman et al., 2000; Jiang et al., 2008; LaDu et al., 1994; Mahley et al., 2006). However, the clear mechanism of these ApoE alleles in AD pathogenesis remains to be established. Nevertheless, this plethora of genetic studies on AD has revealed potential therapeutic windows for addressing various aspects of AD treatment.

1.4 Aggregation of Aβ

Successful isolation and characterization of polypeptide from senile plaques in the brain of AD patients yielded approximately 4 kDa Aβ peptide (Masters et al., 1985). Neuritic plaques have been found to contain predominantly the Aβ42 isoform of the amyloid-β protein, whereas cerebrovascular amyloid deposits contains primarily the Aβ40 variant (Glenner & Wong, 1984; Miller et al., 1993). Fibrils of Aβ isolated from the dense core plaque of AD patients had indistinguishably identical characteristics when compared to fibrils formed *in vitro* (Halverson et al., 1990; Hilbich et al., 1991; Kirschner et al., 1987). These observations incited further studies on the structure, kinetics and molecular events that ultimately lead to fibril formation and the relation to neurodegeneration.

Aβ monomer peptide is soluble in a physiological environments, but due to unclear mechanisms undergoes a self-assembly process to form larger aggregates which are eventually deposited as insoluble fibrils. Aβ42 has a higher tendency to aggregate compared to the shorter peptide Aβ40 (Jarrett et al., 1993; Tamaoka, 1998). This difference is due to two extra carboxy-terminal amino-acids, isoleucine and alanine in Aβ42, making it more hydrophobic compared to the Aβ40 peptide. Importantly, the carboxy-terminus of Aβ42 contains 14 consecutive hydrophobic amino-acid residues

along with β-branched residues valine and isoleucine, with glycine at every fourth amino-acid position. Studies have shown that the propensity of aggregation varies with the length of this hydrophobic chain, with shorter lengths being more soluble and less aggregation-prone. The periodic glycine occurrence has been proposed to impart conformational flexibility for the efficient packing of fibrils (Jarrett & Lansbury, 1992; Liu, Crocker, et al., 2005; Vandersteen et al., 2012). The β-branched residues valine and isoleucine are sterically constrained to extended conformations, facilitating development of β-sheet conformations (Jarrett et al., 1993; Jarrett & Lansbury, 1992).

Various techniques have been employed to study Aβ monomer aggregation. Turbidity testing or recovery of insoluble filtrate, one of the old techniques employed for the aggregation study, fails to reveal early events of aggregation (Jarrett et al., 1993). Extrinsic dye studies such as Thioflavin T (ThT) fluorescence has been used to measure increased fluorescence on binding to β-sheet-rich Aβ aggregated species such as the fibrils. Techniques such as X-ray diffraction, solid state nuclear magnetic resonance (NMR), atomic force microscopy (AFM), and transmission electron microscopy (TEM) have also been used recently to study the structure and aggregation of Aβ but yet these too have limitations (Lee et al., 2011; Paranjape et al., 2012; Petkova et al., 2002; Wu et al., 2009). Recently conformation specific antibodies have been employed as promising sensitive methods to study the Aβ species (Coalier et al., 2013; Kayed et al., 2003).

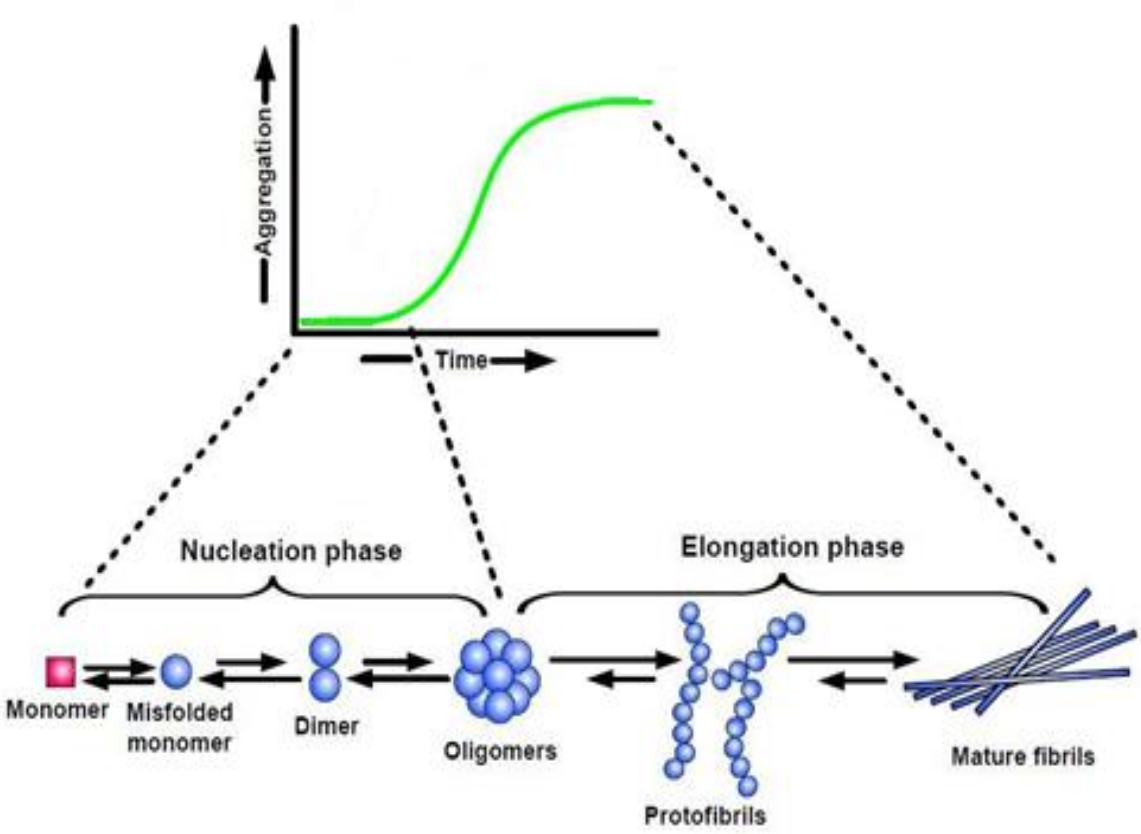


Figure 1.5 Nucleation dependent Aβ aggregation model.

Monomer undergoes conformational change, and associates with other monomers forming oligomeric nuclei or seeds. This phase is referred to as the nucleation or lag phase. The oligomeric nuclei further progresses or matures by addition of monomers into protofibrils, which then advances into fibril formation until saturation is reached. This phase is called the elongation phase. The nucleation phase is slow and thermodynamically unfavored compared to the elongation phase. The rate limiting step in the process is the formation of nuclei or seeds to promote aggregation. Figure 1.5 is modified from (Kumar & Walter, 2011)

Aβ aggregation leading to insoluble fibril formation is nucleation-dependent. This requires formation of a nucleus or seed; this is generally regarded as the lag phase since it occurs slowly (Figure 1.5). Thus formed nucleus, when added to monomers forms bigger soluble units; this is generally regarded as growth phase, which ultimately proceed to fibril formation through its immediate intermediate soluble protofibril species. At this phase monomer and fibrils are in equilibrium and have reached the plateau phase. The lag phase is the rate-limiting step and is mainly Aβ monomer concentration-dependent (Bitan et al., 2003a; Burdick et al., 1992; Jarrett & Lansbury, 1992) (Figure 1.5).

Influence on Aβ aggregation is not limited to presence or absence of additional C-terminal hydrophobic amino-acid residues. Factors such as concentration, pH, temperature and length of incubation time, mechanical or sonic agitation as well as ionization constant or salt concentration modulate aggregation (Burdick et al., 1992; Klement et al., 2007). Increases in salt concentration or acidic pH have been observed to increase the rate of aggregation. More specifically, carrying out aggregation reactions near the isoelectric point of Aβ peptide, around pH 5 to 5.5, promotes insoluble aggregate formation due to the neutrality of the peptide (Burdick et al., 1992; Hortschansky et al., 2005). Various *in vitro* studies have suggested two pathways for fibrillation which differs in nucleation mechanism, based on critical concentration of the protein (Lomakin et al., 1997). In one pathway, when total protein concentration (C) is greater than critical concentration (c), the formation of micelles takes place, increasing the local concentration of monomers and enhancing nucleus formation. This nucleus provides a platform for the further addition of monomers and fibrillation proceeds. Here, equilibrium between monomer and micelle maintains constant monomer concentration,

thus elongation is independent of monomer concentration. When C is less than c, the initial rate of elongation is dependent on total protein concentration and the size of fibrils even exceeds that of the previous condition as concentration goes on decreasing (Harper et al., 1999; Lomakin et al., 1996; Lomakin et al., 1997). However, under biological settings there are various other parameters that influence aggregation kinetics, such as facilitated nucleation through Aβ interaction with lipid membrane (Murphy, 2007; Yip & McLaurin, 2001). Further, concentration *in vivo* is in nanomolar range with Aβ40 having much higher concentration than Aβ42 and local super-saturation is thought to be the driving force for enabling nucleation and subsequent polymerization (Harper et al., 1997; Jarrett & Lansbury, 1992; Lomakin et al., 1997).

1.5 Different forms of Aβ

1.5.1 Aβ oligomers

The profound neurotoxic effect of the soluble form of Aβ has caused an emphasis on studies aimed at characterization of the soluble Aβ species. Oligomers are intermediate soluble species that are formed on the way to Aβ fibril formation. The term oligomer encompasses a broad range of Aβ species in terms of morphology and size. Most biophysical characterization is done using techniques like Atomic Force Microscopy (AFM), Electron Microscopy (EM), and Size-Exclusion Chromatography, as well as Gel electrophoresis (Kayed et al., 2003; Lambert et al., 1998; Stine et al., 2003).

Gel studies have shown oligomers to be dimers, trimers, tetramers to nanomers (also called as Aβ-derived diffusible ligands or ADDLS), as well as dodecamers, with sizes ranging from 10 kDa to 210 kDa for very large oligomeric aggregates. However, most of the studies have shown the common species near a mass of approximately 40

kDa (Lambert et al., 1998; Prangkio et al., 2012; Stine et al., 2003). Immunoreactivity studies have shown that this is the minimum mass for anti-oligomeric antibody recognition that can provide structural motif requirements formed from amyloid backbone. There seems to be a common structural motif requirement for antibody recognition regardless of amino-acid sequence specificity among different amyloidogenic proteins such as polyglutamine and islet amyloid polypeptide (IAPP) (Kayed et al., 2003). AFM images of oligomer showed diameters concentrated between 2 to 5 nm (Dahlgren et al., 2002; Stine et al., 2003). The structures vary from punctate globular, and annular structures, as well as spherical micelles formations due to amphipathic nature of Aβ peptide (Kayed et al., 2003; Prangkio et al., 2012; Soreghan et al., 1994). These variations in structure may depend upon factors like pH, ionic strength, or presence of metal ions during the oligomerization reaction. One AFM study showed the presence of large, loose, rod-like oligomers at pH 5.0 while more globular and spherical species were observed at pH 7.4, raising the possibility of multiple mutually exclusive mechanisms of oligomer formation (Klug et al., 2003).

All else being equal, oligomer preparation of Aβ(1-40) displayed similar globular, spherical structure to preparations of Aβ(1-42), differing only in an increased incubation time for Aβ(1-40). However, Aβ(1-42) oligomer species were found to be more potent reducers of neuronal viability compared to Aβ(1-40) (Dahlgren et al., 2002). Animal studies have shown both cell-derived and synthetic Aβ-derived oligomers capable of causing cognitive impairments, synaptic toxicity, and neuronal toxicity (Lambert et al., 1998; Reed et al., 2011). Using NAB61 oligomer binding antibodies, multiphoton *in vivo* imaging displayed NAB61 antibodies surrounding the dense plaques in brain, forming a

halo with neuronal loss in the vicinity (Koffie et al., 2009). However, the mechanism for Aβ oligomer-induced neuronal loss is still elusive. A few suggested mechanisms involve oligomer interaction with neuronal receptors, the generation of cytotoxic pores in cell membranes, and destabilization of membrane integrity (Benilova et al., 2012; Prangkio et al., 2012).

1.5.2 Aβ protofibrils

With most of the studies being focused on Aβ fibrillation as central to AD, several studies have been conducted to identify the intermediate species in the process (Hartley et al., 1999; Walsh et al., 1997). The protofibril species as an intermediate in fibril formation was first proposed by Harper and his colleagues in 1997. Their AFM images of Aβ(1-42) and Aβ(1-40) contained species termed elongated oligomers or protofibrils. Samples at different incubation durations during fibrillation, displayed diameters around 3.1 nm and 4.2 nm respectively, with a similar periodicity of around 20 nm (Harper et al., 1997). Further characterization of protofibrils by EM showed curvilinear structures with length less than 200 nm. Further hydrodynamic radii (R_H) of Aβ(1-40) and Aβ(1-42) protofibrils were determined as 27.8±1.8 nm and 21±6.0 nm respectively (Hartley et al., 1999; Paranjape et al., 2012; Walsh et al., 1999).

Kinetic studies showed disappearances of protofibril species on longer incubation times with the appearance of straighter fibrils of length greater than 1 μm suggesting protofibrils to be intermediate species in the process of fibril formation (Harper et al., 1997). SEC studies of protofibrils peak showed first a decrease in monomer peak, followed by decrease in protofibril peak on longer incubation of Aβ(1-42) monomer, supporting this notion (Paranjape et al., 2012). Thioflavin T, Congo red, and CD studies

of Aβ protofibril showed high β-sheet secondary structural contents similar to fibrils. Residual content of α-helix seen during CD studies could be from the sub-population of protofibrils, or low molecular oligomers, or the transitory one observed during conformational change from predominantly random coiled smaller species to protofibrils, which ultimately progress to fibrils (Paranjape et al., 2013; Walsh et al., 1999; Wu et al., 2012).

The transition towards β-sheet-rich protofibrils or fibril formation displays hydrophobic core formation and its conservation during fibrillation. Stabilization of this hydrophobic core is believed to be contributed by increases in hydrogen bond formations, along with β-sheet formation between the core amide protons (Kheterpal et al., 2003). Furthermore, destabilization of β-sheet structure or delay in protofibril formation was observed when core amino-acids were replaced with glycine or proline indicating the importance of hydrophobic core protection (Hartley et al., 1999; Kheterpal et al., 2003; Walsh et al., 1999).

Several possible mechanisms proposed for elongation of protofibrils involve addition of monomeric species at the ends, end-to-end annealing, or lateral association of protofibrils. The extent of elongation as well as its rate has been shown to be dependent on pH, temperature, and ionic strength, as well as critical concentration of Aβ (Srinivasan et al., 2003; Walsh et al., 1999). However, the dynamics between low molecular weight oligomers, protofibrils, and fibrils does add complexity to the actual phenomenon (Walsh et al., 1999).

Aβ protofibrils have been shown to be bio-active species in bringing about inflammatory responses, stimulating immune cells like microglia and bringing

electrophysiological changes by altering membrane conductance, ion flux, and neuronal toxicity (Hartley et al., 1999; Paranjape et al., 2012). Therefore, Aβ protofibril species can be a therapeutic target.

1.5.3 Aβ fibrils

X-ray diffraction and Electron Microscopy Analysis as well as solid state nuclear magnetic resonance (SSNMR) of fibrils have been the basis for describing secondary structural conformation from the very beginning, in terms of β-pleated sheet rich secondary structure arranged laterally in antiparallel orientation. In this structure, β-sheet forming peptides fold on to themselves, making the peptide axis perpendicular to the fibrillary axis, while hydrogen bonds formed between the backbones of peptides run parallel to the fibrillary axis (Eanes & Glenner, 1968; Serpell et al., 2000; Sunde & Blake, 1997).

The insoluble and non-crystalline nature of Aβ solution, has posed difficulties in carrying out NMR and X-ray diffraction studies. However, solid state NMR studies have their own limitations, specifically problems with nuclear label incorporation at appropriate positions (Benzinger et al., 1998). Further, resolution issues have been approached via studies on shorter peptides, especially with the X-ray diffraction method (Benzinger et al., 1998; Serpell et al., 2000). Structural studies on shorter analogs of Aβ peptides—more specifically Aβ(34-42), Aβ(16-22), and Aβ(11-25)—showed antiparallel β-sheet structures with alternating hydrogen bonding. (Balbach et al., 2000; Lansbury et al., 1995; Petkova et al., 2004).

Early Infrared absorption spectroscopy studies showed strong amide bands for full length Aβ fibrils at 1,630 cm^{-1} and weak bands at 1690 cm^{-1}, characteristic of anti-parallel

β-sheets (Antzutkin et al., 2000). However, later studies with longer version of the peptides specifically Aβ(10-35) as well as the full length Aβ(1-40) and Aβ(1-42) showed the presence of parallel β-sheet structures (Antzutkin et al., 2000; Antzutkin et al., 2002; Benzinger et al., 1998). Though the exact explanations for these differences are not known, it has been attributed to differences in the peptide folds and amphipathic characteristic of individual peptides (Serpell et al., 2000; Soreghan et al., 1994).

The most widely accepted structural model was put forward based on SSNMR study of Aβ(1-40) fibrils by Petkova et al in 2002 (Figure 1.6). This conformation has a 180° turn forming two β-strands of residues 25-29 that interact through side chain-side contacts. The core of this double-layered structure is formed by some neutral and more importantly hydrophobic amino acid residues. Side chain of D23 [aspartic acid and K28 (lysine)] forms salt bridge across the bend of the fibril. Hydrophobic amino-acids at positions 30, 32, 36 and 40 form hydrophobic face, whereas other charged and polar side-chain amino-acids form outer the convex surface of the fibril (Petkova et al., 2002) (Figure 1.6). Further structural studies on Aβ fibrils have displayed subtle differences in morphologies. These differences could be due to variation in the nucleation stages, different modes of protofibril association for fibril formations, pH, temperatures, and critical concentrations (Petkova et al., 2004; Petkova et al., 2005; Stine et al., 2003; Williams et al., 2006).

1.6 Amyloid Cascade Hypothesis

The amyloid cascade hypothesis, forwarded first by Hardy and Higgins in 1992, was devised primarily to address clinical adversity related to Alzheimer's disease. They proposed that deposition of Aβ in the brain, the main component of senile plaques, leads

to the development of neurofibrillary tangles (NFT), vascular damage, neuronal loss, and dementia. Therefore, two events, generation of intact Aβ or fragments from APP protein and its accumulation, must precede NFT formation and cell death (Hardy & Higgins, 1992).

A positive correlation between cognitive decline and Aβ plaque deposition has been shown both in humans and in mice models (Chen et al., 2000; Cummings & Cotman, 1995). Further *in vitro* studies also showed that the fibrillar form of Aβ is a necessity for neurotoxicity. Hyper-phosphorylated tau protein formation though calcium homeostasis destabilization is equally necessary, as it eventually makes tau protein lose the capacity to bind microtubules and induce NFT formation (Busciglio et al., 1995; Lorenzo & Yankner, 1994; Mattson et al., 1993). Some of the genetic studies—specifically mutations within Aβ peptide that resulted increased fibrillation tendency—also support Aβ deposition to be an early event in the disease (Rentz et al., 2010; Wisniewski et al., 1991). However, with further development in the field of AD research, significant deviations have been observed from original amyloid cascade hypothesis. The most important deviations are poor or no correlation found between the amyloid plaque burden and cognitive decline in patients. Some patients with high amyloid plaque burden have been found to be cognitively normal (K. E. Pike et al., 2007; Rentz et al., 2010).

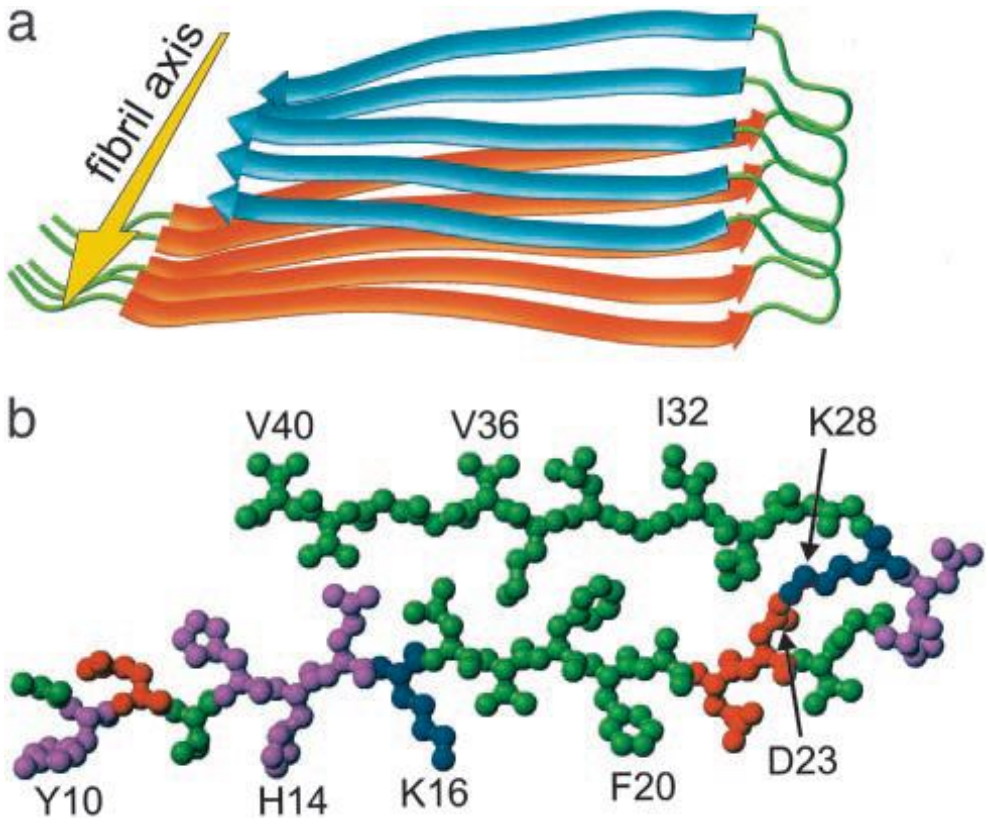


Figure 1.6 Structural model for Aβ(1-40) fibrils.

Panel A represents single molecular layer, or cross-β unit. The yellow arrow indicates the direction of the long axis of the fibril, which coincides with the direction of intermolecular backbone hydrogen bonds. The cross-β unit is a double-layered structure, with in-register parallel β-sheets formed by residues 12-24 and 30-40. Panel B shows 10 to 40 amino-acid residue of Aβ(1-40) viewed down the long axis of the fibrils. Color coding scheme follows nature of amino-acid side chain as hydrophobic (green), polar (magenta), positive (blue), or negative (red). Figure adopted from (Petkova et al., 2002).

Further molecular studies are showing early soluble Aβ intermediate species (such as oligomers and protofibrils) to be the most toxic species other than fibrils (Glabe & Kayed, 2006; Paranjape et al., 2012).

Several hypotheses have been put forward in order to have a better understating of the events in AD. Some are the oligomeric hypothesis, mitochondrial dysfunction hypothesis, cholinergic hypothesis, inflammation, oxidative damage, and neuronal cytoskeletal degeneration hypothesis. However, these are not necessarily mutually exclusive. Still important questions under active investigation are the cause of Aβ peptide deposition into senile plaque, the identification of specific Aβ toxic forms, and connections between different molecular events occurring in the AD brain (Tiiman et al., 2013).

Due to results contradicting the amyloid cascade hypothesis and the failure of clinical trials focused on developing therapeutics targeting only Aβ, it became necessary to revise the hypothesis in order to have a better clinical approach. With this context, Hyman in 2011, developed a remodeled amyloid hypothesis. According to him, AD should be seen as a two-phase process where the role of Aβ is dominant in the first early phase. This involves the role of intermediate soluble or insoluble Aβ species deposition bringing about local changes, including disruption of neuropil, loss of dendritic spines remodeling of neurites, and inflammatory response. The early Aβ response is followed by the second phase with pathophysiological consequences such as tangles development, neuronal loss, synaptic loss, glial responses, neurodegeneration, and ultimately full AD phenotype. In this later phase Aβ deposition has reached a plateau, while the neurodegenerative condition and cognitive decline continue to worsen. This could be the

explanation for the therapeutic failure seen among most second patients with cognitive decline when only anti-Aβ strategy is implemented. Therefore, it becomes very important to identify the stage of the AD patients and devise therapeutic approaches accordingly (Hyman, 2011).

1.7 Inflammation based on AD

Inflammation is a complex series of molecular and cellular event initiated in response to various stimuli such as stress, cell injury, or infection, and is responsible for removing the necrotic cells and tissue as well as initial insults. Thus, it is an attempt to restore the healthy condition. However, inflammation can become chronic and continuously erode the surrounding tissues if the initiating insult is not overcome (Lee et al., 2010). Brain inflammation has now become accepted as one of the hallmark traits of AD (Akiyama et al., 2000).

The inflammation hypothesis posits that the unmanageable nature of Aβ plaque stimulates the chronic inflammation as an attempt to remove the plaque (Town et al., 2005). This is characterized by gliosis, involving the activation of brain-resident immune cells including microglial cells, astrocytes, macrophages, and lymphocytes, which release inflammatory mediators such as cytokines, chemokines, neurotransmitters and reactive oxygen species. The release of such chemicals induces recruitment of monocytes and, lymphocytes through the blood brain barrier (BBB), further fuel activation of microglia, and release of pro-inflammatory factors in a vicious cycle (Lee et al., 2010).

Early studies displayed the presence of immunoglobulins and complement factors present in the Aβ senile plaques, though no activated immune cells were reported (Eikelenboom & Stam, 1982). However, mRNA studies did show the presence of pro-

inflammatory mediators produced by microglia and astrocytes (Finch & Marchalonis, 1996). Later, several imaging studies showed clear microglial accumulation surrounding the amyloid plaque as well as pro-inflammatory markers with microglial burden correlating with declining cognition (Barton, 2006; Edison, Archer, Gerhard, Hinz, Pavese, Turkheimer, Hammers, Tai, Fox, & Kennedy, 2008; Rogers, 2008).

Complement system activation has been shown to decrease Aβ deposition (Golde, 2002). Additionally, activation of Aβ plaque induced inflammatory response has been shown to activate APOE and α-1 anti-chymotrypsin, and these have been shown to enhance Aβ deposition (Golde, 2002). These studies demonstrate the possibility of a fine balance existing between the beneficial and harmful aspects of the AD inflammatory response, but does not clearly identify the dominant side or provide a clear position for inflammation within the progression of AD (Golde, 2002). Nevertheless, epidemiological studies targeting the inflammatory aspect of AD with non-steroid anti-inflammatory drugs (NSAIDs) have demonstrated success in slowing down the progression of AD, especially in high-risk patients, emphasizing the clinical significance of inflammation in AD (Rubio-Perez & Morillas-Ruiz, 2012).

1.8 Microglial activation

Microglia are the principle immune cells for defending against pathogens in the brain(Gonzalez-Scarano & Baltuch, 1999). These cells were first identified by Pio del Rio-Hortega in 1932, but their existence remained a matter of debate for many years (Kettenmann et al., 2011). They are mesodermal in origin, displaying dual characteristics of both macrophages and monocytes in terms of molecular marker expression. Most believe the myeloid-monocytic hypothesis of microglial origin, but the correct progenitor

cells still remain uncertain. However, it has been shown that monocytes can migrate from peripheral blood circulation and disseminate into the brain parenchyma, crossing the blood brain barrier (BBB), in response to chemoattractant or various inflammatory stimuli. These ultimately differentiates as microglia with immunological characteristics similar to brain resident microglial cells (Malm et al., 2005; Simard & Rivest, 2004; Simard et al., 2006; Tambuyzer et al., 2009). Simard and his colleagues showed infiltration of peripheral bone marrow cells, expressing green fluorescent protein in the vicinity of amyloid plaques in brains of AD mouse models (Barton, 2006)

Microglial cells display phenotypic flexibility according to age as well as changes to the micro-environment that they constantly survey. At resting stage, they show ramified morphology during phagocytic response, displaying amoeboid (Caldeira et al., 2014; Hart et al., 2012; Kettenmann et al., 2011). An additional rod-shaped form has also been discovered, but their functional aspect in this form remains enigmatic (Taylor et al., 2014).

Microglia respond not only to early stages in brain injury or changes in its structural integrity, but also to changes in their micro-environment such as presence of pathogen-associated molecular patterns (PAMPS) or danger associated molecular patterns (DAMPS), for example, misfolded proteins (Kigerl et al., 2014; Kreutzberg, 1996). This response includes the migration of microglia to the site of injury, proliferation, and activation, expressing a high level of antigen-presenting proteins, thus becoming phagocytic in nature. Further, they also release cytokines such as tumor necrosis factor–α (TNFα) or interleukin-1β (IL-1β) which potentiate inflammatory signals by recruiting more microglia at the site of injury (Gonzalez-Scarano & Baltuch,

1999; Wojtera et al., 2012). Microglial activation can be recognized by detecting specific expressed markers such as CD-68, CD11b, CD-45, and benzodiazepine receptors (Colton & Wilcock, 2010).

Several studies have shown microglia activation in response to amyloid beta deposits (Cameron & Landreth, 2010; Edison, Archer, Gerhard, Hinz, Pavese, Turkheimer, Hammers, Tai, Fox, Kennedy, et al., 2008; Jung et al., 2015). Development in *in vivo* imaging methods such as multiphoton microscopy, have allowed direct observation in mouse models, of microglial recruitment to amyloid plaques. Studies have also demonstrated positive correlation between an accumulation of activated microglia in the AD brain cortex and cognitive decline. Similar correlation was observed by positron emission tomography imaging of peripheral benzodiazepine receptor (Cagnin et al., 2007; Meyer-Luehmann et al., 2008). In the AD brain, microglia has been shown to be involved in clearing Aβ deposits in addition to expressing pro-inflammatory molecules (Liu, Walter, et al., 2005; Morgan, 2009; Rogers et al., 2002; Simard et al., 2006). Furthermore, this receptor-mediated mechanism is independent of the classical phagocytic pathways primarily for fibrillar amyloid-beta (Koenigsknecht & Landreth, 2004).

With soluble Aβ, fluid phase macropinocytic uptake is considered the main pathway dependent on actin and tubulin dynamics. This mechanism is evidenced by co-localization of fluorescently labelled soluble Aβ and micropinocytic vesicles followed by subsequent trafficking to the endo-lysosomal compartment for degradation (Mandrekar et al., 2009). Conversely, when microglial functionality is suppressed in a mouse model,

expressing human APP with Swedish mutation, Aβ burden is increased, with rescue of non-pathological condition attained by Aβ vaccination (Krabbe et al., 2013).

1.8.1 Receptors found on microglial cells

Innate immunity represents the first line of defense against pathogens or danger-associated molecules. Inflammation in AD has emerged as one of the major events necessary for disease progression. Increased Aβ production drives activation of the innate immune response through a family of receptors collectively called as pattern recognition receptors (PRRs) (Kielian, 2006; Kigerl et al., 2014). PRRs recognize common structures present in exogenous pathogens, referred to as pathogen-associated molecular patterns (PAMPs), or in endogenously produced molecules known as danger associated molecular patterns (DAMPs).

The Toll-like receptors (TLRs) family is a large subgroup within the PRR family. The TLR extracellular domain possesses leucine rich repeats which are considered responsible for PAMPSs/DAMPs recognition. TLRs' activation induces intracellular protein kinase signaling, ultimately leading to expression of pro-inflammatory genes such as NF-kB, IRF-3, and AP-1. (Doens & Fernández, 2014; Kielian, 2006; Salminen et al., 2009). Mouse microglia express at least 9 homologs of human TLRs: TLR1, TLR2, and so on, up to TLR9. This has facilitated many studies, which have identified multiple TLRs involved in the microglial response to Aβ. Conventionally, TLR4 is a receptor for lipopolysaccharide (LPS) derived from the gram-negative bacterial outer membrane. However, TLR4 along with CD14 and myeloid differentiation factor 2 (MD-2) is also involved in Aβ-induced microglial activation (Fassbender et al., 2004; Walter et al., 2007).

Decreased levels of cytokine expression in scavenger receptor CD36 knock out mice in response to fibrillar Aβ, while its upregulation in response to Aβ deposits demonstrated the role of this receptor in AD (El Khoury et al., 2003; Ricciarelli et al., 2004). Furthermore a cell surface receptor complex involving CD36, integrin associated protein CD47, and membrane integrin α6β1 was discovered to be involved in the fibrillar Aβ-induced microglial pro-inflammatory response (Bamberger et al., 2003). Our lab demonstrated that TLR receptors (specifically TLR2 and TLR4) in human monocyte derived from THP-1 cell, mediate monocyte activation by Aβ42 fibrils. Three-way, antibody neutralization against TLR2, TLR4 and co-receptor CD14 provided greater reduction of this response than did blocking any the three receptors individually (Udan et al., 2008).

Later, a similar study utilizing microglial cells showed CD14, TLR4 and TLR2 functioning in parallel with CD36, CD47, and integrin protein α6β1 as a fibrillar Aβ42 receptor complexes to induce intracellular activation of reactive oxygen species (ROS) production, phagocytosis and NF-kB activation (Reed-Geaghan et al., 2009). Additional studies indicated CD36 mediated TLR4 and TLR6 heterodimerization induced by fibrillar Aβ (Stewart et al., 2010). These studies suggest TLR2, TLR4 and TLR 6 are the major players for bringing about fibrillar Aβ-induced proinflammatory responses in microglial cells. Additional receptors, such as those for advanced glycosylation end products (RAGE), have been implicated with transport of Aβ across the blood brain barrier while transmembrane protein CD33 expression is involved in decreased microglial Aβ phagocytosis (Doens & Fernández, 2014).

1.9 NLRP3 inflammasomes

Inflammasome (approximately 700 kDa) is a multi-protein complex platform required to process precursors to mature cytokines such as IL-1β, when an innate immune response is triggered though TLRs or NLRs (NOD-like receptors) by PAMPS or DAMPS (Doens & Fernández, 2014; Kigerl et al., 2014). This protein complex consists of NLR protein, pro-caspase-1, and adaptor protein ASC (apoptosis- associated spec-like protein). Although there are multiple types of NLRs genes responsible for inflammasome formation, NLRP3 is the one best characterized. The structural organization of NLRP3 includes N-terminal pyrin domain and a caspase recruitment domain (CARD): central nucleotide-binding oligomerization domain (NACHT), which is common to all NLR proteins, and C-terminal leucine rich domain (Ting et al., 2008).

The pyrin-pyrin interaction between CARD domains present in NLRP3 and pro-caspase-1 brings them together, thereby forming an inflammasome complex which then converts pro-caspase-1 to active caspase-1 via proteolytic cleavage. This activated caspase then in turn processes immature pro-IL-1β to mature IL-1β (Liu & Chan, 2014). Initiation of this process appears to require two signals. The first signal is the priming event triggered by known TLR ligands such as LPS, which lead to activation of NF-κB, leading to upregulation of pro-IL-1β expression and translation. This signal also induces up regulation of NLRP3 components both at the transcriptional as well as the translational level (Sutterwala et al., 2014).

The second signal required for complex formation and subsequent activation can be achieved by varieties of molecules including toxins (e.g. nigericin), ATP, urate, Aβ, asbestos, silica, potassium ion efflux, and alum (Doens & Fernández, 2014; Hanamsagar

et al., 2012; Sutterwala et al., 2014). Because of such diverse species potent to to activate NLRP3, investigations are more focused towards finding cognate or common mechanisms that involves direct interaction with NLRP3 inflammasome.

Halle et al were the first to show the role of the NLRP3 inflammasome dependent IL-1β production in AD in 2008. Their studies showed fibrillar Aβ phagocytosis mediated release of the cathepsin-B (serine protease enzyme) from lysosomal damage was necessary for mature IL-1β production in microglial cells (Halle et al., 2008). NLRP3 as well as ASC dependent IL-1β production was also illustrated by comparing levels of IL-1β production in NLRP3 knock out and ASC knock out primary microglial cells with wild type microglial cells. The IL-1β level was significantly reduced in both knockout microglial cells. In addition, purinergic receptor P2X7 and dependent ATP induced IL-1β production in these knock out microglial cells was shown to be an independent mechanism of IL-1β production (Hanamsagar et al., 2012). Studies on oligomeric Aβ induced IL-1β production showed dependence on caspase-1 activation, which surprisingly were independent of phagocytosis, lysosomal degradation, and cathepsin-B release, unlike previous report on fibrillar Aβ. This study also displayed mitochondrial reactive oxygen species (mROS) augmenting oligomeric Aβ induced IL-1β production (Parajuli et al., 2013). Another study using reactive oxygen species (ROS) inhibitor, N-acetyl cysteine, and cathepsin- B inhibitor indicated that Aβ oligomer induced IL-1β production is dependent on ROS but surprisingly, cathepsin-B dependent, contradicting results from the Parajuli group. There was no clear explanation for this contradiction, but attributed to probable different oligomeric composition or structures (Taneo et al., 2015).

From our lab, recent studies on soluble Aβ42 protofibril induced IL-1β production showed that Aβ42 protofibrils were able to elicit both mature and pro-form of intracellular IL-1β production, indicating these Aβ species can provide both priming and secondary signals. Surprisingly, this increasingly accumulated intracellular level of mature IL-1β did not correlate with secreted levels, but rather IL-1β secretion occurred in quantized amounts. This indicated the possibility of discrete regulatory event for mature IL-1β secretion (Terrill-Usery et al., 2014).

1.10 Cytokines as pro-inflammatory mediators

Inflammatory activation has come to be recognized as one of the characteristic features of AD brain. It involves activated microglial cells and astrocytes along with production of a number of cytokines. Cytokines are small protein molecules ranging from 8 kDa to 40 kDa and are produced by immune cells in response to antigen. Their biological functions are diverse, and include cell proliferation, differentiation, inflammation, and apoptosis.

Whether AD-related inflammation is a cause or consequence, as well as its role in the disease progression has yet to be delineated (Reale et al., 2012; Rubio-Perez & Morillas-Ruiz, 2012). The major pro-inflammatory cytokines promoting inflammation, found to be upregulated in activated microglia as well as amyloid plaques and CSF are TNFα, IL-1β, IL-1α, and IL-6. IL-6 secretion is induced by IL-1 in both paracrine or autocrine fashions by astrocytes, and is believed to amplify inflammatory signaling. However, increased expression of IL-6 has also been shown to reduce amyloid deposition (Cacquevel et al., 2004; Hensley, 2010). Expression of these cytokines has also been shown to be upregulated in a TLR4-dependent manner in AD mouse models.

Also in AD, secretion of these cytokines has not only been linked to microglial activation as a chemoattractant, astrogliosis, and APP expression, but also induction of further cytokine production—thereby propagating the vicious cycle of inflammatory response causing neurodegeneration (Griffin, 2006; Ho et al., 2005). The roles of most studied pro-inflammatory cytokines TNFα and IL-1β are described in next sections.

1.10.1 Interleukin-1β (IL-1β)

Interleukin-1 (IL-1) is an important pro-inflammatory cytokine that mediates numbers of functions including acute phase proteins productions, lymphocyte activation, the production of other cytokines involved in immune function, and the induction of fever and hypotension when released in blood (Glaccum et al., 1997; Sardi et al., 2011). The gene for IL-1 synthesis is located on chromosome 2, and it codes for two isoforms of IL-1: IL-1α and IL-1β. These isoforms exert their biological activity through native 80kDa cell surface receptors called type I IL-1 receptors (IL-1RI). However they are also type II IL-1 receptors, which are decoy receptors that do not transduce signal. The cerebrospinal fluid of AD patients has high levels of these type II IL-1 decoy receptors, which could be a response to high IL-1β secretion in chronic inflammatory conditions (Sardi et al., 2011; Sciacca et al., 2003; Shaftel et al., 2008).

In connection with AD, IL-1β was initially shown with immuno-staining of microglia, surrounding thioflavin-positive senile plaques, and seems to enhance the metabolism of APP, thereby increasing amyloid deposition and plaque formation (Benzing et al., 1999; Sardi et al., 2011). Increased IL-1β levels in cerebrospinal fluid and CNS have been related to neurodegeneration as well as cognitive decline in AD (Blum-Degena et al., 1995; Holmes et al., 2003). The mature form of IL-1β production involves

the primary signaling through TLR ligands such as LPS, causing an increase in translational pro-form of IL-1β (31 kDa), which in turn is processed into the mature form of IL-1β (17kDa) by inflammasome mediated caspase-1 activation from a secondary signal such as Aβ (Hanamsagar et al., 2012; Sutterwala et al., 2014; Terrill-Usery et al., 2014). In most studies, IL-1β expression is elevated in patients with AD. Sustained production of cytokines reflecting the scenario of chronic inflammation is subjected to the adverse effects in AD and other neurodegenerative diseases (Griffin et al., 1989; Rubio-Perez & Morillas-Ruiz, 2012).

Studies have also shown a neuroprotective effects of IL-1β overexpression. AD mice models (APP/PS1) studies engineered to overexpress IL-1β in the hippocampal region showed a reduction in plaque burden. This was likely implicated to increase Aβ plaque degradation by activating microglial cells (Shaftel et al., 2008). This can also explain the possible recent failure in therapy involving an anti-inflammatory approaches to AD treatment. However, another AD mice model study where IL-1RI receptor was not expressed showed no difference in Aβ deposition, when compared with AD mice expressing this receptor for IL-1β (Das et al., 2006). Therefore, a clear mechanism for the positive role of IL-1β in reducing AD pathology remains elusive.

1.10.2 Tumor necrosis factor-α (TNFα)

TNFα is a pro-inflammatory cytokine produced by immune cells in response to various substances having pathogenic properties. Its functions are diverse and include cell proliferation, differentiation, and apoptosis or necrosis. It is also induces other cytokine production such as IL-1, IL-6, IL-8 and granulocyte colony-stimulating factor. In AD brains, this cytokine has been found upregulated where glial cells (more specifically

microglia) are the major contributors, while neurons also produce decent TNFα levels. However, the role of this cytokine has remained controversial in AD. *In vitro* and *in vivo* studies show neuro-protectiveness against Aβ toxicity, but TNFα have also been implicated in neuronal degeneration (Carriba et al., 2015; Fillit et al., 1991; Medeiros et al., 2007; Perry et al., 2001).

TNFα is expressed as a membrane-bound precursor molecule which is cleaved by metalloprotease TNFα converting enzyme (TACE) into active soluble 51kDa homotrimeric form. Each 17kD monomer subunit is composed of ten β-sheet strands arranged in antiparallel orientation, three of which associate to form a bell-shaped trimer (Eck & Sprang, 1989; Wajant et al., 2003). The diverse cellular effects of this cytokine are propagated through two cysteine rich receptors: TNF receptor type 1 (TNFR1) and TNF receptor type II (TNFR2), belonging to the TNF superfamily. TNFα acts on both receptors. TNFR1 is the primary receptor that can induce survival as well as cell death signals. Downstream activation of NF-κB transcription factors or mitogen-activated protein kinase (MAPK) have been linked to cell survival signaling through TNFR1; blocking the NF-κB pathway, causes TNFα-induced neuronal death. TNFR1 has a death domain that interacts with death domains of other adaptor proteins forming death-induced signaling complexes, ultimately leading to initiator caspase-8 activation followed by effector or executional caspase-3 activation and thus mediating apoptosis (Carriba et al., 2015; Van Antwerp et al., 1996; Wajant et al., 2003; Zhao et al., 2003).

Studies have also shown APP and tau protein are substrates for caspases, and linked to hyper phosphorylation of tau protein (Marks & Berg, 1999). TNFR2 does not have a death domain, and is linked to cell survival signaling through interaction with fas-

associated death domain like interleukin-1 beta-converting enzyme inhibitor protein (FLIP), which in turn interacts with TNFR-associated death domain(TRADD) and prevents the recruitment and activation of caspase-8 (Wajant et al., 2003; Zhao et al., 2003).

Recent study has shown reduced expression of neural protein known as fas apoptotic inhibitory molecule (FAIM) in AD hippocampal samples. The increased expression of this protein showed a protective effect of TNFα in Aβ-induced neuronal death, providing a potential regulatory target for neuronal survival (Carriba et al., 2015). Further, TNFα is also known to cause cell death by necrosis, without involving ATP or caspase. However, ROS produced can directly activate apoptotic caspases, as well as play role in necrotic pathways, making indirect connections between these two pathways (Wajant et al., 2003).

Studies in TNFα receptor knock-out mice showed increased neurodegeneration when such mice were subjected to ischemic injury. Further, the level of anti-oxidant was found to be reduced in such mice. This indicated a protective role of TNFα, likely through anti-oxidant pathway activation (Bruce et al., 1996). TNFα has also been shown to exert its neuro-protective effect against Aβ toxicity through increased microglial chemotactic receptor expression for fibrillar Aβ uptake and subsequent clearance (Cui et al., 2002). However, another study using pharmacological and genetic inhibition of TNFα production after injection of Aβ into AD mouse model brain, reduced the cognitive dysfunctions (Medeiros et al., 2007). Therefore, still more rigorous studies are needed to identify a definitive therapeutic approach to regulating TNFα-mediated neuroprotective effect in AD treatment.

CHAPTER 2. METHODS

2.1 Isolation and plating of primary mouse microglial cells

Wild type, MyD88$^{-/-}$, and CD47$^{-/-}$ primary microglial cells were obtained from neonatal C57BL/6 mouse pups (Harlan laboratories Inc., Kielian's Lab University of Nebraska Medical Center; and Jackson laboratories, United States, respectively) under sterile conditions. Whole brains from euthanized (isoflurane inhalation) 3 to 4 days old post-natal mice pups were isolated. The isolated brains were rolled over gauze, slightly moistened with phosphate buffered saline (PBS) in a petri dish to remove the meninges, and then placed in an ice-cold 50 ml sterile conical tube containing 20 mL of phosphate buffered saline (PBS, Hyclone) supplemented with antibiotics.

The collected brains were transferred to a 100 mm cell-culture dish and minced using the sharp edge of two separate forceps in a scissor action. Then, minced tissues were re-suspended in 5 mL of 0.5% trypsin (Hyclone) and incubated at 37 °C, 5% CO_2 for 20 minutes on a shaker to facilitate further tissue dissociation. Then, cells were suspended again in 10 mL of complete Dulbecco's modified Eagle's medium without pyruvate (DMEM, 4.5 g/l glucose, Hyclone) containing 10% heat-inactivated fetal bovine serum (FBS, Hyclone), 4mM L-glutamine, 100U/ml penicillin, 0.1 mg/ml streptomycin and 0.25 µg/mL amphotericin-B (P/S/A triple antibiotic mixture, Fisher Scientific), 10 µM oxaloacetic acid, 4.5 µM pyruvate, 0.002 U/mL insulin (OPI medium supplement, Sigma-Aldrich), and 0.5ng/ml recombinant mouse GM-CSF (Invitrogen). To get rid of

the debris, the cell suspension was triturated using a 10 mL pipette and then filtered through a 70-μm cell strainer to remove tissue debris (Fisher Scientific). The cell suspension thus obtained was centrifuged at 200 x *g* for 5 minutes at 25 °C, suspended again in complete medium, and seeded into a 150 cm^2 flasks (Corning). Finally, each 150 cm^2 flask contained 30 mL of total cells.

Depending upon size of the litter, the number of flasks per single litter of mouse pups was determined. Mostly each flask contained cells from 3-5 isolated brains. The cells were further cultured at 37 °C in 5% CO_2 until they became confluent in 1 to 2 weeks. Under a light microscope of 10 times magnification, microglial cells appeared as round cells resting on the top of a granular astrocytic layer. For selective microglial harvesting, the flask was shaken overnight at 37 °C in 5% CO_2 (at a speed between 4 and 5 on the shaker in the cell culture facility) followed by medium collection. The flasks were immediately replenished with a fresh growth medium to allow for proliferation of remaining microglia. Commonly, this procedure was repeated 3-4 times for one flask (Esen & Kielian, 2007) within a period of one and half months before discarding the flask.

2.2 Aβ preparation

Aβ has a high tendency to aggregate into polymorphic species, mainly through undergoing nucleation-dependent polymerization driven by non-covalent interactions. The minimum structure required to enhance aggregation kinetics (seeds) or nuclei is thought to be formed initially, allowing aggregation to progress and ultimately form higher molecular aggregates such as protofibrils and fibrils (Bitan et al., 2003; Harper et al., 1999; Jarrett et al., 1993; Jarrett & Lansbury, 1992). Therefore, it becomes important

to remove any pre-formed seeds to obtain homogenous Aβ solution, and this is done by dissolving Aβ in 100% hexafluoroisopropanol (HFIP).

50 mg of Aβ(1-42) or Aβ(1-40) peptides (Keck Center, Yale University) were dissolved in 100% HFIP (Sigma) resulting a concentration of 1mM for an hour at room temperature. The Aβ solution then was aliquoted into sterile microcentrifuge tubes, evaporated overnight under the fume hood without lid at room temperature, and followed by vacuum centrifugation to remove any residual HFIP and was stored at -20 °C.

2.3 SEC-Purified Aβ monomer and protofibril preparation

Initially Walsh and his group prepared Aβ protofibrils by resuspending the Aβ peptide in a solution of NaOH, followed by dilution into PBS, and separating the aggregates by using SEC (Walsh et al., 1997). For our study, Aβ(1-42) protofibril was isolated from monomer by using AKTA-fast protein liquid chromatography (FPLC) along with SEC. SEC separates proteins based on their shape and size. More specifically, larger protein molecules with high molecular weight elute out from the column first in the void peak, and then smaller ones come in the later included peak.

For our experiments, about 1.1 mg of lyophilized Aβ(1-42) peptide was dissolved in 50 mM sodium hydroxide (NaOH, Fisher Scientific) resulting in a 2.5 mM Aβ solution. The solution was pipetted up and down gently to make sure all the peptides dissolved. The Aβ solution was further diluted to 250 μM with pre-filtered (0.2 μM filter, Thermo Scientific) artificial cerebrospinal fluid (aCSF, 15 mM $NaHCO_3$, 1 mM Na_2HPO_4, 130 mM NaCl, and 3 mM KCl at pH 7.8). aCSF composition was slightly modified from the protocol described by Tzounopoulos et al (Tzounopoulos et al., 2004). The diluted Aβ solution was then centrifuged at 18,000 x *g* for 10 minutes with a

Beckman-Coulter Microfuge® 18. The supernatant was injected into the SEC. Incubation of Aβ solution was carried out for longer time when more protofibril was required to be produced. Also, before injecting Aβ solution, the Superdex75 HR 10/30 column was coated with 2 mg/ml bovine serum albumin (BSA, Sigma) in order to reduce the chance of non-specific Aβ binding to the column resin, thereby enhancing the total yield of the protein.

Aβ eluted at 0.5 ml/min while elution profile was continuously being monitored through UV absorbance at 280 nm. The eluted fractions collected were placed immediately on ice. Depending upon the high UV peak absorbance, the fractions were selected. The fractions eluting in the void peak were labeled as protofibrils, while fractions eluting in the included peak were labeled monomer. Depending upon the requirement, the individual fractions were directly used for the cell treatment or pooled together before cell treatment. Concentrations of the individual fractions were directly determined from the absorbance trace using an extinction coefficient of 1450 cm^{-1} M^{-1} at 280 nm, which was mainly based on tyrosine residue (Nichols et al., 2002).

For Aβ(1-42) and Aβ(1-40) monomer purification, a separate protocol was used. Primarily in order to obtain monomer, about 1.1 mg of lyophilized Aβ aliquot was reconstituted in a mixture of 6 M guanidinium hydrochloride (GuHCL, Fisher Scientific) and 10 mM ammonium hydroxide (NH_4OH, Fisher Scientific), yielding a total volume of 1.0 ml. Aβ solution was then subjected to SEC and eluted following the conditions described for protofibril purification. Here also, the solution was centrifuged, supernatant was injected into the column, and elutions were collected as 0.5 mL fractions. With this preparation, the dominant peak we obtained was Aβ monomer in the included peak.

Before injecting Aβ, we coated the column with BSA as previously described in protofibril preparation. Thus, obtained Aβ fraction is not pooled if high concentration is needed, but is pooled if high volume of SEC purified Aβ is needed. Again, monomer concentration is determined from UV absorbance elution profile as previously described. The SEC purified Aβ monomer was then used either to treat the cells for comparing cellular cytokine responses or to perform aggregation studies.

2.4 Microglial stimulation studies with Aβ

Several of the studies led have identified TLR receptors, such as TLR2, TLR4 and TLR6, as being involved in Aβ mediated pro-inflammatory response (Bamberger et al., 2003; Stewart et al., 2010; Udan et al., 2008). Therefore, known TLR ligands like LPS are used in our cellular stimulation studies as positive control for bringing about cytokine responses (Figure 2.1). For Aβ-used cellular studies, primary microglial cells (WT or $CD47^{-/-}$ or $MyD88^{-/-}$) were detached from the astrocyte layer into the growth medium by overnight shaking of the culture flask as stated above. Then, a medium containing primary microglial cell suspension was collected in 15 mL conical flask and centrifuged at 200 x *g* for 10 minutes at room temperature. The spent media was discarded, and a cellular pellet was suspended again in a fresh complete microglial growth medium containing 10% FBS and 50 ng/ml GM-CSF. Cell concentration was determined by counting cells under a microscope in Neubauer chamber, and cells were plated in a 96 well sterile culture plate at 5×10^5 cells per mL with volume of 100 µL in each well of the culture plate. The plate was then left incubated overnight (24 hours) at 37 °C and 5% CO_2 for allowing microglia adhere to the plate.

The next day, the spent media was removed and washed with microglial assay media, which is the same as complete microglial growth media, but lacks FBS and GM-CSF. Depending upon the concentration of the pooled or individual fractions of freshly purified Aβ (monomer or protofibril), the required volume of Aβ added to the microglia was calculated making the final treatment concentration 15 μM in 100 μL in total volume. The remaining volume was filled with assay media. For every treatment, we also carried out negative control treatment in parallel.

For negative control, instead of Aβ monomer or protofibril, an equal volume of aCSF buffer would be added, and the remaining volume would be assay media. For positive control, cells were also treated with LPS at a final concentration of 5 ng/ml with a volume of assay media equal to the other treatments done with Aβ. Cells were then incubated for a specific time period at 37 °C and 5% CO_2.

For extracellular cytokine measurement, the cellular medium after the treatment with Aβ or LPS or aCSF was collected and stored at -20 °C until analyzed by enzyme-linked immunosorbent assay (ELISA). Further, in order to assay intracellular cytokine, more specifically IL-1β, cells were first washed with cold, sterile PBS, and lysed with an ice-cold RIPA buffer containing 20 mM Tris, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1 % deoxycholic acid, and 0.1% sodium dodecyl sulfate (SDS). Before adding RIPA buffer to the cells, it was supplemented with a 40X protease inhibitor cocktail (Sigma). The RIPA buffer was allowed to sit for couple of minutes, and finally mixed well by gently pipetting up and down. The buffer solution was then collected and stored at -20 °C until assayed by ELISA.

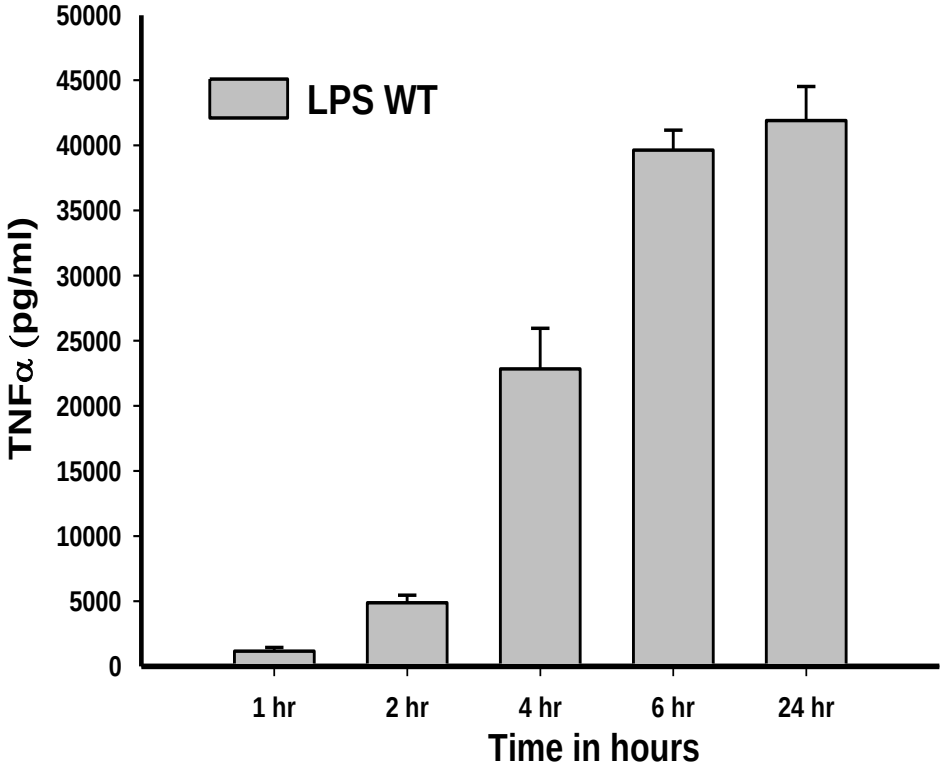


Figure 2.1 LPS induced TNFα secretion in wild type primary microglial cells.

Primary microglia plated in a 96-well sterile plate were incubated with 10 ng/mL of LPS for different time points. Conditioned media from each time point were collected and TNFα ELISA was done. Data bars represent the mean ± std error of n = 3 replicates.

2.5 Antibody neutralization assay

The method was followed similarly to the protocol described in Udan et al, with little modification (Udan et al., 2008). Functional-grade neutralizing anti-mouse CD47 antibody or functional-grade mouse IgG2a isotype control was purchased from eBioscience (San Diego, CA). After overnight incubation of the primary microglial cells was plated in a sterile 96 well plate as described above, and washed with assay media. Depending upon calculated volume of Aβ(1-42) protofibril to be added to the media for final 15 μM treatment concentration, the required volume of assay media was added. 5 μL of antibody (CD47 anti-antibody or IgG2a isotype) was added to the cells making its final concentration either 5 μg/ml or 10 μg/ml, and the cells were incubated for one hour at 37 °C and 5% CO_2. After this pre-treatment, the required volume of SEC-isolated and freshly purified Aβ(1-42) protofibril was added and incubated at 37 °C and 5% CO_2 for another 6 hours.

In parallel negative control, wells containing microglial cells, assay media, an aCSF buffer, positive control wells microglial cells, assay media, LPS, and aCSF were set up and incubated under the same conditions for an equal time period. After incubation, medium was collected and stored at -20 °C until assayed by ELISA for TNFα and IL-1β.

2.6 Treatment with 4N1K peptide

4N1K peptide is ten amino-acid sequence peptides (KRFYVVMWKK) used in studies to inhibit CD47 mediated processes such as cytokine production by binding with CD47 receptors (Bamberger et al., 2003; Brown & Frazier, 2001; Koenigsknecht &

Landreth, 2004). Our intended use of 4N1K was also a CD47 antagonist, and we purchased this peptide from Ana Spec Company (Fremont, CA).

After an overnight incubation of plated primary microglial cells followed by washing and the addition of assay media, 10 μL of 4N1K peptide solution reconstituted in sterile distilled water was added, making the final concentration 100 μM. This concentration was chosen based on studies done by Bamberger and his team (Bamberger et al., 2003).

Incubation at 37 °C in the presence of 5% CO_2 was carried out for 1 hour as pre-treatment. The required volume of freshly SEC-isolated Aβ(1-42) protofibril was added, making a final 15 μM concentration, and further incubation at the same condition was done for 6 hours before the medium was collected and stored, as stated previously for ELISA. For a dose-dependent study of 4N1K treatment, several high-stock concentrations of 4N1K peptide (100 μM, 30 μM, 10 μM, 3 μM, 1 μM and 0.3 μM) solutions were prepared. As described above, pretreatment containing microglial cells, 60 μL microglial assay media, and 6.5 μL 4N1K from stock solution was carried out. After pre-treatment, freshly SEC-purified Aβ(1-42) protofibril (32 μL) or LPS (10 ng/ml final concentration) was added. Further incubation lasted for 6 hours, and the final collection of media was recorded.

2.7 Polymyxin-B sulfate (PMX-B) neutralization assay

PMX-B neutralization assay is a peptide antibiotic used to check contaminations of Aβ solution with bio-active components derived from gram-negative bacteria or other endotoxins. It is employed to test whether the inflammatory response from Aβ is a false positive (Meda et al., 1995; Udan et al., 2008). For treatment after adding the appropriate

volume of microglial assay media, PMX-B (600 μg/ml stock solution in water) was added at a final concentration of 100 ng/ml, Aβ(1-42) or Aβ(1-40) was added and incubation, and the collection of conditioned media was recorded, as described previously for cytokine assessment.

2.8 Cytokine determination

The level of cytokines TNFα and IL-1β was assayed as a marker for proinflammatory response by ELISA. This technique is highly sensitive and efficient for quantifying cytokine levels in a large number of samples (Cooper et al., 1994).

2.8.1 TNFα assay by ELISA

The assay was followed similarly to the one used for assessing human TNFα as described previously, but with few modifications for our mouse TNFα assessment (Udan et al., 2008). In brief, 96 well plates were added with 100 μL of 0.4 μg/ml monoclonal anti-mouse TNFα capture antibodies (stock aliquots were stored at -80 °C at 200 μg/mL in PBS) and incubated overnight at room temperature to allow for the coating of the plate with capture antibodies.

The following day, the antibody coated wells were washed with a wash buffer containing PBS and 0.05% Tween 20. Subsequent blocking with blocking buffer containing 1% BSA, 5% sucrose and 0.05% sodium azide (NaN_3) was performed. After a further washing step, 50 μl of a sample diluent (20 mM Tris base, pH adjusted to 7.3, 150 mM NaCl, 0.1% BSA and 0.05% Tween 20) was added to the wells, followed by adding 50 μl of sample or standard TNFα proteins. We then let it sit at room temperature for two hours. A 100μl of biotinylated polyclonal anti-mouse TNFα detection antibody (stock aliquots stored at -80 °C at 50 μg/ml in detection diluent) diluted in detection diluent (20

mM Tris, pH adjusted to 7.3, 150 mM NaCl and 0.1% BSA) was added to the wells after a wash with washing buffer, followed by incubation at room temperature for 2 hours. 100 µl of streptavidin-horseradish peroxidase (HRP) conjugate diluted in conjugate diluent (PBS containing 1% BSA) was added and allowed to sit for 20 minutes at room temperature. A mixture of HRP substrates 3,3',5,5'—tetramethylbenzidine (TMB)—and hydrogen peroxide in equal volume was added followed by incubation for another 20 minutes. In between these steps, washing with wash buffer was conducted.

Finally, the reaction was stopped by the addition of 1% H_2SO_4 solution. Optical density of the samples was measured by a SpectraMax 340 absorbance plate reader at 450 nm with a reference reading at 630 nm. The unknown TNFα concentration from the samples was determined from a TNFα standard curve of 15 to 2000 pg/ml. However, depending upon the required dilution of the samples or a higher standard curve range, up to 16000 pg/ml was also performed to ensure that the absorbance of the samples fell within the standard curve range.

2.8.2 IL-1β assay by ELISA

IL-1β determination for both intracellular and extracellular samples was done by ELISA. The method is similar to the one described above for TNFα ELISA, with changes in using capture and detection antibodies for IL-1β ELISA. Here also, an overnight coating of 96 well plate with 2.0 µg/mL monoclonal anti-mouse IL-1β capture antibody (stock aliquots were stored at -80 °C at 720 µg/mL in PBS) was done at room temperature. Samples and standards were prepared and added, then followed incubation as described in TNFα ELISA.

After a wash with a washing buffer, a 100 µl of 0.5 µM concentration biotinylated polyclonal anti-mouse IL-1β antibody (stock aliquots stored at -80 °C at 180 µg/mL in detection diluent) diluted in detection diluent was added and incubation at room temperature, and carried out for 2 hours. The next step prior to the addition of 1% H_2SO_4 is same as described for TNFα ELISA. Finally, optical density was read by the Spectra Max 340 absorbance plate reader (Molecular Devices, Union City, CA) for samples and standards at 450 nm, with a reference reading at 630 nm. IL-1β in samples that were calculated from the IL-1β standard curve of range 15 to 2000 pg/ml. However, when needed the standard curve range was increased to 8000 pg/ml or the samples were diluted.

2.9 XTT for assessing cell viability

XTT [2, 3-bis (2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide] is a tetrazolium dye that is reduced by active cells via dehydrogenases enzymes of a mitochondrial electron transport chain into water soluble formazan product in the presence of phenazine methosulfate (PMS) (Roehm et al., 1991) (Figure 2.2). In our study, the viability of primary microglial cells treated with Aβ (1-42) protofibrils alone or in combination with other peptide such as 4N1K was assessed using this assay.

After the necessary treatments of microglial cells, incubation, and collection of supernatants for ELISA, the cells were washed with sterile PBS. In the meantime, a 1 mg/ml solution of XTT in an RPMI-1640 medium devoid of phenol red and 5 mM phenazine methosulfate (Acros, Morris Plains, NJ) solution in water stored at -20 °C were thawed.

The final working XTT solution was prepared by dilution of these stock solutions with RPMI-1640 devoid of phenol red, making final concentration of XTT as 0.33 mg/ml and PMS 8.3 μM. 100 μl of this working XTT solution was added on to the cells and incubated for 2 hours at 37 °C in presence of 5% CO_2. The intensity of color developed due to XTT reduction was measured in the form of absorbance at 467 nm with the Spectra Max 340 absorbance plate reader (Molecular Devices, Union City, CA). This measured absorbance represented the extent of cell viability.

2.10 Dot blot assay

Dot-blot assay are more direct approaches for detecting the protein or antigen of interest through an antigen-antibody specific reaction. They are qualitative assays similar to western blot, which employs the additional steps of electrophoretic separation of protein mixture on polyacrylamide gels and the transfer of the separated proteins to the nitrocellulose membrane—unlike in dot-blot, where the protein sample is directly adsorbed on the nitrocellulose membrane. All procedures of dot-blot in our study were followed as previously described, with little modification (Kayed et al., 2003).

Briefly, nitrocellulose membrane was immersed in water and left to dry for 15 minutes as a membrane activation step. Then, 2 μL of an Aβ sample was spotted onto the membrane and allowed to adsorb for 15 minutes. To prevent non-specific binding, the membrane was immersed in 5.0 ml of blocking buffer containing 10% dried milk in PBST (PBS with 0.2% Tween-20) and gently shaken on rotator for an hour at room temperature. Then the membrane was washed by immersion in PBST with gentle agitation on a shaker for 5 minutes. This was followed by a transfer of the membrane into 5.0 ml of a primary antibody solution—either a conformation specific OC antibody

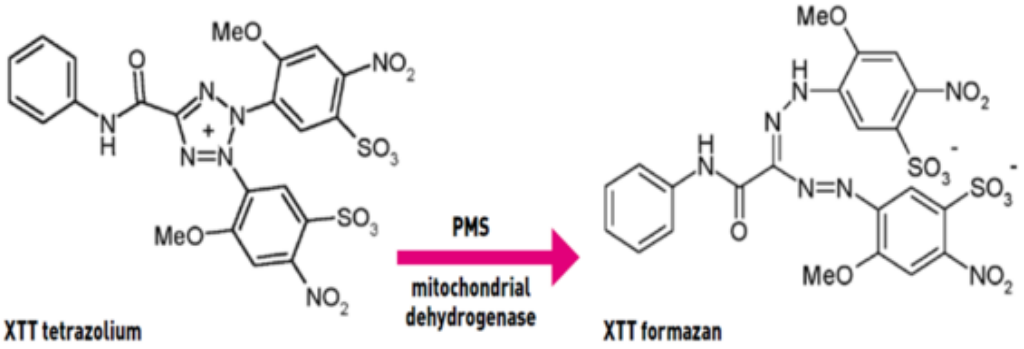


Figure 2.2 Reduction of XTT tetrazolium salt to form formazan derivative.

XTT gets reduced by living cells by mitochondrial enzyme to orange colored water soluble dye which has high absorbance at 467 (nm https://www.applichem.com/en/literature/applications/no-12-cell-proliferation-assay-xtt/)

(gift from R. Kayed, University of Texas Medical Branch, Galveston, TX) with 1:5000 dilution or with an Ab9 antibody (1:5000) (gift from T. Rosenberry, Mayo Clinic Jacksonville, FL). An OC antibody recognizes elements of fibrillar structure across a broad range of Aβ aggregate sizes (Kukar et al., 2005; Wang et al., 2013; Coalier et al., 2013).Ab9 is used as a positive control that first recognizes 16 N-terminal amino acid residues of Aβ regardless of its conformation (Coalier et al., 2013; Kukar et al., 2005; Wang et al., 2013). After treatment, the membrane (with primary antibody) was washed three times with PBST (each washing for 5 minutes in PBST with gentle agitation) to remove excess as well as any non-specifically bound primary antibody. This was followed by incubation for 1 hour at room temperature with gentle shaking, after adding a secondary antibody with a 1:1000 dilution of an anti-rabbit IgG HRP conjugate for the OC primary antibody treated membrane or adding anti-mouse IgG HRP conjugate for the Ab9 treated membrane. Next, a washing step for three times as previously stated and an incubation of the membrane for 1 minute in 6 mL of enhanced chemiluminescent substrate (ECL-Western Blotting substrate, Pierce, Thermo Scientific) with vigorous shaking were conducted. This substrate was prepared by mixing equal volumes of detection reagent 1 with detection reagent 2. Excess ECL substrate was removed by blotting the membrane on Whatman paper. The membrane was then developed by exposing it to the film for 30 seconds.

For our stability assessment of OC-positive Aβ species, 5 μL of SEC-purified Aβ monomer or protofibril was mixed with 5 μL of 4 M urea or 4 M GuHCL or with 5 μL of 1% SDS. The mixture then was allowed to stand for 10 minutes at room temperature. 2 μL of the mixture was spotted on to the membrane, and the steps were followed as

described above. The long term SDS stability of Aβ(1-42) monomer was done on freshly SEC-isolated Aβ stored at 4 °C, carrying out the DOT-blot assay each day by mixing with SDS and following the steps as described above.

2.11 Thioflavin-T fluorescence

Thioflavin T (ThT) is a benzothiazole dye that displays high fluorescence on binding to β-sheet-rich species such as amyloid fibrils; therefore, it indirectly represents the extent of β-sheet-rich species (LeVine, 1999; Paranjape et al., 2012).

In our study, we used ThT fluorescence to monitor aggregation of 20 μM, Aβ(1-42) monomer stored in the refrigerator at -4 °C for a number of days. For the fluorescence measurement, 17.5 μL of Aβ(1-42) solution from 20 μM stock solution was diluted to a final concentration of 5 μM in 48.5 μL of F-12 cell culture medium (Bio World, Dublin, OH) and added with 4 μL of 100 μM stock ThT, final 5 μM ThT concentration, yielding total volume of 70 μL. A ThT-fluorescence scan (460-520 nm) of the solution was done using a Cary Eclipse Fluorescence spectrophotometer at an excitation wavelength of 450 nm. The fluorescence scan was integrated between 470 nm to 500 nm. As a background control, 66 μL of F-12 medium was added with 4 μL of ThT (100 μM stock concentration), and the emission scan integrated value was subtracted from the Aβ sample scan.

ThT fluorescence was also used to monitor *in situ* Aβ(1-42) monomer aggregation and the formation of possible β-sheet-rich aggregated species in presence of primary microglial cells. For this purpose, we plated cells in a black Nunclon 96 well sterile plate at 5×10^5 and incubated them overnight as described in the microglial stimulation studies section. Depending upon the stock Aβ(1-42) monomer concentration, the volume

required for final 15 μM Aβ concentration was calculated, and the volume of microglial assay media was added accordingly, making the final total volume 100 μL in the microglial cells plated well. Incubation was done at 37 ℃ and 5% CO_2.

At different time points 5.2 μL of ThT from 100 μM stock solution was added, and a fluorescence reading was taken with a Perkin Elmer plate reader (Wallac Victor 3, 1420 multilabel counter, 20101 Turku Finland). The excitation and emission filter used was of 450 nm (band width of 8) and 485 nm (band width of 10). For control, the same amount of ThT was added to the microglial wells, having only a microglial assay medium, and a fluorescence reading was taken. Further, the media was also collected for TNFα assessment by ELISA.

2.12 Bis-ANS (4, 4'-Dianilino-1, 1'-binaphthyl-5, 5'-disulfonic acid) fluorescence

4, 4'-Dianilino-1, 1'-binaphthyl-5, 5'-disulfonic acid dye has mostly been used to study the conformational changes during protein folding and binds to the solvent-exposed hydrophobic patches on the partially folded proteins or possible early aggregates. The Bis-ANS fluorescence scan shows the blue-shift with a decrease in polarity of the solvent. However as protein aggregation proceeds with higher protein aggregate formations, the burial of the hydrophobic surface is therefore not accessible for bis-ANS binding and the fluorescence emission is decreased (Brzyska et al., 2006; Chen & Glabe, 2006; Hawe et al., 2008; Kremer et al., 2000).

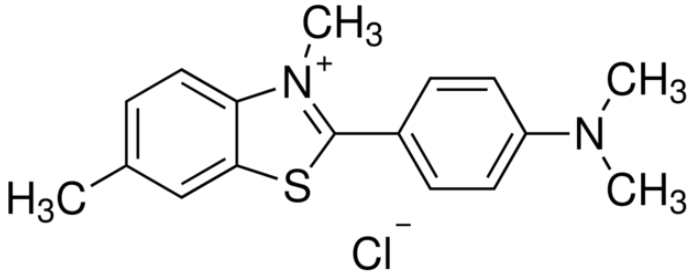
In our study we used this dye to monitor the formation of hydrophobic surface exposing early Aβ(1-42) aggregates during *in situ* aggregation carried out in presence of primary microglial cells. 5 mg of bis-ANS (Sigma) powder was dissolved in 130 μL lab grade methanol, and then further diluted to 100 mM by adding 7.3 mL deionized water. A

final 1 mM solution was stored in water; when necessary, this stock was diluted to a 100 μM working stock solution by adding deionized water.

For our experiment, microglial cells were pleated on a black Nunclon 96 well sterile plate and after overnight incubation, washing and replacing the spent media with microglial assay media Aβ(1-42) monomer. This monomer was added to the cells, making the final Aβ concentration 15 μM, exactly as described above for *in situ* Aβ aggregation monitored using ThT fluorescence. After the addition of the Aβ monomer, the plate was incubated at 37 °C and 5% CO_2. At different points in time, 11 μL of bis-ANS (from 100 μM working stock solution) was added to the wells, making the final concentration 10 μM, and a bis-ANS fluorescence reading was taken with Perkin Elmer plate reader (Wallac Victor 3, 1420 multilabel counter, 20101 Turku, Finland). The excitation and emission filter used was of 405 nm (band width of 10) and 485 nm (band width of 10). In control wells containing microglial assay media devoid of Aβ, 11 μL bis-ANS was added, and a fluorescence reading was recorded in the plate reader. Conditioned media was also collected for TNFα assessment using ELISA.

Further, a separate comparative *in situ* aggregation study between Aβ(1-40) and Aβ(1-42) monomer was also carried out indirectly through cytokine assay at different point of aggregation. As previously described, primary microglial cells plated in sterile 96 well plate were treated with both forms of Aβ monomers in a separate set of wells in triplicate, making 15 μM Aβ the final monomer treated concentration, follow by incubation at 37 °C in presence of 5% CO_2. At different times during incubation, conditioned media was collected for TNFα and IL-1β quantification by ELISA.

A)



B)

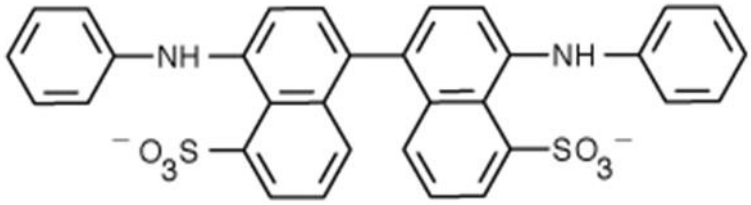


Figure 2.3 Structure of Thioflavin-T (panel A) and 4,4'-bis-1-Anilinonaphthalene-8-sulfonate (BIS-ANS) in panel B.

CHAPTER 3. THE ROLE OF CD47 IN Aβ-TRIGGERED MICROGLIAL ACTIVATION

3.1 Introduction

Alzheimer's disease is a neurodegenerative disease highlighted by the accumulation and deposition of aggregated amyloid-β (Selkoe, 2011). The histopathological signature of AD includes extra-neuronal senile plaques consisting of mainly fibrillar amyloid-β protein and intraneuronal lesions of filamentous neurofibrillary tangles (NFTs) formed by the microtubule associated protein tau (Masters & Selkoe, 2012; Selkoe, 2001). Several early and recent studies support the idea of Aβ aggregation and accumulation as the initiators or causes of AD, while tau protein aggregation drives the clinical manifestations that can involve impairment in memory, language, and higher cognitive functions at later stages (Dickson, 2004). Though senile plaques have been the well-established pathological feature in AD, recent data has shown Aβ toxicity is more related to soluble oligomeric intermediates found in the AD brain and CSF when compared to insoluble fibrillar Aβ and its subsequent effect on synaptic function (Ferretti et al., 2012; Haass & Selkoe, 2007; Kayed et al., 2003).

In AD brains, various morphologies of Aβ exist. However, the core of the senile or neuritic plaque is predominantly composed of Aβ(1-42) peptide fragments when compared to Aβ(1-40) peptide fragments (Selkoe, 2001, 2011). Aβ peptide is derived

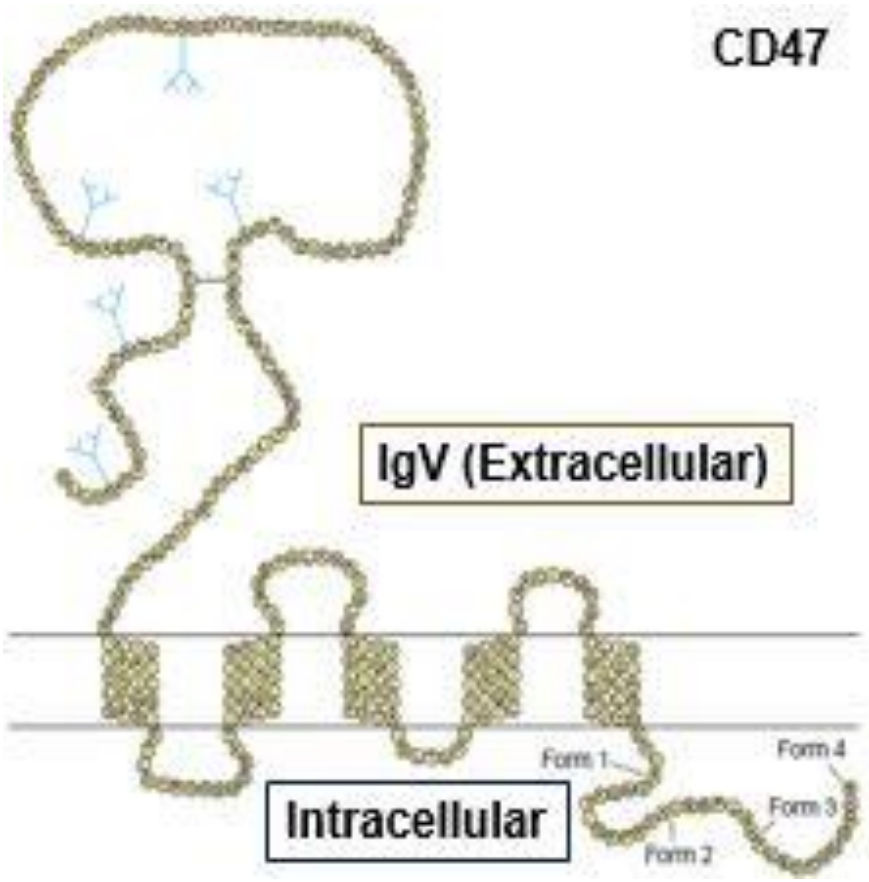


Figure 3.1Structure of integrin-associated protein CD47.

Extracellular heavily glycosylated immunoglobulin variable (IgV) domain is followed by five probable transmembrane segments terminating in a cytoplasmic tail that is alternatively spliced, giving rise to four isoforms. Modified from (Brown & Frazier, 2001).

from proteolytic processing of APP that involves sequential cleavage of APP by β- and γ-secretase as described previously in section 1.2.

In vitro studies show that Aβ monomeric forms can undergo nucleation-dependent aggregation, which involves conformational change through non-covalent association with random coiled monomers to different forms of β-sheet-rich soluble oligomeric species and protofibrils. Protofibrils are immediate soluble precursors that progress to insoluble fibril formations, and can provide important therapeutic opportunity (Bitan et al., 2003; Harper et al., 1997; Jarrett et al., 1993; Lannfelt et al., 2014; Walsh et al., 1997). Aβ(1-42) has a higher tendency to aggregate compared to Aβ(1-40), which is attributed to two extra-hydrophobic amino acids present in the former (Bitan et al., 2003; Lansbury et al., 1995; Tamaoka, 1998).

Neuroinflammation is a prominent feature of AD pathology. The presence of activated microglial cells surrounding the Aβ plaques, along with the detection of released proinflammatory cytokines like tumor necrosis factor-α and interleukin-1β provide the connection between inflammation and AD (Dickson et al., 1993; Golde, 2002; McGeer et al., 1987). However, the exact sequence of events leading to full development of AD is not known. It is proposed that the subsequent cytokine production during microglial activation and proliferation contribute to neurodegeneration (Golde, 2002). For activation of glial cells such as microglial cells or astroglial cells, there should be interaction between Aβ and the cell surface receptors. Therefore, attention has been focused on investigating the relationship between these receptors, Aβ aggregates, and inflammation.

Multiple glial cells surface receptors have been found mediating Aβ-induced proinflammatory response. Not only TLRs such as 2, 4 and 6, but also other cell surface receptor complex including scavenger receptor CD36, CD14, integrin associated protein CD47, and $\alpha_6\beta_1$ integrin have been found mediating Aβ-induced cytokine production. Mainly, TNFα and IL-1β have been assessed as markers for proinflammatory responses in these studies (Bamberger et al., 2003; Fassbender et al., 2004; Reed-Geaghan et al., 2009; Stewart et al., 2010; Udan et al., 2008).

CD47 is an integrin-associated, approximately 50 kDa, transmembrane protein copurified with integrin protein $\alpha_v\gamma_3$ (Brown et al., 1990). As shown in figure 3.1**,** it has five membrane-spanning segments, a heavily glycosylated extracellular immunoglobulin domain (IgV), and an intracellular cytoplasmic domain that is alternatively spliced to give its various isoforms. An IgV domain interacts with a variety of other integrin proteins such as αvγ3 or $\alpha_2\beta_1$, as well as ligands such as signal regulatory protein-α and thrombospondin-1(TSP-1) present in macrophages, astrocytes or platelets; therefore, it is involved in various physiological and pathological functions including cell spreading, platelet activation, cell adhesion, signaling apoptosis, migration, and ischemia-reperfusion injury (Brown & Frazier, 2001; Rogers et al., 2012; Sick et al., 2012).

Several studies showed the possible role of CD47 for Aβ-induced proinflammatory response as well as in Aβ uptake take, hence phagocytosis induction. Most of these studies utilized 10 amino-acid peptides 4N1K (KRFYVVMWKK) as a CD47 antagonist that would probably ligate with CD47 a receptor, and therefore suppress CD47 mediated cytokine production in macrophages and dendritic cells and microglial interaction with

Aβ (Bamberger et al., 2003; Brown & Frazier, 2001; Floden & Combs, 2011; Koenigsknecht & Landreth, 2004).

4N1K is derived from the carboxyl-terminal domain of its natural ligand TSP-1.This peptide acts as a CD47 antagonist, and therefore hinders the interaction of CD47 with other ligands. (Brown & Frazier, 2001; Koenigsknecht & Landreth, 2004). The important role of CD47 in immune cell inflammatory signaling and its potential involvement in AD-related inflammatory mechanisms has led us to characterize the role of CD47 in Aβ-induced microglial activation.

Recently, our lab has shown the Aβ(1-42) protofibril species to be a strong inducer of proinflammatory responses in primary microglial cells (Paranjape et al., 2012). Therefore, we chose Aβ(1-42) protofibril species to study the CD47 role in microglial activation induced by the protofibrils. However, after studies through various techniques, we find this receptor is not involved, at least with Aβ(1-42) protofibril mediated microglial proinflammatory response. Studies also confirmed that 4N1K inhibition study results should be interpreted very carefully.

3.2 4N1K peptide inhibition of Aβ(1-42) protofibril-induced proinflammatory response

Preparation of Aβ(1-42) protofibrils was performed in modified aCSF buffering system followed by its separation by SEC according to the previously defined protocol (Paranjape et al., 2013). In order to study the CD47 role in Aβ(1-42) protofibril-induced microglial activation, TSP-1 derived peptide 4N1K was used. Pre-treatment with 4N1K (100μM) or buffer control was conducted for 1 hour prior to 6-hour Aβ(1-42) protofibril (15μM) treatment. Aβ protofibrils by themselves stimulated significant levels of cytokine TNFα and IL-1β secretion (Figure 3.1) in the medium compared to the unstimulated

control. On the other hand, the response was completely suppressed in microglial cells subjected to 4N1K pretreatment, suggesting a possible role for CD47 in mediating Aβ(1-42) protofibril-induced proinflammatory response.

As mentioned in section 1.9, LPS mediates its intracellular signaling cascade for NF-κB-dependent TNFα production through the TLR-4 receptor. In our lab, we typically use LPS as a positive control for microglial-induced cytokine production. However, in our current study, LPS response was used as negative control for 4N1K, because until now CD47 has not been reported as being involved in LPS-induced cytokine production. Unexpectedly, we observed an inhibition of LPS-induced microglial TNFα secretion by pretreatment with 4N1K in our dose dependent study. Similarly, Aβ protofibril-induced TNFα secretion was also dose-dependently inhibited by 4N1K.

Curves obtained by fitting the data to the equation (y = min + [(max - min)/1 + (x/IC_{50}) slope]) (using SigmaPlot 10.0) gave IC_{50} values of 0.52 μM and 2.5 μM for LPS and Aβ protofibril-induced microglial stimulation, respectively (Figure 3.3). This data confirmed 4N1K as being effective at inhibiting Aβ protofibril-induced proinflammatory responses; LPS inhibition, however, also showed the possible CD47 independent mechanism of 4N1K to exert its inhibitory effect. Further these results decreased the probability of CD47 being involved in Aβ protofibril induced proinflammatory response. Recent study reported the possibility of 4N1K non-specific effects through immunoglobulin antibody binding (Leclair & Lim, 2014).We wanted to confirm that cytokine response reduction in treating microglial cells with 4N1K was not due to the binding of 4N1K with TNFα or IL-1β capture antibodies during ELISA, since in the medium collected after microglial treatments contained our added 4N1K peptide.

However, this possibility was ruled out, as we found no change in the TNFα and IL-1β ELISA standard curves when used with 4N1K (Figure 3.4). The use of 4N1K peptides at 100 μM concentration alone or in combination with the protofibril during microglial cell treatment raised concern over cell viability. Therefore, we conducted an XTT cell viability assay under both circumstances to eliminate the toxicity case and confirm our previous findings. We did not find cell toxicity in treating microglial cells with 100 μM 4N1K peptides alone or in combination with Aβ protofibrils. Therefore, these results indicated that the 4N1K suppression of Aβ protofibril-induced cytokine production was not due to toxicity effects from 4N1K—at least for 100 μM 4N1K treatments. However, at higher concentrations of 4N1K microglial viability was significantly reduced (Figure 3.5).

3.3 CD47 antibody neutralization studies

In order to further investigate the role of CD47 receptors, an anti-mouse CD47 antibody was used. This was done in order to prevent any possible interaction between CD47 and Aβ protofibril, possible through ligation of anti-CD47 antibodies with the receptor. Therefore, if CD47 is involved the Aβ protofibril-induced microglial cytokine response would have been significantly diminished upon using anti-CD47 antibodies. Previously, our lab used this technique in order to assess the involvement of TLRs and CD14 in Aβ-induced TNFα production in monocytes (Udan et al., 2008). Similar to our previous results, Aβ protofibrils induced TNFα significantly compared to our buffer controls (Figure 3.6). Microglial cells were pretreated with 5 μg/mL anti-CD47 antibody or the Ig2a negative control.

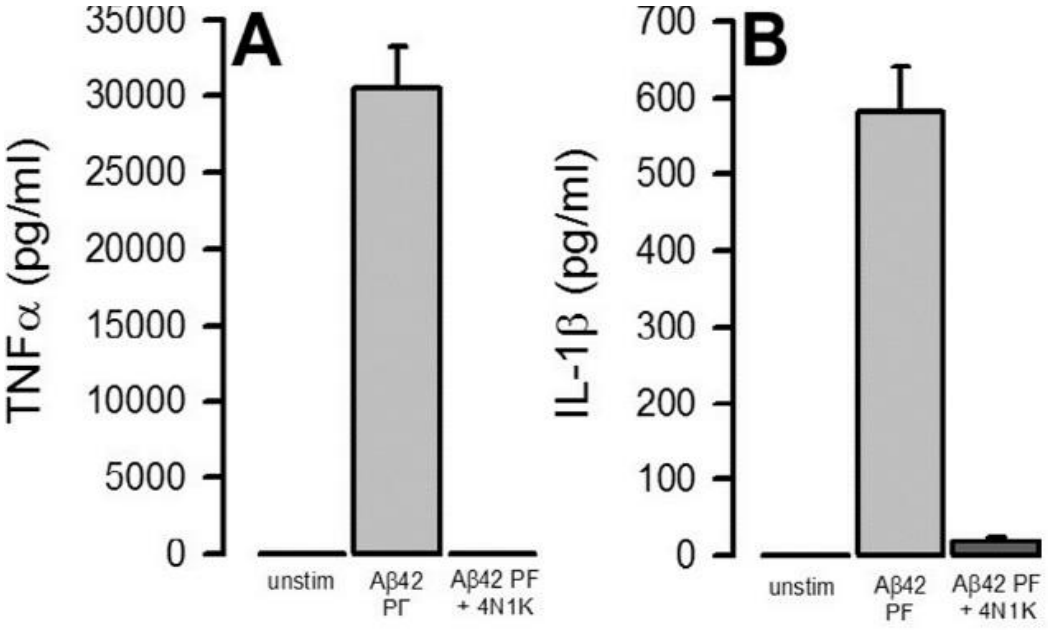


Figure 3.2 Inhibition of Aβ (1-42) protofibril induced TNFα and IL-1β by 4N1K treatment.

Primary microglial cells, isolated from 3 to 4 old mice pups, plated on sterile 96 well plate and incubated overnight. The cells were pretreated with 4N1K peptide (100 μM) or vehicle control in serum free medium for 1 hr followed by incubation with Aβ(1-42) protofibrils (15 μM) or vehicle controls (unstimulated aCSF or H2O) for additional 6 hours. Conditioned media was retained and was subjected to ELISA for TNFα (Panel A) and IL-1β (Panel B) quantification. Error bars represent standard error measure (n=3 trials).

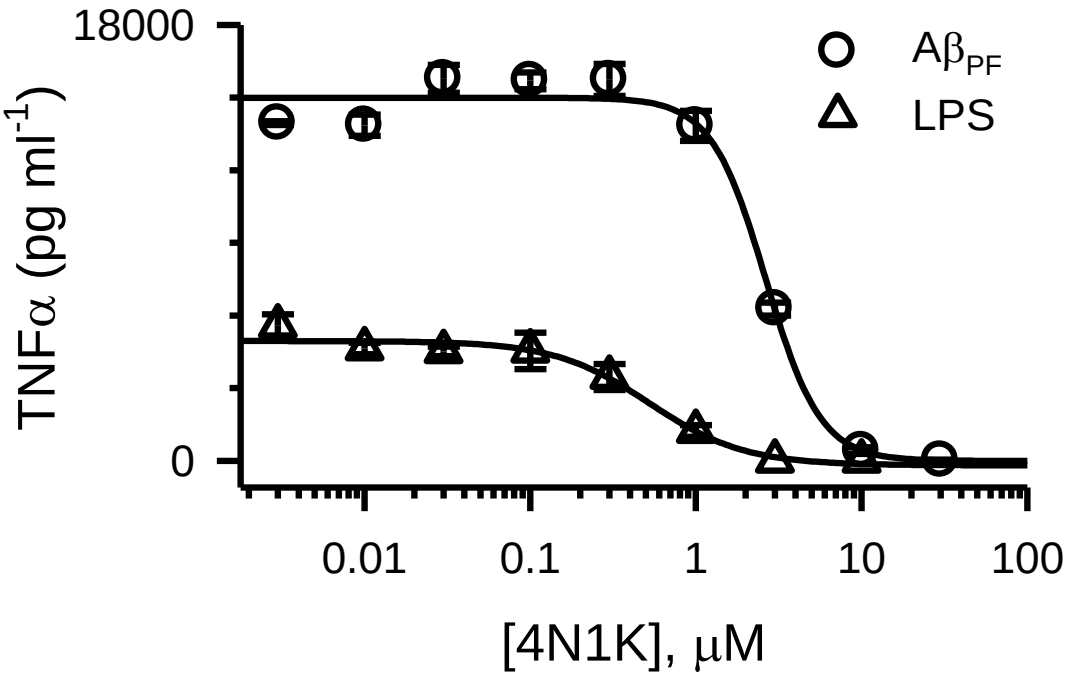


Figure 3.3 4N1K dose dependent inhibition of LPS and Aβ(1-42) protofibril induced TNFα secretion.

Primary microglial cells were pretreated with 4N1K peptide (0.01-100 μM) or vehicle control in serum free medium for 1 hr followed by incubation with Aβ(1-42) protofibrils (15 μM), LPS (10ng/ml) or vehicle controls (aCSF or H_2O) for additional 6 hours. Conditioned media was retained and was subjected to ELISA for TNFα (Panel A) and IL-1β (Panel B) quantification. Error bars represent standard error measure (n=3 trials). Fitting the data to the following equation, y = min + [(max min)/1 + (x/IC50) slope] using Sigma Plot 10.0.

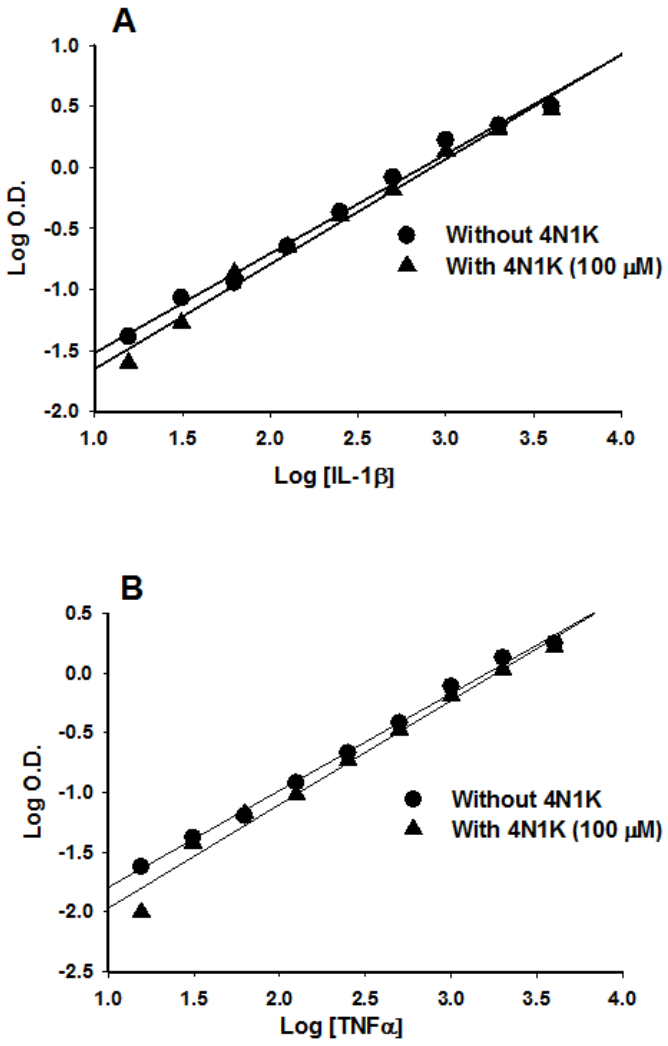


Figure 3.4 IL-1β and TNFα standard curves with and without 4N1K peptide.

Serial dilutions of IL-1β and TNFα standard proteins were carried out in the presence and absence of 4N1K (100 μM) and ELISA was carried out. Panel A shows the standard curve for IL-1β with 100 μM 4N1K (triangles) providing correlation coefficient of 0.9876 and slope of 0.8569. Without 4N1K (circles) curve gave correlation coefficient of 0.9905 and slope of 0.8931. Panel B shows standard curves for TNFα with 100 μM 4N1K (triangles, correlation coefficient 0.9825 and slope 0.8698) and without 4N1K (circles, correlation coefficient 0.996 and slope 0.8114).

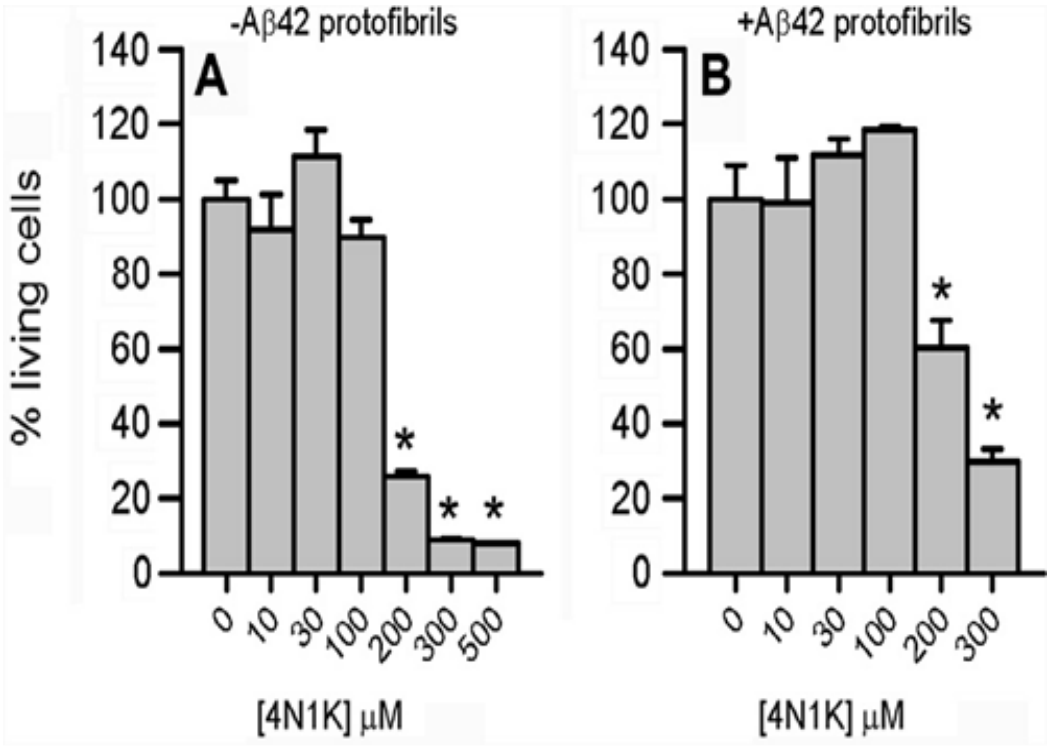


Figure 3.5 Toxicity of 4N1K peptide to primary murine microglia.

Primary microglia plated in a 96-well sterile plate were incubated with increasing concentrations of 4N1K peptide for 1 hour at 37°C in 5%CO_2 followed by addition of aCSF (Panel A) or SEC-isolated Aβ(1-42) protofibrils (Panel B) for an additional 6 hours. An XTT cell viability assay was then conducted on microglia as described in method section. Microglia viability measured as absorbance of reduced XTT compared between microglial exposed to 4N1K and or Aβ(1-42) protofibrils and those with aCSF treated only as normal viable cells. Data is presented as the percentage of reduced XTT absorbance for treated cells compared to untreated cells (no 4N1K or protofibrils) and reported as % living cells. Data bars represent the mean ± std error of n = 3 replicates. Statistical differences ($p < 0.05$) in cell viability caused by 4N1K are denoted with an asterisk.

Then subsequent treatment with the protofibrils was performed (Figure 3.6 A). We also did the treatment with a higher concentration of anti-CD47 antibody or IgG2a isotype (10 μg/mL).In both the cases no significant difference in the level of secreted TNFα was observed between the exclusively Aβ protofibril treated microglial cells and anti-CD47 pretreated microglial cells treated subsequently with the Aβ protofibril (Figure 3.6A and 3.6B).

Additionally we further carried out neutralization studies by conducting experiments in parallel with 4N1K peptide treatments. Again pretreatments of microglial cells with an anti-CD47 antibody (10 μg/mL) or an IgG2a isotype control, or a 4N1K peptide (100 μM) was conducted followed by treatment with Aβ protofibril. Like in previous results, treatment with anti-CD47 antibodies or IgG2a isotype control decreased the response of neither Aβ(1-42) protofibril-mediated TNFα (Figure 3.7A) nor IL-1β (Figure 3.7B). However, 4N1K again showed its inhibitory effect for the protofibril-mediated cytokine response. Overall, the results supported the possible CD47-independent mechanism of Aβ(1-42) protofibril-induced proinflammatory event.

3.4 CD47 knockout microglia studies

In order to further confirm for any possibility of CD47 involvement in mediating Aβ(1-42) protofibril induced proinflammatory activation, comparative proinflammatory response study between $CD47^{-/-}$ microglial cells and wild type primary microglial cell was carried out. For this primary wild type and $CD47^{-/-}$ microglial cells were isolated from their respective mice pups as described in method section. Both the types of microglial cells were exposed to Aβ(1-42) protofibrils for different time points. The protofibril response was similar to our previous work (Terrill-Usery et al., 2014) showing

little TNFα secretion at early time point (2 hours) while significant increase at longer time point (6 hours) (Figure 3.8 A) from wild type microglial cells. Under the same time frame, secreted TNFα level from $CD47^{-/-}$ microglia on protofibril stimulation was observed similar to wild type microglia (Figure 3.8A). The experiment was repeated in parallel with 4N1K pretreatment on both the cell types to examine the 4N1K effect. Again, the protofibril induced stimulation resulted in significant TNFα secretion in both types of microglia whereas in 4N1K pretreated cells, Aβ(1-42) protofibril induced TNFα secretion was completely diminished (Figure 3.8B). These results substantiated the idea that CD47 does not mediate Aβ(1-42) protofibril elicited microglial cytokine secretion. Further the results also show that 4N1K inhibitory action for the protofibril elicited cytokine secretion is independent of CD47 mechanism.

4N1K dose-dependently inhibited TNFα secretion on exposure to both LPS and Aβ(1-42) protofibril in $CD47^{-/-}$ primary microglia with similar IC_{50} values for 4N1K inhibition (~3μM) for both treatments (Figure 3.9A). This might indicate common non-CD47 target in proinflammatory signaling mechanism for LPS and the protofibril. Interestingly, similar IC50 values for 4N1K inhibition of TNFα release elicited by Aβ(1-42) protofibril in CD47-/- and wild type primary microglial cells showed similar 4N1K inhibitory potency (Figure 3.3 and 3.9A). IL-1β secretion induced by Aβ(1-42) protofibril in addition was also attenuated by 4N1K in CD47-/- microglia (Figure 3.9B). Altogether these results not only confirm the CD47 independent mechanism for soluble Aβ(1-42) protofibril induced proinflammatory events but also for 4N1K inhibitory action.

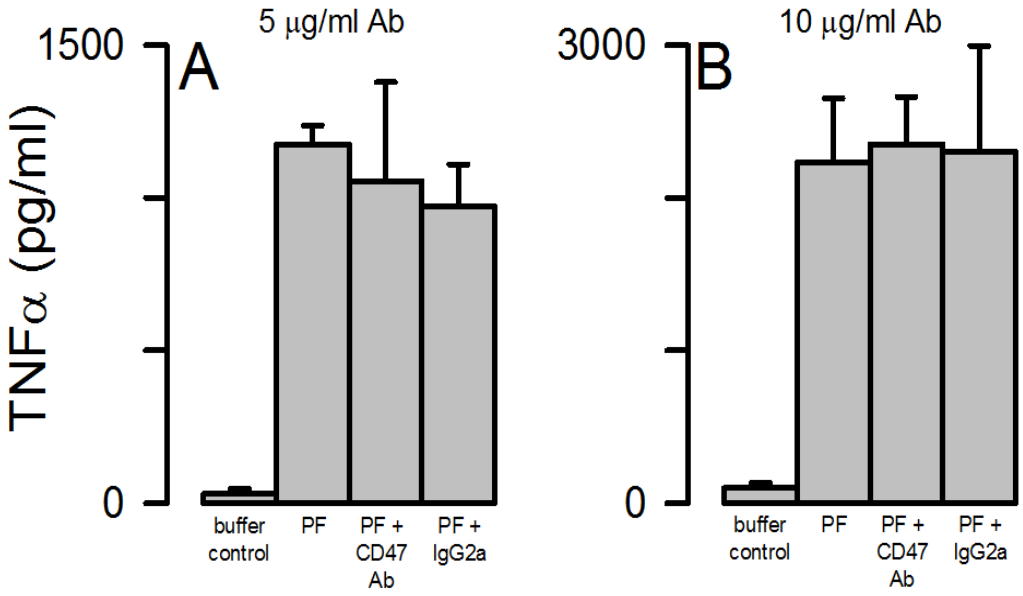


Figure 3.6 Neutralization with CD47 antibodies.

Primary murine microglia were pretreated with 5 μg/mL (Panel A) or 10 μg/mL (Panel B) anti-mouse CD47 antibody or the corresponding isotype control (IgG2a) for 1 h followed by replacement of the medium and addition of SEC isolated Aβ (1–42) protofibrils (15 μM) for 6 h. Secreted TNFα (Panels A–B) levels were determined by ELISA in the conditioned medium Error bars represent standard error measure (n=3 trials).

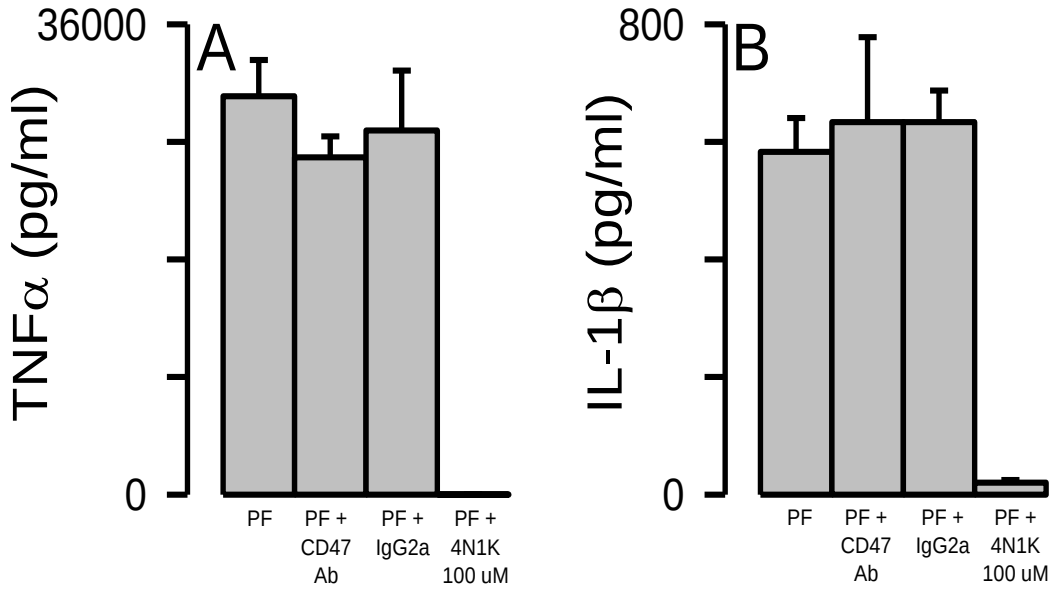


Figure 3.7 Neutralization with CD47 antibodies and 4N1K pretreatment.

Primary murine microglia were pretreated with 10 μg/mL anti-mouse CD47 antibody or the corresponding isotype control (IgG2a) for 1 h followed by replacement of the medium and addition of SEC isolated Aβ(1–42) protofibrils (15 μM) for 6 h. Secreted TNFα (Panels A) or IL-1β (Panel B) levels were determined by ELISA in the conditioned medium Error bars represent standard error measure (n=3 trials).

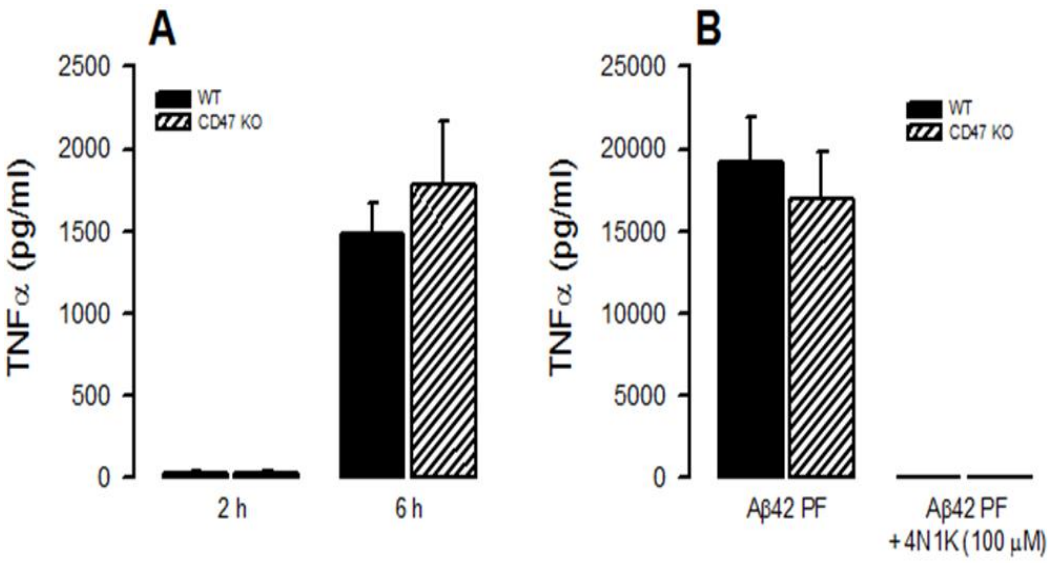


Figure 3.8 TNFα response between CD47-/- and Wild type microglial cells from Aβ(1-42) protofibril response.

Primary murine microglia from CD47-/- and wild type were treated with Aβ(1-42) protofibril (15 μM) only and TNFα response for 2 hours and 6 hour time points were determined by ELISA (Panel A). Pretreatment with 4N1K (100 μM) was followed with Aβ(1-42) protofibril treatment to wild type murine primary microglia and CD47-/- microglia or was treated only with Aβ(1-42) protofibril (Panel B) for 6 hours. Conditioned medium at different time point was retained and TNFα levels were determined by ELISA. Error bars represent standard error measure (n=3 trials). Secreted TNFα elicited by Aβ protofibrils at 6 h in WT or CD47-/- microglia was not significantly different ($p > 0.05$) in Panels A and B.

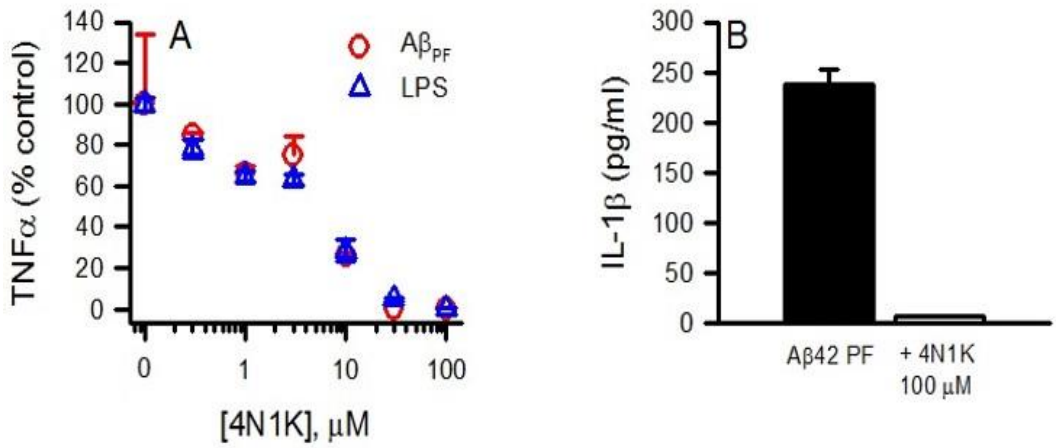


Figure 3.9 4N1K dose dependent inhibition of LPS and Aβ(1-42) protofibril induced TNFα secretion in CD47-/- and Aβ(1-42) protofibril triggered IL-1β response.

Primary murine microglial cells from CD47-/- pretreated with various 4N1K peptide concentrations (0.01-100 μM) for panel A or 100 μM 4N1K peptide (panel B) in serum free medium for an hour followed by incubation with Aβ(1-42) protofibrils (15 μM) or LPS (panel A) for additional 6 hours. Conditioned medium after 6 hour time point was retained and TNFα or IL-1β levels were determined by ELISA. Error bars represent standard error measure (n=3 trials).

3.5 Discussion

Various receptors and receptor complexes have been shown to be implicated in the microglial activation of AD. TLRs are one such set of receptors. Along with TLR4 and co-receptor CD14, TLR2 has been shown to be the primary receptor for Aβ peptides for neuro-inflammation. Further investigation has identified additional receptor complexes such as CD36, TLR4, and TLR6, as well as new cell surface receptor complexes involving CD47, α6β1 integrin protein, and CD36 involved in fibrillar Aβ-mediated microglial activation (Bamberger et al., 2003; Stewart et al., 2010; Udan et al., 2008; Yu & Richard, 2014). Later studies have shown the CD47 receptor as being engaged in the phagocytosis of fibrillar forms of Aβ (Bamberger et al., 2003; Floden & Combs, 2011; Koenigsknecht & Landreth, 2004). Studies from our lab have shown soluble that Aβ(1-42) protofibrils, rather than fibrils, are strong stimulators of microglial cytokine production (Paranjape et al., 2012). Further studies showed that the protofibril-stimulated TNFα production occurs mainly through TLR/MyD88 dependent pathways (Terrill-Usery et al., 2014).

The main objective of our study was to determine the possible significant role of CD47 in mediating the microglia proinflammatory response to protofibrils. Utilization of 4N1K peptides as CD47 antagonist peptides and anti-CD47 antibody experiments showed that CD47 does not mediate Aβ(1-42) protofibril-induced cytokine production. To further confirm these initial results, we employed a direct approach of using $CD47^{-/-}$ microglial cells. Investigations using $CD47^{-/-}$ also demonstrated our previous findings of CD47-independent mechanisms of Aβ(1-42) protofibril-mediated proinflammatory

events. In addition to this, we also found 4N1K inhibitory action being independent of CD47 involvement.

Anti-CD47 antibody neutralization studies did not block Aβ protofibril-triggered cytokine production while using 4N1K in wild type microglial cells and completely attenuated the cytokine production (Figure 3.6 and 3.7). 4N1K peptides potently inhibited Aβ protofibril-induced cytokine responses in $CD47^{-/-}$ microglia, as well (Figure 3.8 and 3.9). 4N1K peptides have been used in several previous studies as CD47 antagonist peptides (Chung et al., 1997; Koenigsknecht & Landreth, 2004). However, based on the non-specific action of this peptide, definite caution is advised for the interpretation of data using CD47.

Recently published work by Leclair and his group showed non-specific activity of 4N1K peptides. They did their work on Jurkat T-cell line derivatives where two types of these cell lines—one expressing CD47 and the other CD47-deficient adhered to a 4N1K-coated surface—in a similar manner. Further, both of these cells showed similar levels of adhesion in fibronectin-coated surfaces when these cells were treated with 4N1K peptides in a CD47-independent manner (Leclair & Lim, 2014). In conclusion, CD47 does not seem to be a possible candidate for soluble Aβ protofibril recognition and subsequent proinflammatory signal transduction.

CHAPTER 4. STABILITY OF NASCENT Aβ(1-42) AGGREGATES

4.1 Introduction

Insoluble fibrillar Aβ, the major constituent of plaques, has long been thought to be the most neurodegenerative species in AD (Selkoe, 1994, 2011). Surprisingly, rather than senile plaques, neurofibrillary tau tangles, another pathological hallmark of AD, correlate well with cognitive decline and AD severity. However, until now aggregation of Aβ is the accepted early event that triggers the neurodegenerative process (Brion, 1998; Pike et al., 2007; Rentz et al., 2010; Selkoe, 2011). Additionally, AD neuroinflammation occurrence has been supported by the presence of activated glial cells, such as microglia and astrocytes that are observed surrounding the amyloid plaques. In this environment increased levels of proinflammatory cytokines and its autocrine action on glial cell and neuronal cell has been studied (Bamberger et al., 2003; Barton, 2006; Fassbender et al., 2004; Rogers, 2008; Stewart et al., 2010).

Recent AD studies have shifted more towards early events in Aβ aggregation as well as identification and characterization of soluble intermediate species such as oligomers and protofibrils formed during the aggregation process which have been shown to be more neurotoxic than fibrils (Glabe & Kayed, 2006; Paranjape et al., 2012; Tiiman et al., 2013). In fact, detailedstudies on amyloid plaques have shown the presence of diffuse soluble oligomeric species surrounding Aβ plaques in mouse models that are highly synaptotoxic and neurotoxic species (Haass & Selkoe, 2007; Koffie et al., 2009).

Earlier studies have also shown results in support of harmful effects of soluble aggregates or oligomers such as cellular dysfunction and toxicity in cultured cells, inhibition of hippocampal long term potentiation in transgenic mice models, memory deficits, electrophysiological alterations in neurons as well as profound decrease in neuronal viability when compared to fibrils (Dahlgren et al., 2002; Gonzalez-Velasquez et al., 2008; Hartley et al., 1999; Lesné et al., 2006).

Aβ protein has a high tendency to undergo aggregation leading to fibril formation. *In vitro* studies have shown that Aβ follows nucleation-dependent aggregation kinetics where monomer undergoes a self-assembly process involving non-covalent interactions forming soluble intermediates such as oligomers, protofibrils and ultimately insoluble fibrils. (Bitan et al., 2003; Burdick et al., 1992; Jarrett et al., 1993; Jarrett & Lansbury, 1992; Kumar & Walter, 2011). However, between Aβ(1-42) and Aβ(1-40), the two main components of amyloid plaques, it is Aβ(1-42) that has the higher propensity to aggregate due to the presence of two hydrophobic amino-acid residues at the C-terminus. Further studies have also indicated the importance of Aβ(1-42) to be greater in AD compared to Aβ(1-40). This is not only due to far higher Aβ(1-42) levels, as shown by many AD genetic mutations studies and in neuritic plaque composition, but also its ability to form numerous varieties of oligomeric species (Bitan et al., 2003; Gravina et al., 1995; Hardy, 1997).

Since most of the studies point towards a toxic effect of soluble oligomeric species or early soluble Aβ aggregates with progression and severity of AD, it has become a challenge to exactly detect and identify the size of these nascent Aβ species with intact structural properties especially since they are highly unstable. This is

important for studying the aggregation process as well as to devise selective therapeutic treatments targeting the pivotal oligomerization process (Pryor et al., 2012). These objectives have been hindered by various limitations of the techniques used. A technique such Thioflavin T binding is not sensitive for oligomeric species, whereas microscopic analysis is not suitable for studying lower molecular weight oligomers. Further techniques such as SDS-PAGE or native PAGE have their own limitations like band resolution and the role of SDS in promoting fibrillation or aggregated conformation destabilization (Pryor et al., 2012). However, the use of conformation-specific antibodies can be a useful technique and has shown to be sensitive for detecting aggregated soluble species in solution as well as in biological samples (Kayed et al., 2003; Lee et al., 2006). For our study we are using OC antibody to characterize nascent Aβ(1-42) aggregates as well as show differences between Aβ(1-40) and Aβ(1-42). This OC antibody recognizes elements of fibrillar structure across a broad range of Aβ aggregate sizes (Kayed et al., 2007).

4.2 Reactivity of OC antibody with freshly SEC-purified Aβ(1-42) and Aβ(1-40) species

SEC-isolated Aβ(1-42) and Aβ(1-40) species were treated with Ab9 antibody as well as OC antibody. Ab9 antibody treatment is done as a positive control for presence of Aβ protein and this antibody recognize the first N-terminal 16 amino-acid residues regardless of conformation, whereas OC antibody recognizes fibrillar structural elements across a wide range of aggregate sizes (Kayed et al., 2007; Kukar et al., 2005). SEC-purified Aβ(1-42) showed OC-positive reactivity, whereas Aβ(1-40) monomer fractions did not (Figure 4.1).

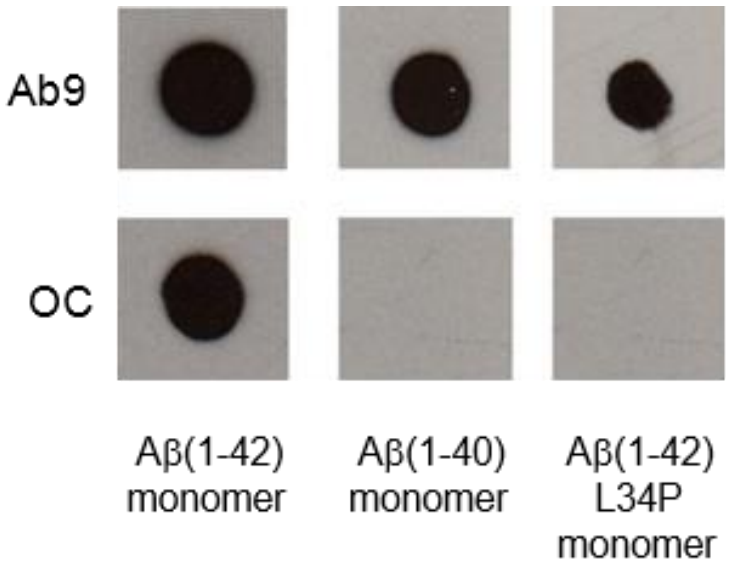


Figure 4.1 An OC-positive species is observed in freshly-purified Aβ(1–42) monomer fractions.

Aβ monomer fractions from separate SEC purifications were placed on ice immediately after elution from a Superdex 75 column and examined by dot blot analysis within 30 min. The concentration for monomer fractions of Aβ(1–42) and Aβ(1–42) L34P eluted in 50 mM Tris–HCl pH 8.0 was 20 μM and 40 μM respectively, while Aβ(1–40) monomer eluted in PBS was 78 μM.

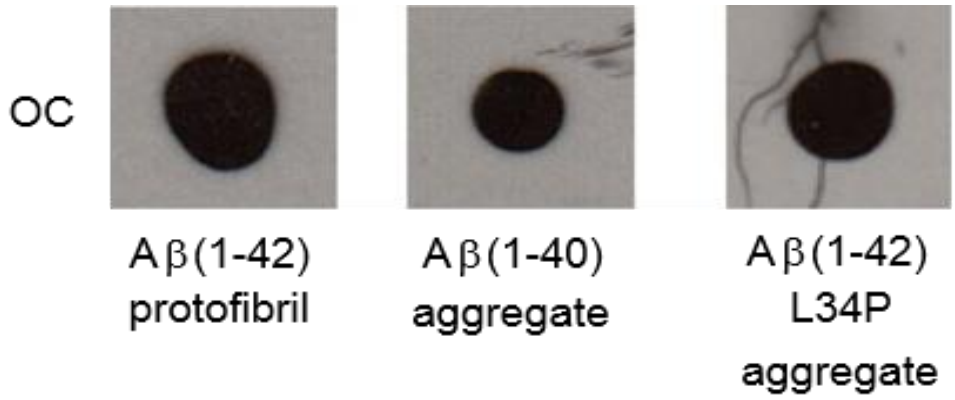


Figure 4.2 OC-negative monomer fractions develop OC immunoreactivity.

Aβ(1–40) and Aβ(1–42) L34P monomer fractions from Fig. 1 were incubated quiescently at 37 °C for 6 and 13 days respectively and subjected to dot blot analysis. For comparison, an Aβ(1–42) protofibril fraction eluted in the Superdex 75 void volume in F-12 medium without phenol red was also analyzed. 2 μL of each sample at final concentrations of Aβ(1–42) (28 μM), Aβ(1–40) (39 μM), and Aβ(1–42) L34P (20 μM) was used.

The data overall indicated that Aβ(1-42) monomer fractions must have undergone conformational changes to obtain elements of fibril structure after SEC isolation, unlike the slow-aggregating Aβ(1-40). Ab9 antibody recognized both Aβ(1-42) and Aβ(1-40) sample fractions.

4.3 Aβ(1-40) fractions develop OC- antibody reactivity

OC antibody reactivity tests carried out on SEC- isolated Aβ(1-40) monomer samples incubated at 37°C for 1-2 weeks finally gave positive blot indicating that Aβ(1-40) did obtain the structural components necessary for the OC-positivity result (Figure 4.2). Pre-assembled or aggregated forms of Aβ(1-42) protofibrils, shown to be potent microglial proinflammatory stimulants, displayed OC reactivity (Figure 4.2) (Ajit et al., 2009; Paranjape et al., 2012). All the samples probed with Ab9 displayed positivity. These data support that Aβ(1-42) monomer fractions display early and rapid aggregation compared to Aβ(1-40) monomers (Figure 4.1 and 4.2).

4.4 Effect of chaotropic agent on stability of freshly purified OC-positive Aβ(1-42) aggregates.

We wanted to further probe OC-detected early Aβ(1-42) aggregation species. For stability studies, the chaotropic reagent urea was used. Freshly SEC- isolated Aβ(1-42) monomer fraction was incubated with 4 M urea and was probed with OC antibody. The positive OC reactivity with the monomer fraction indicated no disruption by the urea of conformational or structural elements, showing that the early OC-positive species is stable in urea (Figure 4.3). Not surprisingly Aβ(1-42) protofibrils were resistant to urea (Figure 4.3). However, Aβ(1-42) aggregates displaying significant ThT fluorescence

were sensitive to urea, showing distinct stability differences between Aβ(1-42) and Aβ(1-40) species (Figure 4.3).

4.5 Effect of SDS in stability of freshly-purified OC-positive Aβ(1-42) aggregates.

Further stability testing for OC-positive Aβ(1-42) aggregated species was carried out by incubating freshly-isolated Aβ(1-42) from SEC monomer fractions with 1% SDS for 10 minutes at room temperature. In the presence of SDS, OC- immunoreactivity with Aβ(1-42) was absent (Figure 4.4A) but with Ab9, reactivity was consistently positive. This indicated that SDS detergent was able to disrupt the OC-positive Aβ(1-42) conformation or structure. Interestingly, continuous follow up of dot blot with 1% SDS on the same Aβ(1-42) monomer fraction incubated at 4°C for several days displayed resistance to SDS though the fraction was initially SDS sensitive and OC-positive (Figure 4.4A). The Aβ(1-42) monomer fraction's resistance to SDS increased as the time of incubation progressed. All samples were Ab9 positive. The Aβ(1-42) monomer fraction was immediately placed on ice after SEC elution and stored at 4°C in order to slow down the kinetics of aggregation and hence allow better observation of physical changes on follow-up. The OC-positive Aβ(1-42) sample began showing resistance to SDS around 4 days with no sign of sensitivity by 8 days. We also followed ThT fluorescence on the same Aβ(1-42) SEC-monomer fractions for several days. Interestingly, there was no significant ThT fluorescence increase during the transition from SDS-sensitive to SDS-resistance. Finally ThT fluorescence significantly increased in around 15 days (Figure 4.4B). We carried out repeated experiments in freshly-isolated Aβ(1-42) monomer fractions to monitor and confirm transition from SDS-sensitive to SDS-resistant OC-positive species.

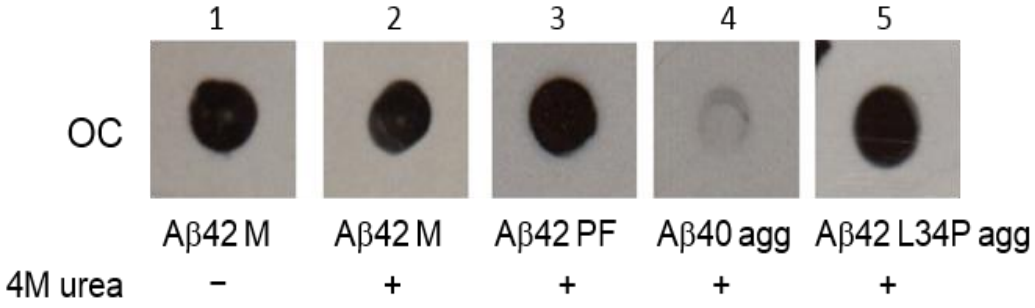


Figure 4.3 Early OC-positive Aβ(1–42) species is resistant to chaotropic reagents. Stability studies were conducted on samples taken from (1) a freshly-isolated Aβ(1–42) monomer (M) fraction (1) or protofibril (PF) fraction (2), aggregated (agg) Aβ(1–40) (3) and aggregated Aβ(1–42) L34P (4). The latter three samples are described in Fig. 2 legend. The samples were incubated with 4 M urea for 10 min (room temperature) at a final Aβ concentration of (1) 20 μM, (2) 28 μM, (3) 39 μM and (4) 20 μM. Dot blot analysis was then performed with both Ab9 and OC antibodies.

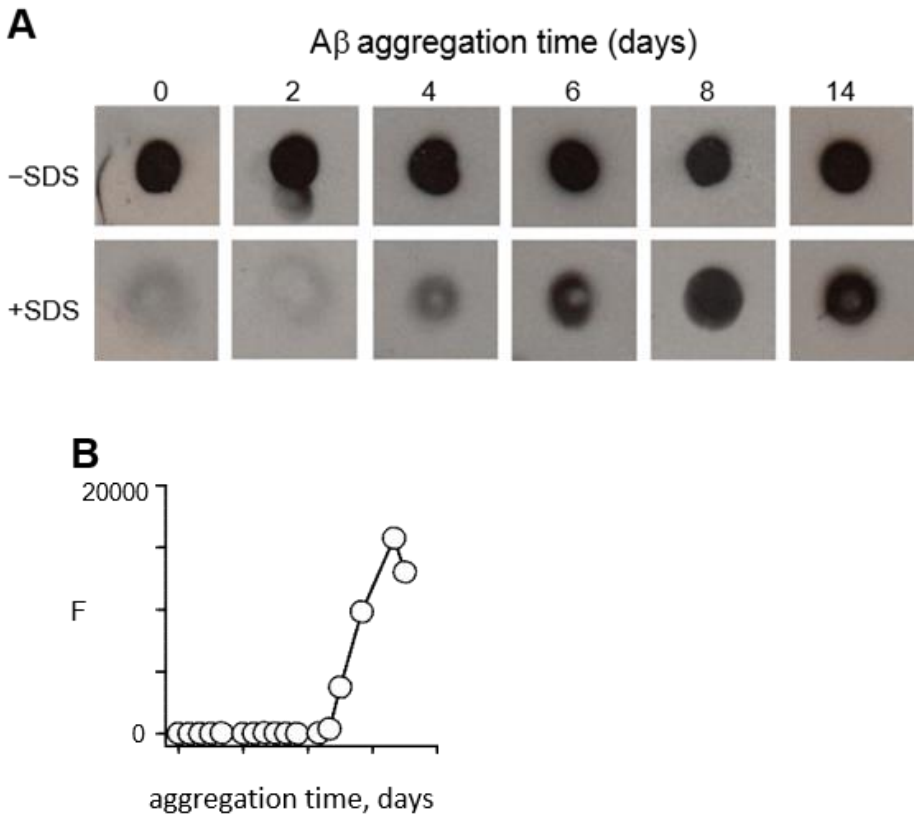


Figure 4.4 Early OC-positive Aβ(1–42) species becomes resistant to SDS over time. Freshly-isolated Aβ(1–42) from a SEC monomer fraction in F-12 medium without phenol red was stored at 4 °C without disturbance over 24 days. Panel A. At chosen time points after isolation the Aβ(1–42) solution (final concentration 20 μM) was incubated with or without 1% SDS for 10 min at 25 °C and then analyzed by dot blot with Ab9 and OC antibodies. Panel B. Thioflavin T fluorescence measurements were taken from stored Aβ(1–42) solution at various time points.

We saw variation in the time required to develop the resistance. However this transition always occurred before the onset of significant ThT fluorescence increase.

4.6 Discussion

SEC is becoming a common technique for Aβ preparation as it removes preexisting aggregated material, producing a homogenous protein solution by separating protofibrillar and monomeric Aβ species (Jan et al., 2010; Teplow, 2006; Walsh et al., 1997). Our current report describes use of OC antibodies to observe very early aggregation events during Aβ fibrillogenesis. Clear differences were obtained between freshly SEC-purified Aβ(1-40) and Aβ(1-42) in terms of rates of early OC-positive species formation and their stability (Figure 4.1). These differences were observed well before significant ThT fluorescence observation (Figure 4.5 and 4.4) which commonly detects β-sheet rich species such as fibrils (Paranjape et al., 2012). The isolated monomer solution is the predominant species yet has been shown to attain rapid equilibrium with lower-order oligomers and at higher concentration (μM) with higher-order oligomers (Teplow, 2006).

The rapid oligomerization of Aβ(1-42) when compared to Aβ(1-40) as well as the difficulty in isolating exclusively monomeric Aβ(1-42) fractions has been previously described (Bitan et al., 2003). Our results also revealed this very early Aβ(1-42) aggregation nature that includes fibrillar structural component formation (Figure 4.1 and 4.2). To support this analysis we carried out OC-reactivity with SEC-purified Aβ(1-42) L34P monomer and the result was a lack of OC-reactivity. This is possibly due to the absence of OC detectable new oligomeric species formation with components of fibril structure in pure monomeric solution (Figure 4.1 and 4.2). Leucine substitution with

proline should restrict aggregation in Aβ(1-42) L34P as this substitution seems to destabilize fibrillar structure, hence possibly enabling it to remain monomeric for a longer time (Williams et al., 2004).

The stability studies also showed a remarkable difference between Aβ(1-40) and Aβ(1-42) species. In the presence of 4 M urea Aβ(1-42) fibrillar oligomers displayed significant stability unlike longer-term Aβ(1-40) aggregates (Figure 4.3). This stability was shown by the Aβ(1-42) monomer fraction within minutes after SEC-isolation (Figure 4.3). This rapid OC-positivity due to probable fibril-like structure formation in Aβ(1-42) oligomers and their resistance to denaturants such as urea displays significant difference between Aβ(1-40) and Aβ(1-42) and emphasizes the intervention strategies that reduce the Aβ(1-42):Aβ(1-40) ratio.

The possible structural or conformational changes likely to occur in the early stage of aggregation are reflected indirectly through transition of the OC-positive, SDS-sensitive SEC-isolated Aβ(1-42) monomer solution to SDS-resistant species (Figure 4.4). Some of these changes can include formation of hydrogen-bonded β-sheet rich conformations that either goes on to form the core of the mature fibrils or simply provides platform for continued addition of monomers and/or coalescence of separate fibrillar oligomers. These changes occur before the significant ThT increase (Figure 4.4).

The study presents conformation-specific antibody usage as an important strategy for examining structural aspects in the early stages of protein aggregation. More importantly, the higher sensitivity with this approach renders an advantage over the other frequently used techniques such as ThT fluorescence, circular dichroism, light scattering and microscopy. Additional information about the structure of the oligomeric species and

the epitope of the antibody can further shed light on Aβ assembly pathways. In conclusion, our study shows *de novo* formation of the earliest elements of fibril structure in SEC-purified monomeric Aβ(1-42) solutions based on recognition by OC antibody. However, rapid formation of such elements were not seen with similarly-prepared Aβ(1-40) solutions, showing differences between the two peptides at the earliest stages of aggregation. This study demonstrates stability differences between OC antisera-positive Aβ(1-42) and Aβ(1-40) oligomers containing elements of fibril structure as well as shows time-dependent development of SDS-resistant stability in early stages of Aβ(1-42) oligomer formation.

CHAPTER 5. Aβ STRUCTURE-DEPENDENT ACTIVATION OF INFLAMMATORY PATHWAYS

5.1 Introduction

Aβ neuritic plaques, extracellular proteinaceous deposits representing a diagnostic characteristic of AD, are primarily composed of fibrillar Aβ (Selkoe, 2011). The protein Aβ is produced through proteolytic cleavage of the larger amyloid precursor protein (APP) in sequence by secretase enzymes named as α, β and γ (Dawkins & Small, 2014; O'Brien & Wong, 2011). The high self-aggregating nature of Aβ has been implicated in AD pathology (Reinke & Gestwicki, 2007). Under normal physiological conditions the randomly coiled monomeric Aβ undergoes a self-assembly process forming a polydisperse array of soluble oligomeric intermediates that may end up in β-sheet rich insoluble fibrils (Bitan et al., 2003; Jarrett et al., 1993; Jarrett & Lansbury, 1992). Such structural polymorphism is a characteristic feature of Aβ aggregation events and can have diverse biological implications (Ajit et al., 2009). However, all the Aβ forms do not show a toxic effect. More specifically monomeric, oligomeric, Aβ derived diffusible ligands, protofibrillar and fibrillar Aβ have been shown to exert different extents of neuronal toxicity and other immunological responses (Dahlgren et al., 2002; Deshpande et al., 2006; Harper et al., 1999; Pike et al., 1991).

Neuroinflammation is a separate pathological aspect of AD. Several components from injured cells or the presence of abnormal materials can act as stimulants for inflammation. The pathological hallmarks of AD such as Aβ plaques, neurofibrillary tangles and cellular debris from neurodegeneration can be stimulants for inflammation in AD brain (Akiyama et al., 2000). More importantly, these plaques have been found surrounded by activated microglial cells, astrocytes, dystrophic neuritis and synaptic losses, indicating their important role in AD pathology. *In vitro* studies, APP transgenic mice models and postmortem brains from AD patients have shown to have increased levels of inflammatory cytokines and chemokines such as IFNγ, TNFα, IL-1β and IL-6 along with activated microglial cells and reactive astrocytes (Akiyama et al., 2000; Golde, 2002; Mandrekar & Landreth, 2010). The cytokines have been proposed to be inflammatory mediators maintaining a state of prolonged immune response. They are also known to influence Aβ aggregation and neurodegeneration leading to disease progression (Akiyama et al., 2000; Glass et al., 2010; Golde, 2002; Mandrekar & Landreth, 2010). Still debate exists about the initial recruitment of microglial cells in AD brain since senile plaques may not alone be enough for microglial recruitment. Instead, chemoattractant from early dystrophic events might provide the initial trigger for immune cell recruitment (Griffin et al., 1995; Jung et al., 2015). This notion can also be supported by the study in which soluble Aβ oligomers and ADDLs (Aβ derived diffusible ligands) were found to bind with high specificity at synaptic sites in human cortical neuronal cultures (Deshpande et al., 2006). Nevertheless, it is widely becoming accepted that microglial activation is secondary to Aβ aggregation (Schwartz & Shechter, 2010).

Several *in vitro* aggregation studies displayed the existence of varieties of soluble oligomeric intermediate species with diverse conformations. Commonly, SDS PAGE, electron microscopy and atomic force microscopy have been used to characterize them as punctate, globular, annular or spherical micelles (Deshpande et al., 2006; Kayed et al., 2003; Lambert et al., 1998; Prangkio et al., 2012; Soreghan et al., 1994; Stine et al., 2003). However, their *in vivo* presence was shown in a transgenic AD mice model using an oligomer-binding antibody. Therefore it becomes important to elucidate the role of Aβ and its different conformations in AD progression (Deshpande et al., 2006; Koffie et al., 2009; Oddo et al., 2006). Correlation of the presence of soluble protein oligomers with the severity of cognitive decline has implicated oligomers as the possible primary toxic species in several neurodegenerative diseases including AD (Deshpande et al., 2006; Lue et al., 1999; Näslund et al., 2000). Interestingly, both AD related and other non-disease related oligomeric peptides displaying toxic effects have been shown react with conformation specific antibody A11, indicating possible consensus conformation (Kayed et al., 2003).

In vitro studies with fibrils and oligomers showed that both species are capable of microglial activation. But depending on the confirmation of the species there was a difference in activation profile. More specifically, activation of Lyn and Syk kinases were specific to Aβ oligomers but not for Aβ fibrils. These observations has raised the possibility that different conformations of Aβ, including soluble and fibrillar Aβ, effect AD pathology through different mechanisms (Bucciantini et al., 2002; Demuro et al., 2005; Deshpande et al., 2006; Kayed et al., 2003; Kayed et al., 2003; Kayed et al., 2004). Previous studies from our lab showed the Aβ(1-42) protofibrils, short curvilinear species,

to be strong stimulators for activating murine primary microglial cells compared to the fibrillar form (Paranjape et al., 2012). In this study we used Aβ(1-42) protofibrils as preassembled or aggregated soluble species with specific conformation for activating microglial cells. The comparative study between Aβ protofibrils and monomer induced microglial activation may provide some insight about the role of Aβ conformation in microglial activation. Most of the *in vitro* studies use isolated Aβ preparations to see an effect on cells. In this study we sought to follow Aβ monomer aggregation and its inflammatory effect directly in the presence of microglial cells. For this purpose, we used the fluorophore bis-ANS to measure the extent of surface hydrophobicity and ThT fluorescence to show the extent of β-sheet rich aggregate formation (Pastukhov & Ropson, 2003; Serpell, 2000). Microglial activation was determined by assessing levels of cytokines, primarily TNFα and IL-1β, by ELISA. Furthermore we made comparisons in the cell response between Aβ42 monomers and Aβ42 protofibrils with particular attention to the time course of microglial activation.

5.2 MyD88 dependent Aβ(1-42) protofibrils induced TNFα production in primary microglial cells

Aβ(1-42) protofibrils were prepared and isolated using SEC in a modified aCSF buffering system as described in our protocol previously (Paranjape et al., 2013). This protocol produces Aβ protofibrils as curvilinear structures less than 100 nm in length with a R_H range of 10-40 nm and average R_H of 21 nm in a reproducible manner. During SEC-isolation the protofibril comes out in a Superdex 75 void-volume fraction whereas Aβ monomer comes out in the included peak in later fractions. Further, these SEC-

isolated protofibrils showed high ThT fluorescence emission at 480 nm, indicating rich β-sheet content when compared with Aβ monomer (Paranjape et al., 2012; Paranjape et al., 2013). Within the same purification, we also obtained SEC-isolated monomer for carrying out aggregation studies in the presence of microglial cells

MyD88 is a cytoplasmic adaptor protein, upstream of NFκB, recruited in a TLR-mediated cell signaling event ultimately triggering pro-inflammatory cytokine production (Jana et al., 2008; Kong & Le, 2011; Valerio et al., 2006). Several TLR receptors, co-receptors and additional cellular components have been shown to get involved in Aβ-mediated inflammatory responses in monocytes/macrophages and microglial cells (Bamberger et al., 2003; Fassbender et al., 2004; Stewart et al., 2010; Udan et al., 2008). We compared TNFα protein secretion between wild type (WT) and MyD88$^{-/-}$ microglial cells by treating them both with SEC-isolated Aβ(1-42) protofibrils. The TNFα level was significantly diminished in MyD88$^{-/-}$ microglial cells compared to the response from WT cells (Figure 5.1). This showed a pivotal role of MyD88 in mediating Aβ(1-42) protofibril-induced TNFα secretion. Similar results have also been published by our lab recently (Terrill-Usery et al., 2014).

5.3 Comparison of Aβ(1-42) protofibril- and monomer-mediated TNFα secretion

SEC- isolated Aβ(1-42) protofibril species were used in the cell treatment as pre-assembled, aggregated, β-sheet rich structured species (Paranjape et al., 2013). In order to show the probable structure dependency in Aβ mediated microglial activation, a comparative time dependent microglial cell treatment study with Aβ(1-42) protofibril and Aβ(1-42) monomer was carried out. Interestingly, compared to monomer, protofibril

induced a relatively early TNFα response in WT microglial cells (Figure 5.2). With Aβ protofibril treatment, there was already a significant TNFα response within an hour of incubation, which increased at later time points. However, for monomer it took almost 4 to 6 hours to show any significant TNFα response. This delay in Aβ(1-42) monomer induced inflammatory response may be due to the lag time needed for the monomer to undergo a nucleation and self-assembly process for forming aggregated soluble species with a bioactive conformation. Soluble protofibrils, since they are already preassembled, may not have required the lag time to induce the significant response. As a quality control assessment of reactivity, microglial cells were treated with 10 ng/ml LPS, and TNFα was assessed (Figure 2.1).

5.4 *In situ* Aβ(1-42) monomer aggregation monitored by ThT and Bis-ANS

Thioflavin-T fluorescence has been used to detect amyloid fibrils. Later its usage expanded for *in vitro* characterization of amyloid fibrils. Apart from a few exceptions, most studies propose that the mechanism of ThT enhanced fluorescence is due to the steric restriction of freely rotating benzyl amine and benzathiole rings of ThT (Figure 2.3A) about their shared carbon-carbon bond. This would therefore enhance quantum yield on excitation when bound to secondary β-sheet rich structured amyloid fibrils (Biancalana & Koide, 2010; Hawe et al., 2008; Wu et al., 2009). Therefore ThT has been commonly used to assess the extent of β-sheet qualitatively and in Aβ aggregation kinetic studies (Mannini et al., 2014; Paranjape et al., 2012; Streets et al., 2013; Younan & Viles, 2015). In our study we used ThT for monitoring the β-sheet rich species formation during *in situ* Aβ(1-42) monomer aggregation.

ThT fluorescence was enhanced as freshly SEC-isolated Aβ(1-42) monomer was incubated for longer time points. This enhancement in fluorescence indicated the probable formation of β-sheet containing species during aggregation progression. When microglia were treated with monomers, a time-dependent TNFα increase was observed in the conditioned medium retained at different time points of incubation, indicating an inflammatory response (Figure 5.3). This study tries to support the other studies where change in secondary structural conformation could be important for inducing cellular response. (Maji et al., 2009; Simmons et al., 1994).

Researchers have tried to correlate surface hydrophobicity of protein with cellular toxicity (Mannini et al., 2014). For this, 4,4'-dianilino-1,1'-binaphthyl-5,5'-disulfonic acid (Bis-ANS) (Figure 2.3B) has been used frequently. Bis-ANS displays enhanced fluorescence on binding to the exposed hydrophobic surface of protein aggregates (Hawe et al., 2008; Younan & Viles, 2015). The dye has been used to characterize soluble Aβ conformations that are distinct from fibrils (Chen & Glabe, 2006). Though this dye is not commonly used and is less characterized, it has been shown to be more sensitive for tau oligomers than tau fibrils (Lasagna-Reeves et al., 2010). In our study we used this bis-ANS dye for monitoring Aβ(1-42) aggregates having solvent-exposed hydrophobic patches. Again, we saw the increase in inflammatory TNFα response significantly as the time of Aβ(1-42) monomer incubation was increased (Figure 5.4). Concomitantly bis-ANS fluorescence was also enhanced in a time-dependent manner, indicating the presence of Aβ aggregates with exposed surface hydrophobicity (Figure 5.5). These results correlate Aβ aggregation with inflammatory response in primary microglial cells. The ThT and bis-ANS fluorescence increases show changes in β-sheet secondary

structure and exposed hydrophobic properties during Aβ(1-42) monomer aggregation. These changes are correlated with the inflammatory response. In addition, quality control assessments of microglial activity were performed via LPS treatment, as previously described. The LPS (10 ng/ml)-induced TNFα response was estimated as 7400 pg/ml by ELISA.

5.5 Direct comparison between Aβ(1-40) and Aβ(1-42) inflammatory response

Aβ(1-40) displays very slow aggregation kinetics compared to Aβ(1-42) and this has been attributed to the presence of two additional C-terminal hydrophobic amino-acids, isoleucine and alanine for Aβ(1-42) (Jarrett et al., 1993; Jarrett & Lansbury, 1992; Vandersteen et al., 2012). We used this difference to show indirectly that aggregation of Aβ is necessary for bringing about an inflammatory response in primary microglial cells. For this purpose we incubated microglial cells with freshly purified SEC-isolated Aβ(1-42) and Aβ(1-40) monomers at a final concentration of 15 μM. At 0 hr, there was no significant TNFα (Figure 5.6A) or IL-1β (Figure 5.6B) response. However, at later time points of incubation, Aβ(1-42) monomer displayed an increased cytokine response (Figure 5.6). As expected, with Aβ(1-40), there was no significant TNFα at early time points and only comparatively very low levels after 48 hours of incubation (Figure 5.6A). Further, no significant IL-1β level was observed for Aβ(1-40) monomer aggregation within the experimental duration (Figure 5.6B). These result indirectly display the necessity of Aβ aggregation to bring about a significant inflammatory response in microglial cells. In addition, quality control assessments of microglial activity were

performed via LPS treatment, as previously described. LPS (10 ng/ml) induced TNFα response was estimated as 38823 pg/ml by ELISA.

5.6 Discussion

Structural polymorphism of Aβ is one of the important characteristics of Aβ fibrillation or aggregation and can be the basis for the pro-inflammatory response from immune cells (Ajit et al., 2009). Initially fibrillar forms of Aβ were shown to be neurotoxic in several studies. However, better correlation of pre-fibrillar soluble Aβ species with memory decline was also observed. Additionally, their presence in the hippocampi of transgenic mice and in post-mortem brain samples from AD patients made researchers focus more on soluble forms of Aβ (Dahlgren et al., 2002; Gong et al., 2003; Klein et al., 2001; Koffie et al., 2009; Lue et al., 1999; Näslund et al., 2000). Similarly microglial activation has not only been shown in response to Aβ fibrils and protofibrils but also to soluble lower order oligomers (Maezawa et al., 2011; Michelucci et al., 2009; Paranjape et al., 2012). In fact these early soluble oligomers have been shown to be toxic to microglial cells (Maezawa et al., 2011). A relationship between the aggregation state of Aβ protein and its ability to promote neurodegeneration and inflammatory processes can provide an important therapeutic window as has been previously suggested (Ajit et al., 2009; Ono et al., 2009; Pike et al., 1993; Simmons et al., 1994). Differential stimulation of microglial cells in response to oligomers and fibrils has also been shown (Dahlgren et al., 2002). Therefore it becomes important to study the structure and activity relationship for Aβ protein.

The earlier TNFα response from Aβ(1-42) protofibril, as a preassembled aggregate, compared to the response from Aβ(1-42) monomer suggests that the Aβ(1-42) monomer undergoes an aggregation process into a bioactive conformation for stimulating microglial cells (Figure 5.2). The study also shows that the majority of this response is attained through the MyD88-dependent pathway (Figure 5.1). This is in support of other studies where MyD88 involvement in Aβ induced cytokine production was shown (Ono et al., 2009; Terrill-Usery et al., 2014). However, some residual response from MyD88$^{-/-}$ cells, suggests the probable existence of a MyD88 independent pathway (Figure 5.1). Similar results have also been reported recently by our lab (Terrill-Usery et al., 2014).

In our study we observed more or less a consistent trend in microglial TNFα response during Aβ(1-42) monomer aggregation. Further, TNFα secretion increased along with an increase in time of Aβ incubation. This increase was correlated with ThT fluorescence increase. Previous aggregation study results from our lab were able to suggest a soluble fibrillar precursor as an optimum Aβ(1-42) intermediate for inducing maximum TNFα production (Ajit et al., 2009). This result might represent a similar observation. Furthermore, decreased ThT fluorescence may occur if significant amounts of insoluble fibrils form during aggregation, thus reducing the effective concentration in solution. However, in this study we cannot exactly suggest a specific aggregation state or specific Aβ aggregate contributing to inflammatory response. A previous study from our laboratory showed the TNFα response from isolated Aβ(1-42) fibrils to be very low, and in fact, as soluble aggregated Aβ intermediates progressed to fibrils, the response declined (Ajit et al., 2009). Since our data show no significant decline in the TNFα response within the experimented time frame (even at the longest time point), we propose

the majority of the Aβ fraction represented soluble Aβ aggregates rather than fibrils (Figure 5.3, 5.4, 5.5).

Several *in vitro* Aβ aggregation studies monitored with circular dichroism (CD) show the conversion of random coiled species to β-sheet structured species (Bartolini et al., 2007; Roychaudhuri et al., 2009). Also, CD-monitored structure-activity studies have shown a correlation between soluble β-sheet conformations and increased neuronal toxicity (Ono et al., 2009; Pike et al., 1995; Simmons et al., 1994). In our aggregation study we also saw a correlation between ThT fluorescence and the TNFα response (Figure 5.3). This suggests that the increase in β-structure as the aggregation proceeded might have a role in influencing the inflammatory response. We also tried to detect qualitative changes in surface hydrophobicity, if any, during *in situ* Aβ aggregation study using bis-ANS. Interestingly, we observed an increase in bis-ANS fluorescence in a time-dependent fashion and this increment also correlated with an increase in TNFα response (Figure 5.4). Change in solvent exposed hydrophobicity between Aβ(1-40) and Aβ(1-42) during aggregation has also been reported in another study (Chen & Glabe, 2006). Such changes have also been related with increased ability of aggregated Aβ protein to cause cellular dysfunction. However, the exact mechanism of this effect is not known. Some have linked such deleterious effect on cells with increased capacity to affect the fluidity of model membranes (Kremer et al., 2000; Mannini et al., 2014).

To study the dependence of microglial stimulation on Aβ aggregation, a comparative *in situ* aggregation between Aβ(1-40) and Aβ(1-42) monomer was done in the presence of microglial cells. The larger TNFα and IL-1β responses from Aβ(1-42) monomer, compared to Aβ(1-40) monomer treatment, is attributed to the slower

aggregation kinetics of Aβ(1-40) (Figure 5.5). This could also be presumed to be related with stability differences between Aβ(1-42) and Aβ(1-40) aggregates (Chen & Glabe, 2006). Additionally, our stability studies also revealed Aβ(1-40) aggregates as sensitive to chaotropic reagents like urea, unlike aggregated Aβ(1-42) (Coalier et al., 2013). Also, an Aβ(1-40) aggregation study from our lab showed no significant TNFα response in monocytes despite Aβ(1-40) monomer progressing to fibril formation at 30 °C (Ajit et al., 2009). Though our study tries to elucidate Aβ structure-dependent inflammatory response, there are several caveats to consider. Firstly we saw TNFα response being not consistent for the given time frame of study (Figure 5.2, 5.3, 5.5). Recently, with our newest Aβ batch, we have observed delayed Aβ(1-42)-induced microglial TNFα response on carrying out aggregation studies. This could result from batch-to-batch variation between chemically equivalent Aβ peptide supplies and therefore is one of the important limitations for reproducible data (Jan et al., 2010; Simmons et al., 1994). The delay in response can also be due to variability in microglial cells, or time needed for cell signaling processes, including cytokine transcription and translation. In all microglial cell studies, we tried to conduct our treatments within 40 days of microglial cell isolation. This is because as microglial cells age, their potential TNFα response declines. Secondly, other studies have shown that intracellular Aβ aggregation occurs in AD brain (Gouras et al., 2005). In fact, occurrence of intraneuronal Aβ oligomerization has been shown in a transgenic AD mouse model, and, more specifically, in endosomal compartments (Oddo et al., 2006; Takahashi et al., 2004). Further, lipid membranes have also been shown to modulate Aβ aggregation, accelerating fibrillogenesis depending upon lipid composition

(LaFerla et al., 2007). However, in our study, we cannot specify the exact location of Aβ aggregation occurrence and this has been our major limitation.

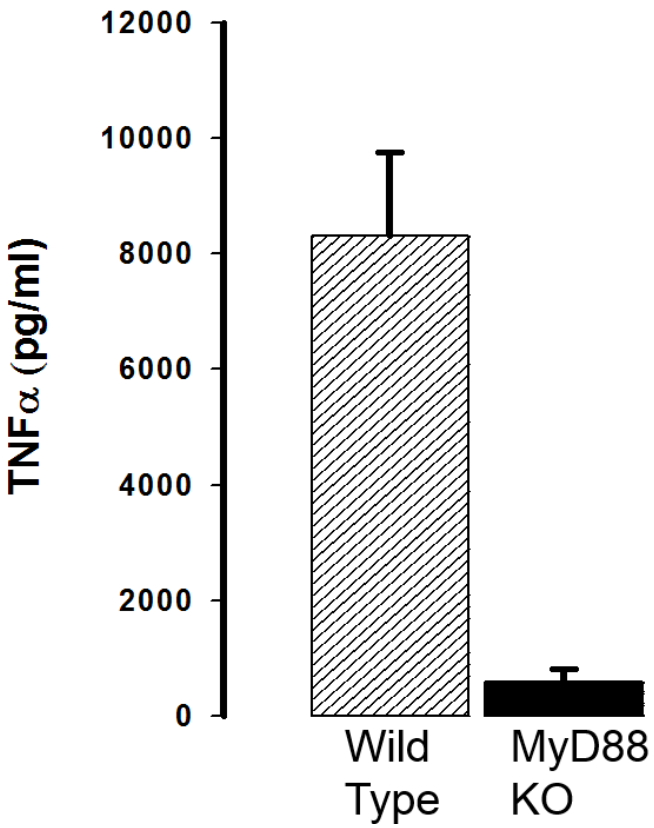


Figure 5.1 MyD88 dependent Aβ(1-42) protofibril induced TNFα production.

SEC-isolated Aβ(1-42) protofibrils (15 μM) were incubated with WT and MyD88$^{-/-}$ primary murine microglial cells for 6 hours. The incubation was carried out at 37°C and 5 % CO_2. Secreted TNFα level was measured by ELISA in the conditioned medium. Data bars represent the average ± standard error for n=3 trials. For the unstimulated WT and MyD88$^{-/-}$ cells TNFα values obtained were 86.7 ± 6.5 pg/ml and 130.8 ± 56.7 pg/ml respectively.

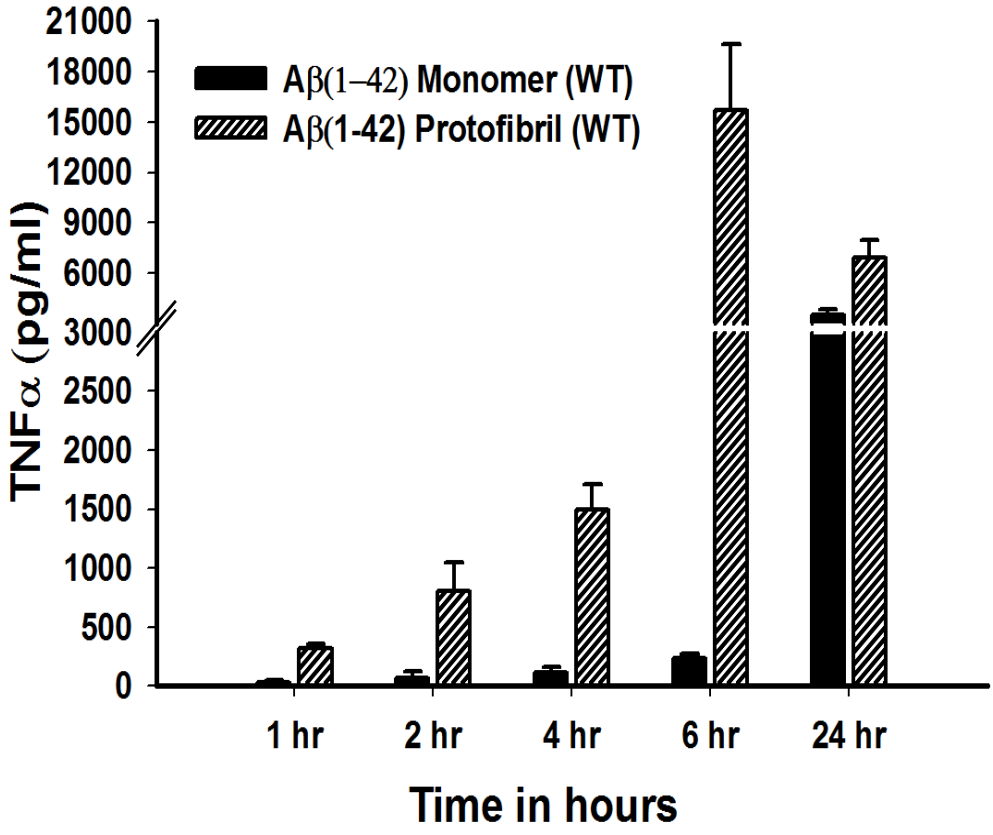


Figure 5.2 Time-dependent Aβ(1-42) monomer and Aβ(1-42) protofibril induced TNFα production.

Freshly prepared SEC-isolated Aβ(1-42) protofibrils and Aβ(1-42) monomers were incubated with microglial cells at final concentration of 15 μM. The incubation was carried out at 37°C and 5 % CO_2. At different time points conditioned medium from the treatment were collected and secreted TNFα level was measured by ELISA. The level of TNFα for unstimulated control were 1.8 ± 0.0 pg/ml, 10.6 ± 8.8 pg/ml, 45.6 ± 13.3 pg/ml, 101.6 ± 11.6 pg/ml and 119.7 ± 32.4 pg/ml for 1 hr, 2 hr, 4hr, 6hr and 24 hr respectively. Data bars represent the average ± standard error for n=3 trials.

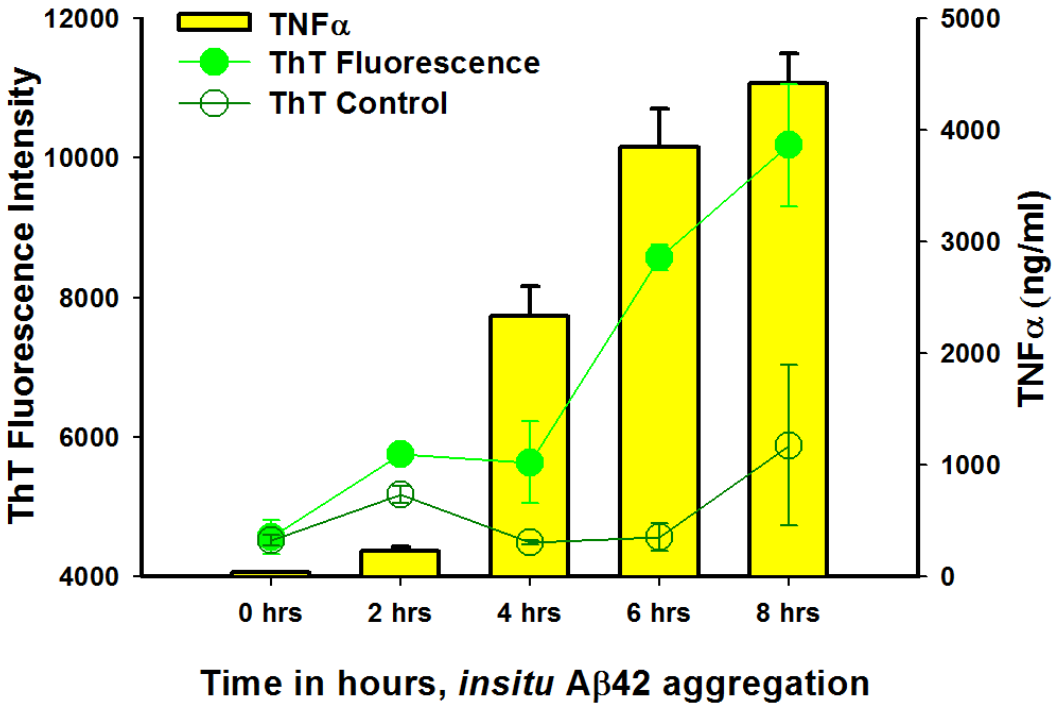


Figure 5.3 ThT monitored *in situ* Aβ(1-42) monomer aggregation.

Freshly prepared SEC-isolated Aβ(1-42) monomers were incubated with microglial cells at final concentration of 15 μM in a 96 well sterile plate. The incubation was carried out at 37°C and 5 % CO_2. ThT (5 μM) was added into the well after required incubation time. Immediately a reading was taken in a Wallac Victor 3 plate reader. Conditioned medium from the treatments were collected at each time point after the ThT fluorescence reading and secreted TNFα level was assayed by ELISA. The level of TNFα for unstimulated control were 6.2± 1.8pg/ml, 23.1 ± 12.6 pg/ml, 150.5 ± 23.3pg/ml, 220.8 ± 23.1pg/ml and 279.5 ± 28.2pg/ml for 0 hr, 2 hr, 4hr, 6 hr and 8 hr respectively. Data bars represent the average ± standard error for n=3 trials.

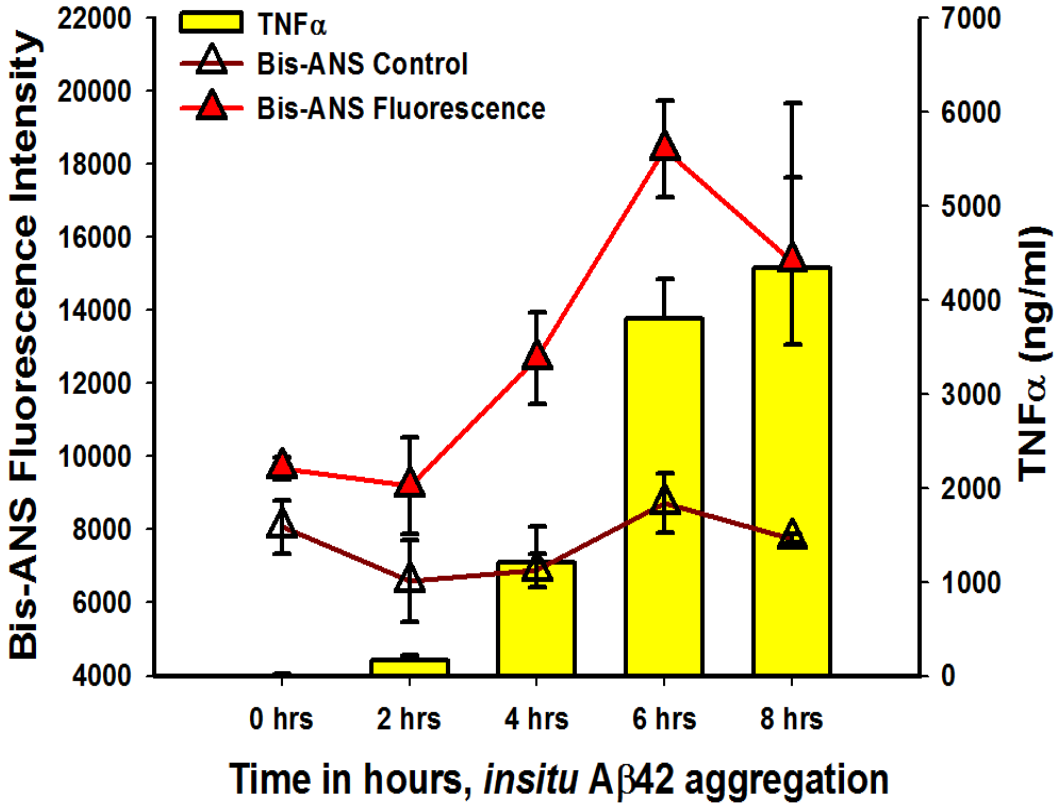


Figure 5.4 Bis-ANS monitored in situ Aβ(1-42) monomer aggregation.

Freshly prepared SEC-isolated Aβ(1-42) monomers were incubated with microglial cells at final concentration of 15 μM in a 96 well sterile plate. The incubation was carried out at 37 °C and 5% CO_2. Bis-ANS (10 μM) was added into the well after required incubation time. Immediately a reading was taken in a Wallac Victor 3 plate reader. Conditioned medium from the treatment were collected at each time point after the Bis-ANS fluorescence reading and secreted TNFα level was assayed by ELISA. The level of TNFα for unstimulated control were 3.2 ± 0.0 pg/ml, 6.4 ± 3.9 pg/ml, 7.9 ± 3.3pg/ml, 277.0 ± 99.1pg/ml and 358.6 ± 48.2pg/ml for 0 hr, 2 hr, 4hr, 6 hr and 8 hr respectively. Data bars represent the average ± standard error for n=3 trials.

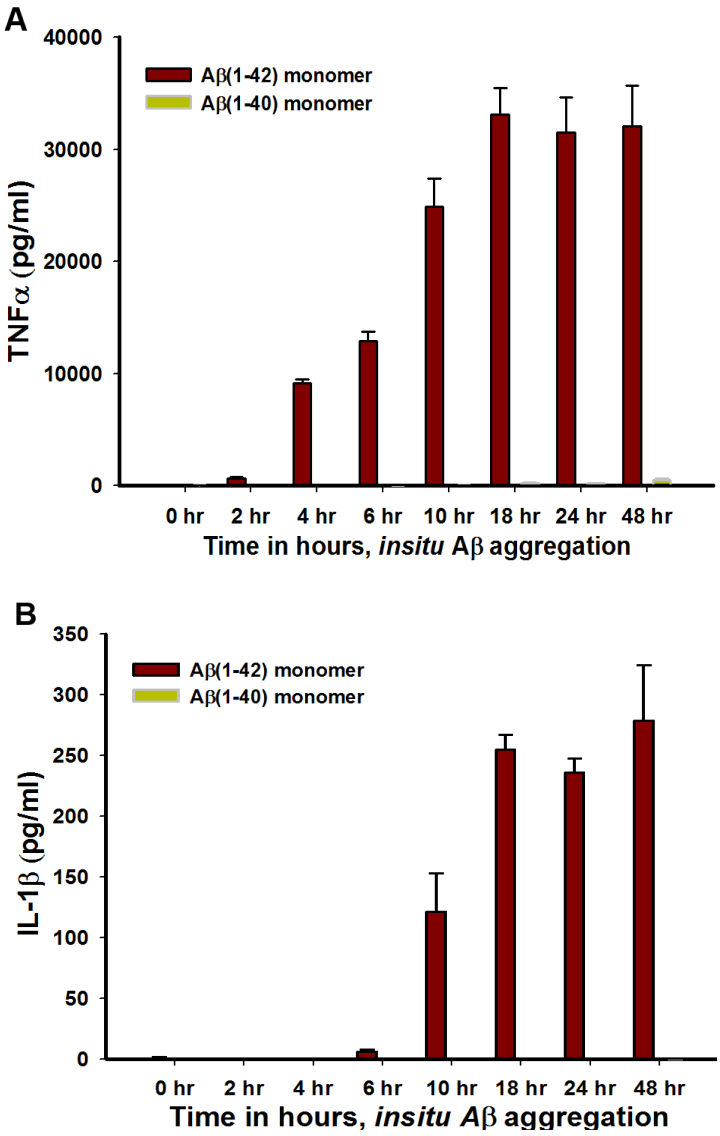


Figure 5.5 Direct comparative inflammatory response between Aβ(1-42) and Aβ(1-40) in primary microglial cells.

Freshly prepared SEC-isolated Aβ(1-42) monomer and Aβ(1-40) monomers were incubated with microglial cells at final concentration of 15 μM. The incubation was carried out at 37 °C and 5% CO_2. At different time points conditioned medium from the treatment were collected and secreted TNFα level (Panel A) and IL-1β (Panel B) was measured by ELISA. The maximum level of TNFα and IL-1β for unstimulated control were 96.9 ± 46.0 pg/ml, 1.0 ± 0.12 pg/ml respectively. Data bars represent the average ± standard error for n=3 trials

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VITA

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