Activation of the Innate Immune Response by the Alzheimer's Amyloid Beta Protein Via Toll-Like Receptors

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ACTIVATION OF THE INNATE IMMUNE RESPONSE BY THE ALZHEIMER’S AMYLOID BETA PROTEIN VIA TOLL-LIKE RECEPTORS

A Dissertation

Submitted to the Faculty

of

University of Missouri-Saint Louis

by

Maria L.D. Udan

In Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy Chemistry and Biochemistry

August 2009

University of Missouri-Saint Louis

Saint Louis, Missouri
For my mama and papa…
ACKNOWLEDGEMENTS

My sincerest gratitude goes to my advisor, Dr. Michael R. Nichols, for giving me continuous support and encouragement throughout my PhD, and for challenging me to think outside the box.

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LIST OF ABBREVIATIONS

Aβ  Amyloid beta
AD  Alzheimer’s Disease
ADDL  Aβ-derived diffusible ligands
AFM  Atomic force microscopy
APP  Amyloid precursor protein
BBB  Blood-brain barrier
BPI  Bactericidal/permeability-increasing protein
CD  Circular dichroism
cdk5  Cyclin-dependent kinase 5
CHO  Chinese hamster ovary
CK1  Casein kinase 1
CNS  Central nervous system
CSF  Cerebrospinal fluid
DC  Dendritic cells
DMSO  Dimethyl sulfoxide
ELISA  Enzyme-linked immunosorbent assay
EM  Electron microscopy
EOAD  Early onset Alzheimer’s disease
FAD  Familial Alzheimer’s disease
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
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<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FSL</td>
<td>Pam$_2$CGDPKHPKSF; synthetic diacylated lipoprotein</td>
</tr>
<tr>
<td>FTD</td>
<td>Fronto-temporal dementia</td>
</tr>
<tr>
<td>FTIR</td>
<td>Fourier transform infrared spectroscopy</td>
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<tr>
<td>GS</td>
<td><em>Griffonia simplicifolia</em></td>
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<tr>
<td>GSK-3</td>
<td>Glycogen synthase kinase-3</td>
</tr>
<tr>
<td>HEK</td>
<td>Human embryonic kidney</td>
</tr>
<tr>
<td>HFIP</td>
<td>Hexafluoroisopropanol</td>
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<tr>
<td>IFN</td>
<td>Interferon</td>
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<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IRAK</td>
<td>IL-1R associated kinase</td>
</tr>
<tr>
<td>KC</td>
<td>Keratinocyte chemoattractant</td>
</tr>
<tr>
<td>$K_D$</td>
<td>Dissociation constant</td>
</tr>
<tr>
<td>LBP</td>
<td>Lipid-binding protein</td>
</tr>
<tr>
<td>LMW</td>
<td>Low molecular weight</td>
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<tr>
<td>LP</td>
<td>Lipoproteins</td>
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<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>LPT</td>
<td>Lipopeptide</td>
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<tr>
<td>LRR</td>
<td>Leucine-rich repeats</td>
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<tr>
<td>LTA</td>
<td>Lipoteichoic acid</td>
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<tr>
<td>MAP</td>
<td>Mitogen-activated protein</td>
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<tr>
<td>MCP</td>
<td>Monocyte chemoattractant</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>MIP</td>
<td>Macrophage inflammatory protein</td>
</tr>
<tr>
<td>MS</td>
<td>Mass spectrometry</td>
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<tr>
<td>MTT</td>
<td>3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide</td>
</tr>
<tr>
<td>MyD88</td>
<td>Myeloid differentiation factor 88</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor-κB</td>
</tr>
<tr>
<td>NFT</td>
<td>Neurofibrillary tangles</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>NSAID</td>
<td>Non-steroidal anti-inflammatory drug</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pathogen-associated molecular pattern</td>
</tr>
<tr>
<td>Pam3CSK4</td>
<td>Tripalmytoyl cysteinyl seryl tetralysine</td>
</tr>
<tr>
<td>Pam3Cys</td>
<td>Tripalmytoyl-S-glyceryl-cysteine</td>
</tr>
<tr>
<td>PBM</td>
<td>Peripheral blood monocytes</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PGN</td>
<td>Peptidoglycan</td>
</tr>
<tr>
<td>PKA</td>
<td>cyclic AMP-dependent kinase</td>
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<tr>
<td>PMA</td>
<td>Phorbol 12-myristate 13-acetate</td>
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<tr>
<td>PMS</td>
<td>Phenazine methosulfate</td>
</tr>
<tr>
<td>PMX-B</td>
<td>Polymyxin-B sulfate</td>
</tr>
<tr>
<td>PRR</td>
<td>Pattern recognition receptor</td>
</tr>
<tr>
<td>QLS</td>
<td>Quasielastic light scattering spectroscopy</td>
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<tr>
<td>RAGE</td>
<td>Receptor for advanced glycation end products</td>
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$R_h$  Hydrodynamic radius

$r\text{IgG}$  Rat immunoglobulin G

ROS  Reactive oxygen species

$s\text{APP}_\alpha$  soluble APP cleaved by $\alpha$ secretase

$s\text{APP}_\beta$  soluble APP cleaved by $\beta$ secretase

SD  Standard deviation

SE  Standard error

SEC  Size exclusion chromatography

SPR  Surface plasmon resonance

TGF  Transforming growth factor

TIR  Toll/IL-1 receptor

TIRAP  Toll-1L-1 receptor associated protein

TLR  Toll-like receptor

TNF$\alpha$  Tumor necrosis factor alpha

TRAF  TNF-receptor associated factor 6

TRAM  Toll receptor-associated molecule

TRIF  Toll-associated activator of IFN

WT  Wildtype

XTT  2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide

293-hRTLR2  HEK 293 cells transfected with TLR2

293-hTLR2/CD14  HEK 293 cells transfected with TLR2 and CD14
ABSTRACT

Udan, Maria L.D. PhD., University of Missouri-Saint Louis, August 2009. Activation of the innate immune response by the Alzheimer’s amyloid beta protein via Toll-like receptors. Major Professor: Michael R. Nichols

Alzheimer’s Disease (AD) is the most common form of neurodegenerative disease characterized by the generation and deposition of amyloid beta plaques and the formation of neurofibrillary tangles. A wealth of data now demonstrate that inflammation is a prominent feature in AD pathology and a potential therapeutic target for the treatment and prevention of the disease. The emergence of evidence linking amyloid beta protein (Aβ), the primary component of senile plaques, to inflammation has led to new insights into understanding AD pathology. Aβ, a protein fragment resulting from cleavage of human amyloid precursor protein (APP), primarily exists in two forms: a slower-aggregating 40-amino acid long peptide (Aβ(1-40)), and a faster-aggregating 42-residue peptide Aβ(1-42). This investigation focused on elucidating the mechanism by which Aβ provokes an inflammatory response in AD. For this study, we utilized THP-1 human monocytes/macrophages as an inflammatory model system due to their sensitivity to Aβ. We hypothesized that fibrillar Aβ(1-42) may utilize Toll-like receptors (TLRs), a family of transmembrane receptors that mediate recognition of certain conserved structural motifs in pathogens, for production of proinflammatory products and activation of the innate immune response. Biophysical characterization of the bioactive species of Aβ(1-42) revealed that a soluble yet fibrillar species of Aβ(1-42) invokes tumor necrosis factor alpha (TNFα) production in THP-1 monocytes/macrophages. Moreover, using a TLR antibody neutralization assay, whereby receptor blockade inhibits cell responsiveness to TLR ligands, we showed that both TLR2 and TLR4 were highly involved in Aβ(1-42)-induced TNFα production. The role of TLR2 in Aβ-induced innate immune response was further substantiated by the production of proinflammatory interleukin-8 (IL-8) in transfected HEK293 cells, a mammalian cell line that does not express TLR2, after stimulation with Aβ(1-42). Furthermore, our results suggest the possible involvement of TLR2/TLR1 or TLR2/TLR6 for the Aβ-induced activation of TLR downstream signaling. Taken together, our findings provide strong correlation between Aβ and innate immune response activation via TLR2 and TLR4. The identification of TLRs that recognize Aβ has opened new venues for understanding the mechanism of Aβ-induced inflammatory response and may thus be a new therapeutic target for AD.
PUBLICATION
Toll-like receptors 2 and 4 mediate Aβ(1–42) activation of the innate immune response in a human monocytic cell line

Maria L. D. Udan, Deepa Ajit, Nikkilina R. Crouse and Michael R. Nichols
Department of Chemistry and Biochemistry, University of Missouri, St Louis, Missouri, USA

Abstract
The primary mediators for mediating the innate immune response are the Toll-like family of receptors (TLRs). Recent work has established that amyloid-beta (Aβ) fibrils, the primary components of senile plaques in Alzheimer’s disease (AD), can interact with the TLR2/4 accessory protein CD14. Using antibody neutralization assays and tumor necrosis factor alpha release in the human monocytic cell line THP-1 cell line, we determined that both TLR2 and TLR4 mediated an inflammatory response to aggregated Aβ(1–42). This was in contrast to exclusive TLR ligands lipopolysaccharide (LPS) (TLR4) and triamethoxy cinnamaldehyde (Pam3CSK4) (TLR2). Atomic force microscopy imaging showed a fibrillar morphology for the pathologically relevant Aβ(1–40) species. Pretreatment of the cells with 10 μg/mL of a TLR2-specific antibody blocked ~50% of the cell response to fibrillar Aβ(1–42), completely blocked the LPS response, and had no effect on the Pam3CSK4-induced response. A TLR4-specific antibody (10 μg/mL) blocked ~20% of the cell response to fibrillar Aβ(1–42), completely blocked the LPS response, and had no effect on the Pam3CSK4 response. Poly (I:C) also abolished the LPS response with no effect on Aβ(1–42) ruling out bacterial contamination of the Aβ samples. Combination antibody pre-treatments indicated that neutralization of TLR2, TLR4, and CD14 together was much more effective at blocking the Aβ(1–42) response than the antibodies used alone. These data demonstrate that fibrillar Aβ can trigger the innate immune response and that both TLR2 and TLR4 mediate Aβ-induced tumor necrosis factor alpha production in a human monocytic cell line.

Keywords: aggregation, Alzheimer’s disease, amyloid-β peptide, inflammation, innate immunity, Toll-like receptors.


Alzheimer’s disease (AD) is a progressive neurodegenerative illness diagnosed clinically by cognitive decline and pathologically by the presence of extracellular neuritic plaques in limbic brain regions and intracellular neurofibrillary tangles (Selkoe 2001). The primary component of neuritic plaques is amyloid-β protein (Aβ) (Ghetti and Wong 1984), a 40- or 42-residue peptide derived from proteolysis of the amyloid-β precursor protein. A large body of evidence supports the fundamental role of Aβ in AD etiology. The monomeric form of Aβ circulates ubiquitously in plasma and cerebral fluid yet an aggregated insoluble fibrillar form comprises the characteristic AD deposits (Selkoe 2004). In vitro studies have shown that Aβ monomer will undergo non-covalent self-assembly (Jarrett et al. 1993) to form a polydisperse mixture of soluble oligomers (Dahlgren et al. 2002) and protofibrils (Harper et al. 1999) that are enriched in β-sheet structure (Wash et al. 1999) and ultimately insoluble fibrils (Harper et al. 1999a). The types of intermediates formed during fibrillogenesis are dependent on the solution conditions (Harper et al. 1990; Dahlgren et al. 2002). Cellular studies have shown that fibrillar forms of Aβ are toxic to neurons compared with the benign monomer (Yankner 1994) yet difficulties have been encountered trying to correlate insoluble fibrillar Aβ with memory loss in a transgenic mouse model (Westman et al. 2002). Therefore, much of the recent investigative focus has shifted to soluble Aβ aggregates as early toxic agents (Haass and Selkoe 2007). Further research will clarify if one species can be implicated.

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E-mail: mtnmole@umcei.edu

Abbreviations used: AD, Alzheimer’s disease; Aβ, amyloid-β; Aβ42, bovine serum albumin; Aβ40, poly(b-amyloid); PAM3CSK4, triamethoxy cinnamaldehyde; Pam3;CSK4, poly(b-amyloid); Pam3CSK4, triamethoxy cinnamaldehyde; Pam3CSK4, poly(b-amyloid); TLR2, Toll-like receptor; TNF-α, tumor necrosis factor alpha; XTT, 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-phenyl formazan.
as the primary toxic agent in AD but it is becoming evident that morphologically diverse aggregation species can cause different harmful effects (Duff and Duff 2006). The overall findings suggest that distinct toxic and biological mechanisms are dependent on a specific Aβ structure or the extent of oligomerisation.

One such biological activity of Aβ is a proinflammatory stimulus. It has been well documented that inflammatory markers such as activated microglia and proinflammatory cytokines have been observed surrounding Aβ lesions in the human AD brain (McGee et al. 1987). The presynaptic microglia are believed to originate from peripheral cells of the mononuclear/macrophage lineage which infiltrate the parenchyma and differentiate into microglial cells (Wegiel et al. 2004). The in vitro inflammatory response to Aβ has been recapitulated in numerous in vitro cell model systems including both microglial and monocytic cells (Eugster et al. 1992; Yates et al. 1990; Combs et al. 2001).

The mechanism by which Aβ evokes a proinflammatory response appears to be quite complex. Fibrillar Aβ serves as a ligand for both the scavenger receptor class A R (Klorey et al. 1996; Parsche et al. 1996) and receptor for advanced glycation end products (Yan et al. 1996) although neither is linked to a proinflammatory response. A multiceptor complex comprising the scavenger receptor class B receptor CD36, α9β2 integrin, and the integrin-associated protein CD47 has been identified in mediating fibrillar Aβ induction of human THP-1 monocyte and murine microglial proinflammatory events (Bamberger et al. 2003) and phagocytosis of fibrillar Aβ via atypical phagocytic mechanisms (Koepnik and Landreth 2004).

Increasing evidence suggests that the human innate immune response may be triggered by aggregated Aβ. Human innate immunity is an important line of defense during bacterial, fungal, or viral invasion and can involve protection of proinflammatory cytokines, anti-microbial peptides (Hoffmann et al. 1999), and proteases (Mur-Bryce et al. 2002) to neutralize pathogens. The ability of phagocytic and immune cells to recognize these pathogens is due to motifs called pathogen-associated molecular patterns (PAMPs) which bind pattern recognition receptors. The primary mediators of the innate immune response are a family of transmembrane pattern recognition receptors termed Toll-like receptors (TLRs) which recognize PAMPs (reviewed in Hoffmann et al. 1999; Adelman and Ulevitch 2006). There are currently 11 human TLRs and they vary in their cellular localization and ability to detect distinct pathogens (Adelman and Ulevitch 2006; Octavio and Compton 2006). The most widely studied PAMP is bacterial lipopolysaccharide (LPS), an outer-membrane component of Gram-negative bacteria. LPS can activate TLR4 via complex formation with LPS-binding proteins and CD14, a glycosyl-phosphatidylinositol-anchored but not membrane-spanning receptor (Bohme and Compton 2006). The function of CD14 appears to be ligand-binding and presentation to membrane TLR4 and/or TLR2 (Kilian et al. 2006) which transduce the signal through the membrane and initiate the intracellular innate immune response pathways. More recently, CD14 was shown to mediate uptake of double-stranded RNA and directly interact with intracellular TLR3 (Lee et al. 2006).

A growing number of endogenous human molecules now appear to activate the innate immune response (Kilian et al. 2006) including the Aβ peptide. A physical and functional interaction was demonstrated between fibrilar Aβ (1-42) and CD14 that resulted in the release of inflammatory products in primary murine microglial cells and human peripheral blood mononuclear cells (Passkind and others 2004). Furthermore, CD14-mediated internalization of fibrilar Aβ (1-42) by a phagocytic mechanism in microglia and increased CD14 immunostaining was observed in AD brain slices compared with age-matched controls (Li et al. 2005). As CD14 can interact with both TLR4 and TLR2, we sought to determine which transmembrane TLR plays a functional role in transmitting the Aβ-induced innate immune signal through the membrane.

Materials and methods

Cell culture and cellular assays

The THP-1 cells were obtained from ATCC (Manassas, VA, USA) and maintained in RPMI-1640 culture medium (HyClone, Logan, UT, USA) containing 2 mM L-glutamine, 25 mM HEPES, 15 µM sodium bicarbonate, 10% fetal bovine serum (HyClone), 50 U/ml penicillin, 50 µg/ml streptomycin (HyClone), and 50 µM β-mercaptoethanol at 37°C in 5% CO2. For cellular assays, THP-1 cells were centrifuged, washed, and resuspended in reduced fetal bovine serum (0%) growth medium. Cell concentrations were adjusted to 1 x 10⁶ cells/ml and 0.3 µl was added to individual wells of a 48-well culture plate. Proinflammatory mediators in tissue culture supernatants (LPS, EnvironGen, San Diego, CA, USA) and polyinosinic Ri (MYC-Ri) sulfates (Sigma, St Louis, MO, USA) were added directly to cells and incubated at 37°C. Following incubation, the content of each well was removed, centrifuged at 2500 g for 10 min, and the supernatant was frozen at −20°C for subsequent analysis. Concentration-dependence data for TLR agonists were fit to a sigmoidal three-parameter equation using Sigmaplot graphing program to determine EC50 values. Aβ (1-42) induced THP-1 cell adherence was measured by direct counting. At various time points the medium containing remaining suspension cells was removed, adherent cells were washed with phosphate-buffered saline (PBS) and removed with 0.25% trypsin-EDTA (HyClone). The medium, PBS wash, and removed adherent cells were counted under a microscope using a hemocytometer.

Preparation of Aβ peptide

Aβ (1-42) peptides (Peptides, Bogart, GA, USA) were dissolved in 100% hexafluoroacetone (Sigma) for 1 h, aliquoted into sterile...
measurement titrages, dried in a vacuum centrifuge, and stored at -20°C. Prior to cell treatment the lyophilized peptides were resuspended in sterile water to 100 μM; peptide concentration and incubated at 4°C. Finally reconstituted AK(1–42) in water was allowed to incubate at 4°C prior to cell application. Numerous experiments indicated that the peak cell response occurred between 48 and 96 h after AK(1–42) aggregation. Cells were exposed to a final concentration of 15 μM AK(1–42). Commercial Aβ lot were endotoxin-tested prior to shipment and demonstrated to be <0.125 USP. This translates into an effective LPS concentration of 1 ng/ml, based on the calculations described in (Gao and Tian 2003). LPS concentrations at this level were ineffective at stimulating THP-1 cells. Mouse monoclonal treatment of a β-amyloid peptide prior to shipment reduced the endotoxin levels to undetectable. AK(1–42) preparations were also routinely tested for contamination using an 2,3,4,6-tetra-O-acetyl-3,4,6-tri-O-methyl-a-D-glucopyranose 4-carboxylic acid (GlcNAc), a potent inhibitor of AK(1–42) aggregation. Cells were maintained in medium containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (pen/strep) for 48 h before treatment. Cells were then treated with various concentrations of AK(1–42) for 24 h and the supernatants were collected and assayed for TNF-α and IL-6 levels.

Atomic force microscopy

Aβ (1–42) aggregation solutions (100 μM) were diluted to 1 μM in water. Grade V1 mice (Ted Pella, Inc. Redding, CA, USA) were used in 11 mm diameter wells that were coated with 10 μM metal (Cu). Aβ (50 μM) was applied to freshly cleaved mica, allowed to adsorb for 15 min, washed twice with water, air dried, and stored in a container with desiccant. Images were obtained with a Nanoscope III multimode atomic force microscope (Digital Instruments, Santa Barbara, CA, USA) in TappingMode™. Height analysis was performed using Nanoscope III software on fastened height mode images.

Results

Human THP-1 monocytes cells have been a useful model for Aβ preinflammatory activity (Elegies et al. 1997; Yavas et al. 2006) and exhibit responses to stimulate similar to those of microglia (Combs et al. 2001). These cells play a critical role in the innate immune response and phagocytic cells express the larger repertoire of TLRs (Boulton and Compton 2004). Our initial studies confirmed the sensitivity of the THP-1 monocytes to TLR agonists. Commercial preparations of the TLR4 agonist LPS (ultrapure E. coli K12) and the TLR2 agonist Pam3CSK4, a synthetic lipopeptide, were tested for their ability to stimulate TNFα production from THP-1 cells (data not shown). TNFα is an important product of the MyD88-dependent innate immune response (Kielian 2006). THP-1 cell supernatants were collected 6 h post-stimulation and secreted TNFα measurements revealed a concentration-dependent response for both compounds. 6 h incubation times were chosen based on separate time-dependent experiments showing maximal TNFα production by LPS and Pam3CSK4 after 6 h of cell exposure (data not shown). Curve fitting of the concentration-dependence data was performed as described in the Materials and methods and produced IC50 values of 5 ng/ml for ultrapure K12 LPS and 1 ng/ml for Pam3CSK4.

Human monocytes produce a significant proinflammatory response to aggregated Aβ and our studies supported these findings using the THP-1 monocyte cell line. AK(1–42) aggregates were prepared by reconstitution in water and quiescent incubation at 4°C. The aggregation was monitored by atomic force microscopy (AFM) (Fig. 1). Freshly reconstituted Aβ (1–42) monomers (Fig. 1, left panel) showed a dense field of small punctate species that had little stimulatory effect on the cells. The heights for the vast majority of
those adsorbed peptide species were < 2 nm. A lesser population of small spherical species was also observed in the freshly reconstituted Aβ(1–42) sample. The heights for these ranged from 2 to 5 nm with an average of 3.2 ± 0.3 nm (SD) for n = 115 measurements suggesting that they may be fibrillar precursors. The few bright spots (heights > 20 nm) in the 0 h image may represent the rapid formation of amorphous aggregates immediately following reconstitution. Continued incubation of the Aβ(1–42) solution produced thin flexible fiber-like structures (Fig. 1, middle panel), which coincided with the ability of the peptide to provoke a marked increase in TNFα production. Using the conditions described in the Materials and methods, an incubation time of typically 48 h was necessary to produce an Aβ(1–42) aggregated species that induced a significant cell response. AFM height measurements of the Aβ(1–42) fibers were plotted as a histogram (Fig. 1, right panel) and fitted for multiple peaks. Peak-fitting analysis subdivided the fibers into two populations. The first, and most populated peak, had a mean height and SE of 4.4 ± 0.1 nm. The second, less populated peak had a mean height of 7.9 ± 0.6 nm (SE). These values were similar to previous AFM-based morphological analyses of type I and II fibrillar Aβ (Harper et al. 1997a,b; Stine et al. 2003) and agreed with an earlier report by Fuehnder et al. linking fibrillar Aβ(1–42) with activation of the innate immune response (Liu et al. 2005). Our results demonstrated that fibrillar Aβ(1–42) was largely responsible for THP-1 cell activation.

The THP-1 TNFα production versus cell exposure time to 15 μM fibrillar Aβ(1–42) was slightly different from that of LPS and Pam3CSK4 in that it peaked consistently at 10 h (Fig. 2, circles) compared with 6 h. THP-1 cells were not pre-treated with a differentiating agent in those studies although we observed that Aβ(1–42) rapidly and effectively converted the suspension monocytes into adherent cells. A representative cell adherence time course is shown in Fig. 2 (triangles). Multiple experiments found that fibrillar Aβ(1–42) at 48 h of aggregation typically induced 74 ± 4% (SE)
adherence of the THP-1 cells after 6 h of Aβ(1–42) exposure (five separate experiments, nine trials total, data not shown). The observation that a longer cell exposure time was necessary to reach peak Aβ(1–42)-induced TNFα production suggests that THP-1 cell adherence or differentiation is important for Aβ responsiveness. Subsequent experiments in this report utilized a 6 h cell exposure to fibrillar Aβ(1–42) to limit any long-term proinflammatory effects to the cells. The Aβ(1–42) concentration (15 μM) that was used is based on monomeric units and does not reflect the actual fibrillar Aβ(1–42) concentration which is likely much lower. Aβ(1–42) concentrations below 15 μM did not effectively induce TNFα production from the THP-1 cells.

To delineate differences in the fibrillar Aβ(1–42) response compared with ultrapure K12 LPS and rule out the presence of small traces of contaminating LPS, the compound PMX-B was tested for its effect on both stimuli. PMX-B neutralizes the endotoxicity of LPS by binding directly with the LPS lipid A moiety and disorganizing the outer bacterial membrane (Vaisan 1992, Tugoby et al. 2006). The inclusion of 0.1 μg/mL PMX-B with ultrapure K12 LPS and Aβ(1–42) had dramatically different effects on the two proinflammatory molecules (Fig. 3). Greater than 98% of the K12 LPS signal was blocked by PMX-B with little effect on the Aβ signal. PMX-B (0.1 μg/mL) also had a small effect on the THP-1 response evoked by 300 pg/mL Pam3CSK4 (data not shown) suggesting that PMX-B may have some non-specific effects or there are subtle structural similarities between the three proinflammatory stimuli used in these studies. Aβ/PMX-B and LPS/PMX-B samples were included in all experiments for continual monitoring of LPS contamination. Some experiments showed a greater than ±10% effect of PMX-B on the Aβ response although XTT cell proliferation measurements indicated there was no detectable presence of bacterial contamination within the Aβ sample. For clarity, those experiments were not included in the TLR antibody neutralization studies described in the ensuing sections.

A TLR antibody neutralization assay was developed to investigate which transmembrane TLRs mediate the Aβ(1–42) proinflammatory response. The assay was initially tested on the TLR agonists LPS and Pam3CSK4 to determine and demonstrate the sensitivity of the antibody neutralization approach. The ultrapure K12 LPS from Invivogen has been stringently purified by double phenol extraction of a 0.5% tributylamine/0.5% deoxycholate aqueous phase. This procedure has been shown to remove contaminating lipoproteins responsible for TLR3-mediated signaling (Hirschfeld et al. 2008). Our TLR antibody neutralization results were consistent with those findings. The ultrapure K12 LPS response was significantly attenuated by CD14 (74% inhibition) and TLR4 (87% inhibition) antibodies (Fig. 4, black bars) with no effect by the TLR2 antibody (12% inhibition) compared with the IgG isotype control (0% inhibition). The CD14 isotype control, IgG1, inhibited just 5% of the K12 LPS response. Antibody neutralization of the TLR2 agonist Pam3CSK4 was clear. Only the CD14 and TLR2 antibodies had blocking activity (Fig. 4, gray bars). The TLR2 antibody was extremely effective and blocked 96% of the Pam3CSK4
response while neutralization with the CD14 antibody blocked 90% of the response. Neutralization of LPS and Pam3CSK4 with anti-TLR2 and −TLR4 antibodies respectively was not statistically different from IgG2 control (p < 0.025).

The TLR antibody neutralization studies were conducted against the Aβ(1-42) proinflammatory response to elucidate which CD14+TLR receptor combination was mediating Aβ(1-42)-induced NFκB production. Pre-treatment of the THP-1 cells with a CD14 antibody effectively attenuated the Aβ(1-42) response (52% inhibition relative to Aβ/IgG1 control) (Fig. 5) consistent with a previous report (Fassbender et al. 2004). Suprisingly, both TLR4 (50% inhibition) and TLR2 (50% inhibition) antibodies were effective at signal attenuation compared with the rat IgG control with TLR2 possessing greater blocking activity. Statistical analysis indicated significant inhibitory differences between the antibodies and their respective IgG1 controls (p < 0.001).

These data indicate that multiple TLRs may interact with fibrillar Aβ(1-42). PMX-2 had an average attenuation of 4 ± 3% SE on the Aβ(1-42) response in these experiments (see Fig. 3). A different set of TLR antibodies and isotype controls were required for the Aβ-IgG2 neutralization studies because of significant attenuation of the Aβ(1-42) response by the mouse IgG2 control used in the LPS and Pam3CSK4 studies (Fig. 4). For this reason we utilized TLR2 and 4 antibodies produced in rat (InvivoGen). Furthermore, the mouse IgG1 isotype control at 16–30 μg/mL showed a consistent 20–30% stimulation of the Aβ(1-42) response (Fig. 5), which may have offset some of the effectiveness of the anti-CD14 neutralization. These issues underscore the importance of including IgG isotype controls in antibody neutralization experiments for proper interpretation. Because of these IgG effects, we were careful not to assign too much importance to differences between TLR2, TLR4, and CD14 antibodies in Aβ(1-42) neutralizing ability. The antibody neutralization results demonstrated that multiple TLRs mediate the Aβ(1-42) innate immune response and that both TLR2 and TLR4 have active roles in the pathway. Antibody blockade of the Aβ(1-42) innate immune response was dose dependent in separate experiments. TLR2 and TLR4 antibodies concentrations were evaluated at 5 and 20 μg/mL compared with 10 μg/mL antibody in Fig. 5. Neutralization effectiveness increased with higher concentrations of TLR2 and TLR4 antibodies (Fig. 6). Similar to Fig. 5, the TLR2 antibody again was a slightly more effective blocker of the Aβ(1-42) response than the TLR4 antibody. Overall, the individual TLR antibodies clearly blocked Aβ(1-42) activity but to a certain extent of around 50–70% inhibition. The results presented here and those of Fassbender et al. (2004) suggest that a significant amount of the Aβ-induced proinflammatory response is

![Graph](image-url)
controlled by innate immune receptors. One explanation for the remaining unblocked activity may be contributions from other receptors that mediate the Aβ proinflammatory response such as the TLR1/2, TLR2, TLR4, and CD40 receptor complex (Baemler et al., 2003).

A second explanation may be that, since both TLR2 and TLR4 mediate Aβ-induced TNFα production, one receptor may compensate for the other when either one is inaccessible. To test this idea, a neutralization assay utilizing combinations of TLR2, TLR4, and CD40 antibodies was conducted to assess the effect of blocking multiple TLR pathways mediating the Aβ(1-42) response. All four preparations were examined at 5 μg/mL of each antibody. A lower antibody concentration was used to better observe the effect of combination antibody treatment. Furthermore, each cell treatment was supplemented up to 10 μg/mL rat IgG and 5 μg/mL mouse IgG to match the triple-combination TLR2:TLR4:CD40 antibody treatment with isotype control amounts. For this reason, direct comparison of the combination antibody treatments in Fig. 7 with those in Figs 5 and 6 is not straightforward. A small reduction of the Aβ(1-42) induced TNFα response was observed in the presence of 5 μg/mL of each antibody (Fig. 7). As noted earlier, the mouse IgG1 isotype control for CD40 augmented the Aβ(1-42) response. Mouse IgG1 (5 μg/mL) was included in each sample that did not contain CD40 and may have masked some of the TLR blocking ability. The difference in neutralizing ability of each antibody at 5 μg/mL in comparison with the mouse IgG1 rat IgG control were of small or no statistical importance (Fig. 7 legend). TLR antibody double combinations were much more effective, particularly the combination of TLR2/TLR4 which blocked 60% of the response. Interestingly, TLR2/CD40 and TLR4/CD40 antibody combinations also blocked the response better than the individual antibodies suggesting that Aβ interactions may overlap to some extent with both TLRs and CD40. The most effective neutralization occurred after antibody pre-treatment with a TLR2/TLR4/CD40 triple combination which blocked 70% of the Aβ(1-42) response compared with the mixed mouse IgG1/rat IgG isotype control (Fig. 7).

**Discussion**

The data presented here demonstrate a role for both TLR2 and TLR4 in mediating the TRP-1 monocyte/macrophage proinflammatory response initiated by fibrillar Aβ(1-42). Our results are consistent with a previous report by Faasbender et al. (2004) showing that CD40 antibody neutralization of Aβ-induced microglial activation. Although CD40 functions as a ligand-binding accessory protein for both TLR4 and TLR2, Faasbender et al. proposed that the Aβ cellular activation was likely transmitted by TLR4 based on a positive response obtained in Chasen's hamster (ovary cells lacking a functional TLR2. However, this result was not confirmed in cells that do express TLR2 such as microglia, peripheral blood monocytes, or TH-1 cells and therefore did not exclude a role for TLR2 when present in the cell. In fact, our data indicates that TLR2 and TLR4 can both induce the Aβ signal and may compensate for each other when necessary. The convergence of Aβ and the innate immune system suggests that in some manner fibrillar Aβ(1-42) may act like a PAMP-like infectious agent through TLR activation and a proinflammatory cascade.

Several reports suggest that Aβ activation of the innate immune system may have beneficial aspects. Cultured primary microglial cells prepared from CD44-deficient mice were significantly better at internalizing fibrillar Aβ(1-42) compared with cells prepared from CD44-deficient mice (Li et al., 2003). Furthermore, it was shown that stimulation of BV2 mouse microglial cells with TLR4, TLR2, and TLR9 agonist caused significant uptake of Aβ(1-42) (Tabara et al., 2006). Moreover, the activation of TLR2 is primary mouse microglial cells with TLR2 agonist-papillomycin enhanced...
formyl peptide receptor-like 2-mediated uptake of Aβ(1–42) (Chen et al. 2006). In vivo studies support the cellular studies and have shown an increased Aβ load in Mo/βu APMSw P51Id90 mice with deficiencies in TLR4 (Tahara et al. 2006). The observation of increased CD14 immunostaining in AD brain slices compared with age-matched controls (Liu et al. 2003) has added to the growing body of evidence linking innate immunity with neurodegenerative disease (discussed in Nguyen et al. 2002).

The sensitivity of innate immune receptors to LPS (TLR4) and G-proteins (TLR2) demands that careful consideration be given to experimental preparations. Caution has been suggested when interpreting an innate immune response and potential contamination of the preparation with bacterial components must be ruled out (Kielian 2006). Endotoxin measurements of the commercial Aβs used in these studies found undetectable levels of LPS and the possibility of externally introduced bacterial contamination was routinely monitored using an XTT cell proliferation assay. These tests were substantiated in Fig. 3 where PM-B had no effect on the Aβ(1–42) response in the same experiments in which TLR antibodies showed neutralizing activity. Furthermore, the innate immune response dependency on Aβ(1–42) aggregation time also argued against the presence of contaminants in the Aβ sample. Low levels of TNFα production were typically observed when the peptide was freshly reconstituted. Continued incubation (48–96 h) of the peptide at 4°C produced maximal stimulatory activity followed by a decline to baseline levels upon further aging of the Aβ sample. This type of time course would not be expected if the sample contained contaminating TLR agonists and degradation of the contaminants after 1 week would not be expected either as Aβ(1–42) aggregation solutions were incubated at 4°C until cell exposure. The decline in stimulatory activity upon continued aggregation of the peptide is of interest and currently under investigation in our laboratory. One explanation may be that midway through the aggregation process the actual concentration of fibrillar Aβ is well below 15 μM, which is based on monomer units. Continued formation of fibres may create new effects thereby masking cellular responses. We are currently investigating the structural and biochemical basis for the Aβ aggregation time-dependent response. The cumulative results and analyses indicate that the THP-1 monocyte TLR4 signal is caused by Aβ and not contaminants.

The structural features of PAMPs that confer TLR ligand specificity are not completely understood. The lipid A moiety of LPS is the primary region responsible for LPS activity (Chiller et al. 1971) and is thought to possess the PAMP motif. Aβ is composed of a didecanamino backbone with ester- and amide-linked long-chain fatty acids. The number and length of lipid A acyl chains vary among bacteria and alter LPS potency (Miller et al. 2005). Although some TLR2 antagonists have a protein component that

common structural feature among PAMPs appears to be amphipathic with significant regions of hydrophobicity. Aβ fibrils can be included in this category based on solid state NMR studies indicating a hydrophobic core running along the fiber axis (Fedorka et al. 2002).Crystallographic studies of soluble CD14 show a large N-terminal hydrophobic pocket important for LPS binding (Kim et al. 2005). The binding pocket overlaps with areas of conserved leucine-rich repeats which are also found on TLR4. Aβ fibrils may possess structural components that have similarities with both TLR2 and TLR4 agonists.

The role of innate immunity in the pathogenesis of AD will need further investigation. Effective TLR4-mediated phagocytosis and clearance of Aβ aggregates would theoretically provide beneficial effects although a sustained inflammatory response to aggregated Aβ has been suggested as one of the underlying mechanisms of progressive neurodegeneration in AD (McGee and McGee 1995) and may in fact exacerbate Aβ deposition (Gold et al. 2002). Aβ plaque-induced recruitment of peripheral monocytes into the brain parenchyma and their subsequent differentiation into phagocytic microglial cells may help explain the increased CD14 expression in AD brain slices reported by Liu et al. (2005). The PAMP-like features necessary to activate the innate immune response may not be present in all Aβ aggregate morphologies thereby allowing certain populations to elude recognition and suggesting that not all can trigger a response. This idea is consistent with the observed diversity in Aβ aggregate morphologies in the AD brain. Some of these deposits are surrounded by inflammatory markers while others lack inflammatory cytopathology (Selloes 2004). Furthermore, some Aβ aggregate structures that initiate an innate immune response may be resistant to phagocytic degradation. In summary, the interaction between Aβ aggregates, TLR4, and innate immunity may further explain some of the complexities of AD etiology and provide a potential point of therapeutic intervention.

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References


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1 INTRODUCTION

1.1 Alzheimer’s Disease: Comprehending the etiology of the disease

Advances in scientific and medical research have resulted in a dramatic rise in human life expectancy. In the 20th century, an increasing number of individuals have reached the age at which short-term memory defects linked with normal aging of the human brain has become one of the major concerns. Although this is a common occurrence among older people, the problem arises when a person starts to have trouble following complex discussions and making decisions, and begins to experience a heightened degree of forgetfulness. These are common symptoms of dementia, an illness that is associated with age, in which parts of the brain begin to malfunction causing disruptions and progressive loss in memory, judgement, reasoning and behavioral stability (St George-Hyslop 2000).

Alzheimer’s Disease (AD) is the most common form of neurodegenerative dementia. This illness currently affects about 10% of persons over 65 years of age, and >40% of people over 85 years (Buxbaum and Tagoe 2000). Globally, the disease affects almost 2% of the population in industrialized countries, and it is predicted that the occurrence of AD will increase three-fold within the next fifty years (http://www.alz.org). There is no strong correlation between the occurrence of AD and race or sex type. However, it is more prevalent in women mainly due to the fact that women live longer
than men (Irvine, El-Agnaf et al. 2008). To date, there is still no definitive diagnosis of the disease other than postmortem analysis of the brain (Georganopoulou, Chang et al. 2005). However, the effort to decipher the causes and mechanism of AD has gone a long way since its discovery in 1906 by Alois Alzheimer (Selkoe 2001). Studies done for the past decades have identified two proteins that comprise the classical neuropathological lesions that are diagnostic of AD: the neurofibrillary tangles and senile plaques.

1.1.1 Neurofibrillary Tangles

In an effort to better understand the pathology of AD, researchers have done post-mortem analysis of the human AD brain. Examination of the degenerating neurons in the diseased brain regions showed the presence of nonmembrane-bound clusters of abnormal cytoplasmic fibers ~20-nm length, which are referred to as neurofibrillary tangles (Kosik, Joachim et al. 1986; Selkoe 1996; Selkoe 2001). Furthermore, rigorous immunocytochemical and biochemical analyses identified the microtubule-associated phosphoprotein tau as the main component of neurofibrillary tangles (Grundke-Iqbal, Iqbal et al. 1986; Kosik, Joachim et al. 1986; Selkoe 1996). In the neurons, tau proteins can be found predominantly in axons. Under normal conditions, tau exists as a highly soluble phosphorylated protein, which functions as a stabilizer and promoter of microtubule polymerization (Hanger, Anderton et al. 2009). Microtubules are important for providing routes where nutrients and other molecules can move through cells (St George-Hyslop 2000). However, excessive tau phosphorylation and overexpression was found to be the main cause of transformation of soluble tau into tangles. Moreover,
numerous studies using antibodies specific for various phosphor-tau epitopes have suggested that the disr egulation of tau phosphorylation is due to the augmented activity of certain kinases, such as glycogen synthase kinase-3 (GSK-3), cyclin-dependent kinase 5 (cdk5), casein kinase 1 (CK1) and cyclic AMP-dependent protein kinase (PKA), as well as inactivation of certain phosphatases (Selkoe 1996; Patrick, Zukerberg et al. 1999; Churcher 2006; Hanger, Byers et al. 2007; Hanger, Anderton et al. 2009). So far, one recent therapeutic strategy focuses on how to subtly regulate the activity of kinases. The activation of phosphatases as a therapeutic target seems unlikely due to still poorly understood mechanism and involvement of phosphatases in tau pathology (Hanger, Anderton et al. 2009).

1.1.2. Senile plaques

Another directly observable hallmark of AD is the presence of extracellular senile or amyloid plaques. These clusters of protein accumulate in the spaces between nerve cells and are present extensively in the hippocampus and the cerebral cortex region of the AD brain. Closer analysis showed that these plaques contain extracellular deposition of numerous proteins, the principal of which is amyloid β- protein (Aβ) (Selkoe 2001). Numerous studies have associated both the more common 40-amino acids long Aβ (designated as Aβ(1-40)) and the less common but faster aggregating 42-amino acids long Aβ(1-42) with AD. There are two forms of senile plaques that have been detected in the diseased brain: neuritic plaques and diffuse plaques. Neuritic plaques are compact and contain the fibrillar form of both Aβ(1-40) and Aβ(1-42). Further evidences revealed the
presence of reactive proinflammatory cells called microglia, as well as reactive astrocytes, along with the plaques (El Khoury, Moore et al. 2003). Swollen and deformed neurons in the vicinity of the plaques are also observed. Microscopic analysis of the diseased brain sections showed that the size (diameter) of neuritic plaques vary greatly, from 10 to >120 μm (Selkoe 2001). In contrast, diffuse plaques are composed exclusively of Aβ(1-42). These amorphous plaques are also observed in young individuals afflicted with Down’s syndrome before Alzheimer’s type-dementia is manifested (Irvine, El-Agnaf et al. 2008). Moreover, immunohistochemical studies of patients with Down’s syndrome demonstrated the presence of diffuse plaques at an early age, but neuritic plaques only occur at a later age, along with the presence of abundant neurofibrillary tangles (Lemere, Blusztajn et al. 1996; Selkoe 2001). Due to these findings, scientists considered diffuse plaques to be precursors of the neuritic plaques, and thus called diffuse plaques as “preamyloid deposits”.

1.2 Probing the molecular mechanism of AD: Focus on amyloid β- protein (Aβ)

As mentioned previously, AD is characterized by progressive accumulation of Aβ protein in the brain sections, mainly in the cerebral cortex, hippocampus and other regions of the brain. To understand the protein’s involvement in the disease, it is crucial to fully comprehend the molecular mechanism of Aβ production. A major breakthrough in the study of Aβ and its connection with AD transpired when scientists effectively isolated plaque amyloid deposits from the diseased brain and successfully sequenced the Aβ protein (Selkoe 1996; Mattson 2004). This led to the identification of amyloid
precursor protein (APP) as the major source of Aβ (Selkoe 1998; Castellani, Lee et al. 2008). The subsequent discussions below will focus on the mechanism of Aβ production, Aβ hypothesis, and structural studies of Aβ and how it contributes to AD pathogenesis.

1.2.1 Amyloid precursor protein processing and generation of Aβ

Extensive reviews have been written about the nature of the human amyloid precursor protein. APP was identified as the main source of Aβ that is implicated in AD. Numerous researchers like Dennis Selkoe of Harvard, Mark Mattson of U. Kentucky and Edward Koo of UC San Diego, among others, have focused on elucidating the nature and function of this type-1 integral membrane glycoprotein. The human amyloid precursor protein (APP), which is located on chromosome 21, is composed of a single membrane spanning domain, a large extracellular domain and a shorter (~47 amino acid) cytoplasmic COOH-terminal region (Selkoe 2001; Thinakaran and Koo 2008). Studies now show that approximately half of Aβ(1-40) or Aβ(1-42) sequence lies on the extracellular part of APP (Aβ amino acids 1-17, from amino acids 597-613 of APP that is 695-amino acid long (APP-695)), while the other half, which contains hydrophobic residues, lies within the phospholipid bilayer (Mattson 1997). Among the numerous isoforms, the largest of the known APP splice forms is comprised of 770 amino acids (Suzuki and Nakaya 2008). This particular APP is expressed throughout the body, as well as in neurons. Increasing evidence suggests that APP is important in neuronal growth and survival, synaptic plasticity and cell adhesion (Buxbaum and Tagoe 2000; Mattson 2004; Thinakaran and Koo 2008).
A model of APP processing is illustrated in Figure 1.1 (Mattson 2004). APP trafficking involves transit from the endoplasmic reticulum to the plasma membrane where it undergoes post-translational modification. After which, APP is rapidly internalized and translocated back to secretory vesicles through endocytic and recycling compartments (Selkoe 1998; Thinakaran and Koo 2008). During this trafficking process, APP molecules can undergo specific proteolytic cleavage through the action of enzymes α, β and γ secretases to release the secreted products into the extracellular space. The likely APP cleavage sites by the secretase enzymes that are discussed in this section are based on the sequence of APP-695. The action of α secretase on APP was the first to be identified. Investigations showed that α secretase clips 12 amino acids NH$_2$-terminal to the single transmembrane domain of APP (between amino acids 612 and 613, amino acids 16 and 17 of Aβ) (Sisodia, Koo et al. 1990). This cleavage releases the non-amyloidogenic form secreted APP$_\alpha$ ectodomain from the cell surface, leaving an 83-residue (APP83) COOH-terminal fragment in the membrane. This residue is further acted upon by γ-secretase (cleavage between amino acids 639 and 640 of APP-695, and at the COOH terminus of Aβ) to form a shorter 3 kDa peptide p3 into the extracellular space. Aside from the products produced through the action of α-secretase, alternate APP cleavage occurs 16 amino acids NH$_2$-terminal to the α-cleavage site is mediated by β secretase (amino acid residues 596 and 597 of APP-695 that corresponds to the NH$_2$ terminus of Aβ) (Mattson 1997; Selkoe 1998; Selkoe 2001). Consequently, a smaller fragment (s)APP$_\beta$ is released into extracellular milieu, retaining a 99-residue (APP99) COOH-terminal fragment that contains intact Aβ in the membrane. This COOH-terminal fragment can be further proteolytically cleaved by γ-secretase to generate an intact Aβ.
Figure 1.1. Processing of Amyloid precursor protein (APP). (a) Cleavage of APP involves the activities of α-, β- (BACE1) and γ-secretase. α secretase cleavage results in the release of soluble non-amyloidogenic APP fragment (sAPPα) from the cell surface and leaves an 83-amino acid-C-terminal fragment (C83). BACE cleavage leaves a 99-aa residue, which can be further processed by γ-secretase to generate and liberate a 39-42 residue amyloidogenic peptide. The 99-residue fragment can also be internalized and further processed by γ-secretase to produce Aβ (1-40)/(1-42) in endocytic compartments. C99 cleavage by γ-secretase also liberates an APP cytoplasmic domain that can translocate to the nucleus for modulation of gene expression, i.e., induction of apoptotic genes. Processing of APP/C99 residue by caspases results in the production of neurotoxic peptide C31. (b) Amino acid sequence of Aβ(1-40) and Aβ(1-42). The bold italics represent the part of Aβ that lies partly outside the cell membrane and is being liberated upon cleavage of APP by α secretase (Mattson 1997; Mattson 2004)
peptide 38-42 amino acid residues in length (Mastrangelo, Ahmed et al. 2006; Pearson and Peers 2006). Under normal conditions, APP cleavage by β and γ secretases typically results in the formation of 40-residue Aβ peptide. However, about 10% of the cleavage product is Aβ(1-42) (St George-Hyslop 2000). Because the nature of γ secretase has not been fully understood, further studies are still being done as to the site of cleavage of APP99 and APP83. However, several lines of evidence showed that considerable amount of Aβ(1-40) and Aβ(1-42) are made during internalization and internal processing of the APP COOH fragment (Mattson 1997; Selkoe 2001; Mattson 2004). The formed Aβ(1-40) or Aβ(1-42) is then released from the cell and has likelihood to form fibrils.

The α secretase-catalyzed APP cleavage is believed to be the predominant processing pathway for APP and the APPα have distinct extracellular functions. Numerous in vitro studies reveal increasing number of roles of sAPPα in neurons including cell survival (Mattson, Cheng et al. 1993; Ohsawa, Hirose et al. 1995), stimulation of neurite outgrowth (Clarris, Nurcombe et al. 1994; Ohsawa, Hirose et al. 1995; Furukawa, Sopher et al. 1996), regulation of cell adhesion, and protection against a range of metabolic, excitotoxic and oxidative insults, among others (Mattson, Cheng et al. 1993; Smith-Swintosky, Pettigrew et al. 1994; Selkoe 1998).

The cleavage by β-secretase also normally occurs to produce and release Aβ. Studies indicate that Aβ is being generated constitutively by normal cells in blood and cerebrospinal fluid (CSF) with normal concentrations in the low nanomolar range (3-8 nM for CSF and under 500 pM in plasma) (Seubert, Vigo-Pelfrey et al. 1992; Motter, Vigo-Pelfrey et al. 1995; Scheuner, Eckman et al. 1996; Dumery, Bourdel et al. 2001; Ramsden, Plant et al. 2001). Thus, unaffected individuals normally produce and clear Aβ.
Additionally, the Aβ protein being normally generated is thought to have a normal physiological role. On the other hand, individuals afflicted with AD generate Aβ that forms ordered aggregates, which are deposited as amyloid plaques.

1.2.2 Missense mutations in APP and other mutations cause autosomal dominant AD

Familial AD (FAD) occurs in mid, rather than late, adulthood (Buxbaum and Tagoe 2000). Numerous studies of FAD cases reveal that early-onset AD (EOAD) is caused by mutations in APP and presenilin genes. These mutations affect the metabolism or stability of Aβ and cause autosomal forms of AD (Selkoe 1996; LaFerla, Green et al. 2007).

Several lines of evidence showed that mutations affecting the APP gene (Figure 1.2 adapted from (Selkoe 2001)) are closely associated with AD by increasing local concentration and deposition of Aβ (1-42). Most of the mutations are located within the Aβ sequence or in regions of the β-APP gene that encode amino acids that lie immediately adjacent to the β- or γ-secretase cleavage sites (Chartier-Harlin, Crawford et al. 1991; Goate, Chartier-Harlin et al. 1991; Mullan, Crawford et al. 1992; Dumery, Bourdel et al. 2001). The following discussion enumerates some of the known missense APP mutations that are linked to familial or early-onset AD.

A double mutation (APPΔNL) that alters Lys670Met671 in APP770 to asparagine and leucine, respectively, also known as Swedish mutation, can be found just adjacent to the site of β secretase cleavage and induces heightened β-secretase cleavage to produce more Aβ(1-40) and Aβ(1-42) (Citron, Oltersdorf et al. 1992; Mullan, Crawford et al.
Figure 1.2. APP mutations genetically linked to familial Alzheimer’s disease. The sequence within APP region that contains the Aβ and transmembrane region is shown in expanded form, with a single-letter amino acid code. The underlined residues represent the Aβ sequence. Arrows represent the cleavage sites for α, β and γ secretases. The vertical broken lines indicate the transmembrane region of the APP. Residues in yellow are the known sites for missense mutations, and residues in blue are the amino acids that replace the amino acids in original sequence. These are mutations identified in certain patients with familial Alzheimer’s disease. The three-digit numbers represent the residue number according to the APP-770 isoform. (Selkoe 2001)
1992; Selkoe 2001). On the other hand, the five mutation sites that occur just COOH-terminal to the γ-secretase cleavage sites (some of which are London mutation (Val717Ile), Rouen mutation (Val715Met) and Florida mutation (Ile716Val)) seemingly have an enhancing effect on the production of Aβ(1-42) species (Goate, Chartier-Harlin et al. 1991). The mutations within the Aβ sequence enhance the aggregational properties of all Aβ species. For instance, Dutch mutation (Ala692Gly) results in formation of plaques and tangles associated with dementia, and severe hereditary cerebral hemorrhage with β-amyloidosis (Hendriks, van Duijn et al. 1992; Buxbaum and Tagoe 2000; Selkoe 2001). Afflicted individuals show extensive amyloid deposition in vessel walls of cerebral cortex and leptomeninges (Levy, Carman et al. 1990). In general, vast evidences revealed that APP mutation increases Aβ(1-42) concentration by a factor of 1.5 to 1.9, while Aβ(1-40) concentration remains the same (Findeis 2007).

Aside from APP mutations, mutations in presenilin 1 (PS1, found on chromosome 14) and presenilin 2 (PS2, on chromosome 1) have also accounted for 30% to 40% of all cases of EOAD. Presenilins are expressed in brain and concentrated in neurons (Scheuner, Eckman et al. 1996; Uchihara, el Hachimi et al. 1996). More than 30 mutations in PS1 and 2 in PS2 have been reported (Selkoe 1997) and they generally result in increase of Aβ1-42) production (Scheuner, Eckman et al. 1996; Buxbaum and Tagoe 2000). For instance, immunohistochemical analyses of brains of patients with Glu280Ala PS-1 mutation showed a greatly elevated Aβ(1-42), but not Aβ(1-40), levels in the cerebellum (Lemere, Blusztajn et al. 1996). Evidences also showed that mutations in gene encoding PS lead to a 1.5 to 3-fold increase in the relative abundance of plaques containing Aβ(1-42) peptides in FAD, compared with the levels in sporadic cases of AD.
(Lemere, Blusztajn et al. 1996; Mann, Iwatsubo et al. 1996; Selkoe 2001). Moreover, the rate of \( A\beta(1-42) \) aggregation was significantly enhanced in the presence of PS mutation, compared to that of \( A\beta(1-40) \) peptide (Jarrett, Berger et al. 1993).

Taken together, these genetic studies support the notion that the mutations in APP and PS lead to a significantly increased production of faster-nucleating \( A\beta \) variant \( A\beta(1-42) \) (Jarrett, Berger et al. 1993), and further underscores the idea that the acceleration of amyloid fibril formation is critical for the study of AD (Selkoe 1997).

As discussed in the previous section (1.1), neurofibrillary tangles that contain hyperphosphorylated tau proteins and \( A\beta \)-containing senile plaques are hallmark characteristic features of AD. Over the years, debate has ensued over whether there is a link between \( A\beta \) and tau abnormalities, and whether either contribute to the pathogenesis of the disease. Numerous evidences have demonstrated that mutations in both genes encoding APP and tau result in dementing illness. However, further inquiry of the molecular effects and characterizing the clinical signs and symptoms of these mutations clarified the significance of tau and \( A\beta \) in AD progression. APP mutations were proven to accelerate \( A\beta \) production (as discussed above), and these mutations have been linked to some cases of EOAD (Goate, Chartier-Harlin et al. 1991). EOAD, although rare, reflects the histological profile of plaques and tangles. Furthermore, clinical studies of EOAD showed a characteristic hippocampal-predominant dysfunction as well as dysfunction in other neocortical sites. In comparison, investigations focusing on mutations of gene encoding tau showed that although these mutations promoted tau hyperphosphorylation, they did not lead to AD. Instead, the mutations resulted in the development of fronto-temporal dementia (FTD), which is another type of dementia.
different from AD and characterized by frontotemporal atrophy (Hutton, Lendon et al. 1998). Furthermore, the presence of tangles that appear first in extrahippocampal sites and the absence of plaques make FTD histologically distinct from AD (Small and Duff 2008). These genetic findings led to the proposed “amyloid hypothesis” that Aβ is the primary instigator for pathogenicity in AD, and its accumulation and elevation result in the hyperphosphorylation of tau and other clinical features of AD (Small and Duff 2008).

1.2.3 Amyloid β-peptide

As discussed previously, the heterogeneous cleavage property of β and γ secretase gives rise to a 39 to 42 amino acid long fragment of Aβ, with 40-residue peptide (termed Aβ(1-40)) and 42-residue peptide (Aβ(1-42)) as the most common. The presence of two additional amino acids in Aβ(1-42) has extensive consequence with regards to its tendency to aggregate and form fibrils, with the longer Aβ(1-42) having a faster aggregation rate and being more pathogenic (McLaurin, Yang et al. 2000; Castellani, Lee et al. 2008). In 1985, researchers purified and characterized the peptide from post-mortem brain of AD patients. Using liquid chromatography and western blotting, Beyreuther’s group revealed that the Aβ peptides isolated from diseased patients are 4-5 kDa in size (Masters, Simms et al. 1985).

The presence of Aβ in individuals not afflicted with AD indicates that the protein has a role in the normal physiology of the central nervous system; but the normal function of Aβ is less understood as compared to its cytotoxic effects and its pathological role in AD. Nevertheless, numerous investigations have revealed some of the roles of this
APP fragment in the normal function of neuronal cells. Teng and co-workers suggested that the more predominant form Aβ(1-40) functions as an antioxidant (Teng and Tang 2005). Aβ(1-40) was also shown to counteract the toxic effects of β and γ secretase inhibitors at concentration as low as 10 pM (Plant, Boyle et al. 2003). Further studies have demonstrated that both Aβ(1-40) and Aβ(1-42) were found to moderate potassium channels in neurons (Ramsden, Plant et al. 2001; Findeis 2007).

Despite the normal physiological roles of Aβ, the main focus of investigations is the ability of this protein to form fibrils and its role in neurodegeneration. Increasing interest in structural and functional properties of Aβ led to a better understanding of this protein fragment. Numerous investigators have utilized various methods like circular dichroism (CD), nuclear magnetic resonance (NMR), Fourier Transform Infrared spectroscopy (FTIR) and microscopy techniques, among others, to extensively study the structure of Aβ and its formation of fibrils (Hilbich, Kisters-Woike et al. 1991; Shen and Murphy 1995; Nilsson 2004; Stromer and Serpell 2005).

One challenge that researchers encountered in studying the structure of Alzheimer’s Aβ is the insolubility of the amyloid plaque, and as a result, the analysis of Aβ from the diseased brain proved to be extremely difficult (Serpell 2000). This prompted researchers to concentrate on Aβ fibrils that are formed in vitro. In vitro structural prediction studies revealed that the fibrils were of varying lengths, about 6-8 nm in diameter, and generally possess a parallel β-sheet conformation (as shown in Figure 1.3a and b) in which amino acids 41-42 of one peptide strand interact with amino acids 34-35 of the second peptide monomer (Lansbury, Costa et al. 1995; Mattson 1997;
Figure 1.3. Structural studies of Aβ. (a) Ribbon diagram of residues 9-40 of Aβ (1-40) showing two β-sheet per molecule. Parallel β-sheets also observed on cross-β motif. (b) Atomic representation of fibers, showing the length (in diameter) of about 6-8 nm. (c) Structure prediction of Aβ (1-42), showing residues with high propensity for β-sheet. Highly hydrophobic regions are also shown. (Serpell 2000; Tycko 2004)
Kowalewski and Holtzman 1999; Tycko 2004). For fibrils of relatively shorter peptides (15 residues or less), an antiparallel β-sheet structure was demonstrated (Lansbury, Costa et al. 1995; Balbach, Ishii et al. 2000; Tycko 2004). Moreover, analysis of soluble Aβ peptide uncovered amino acid characteristics that favor β-sheet conformation and revealed that C-terminal residues 28 to 40/42 have the highest probability for β-sheet formation, while residues 9 to 21 showed a lower probability for β-sheet. Further investigations showed that the propensity of residues 28 to 40/42 to form β-sheet is due to its highly hydrophobic property. Residues 17-21 also exhibit greatest hydrophobicity. Two predicted sites for β-turn in the peptide structure can be found between residues 6 to 8, and 23 to 27 (Figure 1.3c) (Serpell 2000).

Using X-ray diffraction, Kirschner et al. revealed the β-sheet conformation of the Aβ fibers, estimating the length to be 80Å long and about 40Å thick (Kirschner, Abraham et al. 1986). These measurements correspond to four pleated sheets, with approximately 16 hydrogen-bonds for each sheet. Halverson et al. (1990) correlated peptide insolubility with β-sheet conformation using Fourier Transform Infrared spectroscopy (FTIR) studies. His group reported that residue Aβ(34-42) possessed antiparallel stable β-sheet structure in the solid state (Halverson, Fraser et al. 1990). It was also shown that the residues 10 to 42 may form the β-sheet core of the fibrils, while fragments 1-9 were not required for fibril formation. Furthermore, this N-terminal region may be exposed on the surface of the fibers and may play a role in interaction between fibrils (Hilbich, Kisters-Woike et al. 1991). In addition, using electron microscopy, Aβ(14-23) was found to be the shortest N-terminal fragment capable of fibril formation, and that deletions or substitutions on this fragment completely abolished or impaired
fibril formation (Tjernberg, Callaway et al. 1999). This led to the conclusion that sequence 14 to 23 of Aβ forms the core of Aβ fibrils.

As shown by evidences that are stated previously, Aβ is a normal product in the brain and cerebrospinal fluid of normal individuals. This signifies that Aβ itself does not lead to neurodegeneration. However, studies showed that the key to neuronal injury seems to lie on the aggregation state of Aβ. Moreover, the ability of synthetic Aβ to form fibrils in vitro may be influenced by various parameters such as variations in pH, temperature, buffers or solvent composition, presence of metals such as iron, copper and zinc, peptide concentration and peptide sequence (Fraser, Nguyen et al. 1991; Shen and Murphy 1995; Mattson 1997; Lansbury 1999; Serpell 2000; Walsh, Tseng et al. 2000; Nichols, Moss et al. 2005). The following discussion will focus on the study of amyloid β fibril formation in vitro.

1.2.4 Amyloid β fibrillogenesis

Why is the study of amyloid β fibrillogenesis relevant? It has been about a decade since the amyloid-β cascade hypothesis was first proposed. According to this hypothesis, deposition and accumulation of fibrillar Aβ in brain tissues are the key causative agent that drives AD pathogenesis (Hardy and Selkoe 2002; Teng and Tang 2005; Castellani, Lee et al. 2008). This led to an increased interest on understanding Aβ fibril formation and how it can be a key to developing therapeutic strategies. Though numerous studies have focused on the biophysical aspects affecting formation of Aβ fibrils, the kinetics or mechanism of Aβ fibrillogenesis was poorly understood. Numerous
laboratories investigated the mechanism governing Aβ fibrillogenesis by utilizing synthetic peptides. However, investigating the kinetics of Aβ fibril formation proved to be a challenging quest due to limitations on available techniques. FTIR spectroscopy, CD, turbidity, thioflavin-T binding or microscopy could not provide detailed information on fibril size, nor was it appropriate for real time analysis. Moreover, these techniques are of limited use in clarifying the structures of fibrillogenesis intermediates (Kirschner, Abraham et al. 1986; Fraser, Nguyen et al. 1991; Hilbich, Kisters-Woike et al. 1991). The in vitro finding that Aβ fibrillogenesis follows a nucleation-dependent polymerization mechanism (as illustrated in Figure 1.4a) was first verified in 1986 when Teplow’s group extensively studied the nucleation and growth of Aβ(1-40) fibrils using quasielastic light scattering spectroscopy (QLS) along with size exclusion chromatography (SEC) and electron microscopy (EM) (Lomakin, Chung et al. 1996). QLS is a useful technique in monitoring the sizes of protein polymers in solution. The combination of QLS, SEC, and microscopy allows a direct and rapid estimation of the Aβ oligomerization state (Walsh, Lomakin et al. 1997).

The Aβ kinetic process is dependent on two parameters, namely the nucleation rate and the rapid elongation or growth rate (Figure 1.4b, (Nilsson 2004)). Nucleation is considered to be an initial rate-limiting step characterized by a lag period in which Aβ monomers self-associate to form micelles of Aβ from which fibrils materialize and elongate (Serpell 2000; Carrotta, Barthes et al. 2007). The lag period of initial association of monomers to form a nucleus is an entropically unfavorable process (Nilsson 2004). However, once nucleus is formed, the aggregation proceeds rapidly to form fibrils. The lag phase can be overcome experimentally by several ways such as seeding and other
**Figure 1.4. Mechanism of Aβ fibril-formation** (a) Process of Aβ fibril-formation from monomers follows a nucleation-dependent polymerization mechanism (illustration from Walsh, Lomakin et al. 1997). (b) The nucleation polymerization model of aggregation follows two processes: nucleation, which is a slow process, and elongation which is rapid. The lag phase in nucleation process can be eliminated using various parameters such as variations in pH, temperature, solvent or buffer system, peptide concentration, or peptide sequence among others (illustration from Nilsson 2004).
biophysical processes (briefly discussed in section 1.4.3) Several laboratories have shown that Aβ fibril polymerization proceeds with the formation of dimers, tetramers, and finally oligomers (Tjernberg, Callaway et al. 1999). Moreover, further structural and kinetic characterization of Aβ fibrillogenesis utilizing X-ray fiber diffraction, light scattering, SEC and microscopy methods revealed that a time-dependent decrease in dimer levels was paralleled by an increase of transient prefibrillar intermediate in the fibril assembly, termed protofibrils (Harper, Wong et al. 1997; Walsh, Lomakin et al. 1997; Harper, Wong et al. 1999; Walsh, Hartley et al. 1999; Serpell 2000). Protofibrils are small elongated oligomers with beaded appearance observed early on in the Aβ fibril formation process, are about 2.7 to 4.2 nm in diameter, and measure <200 nm in length which disappeared immediately with longer incubation time and were replaced by rigid, amyloid-type full-length fibrils (Harper, Wong et al. 1997; Kowalewski and Holtzman 1999; Walsh, Hartley et al. 1999; Dumery, Bourdel et al. 2001; Kayed, Head et al. 2003). Harper et al (1997) investigated Aβ(1-40) protofibril formation by AFM and reported the appearance of small elongated Aβ oligomers with average height of 4.3 ± 0.5 nm and lengths that range from 20-70 nm, characteristic of protofibrils (Harper, Wong et al. 1997). Using QLS, Walsh et al reported a hydrodynamic radius $R_H$ of 27.8 ± 1.8 nm for the initially formed protofibrils, and steadily increased to a maximal value of 80.6 ± 14.4 nm (Walsh, Hartley et al. 1999). Other evidences have demonstrated that protofibril formation was observed at the early stages of both Aβ(1-40)and Aβ(1-42) fibrillogenesis although they have differences in the diameter (~4.2 nm for Aβ (1-42) and ~2.2 nm for Aβ(1-40) ) that may be attributed to the extra two residues of Aβ(1-42) (Harper, Wong et al. 1997). Radiochemical and immunological assays revealed that other short-lived
intermediates are being produced prior to protofibril formation, however, it is the dimer and protofibrils that accumulate during fibrillogenesis substantiating the main idea that protofibrils act as centers of growth of mature fibers (Walsh, Lomakin et al. 1997). AFM analysis demonstrated that protofibril height is 40% that of fibrils (Harper, Wong et al. 1997; Harper, Wong et al. 1999). Several evidences also showed that protofibrils were in equilibrium with lower molecular weight (LMW) Aβ in the course of Aβ fibril formation. Further characterizations revealed that protofibrils are not easily sedimented, are too small to produce turbidity, and have significant β-sheet content, as shown by binding to Congo red and thioflavin T, as well as CD data (Harper, Wong et al. 1997; Walsh, Hartley et al. 1999).

Active research on the mechanism of Aβ fibrillogenesis resulted in several proposed models that may further clarify the transition from protofibril to fibril formation. One possible mechanism introduced is end-to-end association of protofibrils. However, this model is unlikely due to kinetic barriers that may be encountered with regards to proper alignment of protofibril ends. Another possible mechanism is “lateral association” in which protofibrils combine laterally followed by addition of smaller Aβ species (ie. monomers and dimers) to the end. Lateral association of protofibrils followed by end-to-end annealing is another possible alternative explanation for Aβ fibril formation (Burdick, Soreghan et al. 1992; Harper, Wong et al. 1997; Walsh, Lomakin et al. 1997; Walsh, Hartley et al. 1999; Nichols, Moss et al. 2002).

The investigation of Aβ fibrillogenesis proved to be a very challenging endeavor. Numerous investigators reported other structures preceding fibril formation including Aβ-derived diffusible ligands (ADDLs), Aβ*56, “globulomers” and “Aβ oligomers”
(Roychaudhuri, Yang et al. 2009). These fibrillar intermediates differ in morphology and size, but it is still a challenge to distinguish one from the other. AFM analysis showed that ADDLs are small “globules” of diffusible Aβ oligomers that measures 4.8 to 5.7 nm. Further characterization of ADDLs by western blot analysis and SDS revealed a molecular weight that ranges between 17 and 42 kDa, with the predominant species at 27 kDa (Lambert, Barlow et al. 1998). Aβ*56, which has a molecular weight of 56 kDa characteristic of dodecameric species and morphology of prostate ellipsoid, was isolated and identified from brains of Tg2576 mice (Lesne, Koh et al. 2006). AFM analysis of the isolated Aβ*56 measured ~1 nm in height (Cheng, Scearce-Levie et al. 2007). On the other hand, “globulomers”, so called due to the globular oligomeric structure, do not form fibrils despite their ability to form β-sheet structure (Gellermann, Byrnes et al. 2008). Aβ oligomers that were produced in vitro were found to be composed of 15 – 20 monomers, with approximate MW of ~90,000 characteristic of an octadecamer (Kayed, Head et al. 2003). Taken together, these intermediate species were termed “soluble oligomers” (Deshpande, Mina et al. 2006). This general term applies to all forms of Aβ that do not pellet down after high speed centrifugation (typically >100,00g for more than 1h) (Irvine, El-Agnaf et al. 2008).

It is apparent that the extensive efforts of various laboratories to study and elucidate the mechanism of Aβ fibrillogenesis unexpectedly revealed a wide range of fibrillar and oligomeric intermediates that may have a contribution to development of AD. Biochemical and biophysical methods such as size measurement, however, could not sufficiently provide the required sensitivity to distinguish one aggregation state to another. Recent advances in the study of Aβ polymerization have included the use of
conformation-specific antibodies that recognize generic structural features for providing more detailed and sensitive information about the identity of aggregated species (O'Nuallain and Wetzel 2002; Kayed, Head et al. 2003; Glabe 2004). The continuing progress in the research of Aβ polymerization process, and the availability of numerous tools for studying Aβ aggregation, are instrumental for elucidating the pathogenesis of AD and in designing strategies for therapeutic intervention.

1.2.5 Correlation between Aβ assembly and AD

According to the Aβ cascade hypothesis, the overproduction of Aβ or the increased proportion of the Aβ42/Aβ40 ratio is the basic pathophysiological process that causes early-onset AD. Over the years, numerous in vitro and in vivo studies have demonstrated that the fibrillar form of Aβ is toxic to the neurons (Table 1.1 (Mattson 1997)). Several investigators, for instance, Yankner et al (Lorenzo and Yankner 1994) studied the neurotoxicity of different Aβ species by creating nonamyloidogenic amorphous Aβ aggregates and the amyloidogenic Aβ fibrils and comparing their neurotoxic effects to primary rat hippocampal cultures. The prepared peptides were characterized by microscopy and Congo red staining technique. For this study, 20 μM of Aβ was applied to the hippocampal cultures and the biological effects were determined by immunohistochemical process. Their results showed that the fibrillar form caused significant neuronal cell loss and synapse loss while the nonfibrillar form was not toxic. The neurotoxic effects of fibrillar Aβ to neurons were also demonstrated by other laboratories as well (Kowall, Beal et al. 1991; Pike, Burdick et al. 1993; Geula, Wu et al.)
<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Toxic Action</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hippocampal neurons</td>
<td>Cell death, sensitivity to excitotoxicity</td>
</tr>
<tr>
<td>Cortical neurons</td>
<td>Cell death, sensitivity to excitotoxicity, sensitivity to energy depletion, impaired muscarinic signaling</td>
</tr>
<tr>
<td>Neocortical neurons</td>
<td>Cell death</td>
</tr>
<tr>
<td>Neuroblastoma cells</td>
<td>Cell death</td>
</tr>
<tr>
<td>PC12 cells</td>
<td>Impaired mitochondrial function</td>
</tr>
<tr>
<td>Synaptosomes</td>
<td>Impaired glutamate transport, impaired mitochondrial function</td>
</tr>
<tr>
<td>Astrocytes</td>
<td>Reactive phenotype, glutamate transport impairment</td>
</tr>
<tr>
<td>Microglia</td>
<td>Cell activation/injury</td>
</tr>
<tr>
<td>Smooth muscle</td>
<td>Damage and death</td>
</tr>
<tr>
<td>Vascular endothelial</td>
<td>Impaired glucose transport, loss of barrier function, apoptosis influx</td>
</tr>
</tbody>
</table>
However, upon analyzing the post-mortem brains of the diseased patients, there is insubstantial correlation between dementia and the density of fibrillar amyloid (Walsh, Klyubin et al. 2002b; Walsh and Selkoe 2007; Irvine, El-Agnaf et al. 2008). This observation eventually became a flaw of the amyloid-β cascade hypothesis.

In contrast, for the past decade, evidences of the significant connection between soluble Aβ levels and the extent of synaptic loss and cognitive impairment have continuously emerged (Lambert, Barlow et al. 1998; Lansbury 1999; Lue, Kuo et al. 1999; Walsh and Selkoe 2007). The use of synthetic Aβ peptides, cell systems with over-expressed APP, APP transgenic mouse models and human CSF and postmortem brain contributed to the conclusion that soluble Aβ induces neurotoxicity rather than fibrillar Aβ (Kirkitadze, Bitan et al. 2002; Irvine, El-Agnaf et al. 2008). Krafft and coworkers (Roher, Chaney et al. 1996) isolated Aβ from the post-mortem brain of AD patients, characterized the peptides using SEC, mass spectrometry (MS), and microscopy techniques, and applied the peptides to cultures of rat hippocampal neuron glia cells. Their study shows that dimers caused neuronal killing in the presence of microglia.

Similarly, Teplow’s group demonstrated using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay that protofibrils perturb the normal metabolism of cultured rat cortical neurons, which may be an early indicator of neuronal dysfunction and cell death (Walsh, Hartley et al. 1999).

Utilizing Tg(APP'Swe)2576Kahs mice (Tg2576), a well-characterized APP transgenic mouse model that expresses APP mutation that is linked to AD, Lesne and colleagues investigated the cause of memory decline in the absence of neurodegeneration (Lesne, Koh et al. 2006). Using the performance on the Morris-water maze as a measure
of spatial memory, they showed that mice started to develop memory deficits during the middle age (6-14 months). When they analyzed the Aβ species in the forebrain extracts of these mice, they found that the nonameric and dodecameric Aβ species correlated with the impairment of spatial memory.

Despite the emerging evidences implying the toxicity of soluble oligomers and their ability to cause neuronal injury, it must be emphasized that the large insoluble Aβ fibrils have been observed in the vicinity of the plaques and are also likely to be intimately surrounded by a number of soluble oligomers. Taken together, the conclusion that the large insoluble deposits, or the small oligomeric structures are the sole neurotoxic entity is not yet established. Rather, it may be possible that there is a continuous exchange between the two forms and both species are detrimental.

1.3 AD and inflammation

Closer analysis of the senile plaques observed in AD revealed the presence of several cells that include astrocytes and activated microglia (El Khoury, Moore et al. 2003). Astrocytes are the largest population of cells in the central nervous system (CNS). These cells function as major contributors to the structure and preservation of the blood-brain barrier (BBB). They also help in maintaining homeostasis of the extracellular environment (Moore and O'Banion 2002). In the presence of inflammatory stimulus, astrocytes respond by expressing class I and II major histocompatibility molecules (MHC-I and MHC-II, respectively). However, astrocytes are deficient in costimulatory molecules. This deficiency inhibits them from presenting the antigen to naïve T-cells
although they can present antigens to primed memory T cells (Halliday, Robinson et al. 2000).

Microglial cells are the resident immune cells of the CNS with properties and staining characteristics similar to macrophages (Ulvestad, Williams et al. 1994; Halliday, Robinson et al. 2000). The morphology of the microglial cells was first described in 1932 by Rio-Hortega in silver carbonate-stained brain preparations at the light microscope level (Rio-Hortega 1932; Lee, Nagai et al. 2002). Under normal physiological conditions and in the normal adult brain, the microglia are found as “resting” microglia, and adopt a characterized by a small cell body with fine and ramified processes and low expression of surface antigens (Garden and Moller 2006). The role of “resting” microglia is for immune surveillance and host defense (Liu and Hong 2003). Microglial cells are considered the first line of host defense against pathogens. However, when there is an injury or infection in the CNS, the “resting” microglia becomes activated, bringing about a change in morphology from ramified morphology to a more spherical cell morphology and more elongated or extended processes (Fischer and Reichmann 2001). Moreover, similar to activated astrocytes, activated microglia up-regulate a variety of cell-surface receptors, including MHC-II, proinflammatory cytokines and chemokines which include tumor necrosis factor alpha (TNFα), free radicals (NO), reactive oxygen species (ROS) and complement proteins (Moore and O'Banion 2002; Liu and Hong 2003).

Numerous studies of AD have demonstrated that microglia often cluster at sites of extracellular deposits of Aβ (Masumura, Hata et al. 2000). The first animal model evidence linking Aβ plaque formation with microglial activation was reported in 1998 when Cole and co-workers utilized hybrid Tg2576(HuAPPsw) mice (Tg2576 with
Swedish familial K670N/M671L double mutation) in probing microglial response to Aβ formation (Frautschy, Yang et al. 1998). This transgenic mouse model progressively develops Aβ deposits that test positive in Congo red staining between 26 and 32 weeks of age. Swedish mutation also increases the total cerebral burden of Aβ than with the mutant APP (Buxbaum and Tagoe 2000). In the study, Cole et al. used Griffonia simplicifolia (GS) lectin labeling and phosphotyrosine staining to identify microglia. These methods were chosen due to the fact that GS lectin specifically labels microglia in the rodent brain (Kato, Kogure et al. 1995) and plaque-associated microglia express high levels of phosphotyrosine (Akiyama, Barger et al. 2000). The investigators’ results demonstrate that an increased density of enlarged microglia gathered in and around plaques that are present predominantly in the hippocampus and cerebral cortex of 10- to 16-month HuAPPsw mice. This finding is very similar to the microglial activation related to Aβ formation in the AD brain.

Similar transgenic mouse (Tg2576APPsw) model studies showed activation of the microglial cells in and around the fibrillary Aβ plaque perimeter (Apelt and Schliebs 2001; Wegiel, Wang et al. 2001). Moreover, several studies have demonstrated the microlia’s capacity to phagocytose and internally degrade Aβ (Frautschy, Cole et al. 1992; Moore and O'Banion 2002). This phagocytic activity of microglia is considered to be an antigen-presenting ability of the microglial cells and this may be substantial in activation of the immune response.

Evidences have also emerged as to the contribution of Aβ in neurotoxicity and AD pathogenesis. Both in vivo and in vitro studies have shown that Aβ(1-42) induce neuronal apoptosis (Kowall, Beal et al. 1991; Loo, Copani et al. 1993; Pike, Burdick et
al. 1993; LaFerla, Tinkle et al. 1995; Masumura, Hata et al. 2000; Combs, Karlo et al. 2001; Morishima, Gotoh et al. 2001) as manifested by changes in morphology and biochemistry of the neurons, such as membrane blebbing, compaction of nuclear chromatin and intranucleosomal DNA fragmentation (Loo, Copani et al. 1993; LaFerla, Tinkle et al. 1995).

Perhaps the most studied effect of Aβ in neuroinflammatory process is as an inflammatory stimulus. It has been well documented that AD is characterized by a wide array of pro- and anti-inflammatory mediators. Analysis of microglia associated with senile plaques showed the presence of or a significant upregulation of inflammatory activity, such as production of cytokines and chemokines including interleukin (IL) -1β, IL-6, TNF-α, IL-8, transforming growth factor-β (TGF-β), and macrophage inflammatory protein-1α (MIP-1α), as compared to the age-matched, non-demented controls (Table 1.2) (Akiyama, Barger et al. 2000; Halliday, Robinson et al. 2000; Dumery, Bourdel et al. 2001; Perry, Newman et al. 2003). Likewise, several in vitro immunohistochemical studies have demonstrated the same findings of Aβ-induced microglial upregulation of cytokines and chemokines (Meda, Cassatella et al. 1995; Yates, Burgess et al. 2000; Apelt and Schliebs 2001; Floden and Combs 2006). Using an in vitro cellular model of human monocytes/macrophages, Klegeris et al. showed that Aβ peptide induced TNFα secretion on THP-1 cells (Klegeris, Walker et al. 1997). These accumulated data of an increased level of the proinflammatory products in the vicinity of senile plaques suggests that a chronic inflammatory process contributes to the progression of AD. Despite the numerous studies showing that several of these bioactive species promote neurodegenerative mechanisms, others exert beneficial neurotrophic effects (Halliday,
The role of TNFα in AD, for instance, is surprisingly controversial since it has both pro-apoptotic and anti-apoptotic effects. This proinflammatory cytokine, which is a powerful inflammatory mediator, is reported to kill human cortical neurons (Good, Werner et al. 1996; Venters, Tang et al. 1999) and was found to be accountable for the neurotoxic activity of microglia such as an increased expression of inducible nitric oxide synthase (Combs, Karlo et al. 2001). Along with interferon gamma (IFNγ), TNFα is a potent paracrine stimulator of other proinflammatory cytokines (Perry, Collins et al. 2001). On the other hand, TNFα production has also been reported to have a neuroprotective role in neurons by inducing the expression of protective molecules including manganese superoxide dismutase (Akiyama, Barger et al. 2000).

There is strong evidence of increased levels of TNFα in the brain microvessels and cerebrospinal fluid of clinically diagnosed AD patients (Bruunsgaard, Andersen-Ranberg et al. 1999; Tarkowski, Blennow et al. 1999; Tarkowski, Andreasen et al. 2003). For example, Blasko et al. showed that TNFα, in combination of interferon (IFN)-γ, increases the production of Aβ and inhibits the production of soluble APP in human neuronal and nonneuronal cells (Blasko, Marx et al. 1999). These demonstrate the participation of proinflammatory factors in the exacerbation of AD pathology.

Thus, vast data now have convincingly demonstrated that extracellular deposition of Aβ in the AD brains triggers inflammation. How Aβ stimulates microglia at a molecular level is still unclear. Several studies have shown that Aβ induces glial activation through nuclear factor-κB (NF-κB) (Akama, Albanese et al. 1998; Bales, Du et al. 2000; Combs, Karlo et al. 2001). Moreover, several laboratories suggested the
| Surface/membrane receptors                                                                 | MHC class I, MHC class II  
|                                                                                           | Leukocyte common antigen,  
|                                                                                           | CD11a  
|                                                                                           | Complement receptor 3  
|                                                                                           | Complement receptor 4  
|                                                                                           | Vitronecting receptor  
|                                                                                           | Fc-γ receptor  
|                                                                                           | CSF1 receptor  
|                                                                                           | Macrosialin (CD68)  
| Complement and related proteins                                                            | C1q, C3, C5, C6, C7, C8, C9  
|                                                                                           | C3b, C4b and C5b opsonins  
|                                                                                           | C5b9 membrane attack complex  
|                                                                                           | C4 binding proteins  
|                                                                                           | Clusterin (apolipoprotein J)  
| Cytokines and Chemokines (and receptors)                                                     | IL1β  
|                                                                                           | IL6 (IL6R, gp130)  
|                                                                                           | TNFα  
|                                                                                           | TGFβ1, 2 (TGFβRI, TGFβRII)  
|                                                                                           | IL8 (CXCR2)  
|                                                                                           | MIP1α (CCR3, CCR5)  
|                                                                                           | MIP1β (CCR3, CCR5)  
|                                                                                           | MCP1 (CCR3, CCR5)  
| Effector enzymes                                                                            | Cyclooxygenase 2  
|                                                                                           | Inducible nitric oxide synthase  
| Acute phase proteins                                                                        | Plasminogen activator inhibitor-1  
|                                                                                           | α1-Antichymotrypsin  
|                                                                                           | Tissue plasminogen activator  
|                                                                                           | Urokinase plasminogen activator  
|                                                                                           | Protease nexin-1  
|                                                                                           | α2- Macroglobulin  
|                                                                                           | Serum amyloid protein  
|                                                                                           | C-reactive protein  
|                                                                                           | Thrombin  
|                                                                                           | Apolipoprotein E  
|                                                                                           | α2-Antiplasmin  

CD, cluster differentiation; CSF, colony stimulating factor; IL, interleukin, MCP, monocyte  
chemoattractant; MHC, major histocompatibility complex; MIP, macrophage inflammatory  
protein; TGF, transforming growth factor; TNF, tumor necrosis factor
involvement of receptors in Aβ-induced microglial activation. For instance, El-Khoury et al. (El Khoury, Hickman et al. 1996) reported that the scavenger receptor on the surface of microglia binds to Aβ fibrils leading to cell adhesion and activation. Furthermore, other investigators suggested that Aβ-induced microglial activation is due to Aβ binding to the receptor for advanced glycation end products (RAGE) (Yan, Chen et al. 1996; Walsh, Lomakin et al. 1997). Using myeloid cells (e.g., THP-1 monocytes) and microglia, Bamberger et al. reported the involvement of a receptor complex (B-class scavenger receptor CD36, integrin associated protein/CD47 and the α6β1-integrin) for microglial activation and proinflammatory response by fibrillar Aβ (Bamberger, Harris et al. 2003). Recently, Fassbender’s group showed that fibrillar Aβ(1-42) interacts with LPS accessory receptor CD14 and triggers the release of proinflammatory products in primary murine microglial cells and human peripheral blood mononuclear (PBM) cells (Fassbender, Walter et al. 2004). This involvement of CD14 in Aβ-induced microglial activation now presents a possible connection between innate immunity and AD pathology.

1.4 Toll-like Receptors and Innate Immunity

“Immunity” refers to the ability of the host to protect itself from microbes that would otherwise destroy it (Hoebe, Janssen et al. 2004). Immunity can be broadly classified into two inducible systems: the innate immunity (‘natural immunity’) and adaptive immunity (‘acquired immunity’) (Kielian 2006). During infection, these two systems are activated sequentially to fight off and eliminate the microbe. Innate immunity
is the first line of host defense towards these invading microbes while adaptive immunity is activated later, usually about 4-7 days after infection (Albiger, Dahlberg et al. 2007). The adaptive immune response is mediated by clonally distributed B and T lymphocytes and is characterized by specificity and memory (Akira, Uematsu et al. 2006). Microbial recognition involves the production of random and highly diverse antigen T- and B- cell receptors, followed by clonal selection and amplification of these receptors with relevant specificities. This mechanism requires augmentation and differentiation of the specific clones into effector cells before they can contribute to host defense. The whole process takes, as mentioned above, about 4-7 days thus making adaptive immune response a delayed response (Akira, Takeda et al. 2001; Janssens and Beyaert 2003).

Innate immune response, on the other hand, is responsible for early detection of invading pathogens. It is largely mediated by white blood cells (neutrophils and macrophages), natural killer cells, dendritic cells, as well as perivascular macrophages and microglia in CNS (Aderem and Ulevitch 2000; Kielian 2006). Originally thought of as nonspecific, later investigations showed that innate immune response can discriminate self and a variety of potential pathogens. Cells of the innate immunity effectively recognize the antigens by predetermined sets of germline-encoded pattern recognition receptors (PRRs) (Janssens and Beyaert 2003; Lee and Kim 2007). These PRRs are involved in opsonization, activation of complement and coagulation cascades as well as of proinflammatory signaling cascades, phagocytosis and apoptosis (Medzhitov 2001).

Because of limited receptor expression, the cells of the innate immune system recognize the antigen by virtue of highly conserved structures that are expressed on these invading microorganisms. These specific, highly conserved motifs are termed pathogen-
associated molecular patterns (PAMPs) (Aderem and Ulevitch 2000; Kielian 2006; Lee and Kim 2007). PAMPs are produced only by microbes and not by host cells and do not vary between microorganisms of the same class. Moreover, they are vital for microbial survival. These features make them the perfect target for innate immune recognition (Medzhitov 2001). PAMPs recognition by PRRs results in activation of both extracellular (such as complement pathways) and intracellular signaling cascades that eventually culminate in the production of inflammatory response (Lee and Kim 2007).

The innate immune system uses PRRs that are located in three different compartments: those that are secreted into the blood stream and tissue fluids, expressed on the cell surface, or those that are expressed in intracellular compartments (Janssens and Beyaert 2003). PRRs in body fluids functions include PAMPs opsonization, activation of complement pathways and transfer of PAMPs to other PRRs. PRRs on the cell surface presents PAMPs to other PRRs, promotes phagocytosis, and initiates major signaling pathways. The cytoplasmic PRRs, on the other hand, are involved in antibacterial immune response and antiviral defense (Lee and Kim 2007). One of the most important and best characterized pattern recognition receptor families on the cell surface are the Toll-like receptors.

1.4.1 Toll-like receptors (TLR)

Toll like receptors (TLRs) are products of evolutionary process. Analogous receptors are found in plants, insects, worms (*Caenorhabditis elegans*) and vertebrates (Albiger, Dahlberg et al. 2007). The founding member of the Toll family, termed Toll,
was identified in 1996 in the fruit fly *Drosophila melanogaster* (Parker, Prince et al. 2007). Drosophila Toll was initially reported to be responsible for controlling dorsoventral patterning during the fruitfly development (Medzhitov 2001; Kielian 2006; Glezer, Simard et al. 2007). Later, Lemaitre *et al.* (Lemaitre, Nicolas et al. 1996) reported that Drosophila Toll was also involved in antifungal immunity in adult fruit flies. In this study, they utilized Toll-mutant Drosophila and found that these species rapidly succumb to fungal infection due to failure to induce Drosomycin, an antifungal peptide. When the Drosophila Toll was sequenced, Gay and Keith (Gay and Keith 1991) realized that their intracellular domains showed striking similarity with the intracellular signaling domain of the mammalian interleukin-1 (IL-1) receptor. This discovery prompted investigators to search and identify mammalian Toll-like homologues.

TLRs are type I transmembrane proteins that are composed of a highly variable ectodomain of leucine-rich repeats (LRRs) and a highly conserved intracellular or cytoplasmic domain that is homologous to the interleukin-1 receptor (IL-1R) thus called Toll/IL-1 receptor (TIR) domain (Miggin and O'Neill 2006; Trinchieri and Sher 2007). LRR domains consist of 19-25 tandem repeats, each repeat contains 24-29 amino acids and is involved directly or through accessory molecules in ligand binding (Albiger, Dahlberg et al. 2007; Trinchieri and Sher 2007). TIR domain, on the other hand, interacts with TIR-domain-containing adaptor molecules for signal transduction (Janssens and Beyaert 2003). To date, 13 mammalian TLRs have been identified (10 human (TLR1-10) and 12 murine (TLR1-9 and TLR11-13)) (Kielian 2006; Konat, Kielian et al. 2006; Albiger, Dahlberg et al. 2007; Parker, Prince et al. 2007), and at least one agonist has been identified for each TLR, with the exception of TLR10 (Kopp and Medzhitov 2003;
The agonists that are being recognized by TLRs, some of which are listed in Table 1.3, include microbial components in bacteria, fungi, parasites and viruses, including lipid-based cell wall components, microbial protein components and nucleic acids.

TLRs are expressed in several immune cells including human monocytes and macrophages, microglia, astrocytes, oligodendrocytes, dendritic cells (DC), B-cell, specific types of T-cells, as well as nonimmune cells such as fibroblasts and epithelial cells (Andreakos, Foxwell et al. 2004; Akira, Uematsu et al. 2006; Konat, Kielian et al. 2006). Furthermore, expression of TLRs may be extracellular or intracellular. TLRs 1, 2, 4, 5 and 6 are expressed on the cell surface while TLR 3, 7, 8 and 9 are almost exclusively found in intracellular compartments such as endosomes. TLRs that are intracellularly expressed have ligands that are mainly nucleic acids, and these ligands need to be internalized to the endosome before signaling is possible (Kielian 2006; Parker, Prince et al. 2007).

Figure 1.5 (adapted from (Konat, Kielian et al. 2006)) illustrates the general signaling pathway by TLRs. Upon PAMPs recognition, TLR activation results in initiation of the downstream signaling pathway through recruitment and activation of a TIR-domain containing adaptor molecule, myeloid differentiation factor 88 (MyD88). Activation of MyD88 leads to further activation of other adaptor molecules such as serine/threonine kinase IL-1R-associated kinase (IRAK), which is associated with MyD88, as well as TNF-receptor associated factor 6 (TRAF6). This downstream activation cascade eventually leads to the activation of nuclear factor (NF)-κB family of
<table>
<thead>
<tr>
<th>TLR</th>
<th>Major Ligands</th>
<th>Major Ligands and species</th>
</tr>
</thead>
<tbody>
<tr>
<td>TLR1/TLR2</td>
<td>Triacyl lipopeptides</td>
<td>Bacteria and mycobacteria</td>
</tr>
<tr>
<td>TLR2</td>
<td>LTA</td>
<td>Gram-positive bacteria, i.e. <em>Staphylococcus aureus</em>, <em>Streptococcus pneumoniae</em>, etc.</td>
</tr>
<tr>
<td></td>
<td>Atypical LPS</td>
<td>Gram-negative bacteria, i.e. <em>Phorphyromonas gingivalis</em></td>
</tr>
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<td></td>
<td>Porins</td>
<td>Gram-negative bacteria, i.e. <em>Neisseria sp.</em>, <em>Shigella sp.</em>, <em>Haemophilus influenzae</em></td>
</tr>
<tr>
<td></td>
<td>Lipoarabinomannan</td>
<td>Mycobacteria</td>
</tr>
<tr>
<td></td>
<td>Lipopeptides (Pam3CSK4, MALP2)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Peptidoglycan</td>
<td>Gram-positive bacteria</td>
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<tr>
<td>TLR3</td>
<td>dsRNA</td>
<td>Virus</td>
</tr>
<tr>
<td>TLR4</td>
<td>LPS</td>
<td>Gram-negative bacteria</td>
</tr>
<tr>
<td></td>
<td>Fusion protein</td>
<td>RSV</td>
</tr>
<tr>
<td></td>
<td>EDA domain fibronectin</td>
<td>(endogenous)</td>
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<tr>
<td></td>
<td>HSP60</td>
<td>(endogenous)</td>
</tr>
<tr>
<td>TLR5</td>
<td>Flagellin</td>
<td>Flagellated Gram-positive and Gram-negative bacteria</td>
</tr>
<tr>
<td>TLR6/TLR2</td>
<td>Diacylated lipopeptides</td>
<td>Mycoplasma</td>
</tr>
<tr>
<td></td>
<td>Zymosan</td>
<td>Yeast</td>
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<tr>
<td></td>
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<td>Group B streptococci</td>
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<tr>
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<tr>
<td>TLR8</td>
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<tr>
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<td></td>
<td>Herpes virus DNA</td>
<td>Virus</td>
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<tr>
<td>TLR10</td>
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</tr>
<tr>
<td>TLR11</td>
<td>Unknown</td>
<td>Uropathogenic <em>E. coli</em></td>
</tr>
</tbody>
</table>
Figure 1.5. The Toll-like receptor signaling pathway. TLR activation by PAMPs recognition results in the activation of the downstream signaling pathway that culminates in the production of proinflammatory cytokines and chemokines, as well as IFNβ. The initiation of downstream signaling begins by activation and recruitment of adaptor molecules such as MyD88 (as shown). TLR signaling pathway also utilizes a MyD88-independent pathway, wherein TIRAP, TRIF and TRAM adaptor molecules are recruited and activated (not shown). (illustration from Konat, Kielian et al. 2006)
transcription factors, as well as initiation of distinct parallel signaling pathways leading to
mitogen-activated protein (MAP) kinase. Initiation of these pathways subsequently result
in transcription of a myriad of pro- and anti-inflammatory cytokines, chemokines and
costimulatory molecules, such as TNF-α, IL-6, IL-1β and IL-12 (Kielian 2006; Konat,
Kielian et al. 2006; Albiger, Dahlberg et al. 2007; Guo and Schluesener 2007; Parker,
Prince et al. 2007).

TLR also utilizes other adaptor proteins for downstream signaling through a
MyD88-independent pathway. This pathway starts with the TLR recruitment of adaptor
proteins such as Toll-IR-1 receptor (TIR)-associated protein (TIRAP, also known as
MAL), Toll-associated activator of IFN (TRIF) and Toll receptor-associated molecule
(TRAM), which are crucial for the expression of interferon (IFN)-inducible genes (Akira,
Uematsu et al. 2006; Kielian 2006; Konat, Kielian et al. 2006; Miggin and O'Neill 2006).

The study of human TLRs has progressed in the last 5 years (Kielian 2006), and
investigations on TLR expression are rapidly expanding. The following discussion will
focus on the most common and well-defined TLRs, TLR2 and TLR4, as well as TLR
accessory proteins.

1.4.1.1 TLR4

TLR4 is perhaps the most extensively studied PRR. Furthermore, human TLR4
was the first characterized mammalian Toll (Medzhitov, Preston-Hurlburt et al. 1997).
Like other TLRs, TLR4 is expressed in various cell types, predominantly in immune cells
such as macrophages and DCs (Medzhitov 2001). It recognizes a variety of ligands, like
mannan (yeast) and host heat shock proteins and fibrinogen (virus) (Albiger, Dahlberg et al. 2007); however, TLR4 is mostly known to recognize gram negative bacterial cell wall component lipopolysaccharide (LPS) (Hoshino, Takeuchi et al. 1999). Hoshino et al. and several other investigators demonstrated that TLR4 mediates responses to LPS using several mouse models that are TLR4-gene deficient, or LPS hyporesponsive mouse strains (Poltorak, He et al. 1998; Hoshino, Takeuchi et al. 1999; Qureshi, Lariviere et al. 1999). LPS, a major constituent of the outer membrane of the Gram-negative bacteria, consists of three regions (Figure 1.6 (Miller, Ernst et al. 2005)): the O-polysaccharide chain, the core saccharide and the lipid A (Huber, Kalis et al. 2006). The O-specific chain consists of a polymer of oligosaccharides with a repeating unit of one to eight glycosyl residues. Core saccharide (or core region), on the other hand, is made up of heterooligosaccharide that is subdivided into inner and outer core (Rietschel, Kirikae et al. 1994). Lipid A is composed of a diglucosamine backbone containing ester-linked and amide-linked long-chain fatty acids (Aderem and Ulevitch 2000) and functions as a hydrophobic anchor of LPS on the major gram-negative outer membranes (Dixon and Darveau 2005). It was already postulated since the 1950s that the lipid A is the toxic component of LPS. But it was not until the late 1980s when the chemical structure of lipid A was elucidated and chemically synthesized, and the biological activity of synthetic lipid A was compared with bacterial lipid A and LPS that lipid A was shown to be, in fact, the bioactive component of LPS (Loppnow, Brade et al. 1989; Rietschel, Kirikae et al. 1994)

LPS is an amphiphilic molecule and it forms aggregates in solution (Jerala 2007). Numerous evidences have established LPS as a powerful proinflammatory activator of
Figure 1.6 Chemical structure of known TLR agonists. (A) Bacterial lipopolysaccharide (LPS). (B) Synthetic triacylated bacterial lipoprotein tripalmitoyl cysteinyl seryl tetralysine (Pam₃CSK₄). (C) Synthetic diacylated bacterial lipoprotein FSL-1.
mononuclear cells, and TLR4-dependent activation of macrophages and microglia by nanogram quantities of LPS results in the production of myriad cytokines such as TNF-α, IL-1, -6, -8, as well as nitric oxide (NO), and superoxide which are capable of inducing apoptotic cell death (Poltorak, He et al. 1998; Moore, Andersson et al. 2000).

The molecular pathway of TLR4 activation by LPS has been extensively studied and is depicted in Figure 1.7a and 1.7b (Aderem and Ulevitch 2000; Akashi-Takamura and Miyake 2006; Kielian 2006). LPS recognition by TLR4 starts with the lipid A moiety binding to a 58-60 kDa serum protein, human lipopolysaccharide-binding protein (LBP). LBP is a serum glycoprotein belonging to a family of lipid-binding proteins that includes bactericidal/permeability-increasing protein (BPI), phospholipid ester transfer protein and cholesterol ester transfer protein (Gutsmann, Muller et al. 2001). In normal serum, LBP is present at concentrations of ≈5 to 15 μg/ml (Kitchens, Wölbauer et al. 1999). Investigations have shown that the function of LBP in LPS signaling is to convert oligomeric micelles of LPS to monomers, and to shuttle the monomeric LPS to CD14 (Jerala 2007). Disaggregating the LPS increases the transfer and binding of LPS to CD14 (Hailman, Lichenstein et al. 1994; Miller, Ernst et al. 2005).

CD14, a 55-kDa high-affinity LPS receptor, can either be secreted in the serum (soluble or sCD14) or expressed as a glycoprophosphoinositol (GPI)-anchored protein (membrane or mCD14) on the surface of macrophages. Pugin et al reported the sCD14 level in normal serum to be 2 to 3 μg/ml (Pugin, Schurer-Maly et al. 1993). Several biochemical and genetic evidences showed that CD14 binds to LPS with dissociation constant (K_D) between 30 and 74 nM (Miyake 2004) and facilitates its signaling, but it does not appear to be essential in direct LPS response (da Silva Correia, Soldau et al. 2000).
Figure 1.7. TLR2 and TLR4 signaling pathway. (a) TLR4 signaling starts with the binding of LPS, the most common TLR4 ligand, to LBP. LBP then transfers monomeric LPS to CD14. CD14 then presents LPS to TLR4:MD2. The trimeric complex LPS:TLR4:MD2 activates downstream TLR signaling leading to formation of proinflammatory products (Aderem and Ulevitch 2000). (b) Both TLR2 and TLR4 utilize the adaptor molecule MyD88 for downstream signaling pathway. TLR2 forms a complex with either TLR1 or TLR6 for recognition of triacylated or diacylated lipopeptide, respectively (illustration from Kielian 2006).
2001). Although CD14-knockout mouse studies have shown that LPS responses are detectable without CD14, this TLR accessory protein is essential for the initiation of the TRAM-TRIF pathway by TLR4/MD2 (Jiang, Georgel et al. 2005). Moreover, the significance of CD14 in LPS signaling was evidenced \textit{in vivo} by an impaired TNF\(\alpha\) response when CD14-deficient mice were challenged with LPS (Haziot, Ferrero et al. 1996).

\textit{In vitro} studies showed the LPS/CD14 complex utilizes another host-derived soluble adaptor molecule for the activation of downstream TLR signaling. This co-receptor, termed MD-2, is a 25-30 kDa protein, lacks the transmembrane and intracellular region and is expressed on the cell surface in association with the ectodomain of TLR4 (Akira, Takeda et al. 2001; Medzhitov 2001; Akashi-Takamura and Miyake 2006; Kielian 2006). LPS bound to CD14 is transferred to MD-2, which associates with the ectodomain of TLR4. Together, the MD-2/TLR4 oligomer binds LPS with nanomolar affinity and the trimeric complex LPS:MD-2:TLR4 induces LPS signaling, translocation of nuclear proteins and transcriptional activation of genes associated with the inflammatory processes (Viriyakosol, Tobias et al. 2001; Akira, Uematsu et al. 2006; Glezer, Simard et al. 2007).

TLR4 interaction with MD-2 is crucial for efficient responses to LPS. This was supported by experiments that demonstrated unresponsive phenotype of mice carrying knockout mutations in either TLR4 or MD-2 genes (Hoshino, Takeuchi et al. 1999; Shimazu, Akashi et al. 1999; Nagai, Akashi et al. 2002). Recent efforts to elucidate the MD-2:TLR4 binding to LPS showed that the TLR4 region Glu\(^{24}\)-Lys\(^{47}\) is the site for MD-2 binding. Moreover, Cys\(^{29}\) and Cys\(^{40}\) within this region are critical for interaction with
MD-2 and LPS signaling (Nishitani, Mitsuzawa et al. 2006). Furthermore, a model for LPS-induced TLR4:MD-2 dimer was constructed based on mutational analysis wherein the structure formed resembles the ‘m’ shaped dimers (Jin and Lee 2008). Crystal structures of human MD-2 and its complex with LPS lipid A suggested that MD-2 plays a principal role in endotoxin recognition (Ohto, Fukase et al. 2007). Several lines of evidence also showed that TLR4 and MD-2 are important in ligand-recognition specificity (Shimazu, Akashi et al. 1999; Miyake 2004; Prohinar, Re et al. 2007), thus, it is plausible that TLR4 and MD-2 work together for ligand recognition and signal transduction.

1.4.1.2 TLR2, TLR1, TLR6

Among all the TLRs that have been identified, TLR2 is considered the one with the broadest specificity when it pertains to PAMPs recognition. TLR2 has been shown to recognize a broad range of microbial products including peptidoglycan (PGN) from Gram-positive bacteria, bacterial lipoproteins (LP), mycobacterial cell-wall lipoarabinomannan, lipoteichoic acid (LTA), tripalmitoyl-S-glyceryl-cysteine (Pam\textsubscript{3}Cys), glycosylphosphatidylinositol lipid from Trypanosoma Cruzi, a phenol-soluble modulin produced by Staphylococcus epidermidis, zymoan from fungi and glycolipids from Treponema maltophilum (Janeway and Medzhitov 1999; Medzhitov 2001; Kielian 2006). Moreover, studies have also shown that TLR2 recognize atypical LPS, which is structurally different from bacterial LPS by virtue of the number of acyl chains in the lipid A component (Takeda, Kaisho et al. 2003), from Leptospira interogans (Medzhitov
2001) and Porphyromonas gingivitis (Hirschfeld, Weis et al. 2001). The wide spectrum of microbial components that TLR2 can recognize may be due, in part, to the ability of TLR2 to cooperate or complex with at least two other TLRs: TLR1 and TLR6. Therefore, TLR2 dimer formation with TLR1 or TLR6 may dictate specificity of ligand recognition (Ozinsky, Underhill et al. 2000; Takeuchi, Kawai et al. 2001; Akira, Uematsu et al. 2006). For instance, TLR2/TLR1 heterodimers preferentially act as a receptor for triacylated lipopeptides whereas TLR2/TLR6 heterodimers are the receptors for diacylated lipopeptides (Figure 1.6) (Ozinsky, Underhill et al. 2000; Takeuchi, Kawai et al. 2001; Takeuchi, Sato et al. 2002; Dziarski 2003; Omueti, Beyer et al. 2005).

TLR2 is expressed on monocytes, macrophages, microglia, dendritic cells, B cells and, to a lesser extent, on neutrophils and few other cells, whereas it has been shown that both TLR1 and TLR6 are expressed by microglia (Dziarski 2003; Kielian 2006). As presented in Figure 1.7b, TLR2 downstream signaling pathway begins with TLR2 complex formation with either TLR1 or TLR6, after which, the complex utilizes both the intracellular adaptor proteins MyD88 and TIRAP for subsequent induction of target genes such as TNFα and other cytokines and chemokines (Kielian 2006).

Evidences also demonstrate the role of CD14 on TLR2 recognition of gram-positive PAMPs, such as PGN and LTA. Schröder et al. used human embryonic kidney (HEK) cells and Chinese hamster ovary (CHO) cells transfected with both TLR2 and CD14 to show that LTA of Streptococcus pneumoniae and Staphylococcus aureus utilize CD14 and TLR2 to activate immune cells (Schroder, Morath et al. 2003). Moreover, using a CD14 mutant with deletion of the part of possible N-terminal ligand binding pocket, and an anti-CD14 monoclonal antibody, Nakata’s group demonstrated that CD14
binds to triacylated lipopeptides and facilitates its recognition by TLR2/TLR1 complex (Nakata, Yasuda et al. 2006). Other investigations utilizing knockout mice and transfected cell lines also demonstrated the necessity of CD14 for TLR2/TLR1 or TLR2/TLR6 heterodimer recognition of PAMPs (Gupta, Kirkland et al. 1996; Henneke, Takeuchi et al. 2001; Dziarski 2003; Esen and Kielian 2005; Manukyan, Triantafilou et al. 2005).

Earlier studies implicated TLR2, along with TLR4, as a receptor for LPS signaling (Kirschning, Wesche et al. 1998; Yang, Mark et al. 1998; Yang, Mark et al. 1999). However, a closer examination revealed that the original discrepancy on the involvement of TLR2 in LPS activation was due to the contaminating lipoproteins, which are TLR2 ligands, in commercially available LPS preparations (Kielian 2006). In fact, when LPS was repurified and the contaminating lipoproteins were removed, the LPS was unable to signal via TLR2 (Hirschfeld, Ma et al. 2000). Thus, investigations of PAMPs recognition by TLRs require careful scrutiny to ascertain that the TLR agonists are not contaminated with other biologically active PAMPs.

1.4.2 TLRs and Aβ: What is the connection?

In 2003, Fassbender’s group published results demonstrating the role of CD14 in linking Aβ with the innate immunity (Fassbender, Walter et al. 2004). By surface plasmon resonance spectroscopy (SPR), immunoprecipitation and western blotting, they showed that CD14 binds fibrillar Aβ (1-42) with a dissociation constant (K_D) of 1.1 ± 0.1 x10^{-7} M. Moreover, CD14 binding to fibrillar Aβ42 was 20-fold stronger as compared to
CD14 and nonfibrillar Aβ (1-42) (K_D [M] = 2.2 ± 0.7 x10^{-6}). The huge difference in the K_D values suggests that CD14 recognizes the β-sheet structure of the fibrillar Aβ(1-42). The group also tested the role of CD14 in fibrillar Aβ(1-42) – induced microglial activation using primary murine microglia from wildtype (WT) and CD14-deficient mice, treated the cells with fibrillar Aβ(1-42) and interferon (IFN)-γ, and analyzed the proinflammatory marker IL-6. Results showed that CD14-deficient microglia released significantly lower amounts of IL-6 in response to Aβ as compared to WT microglia. A significant reduction of proinflammatory products was also observed when human peripheral blood monocytes (PBM) induced with fibrillar Aβ was treated with anti-human CD14 monoclonal antibody, 3C10. These data further strengthens the interaction between CD14 and fibrillar Aβ(1-42) in induction of proinflammatory products. The authors also showed overexpression of CD14 in APP transgenic mice, which signifies that CD14 significantly contributes to the inflammatory responses in AD.

However, CD14 does not contain a cytoplasmic domain (Haziot, Chen et al. 1988; Muta and Takeshige 2001; Viriyakosol, Tobias et al. 2001; Kim, Lee et al. 2005) that could activate the downstream signaling that induces production of proinflammatory products (see Figure 4). As explained earlier, CD14 functions as a co-receptor for LPS (Wright, Ramos et al. 1990; da Silva Correia, Soldau et al. 2001; Medzhitov 2001), as well as for Gram-positive cell walls and their PGN component (Pugin, Schurer-Maly et al. 1993; Gupta, Kirkland et al. 1996; Henneke, Takeuchi et al. 2001; Muta and Takeshige 2001; Nakata, Yasuda et al. 2006). These PAMPs utilize TLR4 and TLR2 for intracellular signal transduction. Fassbender et al (Fassbender, Walter et al. 2004) proposed that the observed Aβ cellular activation may likely be transmitted by TLR4
based on the positive inflammatory response obtained from CHO cells which lack a functional TLR2.

In this regard, the purpose of this research is to investigate the possible involvement of TLR(s) in Aβ(1-42) induction of the innate immune response. Specifically, this research aims to identify the TLR that functionally interacts with Aβ(1-42). We also aim to investigate the possible involvement of other TLR accessory proteins and TLR complexes in Aβ(1-42) innate immune activation. The field of TLR research is still in its infancy; thus, the information that will be obtained from this research may contribute to increased understanding of how Aβ assemblies interact with TLR family members. Additionally, this investigation will contribute to further understanding the role of Aβ in AD-associated neurodegeneration, and possibly, open a therapeutically relevant perspective for TLRs and their recognition abilities for host-derived pathogens.
1.5 Bibliography


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2 GENERAL METHODS

2.1 Cell Culture

2.1.1 THP-1 monocytes

2.1.1.1 THP-1 storage, growth and culture

The cultured human peripheral blood THP-1 monocytes were obtained from ATCC (Manassas, VA, USA) and maintained in RPMI-1640 culture medium (HyClone, Logan, UT, USA) that contains 2 mmol/L L-glutamine, 25 mmol/L HEPES, 1.5 g/L sodium bicarbonate, 10% fetal bovine serum (FBS) (HyClone), 50 U/ml penicillin, 50 μg/ml streptomycin (HyClone), and 50 μmol/L β-mercaptoethanol (Fisher, Pittsburg, PA) at 37°C in 5% CO₂. For growth and maintenance, THP-1 monocytes were diluted three times a week, with a dilution of 1:1 twice during the week, and 3:10 dilution at the end of the week. For 1:1 dilution, half of the cells were removed from the flask and replaced with the same volume of fresh growth medium for propagation, ensuring that the cell concentration in the flask was maintained at 1 x10⁶ cells/ml. For 3:10 dilution, 3 ml of THP-1 cells were removed from the culture flask and spun at 500 xg for 10 minutes. After centrifugation, supernatant was removed and cells were resuspended in 10 ml of growth medium. Cells were transferred to a new cell culture flask for propagation. Cells
were continuously subcultured for 3 weeks prior to experimentation.

For maintaining a continuous supply of THP-1, THP-1 monocytes were cryopreserved. As soon as a small surplus of THP-1 cells becomes available for subculture, several ampules of cells were frozen. Cells were removed from the flask, centrifuged at 500 xg for 10 minutes and supernatant was removed without disturbing the pellet. The pellet was then resuspended to a final concentration of $5 \times 10^6$ cells/ml in freezing medium (fresh growth medium containing 0.5% sterile dimethyl sulfoxide (DMSO)). The cell suspensions were dispensed into prelabeled ampules (1 ml cell suspension per ampule) and ampules were transferred to an ampule cooler (Nalge Nunc) containing isopropyl alcohol. The specific heat of the coolant in the base of the cooler insulates the container and gives a cooling rate of $\sim 1^\circ\text{C}/\text{min}$ in the ampules (Freshney, 2000). The cooler was placed in a -70°C freezer overnight prior to transfer of ampules in liquid nitrogen. After overnight freezing at -70°C, ampules were rapidly transferred to a cryo-container and THP-1 cells were stored immersed in liquid nitrogen.

Thawing THP-1 ampules needs to be rapid. THP-1 cells were thawed by immersing the lower half of the ampule in 37°C waterbath for 2-3 minutes (but not exceeding 3 minutes). The ampule containing thawed cells was then immersed in 70% ethanol before opening the container. Cells were pipetted out from the ampule and suspended in 9 ml of fresh THP-1 growth medium. Cell suspension was centrifuged, and reseeded in a new cell culture flask, as described previously.

We have previously observed that the proinflammatory response of our THP-1 monocytes to TLR agonists started to deteriorate when THP-1 cells have been subcultured continuously for greater than 2 months. To avoid this problem, we have
staggered our culture of THP-1 monocytes. We constantly maintained two culture flasks of THP-1 monocytes, in which one flask was cultured a month after the initial culture of the first THP-1 flask. Cells were constantly monitored for viability by stimulation with TLR agonists (section 2.3) and measuring TNFα production (section 2.7).

### 2.1.1.2 THP-1 preparation for experimentation

For cellular assays, THP-1 monocytes were removed from the culture flask and centrifuged at 500 x g for 10 minutes. After centrifugation, supernatant was removed without disturbing the pellet. The pellet was washed with THP-1 assay culture medium (THP-1 growth but with 2% FBS), and centrifuged as described above. Afterwhich, supernatant was removed and pellet was resuspended in assay medium. Cell concentration was determined by direct counting of the cells using a hemocytometer. THP-1 cells (with concentration maintained at 1x10^6 cells/ml) were added to individual wells of a 48-well sterile plate to a final volume of 0.3 ml, or 96-well cell culture plate to a final volume of 0.08 ml.

THP-1 monocytes are derived from the blood of a patient with monocytic leukemia (Tsuchiya et al., 1980). The cells grow in suspension, have round morphology, and do not adhere to the plastic surfaces of the culture plates (Takashiba et al., 1999; Zhou et al., 2005). THP-1 can serve as a model of primary human microglia since they acquire a microglia-like morphology when treated with LPS (Yates et al., 2000).
2.1.2 Human Embryonic Kidney (HEK293) cells

Null HEK293 (stably transfected with the pUNO-mcs vector), 293-hTLR2 cells (isolated clone of HEK293 cells stably transfected with human TLR2 gene), and 293-hTLR2/CD14 (isolated clone of HEK293 cells stably transfected with human TLR2 and CD14 genes) were obtained from InvivoGen (San Diego, CA, USA). Null HEK293 and HEK 293-hTLR2 cells were maintained in Dunkelcco’s Modified Eagle’s Medium (DMEM) growth medium (HyClone) containing 4 mmol/L L-glutamine, 4.5 g/l glucose, 10% FBS and supplemented with 10 μg/ml Blasticidin (InvivoGen) at 37°C in 5% CO₂. HEK 293-hTLR2/CD14 cells were maintained in the same growth medium supplemented with 10 μg/ml Blasticidin and 50 μg/ml hygromycin (HygroGold™) (InvivoGen). Cells were subcultured in a T-75 cell culture flask every 4 days. The number of times the cells were subcultured is denoted by passage number. Subculture of HEK 293 cells is illustrated in Figure 2.1 and was done using the following procedure. Briefly, the growth medium in the flask was removed, and the cells were gently washed with sterile phosphate buffered saline (PBS) (Hyclone). After washing, the cells were treated with 2 ml of 0.25% trypsin-EDTA for 5 minutes at 37°C followed by addition of 8 ml of corresponding growth medium. Cells were dispersed by repeated gentle pipetting over the surface bearing the monolayer. Cells were collected, placed in a 15 ml conical tube, centrifuged at 500 xg for 10 minutes, resuspended in appropriate fresh growth medium, diluted to the appropriate seeding concentration, and reseeded in a fresh flask (Freshney, 2000). A surplus of HEK 293 cells were also cryopreserved in a similar manner as in
Figure 2.1 Schematic diagram of the subculture of HEK293 cells. Stages in the subculture and growth cycle of HEK293 cells following trypsinization (Freshney, 2000)
THP-1 monocytes (section 2.1.1.1), in which 1.0 ml aliquots of cells were kept in liquid nitrogen until needed for growth and culture.

For cellular assays, Null HEK293, 293-hTLR2 and 293-hTLR2/CD14 cells were trypsinized, as described above, to dislodge the cells. Cell supernatant was centrifuged at 500 xg for 10 minutes, and cells were resuspended in fresh growth medium. 0.3 ml or 0.2 ml of cells was plated to individual wells of a 48-well or 96-well sterile culture plate, respectively. The cell concentration for a 48-well plate was maintained at 3.0 x10^5 cells/ml, and 2.0 x10^5 for a 96-well culture plate. HEK293 cells were incubated and allowed to adhere for 4h at 37°C, 5% CO_2. After incubation, growth medium was removed and HEK293 cells were resuspended in their respective assay medium (growth medium with reduced (2%) FBS) prior to treatment with effectors.

We have optimized the conditions of HEK 293 growth to achieve the maximal IL-8 concentration. We varied the length of adhesion of our HEK 293 cells to the cell culture plate prior to stimulation with effectors (section 2.3) and measured the IL-8 secretion after incubation (section 2.7). Results in Figure 2.2 showed the highest IL-8 production was achieved when HEK 293hTLR2 was allowed to adhere to the cell culture plate for 4 hours prior to treatment with effectors. Thus, for all experiments with Null HEK 293, HEK 293hTLR2 and HEK293hTLR2/CD14 cells, cells were incubated for 4 hours prior to stimulation with effectors. Similarly, we tested if proinflammatory production by HEK293 cells is affected by the number of times the cells have been subcultured. Figure 2.3 shows that the ability of HEK 293hTLR2 to produce IL-8 upon stimulation with fibrillar Aβ(1-42) aggregated for 216 hours at 4°C significantly decreased as the cells
Figure 2.2. Optimization of HEK 293 adhesion time prior to stimulation with Pam$_3$CSK$_4$. Null HEK293 (light gray bars) or HEK 293hTLR2 (dark gray bars) was prepared for experiment, as described. After resuspension in growth medium, cells were plated in a cell culture plate and allowed to adhere at 37°C, 5%CO$_2$ for 0, 4, 24 or 72 hours prior to stimulation for IL-8 production (in pg/ml). After the given times, growth medium was removed, resuspended in assay medium and cells treated with 1 ng/ml Pam$_3$CSK$_4$. After 24-hour post-stimulation, IL-8 was measured. HEK 293hTLR2/CD14 cells were also tested and gave a trend similar to that of HEK 293hTLR2 (data not shown).
aged. Therefore, for all experiments employing HEK293 cells, we used cells from either passage 15 or 16 to achieve maximal secretion of IL-8.

2.2. Preparation of Aβ peptides

Lyophilized powder of Aβ(1-42) and Aβ(1-40) peptides were purchased from rPeptide (Bogarth, GA, USA). The powder was dissolved in 100% hexafluoroisopropanol (HFIP) (Sigma, St. Louis, MO), and incubated at room temperature for 1 hour. This step is crucial to ensure disaggregation of any pre-formed aggregates. HFIP treatment also allows “normalization” of the properties of different commercial preparations of Aβ (Wood et al., 1996; Zagorski et al., 1999). After incubation, the peptides were aliquotted into sterile microcentrifuge tubes, dried in a vacuum centrifuge, and dried samples stored at -20°C. Before cell treatment, the lyophilized samples were resuspended to 100 μmol/L or 1 mmol/L in sterile water and incubated at 4°C. For studying the effect of temperature in Aβ(1-42) aggregation, prepared Aβ peptides were also stored at 25°C and 37°C. For cell treatment, cells were exposed to a final concentration of 15 μmol/L of Aβ(1-42) or Aβ(1-40).

2.3 Activation of cell model systems

THP-1 monocytes or HEK 293 cells were prepared for experiment as described above, and plated on a sterile 48-well or 96-well cell culture plate. To the wells, pure bacterial LPS (Escherichia coli 026.B6, Sigma), ultrapure bacterial LPS (Escherichia coli
Figure 2.3. Effect of HEK293 passage number on fibrillar Aβ(1-42) response. HEK293 hTLR2 cells were subcultured, as described. The number of times the cells have been subcultured is denoted by passage number. For each subculture, cells are prepared for experiment and stimulated with 15 μM of fibrillar Aβ(1-42) for 24 hours. After post-stimulation, secreted IL-8 was determined. This result represents 1 representative experiment of 3. Error bars represent n= 3 trials (1 experiment). The same experiment was done in Null HEK293 cells and treatment with Aβ failed to stimulate Null HEK293 cells for IL-8 production (data not shown).
K12, InvivoGen), synthetic bacterial lipoprotein tripalmitoyl cysteinylderyl tetralysine (Pam3CSK4, InvivoGen), synthetic Pam2CGDPKHPKS (FSL-1, InvivoGen) (Figure 1.6) or 15 μmol/L Aβ(1-42) or Aβ(1-40) were applied. For HEK 293 experiments, Aβ(1-42) that was allowed to aggregate for 216 hours at 4°C was used. Cells were incubated at 37°C, 5% CO2 at concentrations and incubation times stated in the experiments. After the indicated incubation time, cell supernatants were removed and centrifuged at 2500 xg for 10 minutes (Microcentrifuge® 18 Centrifuge, Beckman-Coulter) to remove cells, and supernatants were collected and stored at -20°C prior to analysis of proinflammatory products (TNFα or IL-8). For concentration dependence experiment, the EC50 values were determined by fitting the concentration-dependence data for the agonists to a sigmoidal three-parameter equation (y = a/ [1 + e^(-(x-x0)/b) ] using SigmaPlot graphing program.

2.4 Conversion of non-adherent THP-1 monocytes to adhering cells

THP-1 cell adhesion was described previously (Crouse et al., 2009). Briefly, THP-1 cells were treated with 10 ng/ml phorbol 12-myristate 13-acetate (PMA, Sigma) for 24 hours, and cells incubated at 37°C, 5% CO2. Vehicle control was 0.0005% DMSO. After incubation, non-adherent cells in the supernatant were removed and adherent cells were washed with assay medium prior to 6-hour stimulation of cells with known TLR agonists or 15 μM Aβ(1-42).

To verify the extent of adhesion, a separate well containing THP-1 cells was induced with PMA, as described above. After incubation, non-adherent cells were
removed, and adherent cells were washed with PBS. The adherent cells were removed from the bottom surface of the cell-culture plate with 0.25% trypsin-EDTA (HyClone), and counted under a microscope using a hemocytometer. Percent adhesion was calculated by the number of adherent cells divided by the plated cell number. Adherent cells with % adhesion range of 75% and above were used for proinflammatory response experiments.

2.5 LPS contamination assay

To test the Aβ preparations for the presence of contaminating bacterial lipopolysaccharide, Aβ(1-42) was routinely tested using Polymyxin B-sulfate (PMX-B) (Sigma). THP-1 monocytes were prepared as described, and plated on a 48-well cell culture plate. Cells were pretreated with 0.1 μg/ml of PMX-B and incubated for 30 minutes at 37°C, 5%CO₂. After incubation, cells were treated with either 10 ng/ml of ultrapure LPS or 100 μmol/L of Aβ(1-42) and incubated further for 6 hours at 37°C. Following incubation, cell supernatants were collected, centrifuged as described above, and supernatants stored at -20°C prior to TNFα measurement.

2.6 TLR antibody neutralization assay

THP-1 monocytes or HEK293 cells were seeded in 48-well or 96-well cell culture plate, and pre-treated with 5-20 μg/ml of TLR antibodies, IgG isotype control or PBS for 1 hour at 37°C, 5% CO₂. TLR antibodies and IgG isotype controls that were utilized in this experiment were functional grade anti-human TLR2 (clone T2.5), TLR4 (clone
HTA125), CD14 (clone 61D3) antibodies, mouse IgG2,κ and IgG1,κ isotype controls from eBioscience (San Diego, CA), polyclonal anti-TLR2, TLR4, TLR1 or TLR6 antibodies from InvivoGen, or rat IgG isotype control from Sigma. Following incubation, cells were treated with either 10 ng/ml ultrapure LPS, 1 ng/ml Pam3CSK4, 3 ng/ml (for THP-1 monocytes) or 1 ng/ml (for HEK293 cells) FSL-1, or 15 μmol/L of Aβ(1-42) and further incubated for 6 or 24 hours in the same conditions. After incubation, cell supernatants were collected as described above for TNFα or IL-8 determination.

2.7 Measurement of proinflammatory products

Secreted TNFα or CXC chemokine IL-8 in the supernatants were determined using Enzyme-linked immunosorbent assay (ELISA). ELISA has become a standard biochemical technique for determination of cytokine concentration, as well as levels of other proteins of interest, in body fluids and culture medium. An advantage of this method is its high specificity when monoclonal antibodies are used. Moreover, it is quick and easy to perform for large number of samples (Turner et al., 2004).

100 μl of 2 μg/ml monoclonal anti-human TNFα/TNFSF1A primary antibody (for TNFα) or monoclonal anti-human CXCL8/IL-8 antibody (for IL-8) (R&D Systems, Minneapolis, MN, USA) was added to 96-well plates for overnight incubation at 24°C. Following incubation, wells were washed with PBS containing 0.05% Tween-20 and blocked with 300μL PBS containing 1% bovine serum albumin (BSA), 5% sucrose, and 0.05% NaN3 for 1 hour at 24°C. After washing, 50μL of standards or cellular supernatant samples were added and the plate further incubated for 2 hours. After successive washing
and additions of 100μL of 0.1 μg/ml biotinylated anti-human TNF-α/TNFSF1A detection antibody (TNFα) or biotinylated anti-human CXCL8/IL-8 antibody (IL-8) (R&D Systems) in 20mmol/L Tris with 150mmol/L NaCl and 0.1% BSA for 2 hours, 100μL of streptavidin-horseradish peroxidase (R&D Systems) diluted 200 times with PBS containing 1% BSA for 20 minutes, and 100μL of equal volumes of 3,3’,5,5’-tetramethylbenzidine and hydrogen peroxide (KPL, Gaithersburg, MD, USA) for 30 minutes, the reaction was stopped by the addition of 1 mol/L H₂SO₄ solution. The optical density of each sample was analyzed at 450 nm with a reference reading at 630 nm using a SpectraMax 340 absorbance plate reader (Molecular Devices, Union City, CA, USA). A TNFα or IL-8 standard curve, with a range of 15 pg/ml to 2000 pg/ml is used to calculate the actual TNFα or IL-8 in the experimental samples. We made certain that the optical density of each sample falls within the standard curve. Samples whose optical density is outside the standard curve were diluted.

2.8 Cell viability assay

Powdered XTT (2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide) (Sigma) was dissolved in RPMI 1640 medium without phenol red (HyClone) supplemented with 2 mmol/L L-glutamine to make a stock solution of 1 mg/ml. The stock solution was kept at -20°C prior to use.

For assessing the viability of THP-1 monocytes, the cells were plated in a 96-well cell culture plate and treated with effectors as described in section 2.3. After incubation, ≈60ml of cell supernatants were treated with 30 ml of thawed 1 mg/ml XTT stock
solution containing 24.9 μmol/L of phenazine methosulfate (PMS) (Fisher Scientific) and cells further incubated for 3 hours at 37°C, 5% CO₂.

For HEK293 cells, cells were plated in a 96-well cell culture plate and treated with effectors as described. Following the desired incubation time, cell supernatants were removed and adherent cells were washed with corresponding assay medium. After removal of the medium, adherent cells were resuspended in 100 ml of HEK293 assay medium containing 0.33 mg/ml XTT and 8.3 μmol/L PMS. Cells were incubated further for 3 hours at 37°C, 5% CO₂.

For both cases, cell supernatants were removed from individual wells after incubation, centrifuged at 2500 xg for 10 minutes to remove cells, and supernatants transferred to a new 96-well plate. XTT reduction was analyzed by reading the absorbance of the solution at 467 nm.

Tetrazolium salts, such as XTT, are commonly used as a measure of the redox potential of cells as a measure of their viability (Braeckman et al., 2002). It is based on the reduction of the colorless XTT tetrazolium salt within active mitochondria of living cells by succinate dehydrogenase to form an orange-colored water-soluble formazan (Figure 2.4) (Braeckman et al., 2002; Brady et al., 2007). The formation of a water-soluble formazan allows direct monitoring of its appearance, and thus, is one of the advantages of XTT over other previous tetrazolium salts like MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), which produces insoluble salt when reduced (Scudiero et al., 1988; Kuhn et al., 2003).
Figure 2.4. Conversion of XTT to a water-soluble formazan salt by viable cells. Metabolically active cells cleave the yellow tetrazolium salt XTT to form an orange formazan dye. XTT reduction is measured by reading the optical density at 467 nm.
2.9 Atomic Force Microscopy

At different aggregation states of the peptide, samples of Aβ(1-42) or Aβ(1-40) (100 μmol/L and 1 mmol/L) solutions were obtained and diluted to 1 μmol/L in water. Grade V1 mica (Ted Pella, Inc., Redding, CA, USA) was cut into 11 mm circles and affixed to 12 mm metal discs. 50 μL of resulting Aβ aliquots were applied to freshly cleaved mica, allowed to adsorb for 15 minutes, washed twice with water, air dried, and stored in a container with desiccant. Images were obtained with a Nanoscope III multimode atomic force microscope (Digital Instruments, Santa Barbara, CA, USA) in TappingMode™. Height analysis was performed using Nanoscope III software on flattened height mode images.

2.10 Statistical Analysis

Data are expressed as mean ± SD. Statistical comparisons were made using Student’s t test (SAS system) (Harris, 2003). Differences between mean were considered significant at p<0.05.
2.11 Bibliography


Mol Biol. 256:870-877.


3 MODULATION OF AMYLOID BETA AGGREGATION MORPHOLOGY AND ITS EFFECT ON PROINFLAMMATORY RESPONSE OF THP-1 MONOCYTES

3.1 Introduction

The brains of individuals with AD are characterized by the presence of two lesions: extracellular deposits of Aβ peptides, so-called neuritic or senile plaques, and intracellular neurofibrillary tangles (NFT) of hyperphosphorylated tau (Selkoe, 2001). Numerous studies have now shown that Aβ plays a very important role in the AD pathogenesis (Walsh et al., 2002a; Walsh et al., 2002b). Although the exact mechanism of neurodegeneration is still uncertain, substantial evidences associate Aβ as fundamental for neurodegeneration in AD (Ramsden et al., 2001). Particularly, several studies with synthetic Aβ pinpoint the fibrillar form similar to those present in amyloid-bearing plaques in AD as neurotoxic both \textit{in vitro} and \textit{in vivo} and causes neuronal dysfunction and loss in AD (Kowall et al., 1991; Pike et al., 1991; Lorenzo and Yankner, 1994; Iversen et al., 1995). However, recent reports suggest that it is the soluble, rather than the insoluble Aβ that is responsible for early dendritic and synaptic injury, and eventually neuronal dysfunction and degeneration (Lambert et al., 1998; Lue et al., 1999; Walsh et al., 2002a; Chromy et al., 2003; Bucciantini et al., 2004).
Besides being known to have direct neurotoxic effect, considerable evidences also favor an indirect effect of Aβ to neurodegeneration based on ability of Aβ to initiate and release inflammatory mediators and neurotoxic factors in microglia, such as secretion of proinflammatory cytokines, respiratory burst activity and increased phagocytosis and chemotaxis (Murphy et al., 1998; Lue et al., 2001a; Lue et al., 2001b; Lee et al., 2002; Floden and Combs, 2006). Consistent with this, reactive microglia has been observed to be in and around Aβ – consisting plaques in AD (Frautschy et al., 1998; Stalder et al., 1999). Thus, these studies suggest that the extracellular deposition of Aβ triggers inflammation in AD brain.

However, despite numerous evidences connecting Aβ to neuronal injury and death, limited information is still available as to the exact mechanism by which Aβ causes neurodegeneration. Similarly, a more complicated question that is a focus of active study and debate is the question of, what Aβ assembly state correlates with the biological activity and contributes most critically to neurological decline in AD. It is thus important to have a clearer understanding of the Aβ structure-function relationship for the reason that determining the trigger of activation will result in a better understanding of the contribution of inflammation in AD, and subsequently, will have important implications to the development of therapeutic strategies.

In this study we investigate the ability of synthetic Aβ peptides to invoke a proinflammatory response in a human monocytic cell line. Moreover, we seek to determine the active Aβ species that induces TNFα production in our THP-1 monocytes. We modulated Aβ aggregation by varying several factors including peptide concentration, peptide length and temperature to examine the Aβ assembly state that
correlates with biological activity. We propose that an Aβ (1-42) fibrillar precursor was largely responsible for THP-1 cell activation. The data presented in this chapter is part of a collaborative study with Deepa Ajit of Department of Chemistry and Biochemistry, University of Missouri-Saint Louis. Additional data, analysis and conclusions will be presented and included in Ms. Ajit’s dissertation.

3.2 Results

3.2.1 Aβ aggregation and proinflammatory response

To study the proinflammatory response of different Aβ aggregation species, we have utilized a well-studied mammalian cell system, THP-1 monocytes. Numerous investigators have utilized THP-1 cells as a model system for the study of proinflammatory production by LPS and Aβ, and have shown that THP-1 cells are morphologically similar to microglia when stimulated with LPS and Aβ, making them a very good model of primary human microglia and for investigating Aβ induced inflammatory activity (Klegeris et al., 1997; Yates et al., 2000; Combs et al., 2001). We prepared the THP-1 monocytes as described in the methods, maintaining a cell concentration of 1x10⁶ cells/ml. The lyophilized Aβ were resuspended in water to a final concentration of 100 μmol/L and kept at 4°C prior to cellular stimulation. The Aβ was allowed to aggregate at 4°C from 0h (freshly prepared) to 216h. In between these aggregation times, Aβ solution was removed and was used to stimulate the THP-1 monocytes to a final Aβ concentration of 15 μmol/L. Cells were incubated for 6h at 37°C,
Figure 3.1 Proinflammatory activity of synthetic Aβ(1-42) at different aggregation. Aβ(1-42) was prepared in water and stored at 4°C, as described in the Methods. THP-1 monocytes were incubated with 100 μmol/L of Aβ(1-42) at different aggregation age to a final Aβ concentration of 15 μmol/L, and cells incubated for 6h at 37°C, 5% CO₂. After post-incubation, supernatants were collected and TNFα production was measured using ELISA. Shown are three representative experiments from different Aβ(1-42) lots.
supplemented with 5% CO₂. After incubation, supernatants were collected and assayed for TNFα production by ELISA. Figure 3.1 illustrates the proinflammatory activity of different aggregates of 100 μmol/L Aβ. Minimal TNFα levels were produced when THP-1 cells were treated with freshly reconstituted (0 hours) Aβ(1-42). However, a steady and significant increase in TNFα production was observed when Aβ(1-42) solution was allowed to aggregate further, with the peak TNFα level observed between 48h and 96h of Aβ aggregation. Interestingly, there was a noticeable decline in stimulatory activity when Aβ sample was incubated for a longer aggregation time. Different representative aggregation age profiles of 100 μmol/L of Aβ were included in the figure to illustrate that there is a lot-to-lot variation in stimulatory activity by synthetic Aβ (May et al., 1992; Zambrzycka et al., 2000). The toxicity of different Aβ aggregates were also monitored using XTT and results showed that 15 μmol/L Aβ aggregates were not toxic to THP-1 cells (data not shown).

We monitored the morphology of the Aβ aggregate species using AFM (Figure 3.2a). The appearance of numerous punctuate species was observed for freshly reconstituted Aβ monomers, with height measurement of <2 nm for majority of the adsorbed species. There was also a noticeable presence of small spherical species in Aβ(1-42) at 0h of aggregation. Height analysis of these species ranged from 2 to 5 nm, with an average of 3.2 ± 0.8 nm (SD) for n = 115 measurements. This suggests that the said spherical species might be fibrillar precursors. Also, a number of bright spots, with height > 20nm, can be detected which may represent the formation of amorphous aggregates immediately following reconstitution of the peptide. Consequently, these spherical species were not able to stimulate THP-1 cells for TNFα production (Figure
The appearance of thin flexible fiber-like structures was observed at 48 hours of Aβ aggregation. Continuous incubation of Aβ increased the appearance of fibrillar structures. When applied to THP-1 cells, these Aβ species invoked TNFα production. Height measurements of the 48h aggregated Aβ fibrillar structure were performed and plotted as a histogram (Figure 3.2b) and fitted for multiple peaks. Using peak fitting analysis, we observed two populations: the first with a peak height and SE of 4.4 ± 0.1 nm, and the second having a mean height and SE of 7.9 ± 0.6 nm (Udan et al., 2008). Our height measurements for the fibers formed at 48h were in agreement with previous AFM measurements describing type I and type II fibrillar Aβ (Harper et al., 1997; Stine et al., 2003). Further incubation of the Aβ samples (216 hours) resulted in the formation of longer fibrillar structures. Interestingly, a marked decrease in the presence of spherical species was observed at this incubation time. The longer, more mature fibril structures present at 216 hours surprisingly were not able to stimulate the THP-1 cells in producing TNFα.

We also varied the length of cell exposure to further analyze Aβ-induced proinflammatory production. We found that maximum TNFα production was achieved when THP-1 cells were exposed to 15 μmol/L of Aβ(1-42) for 10 hours (Figure 3.3a). This trend was slightly different from that of LPS - and Pam3CSK4 - treated THP-1 cells in that the maximal TNFα response was consistently observed after 6 hours of post-stimulation (Figure 3.3b). Because continual exposure of the cells to proinflammatory products may have a toxic effect on the cells, we have utilized a 6-hour cell exposure despite the observed maximal Aβ TNFα response at 10 hour post stimulation.
Figure 3.2. Morphological studies of Aβ(1-42) aggregated species. (A) Aβ aggregation solutions (100 μmol/L) in water were prepared as described in Methods, and allowed to aggregate at 4°C. Aliquots were removed at 0, 48, 96 and 216h, diluted to 1 μmol/L with water and imaged by AFM. Representative Aβ solutions were also used to treat THP-1 monocytes for TNFα production. AFM images are 5μm x 5μm and are shown in ‘height’ mode. (B) Representative frequency histogram from 300 height measurements of Aβ(1-42) aggregated at 48h. Graph was fitted (r² = 0.932) to a two-peak Gaussian area curve using PeakFit software v3.0 (Systat Software, Inc., San Jose, CA, USA). AFM images courtesy of Ms. Deepa Ajit, Univ. of Missouri-St. Louis. Frequency histogram analysis courtesy of Dr. Michael R. Nichols, University of Missouri-St. Louis.
Figure 3.3. Effect of exposure time on Aβ(1-42)-, LPS- and Pam3CSK4-induced TNFα response in THP-1 cells. (A) THP-1 cells were exposed to 15 μM Aβ(1-42) at given times. After post-incubation, TNFα production was analyzed by ELISA. (B) THP-1 cells were exposed to 10 ng/ml ultrapure *E. coli* K12 LPS or 1 ng/ml Pam3CSK4 at given times. Supernatants were collected after stimulation and assayed for TNFα. For both figures, TNFα was expressed as % of the maximum response, which was at 10 hours for Aβ(1-42), and 6 hours for LPS and Pam3CSK4. Error bars for Aβ are standard error for n = 6 trials for 0, 6, 10 and 24h and n = 3 for 48h; and n = 3 for both LPS and Pam3CSK4. Actual maximum averaged TNFα levels are 507 pg/ml for Aβ, 674 pg/ml for ultrapure LPS and 214 pg/ml for Pam3CSK4.
3.2.2. Modulation of Aβ aggregation

Our data suggests that an intermediate Aβ(1-42) species is stimulating our THP-1 monocytes in producing TNFα. However, as the Aβ aged, the species that were produced failed to invoke TNFα production. To further understand the inability of the later aggregated species to induce proinflammatory response in our THP-1 monocytes, we regulated the aggregation kinetics. Several factors can modulate in vitro Aβ fibril formation. One of the factors that affects Aβ fibrillogenesis is the peptide concentration (McLaurin et al., 2000; Taylor et al., 2003). Increasing the peptide concentration considerably enhances the rate of aggregation (Harper et al., 1999; Nilsson, 2004; Chen and Glabe, 2006). For this study, we increased the concentration of Aβ(1-42) stock solution from 100 μmol/L to 1.2 mmol/L, and followed the ability of the Aβ aggregation species to invoke TNFα production in THP-1 monocytes. To compare the effect of concentration, the THP-1 cells were treated with both the concentrated Aβ sample and 100 μmol/L Aβ preparation, to a final concentration of 15 μmol/L. As shown in Figure 3.4a, 1.2 mmol/L Aβ sample invoked TNFα production (24 pg/ml) when it was freshly reconstituted; however, further aging of the concentrated Aβ samples eradicated induction of TNFα response. This response was significantly different from that of 100 μmol/L of Aβ, wherein the peak response was observed at 96h of Aβ aggregation. AFM analysis of the 1.2 mmol/L Aβ solutions revealed that aside from the globular species, the freshly reconstituted peptide solution (Figure 3.4b) already formed numerous long fibrillar structures. A longer, intertwined dense population of fibrils was observed as
Figure 3.4. Proinflammatory activity and morphological studies of concentrated Aβ (1-42) sample. (A) Unlike 100 μM of Aβ (black line), a more concentrated 1.2 mM Aβ(1-42) (circles) failed to induce TNFα response in THP-1 monocytes. Aβ was reconstituted in water to a final concentration 1.2 mM, as described in Methods. THP-1 cells were treated with Aβ solution at different aggregation times, and cells incubated for 6h at 37°C. After incubation, cell supernatants were analyzed for TNFα using ELISA. A representative graph for 100 μM Aβ from Figure 3.1 was included for comparison. (B) AFM of freshly reconstituted and 24h aggregated 1.2 mM Aβ showed a population of fibrillar species. AFM was done as described in methods. AFM images courtesy of Deepa Ajit, University of Missouri-St. Louis.
early as 24 hours of Aβ aggregation, however, these species were not able to invoke
TNFα response. It was not possible to do height analysis on fibrils at 24 hours of
aggregation due to overabundance of intertwined fibrils. Nevertheless, the presented data
suggest that a 12-fold increase in Aβ concentration rapidly diminishes the lag phase for
fibril formation, and also attenuates the ability to induce TNFα response.

We next studied the effect of Aβ incubation temperature in fibril formation and
analyzed the biological activity of the Aβ species that are formed. We resuspended Aβ(1-42)
in water, as described in Methods, to a final concentration of 100 μmol/L and let the
solution aggregate at 4°C, 25°C and 37°C. At different times, solutions were removed and
used for THP-1 treatment. As shown in Figure 3.5a, only the Aβ species formed at 4°C,
and not at 25°C and 37°C, induced TNFα production in THP-1 monocytes. There was a
slight increase in the signal of Aβ at 25°C aggregated for 48 hours, but the increase was
not significant. AFM analysis of freshly prepared Aβ solutions contained spherical
species (Figure 3.5b), which were still present at 48 hours of aggregation for Aβ stored at
4°C, along with a few fibrillar structures. Aβ solution incubated at 25°C and 37°C quickly
formed longer fibrillar structures (data not shown). At 96 hours of aggregation, Aβ
incubated at 4°C contained long flexible fibrils (Figure 3.5b) with a mean height of 5.5 ±
1.6 nm (SD), as well as numerous globular structures, which correspondingly elicited an
increased TNFα production in THP-1 cells. Aβ samples incubated at 25°C showed longer
fibrils, with mean height of 6.9 ± 2.1 nm (SD). Interestingly, a decrease in the presence of
globular structures was also noticeable. Aβ samples at 37°C aggregated much faster, as
shown by an abundance of fibrils formed at 96 hours of aggregation. These data further
suggest that an intermediate fibrillar Aβ species induced TNFα response in THP-1 cells.
Figure 3.5. Accelerated aggregation of Aβ(1-42) by increasing the incubation temperature failed to invoke TNFα response in THP-1 monocytes. (A) 100 μM Aβ(1-42) was prepared in water and incubated at 0°C (circles), 25°C (triangles) or 37°C (diamonds). THP-1 cells were incubated with Aβ solutions to a final concentration of 15 μM for 6 hours, as described in Methods. After incubation, TNFα was assessed by ELISA. (B) Representative AFM images of freshly prepared (a-c) or 96-hour aggregated (d-f) Aβ(1-42) solutions incubated at 4°C (a,d), 25°C (b,e) or 37°C (c,f) were analyzed as described. Only the Aβ sample incubated at 4°C elicited TNFα response in THP-1 monocytes. AFM images courtesy of Deepa Ajit, University of Missouri-St. Louis.
Increased fibril formation diminished the TNFα response, and prolonged and accelerated aggregation failed to induce TNFα production in THP-1 cells.

Aβ(1-42) and Aβ(1-40) are the most predominant variants of Aβ that are present in amyloid plaques (Taylor et al., 2003). The data that we have shown so far indicate that the active species of Aβ(1-42) was effective in inducing TNFα production in THP-1 monocytes. We wanted to know if the species formed during Aβ(1-40) aggregation will also instigate TNFα response similar to that of Aβ(1-42). For this, we prepared 100 μmol/L of Aβ(1-40) in water and treated the THP-1 cells the same way as Aβ(1-42). After 6 hours of post-incubation, supernatants were analyzed for TNFα. Our results showed that Aβ(1-40) samples at 4°C were not effective in inducing TNFα response (Figure 3.6a). Moreover, AFM imaging of the Aβ(1-40) aggregation at 4°C indicated a slower rate of fibril formation (Figure 6b, panels a-d). To hasten Aβ aggregation, samples of Aβ(1-40) were likewise incubated at 25°C or 37°C. AFM analysis showed that although at a much slower rate than Aβ(1-42), Aβ(1-40) also formed fibrils at elevated temperatures (Figure 3.6b, panels e-h, i-l) and longer Aβ incubation (Figure 6b, panels h and l). However, these species were ineffective in inducing TNFα release in THP-1 monocytes (Figure 3.6a).

3.3. Discussion

Inflammation plays an essential role in the brain’s response to injury and pathology (Moore et al., 2002). Growing evidences have linked inflammation with the development of AD. Microglial cells, the resident immune cells of the CNS, play an
Figure 3.6. Aβ(1-40) failed to induce proinflammatory activity on THP-1 cells. (A) 100 μM Aβ(1-40) was prepared in water, and incubated at 4°C (triangle), 25°C (inverted triangle) and 37°C (diamond), as described in Methods. Different aggregation solutions were used to treat the THP-1 monocytes for 6 hours, and TNFα was analyzed after post-stimulation. (B) Representative AFM images of Aβ(1-40) samples at 4°C (a-d), 25°C (e-f) and 37°C (i-l) at different times. AFM images are 5 μm x 5 μm. AFM images courtesy of Deepa Ajit, University of Missouri-St. Louis.
integral role in inflammation. Moreover, activated microglial cells have been found to be closely associated with and the most prominent component of senile plaques (Combs et al., 2000; Masumura et al., 2000; Selkoe, 2001; Mattson, 2004). Numerous cellular studies using microglia and human macrophage/monocytes cell line have demonstrated that Aβ peptides are able to induce these cells to produce significant amounts of proinflammatory cytokines and chemokines (Meda et al., 1995; Yan et al., 1996).

In studying the correlation between different Aβ aggregation species and proinflammatory production, we have utilized THP-1 monocytes for the main reason that THP-1 cells have expanse properties that are analogous to microglia and mature phagocytes when treated with LPS and Aβ (Klegeris et al., 1997; Combs et al., 1999; Combs et al., 2000). Moreover, THP-1 cells express numerous surface markers such as CD11a, CD11b, CD11c, CD18, CD36, CD44 and Fc immunoglobulin receptors that are pronounced on macrophages and microglia (Klegeris et al., 1997). These metabolic and morphological similarities between human monocytic cell line THP-1 and microglia made the THP-1 an appropriate model for the study of Aβ-induced proinflammatory response in primary human microglia.

We have shown that 100 µmol/L of Aβ(1-42) at 4°C was capable of invoking TNFα production in THP-1 monocytes. Although the observed maximum TNFα response varies from lot-to-lot preparation of Aβ, it was still apparent that an intermediate Aβ species activate the THP-1 monocytes and prolonged aggregation (216 hours) of the Aβ was ineffective in stimulating proinflammatory response. Furthermore, enhancing the rate of aggregation and fibril formation by manipulating several factors such as increasing the concentration (Fig. 3.4) or Aβ incubation temperature (Fig. 3.5) produced Aβ species that
were not able to induce proinflammatory response in our THP-1 monocytes. These data suggest that an intermediate Aβ structure, and not the more mature rigid fibrils, act as a proinflammatory stimulus.

Atomic force microscopy (AFM) is an ideal tool to follow the early morphological changes in Aβ fibril formation (Stine et al., 1996). Several investigators have used this technique to understand the process of Aβ fibrillogenesis (Harper et al., 1997; Kowalewski and Holtzman, 1999; Nybo et al., 1999; Mastrangelo et al., 2006). Since we are investigating the Aβ aggregation species that invokes proinflammatory response in our model THP-1 monocytes, it is imperative that we consistently produce an unaggregated starting material. We accomplished this by treating the Aβ with hexafluoroisopropanol (HFIP). HFIP is known to disrupt peptide-peptide interaction thereby disaggregating the pre-formed aggregates (Klein et al., 2004; Findeis, 2007). AFM analysis of our freshly reconstituted Aβ(1-42) samples at 4°C, 25°C and 37°C showed punctate structures that had height measurements of < 2nm, which correspond to monomers (Klein et al., 2004). Moreover, small spherical structures with heights of 2-5 nm were also observed along with the punctuate species. These species were thought to be prefibrillar precursors. These measurements correspond with the findings of Nybo et al. (1999) in their investigation of the early stages of Aβ(1-42) fibrillogenesis. They reported the earliest recognizable ultrastructure of Aβ as globular structures with mean height of 4-5 nm. Moreover, these structures appear to fuse and align in a row, which later becomes fibrils (Nybo et al., 1999). Our results showed that further incubation of Aβ(1-42) at 4°C resulted in formation of two populations of fibrils with mean heights of 4.4 ± 0.1 nm (SE) and 7.9 ± 0.6 (SE). These measurements were consistent with that of
Harper and colleagues (1997) when they analyzed the early steps of Aβ formation in vitro by AFM. They reported height measurements of 7.3 ± 0.53 nm and 3.8 ± 0.43 nm for their Aβ(1-42) species, which correspond to type-1 and type-2 fibrils, respectively (Harper et al., 1997). Interestingly, these fibrils consistently stimulated our THP-1 cells in producing proinflammatory TNFα. More importantly, the more mature fibrils that we have generated in later Aβ aggregation were ineffective in stimulating a response. This further infers that the intermediate fibrillar aggregation structures of Aβ(1-42) promote TNFα secretion, and not the more mature fibrils.

Unlike Aβ(1-42), our Aβ(1-40) preparations were ineffective in activating our THP-1 cells for TNFα response (Figure 3.6). Morphological analysis of these samples showed a very slow progression of fibril formation. Aβ(1-40) and Aβ(1-42) bear different biochemical properties. Numerous biochemical studies have demonstrated that Aβ(1-42) aggregates much more quickly than Aβ(1-40) (Burdick et al., 1992; Jarrett et al., 1993). Moreover, although there is more Aβ(1-40) that is being secreted, Aβ(1-42) is the major component of senile plaques (Miller et al., 1993), as substantiated by recent findings which showed that high levels of Aβ(1-40) alone do not result in observable amyloid pathology, while low levels of Aβ(1-42) result in a wide range of amyloid pathology (McGowan et al., 2005). These findings, together with our observation, suggest that Aβ(1-42) is a major causative agent in pathogenesis of AD due to its enhanced aggregation properties (Chen and Glabe, 2006).

Although instrumental in surveying the morphology of the species formed during fibril formation, AFM analysis must be combined with other in vitro techniques that can further substantiate our finding that intermediate soluble fibrillar structures of Aβ induce
production of proinflammatory products. We have further characterized our Aβ preparation by centrifuging the Aβ samples that were aggregated for 72 or 96 hours at a speed of >100,000g for 1 hour. At this speed, all the rigid fibrillar structures would pellet down and only the soluble oligomers will remain in the solution (Walsh et al., 2002b; Klein et al., 2004; Irvine et al., 2008). After centrifuging the prepared Aβ solution, the supernatant was collected and tested for its ability to invoke proinflammatory response in THP-1 cells. Using AFM, we found that some fibrillar structures were still present in the supernatant after the Aβ samples were spun. Yet, supernatant still invoked a TNFα response (data not shown). Furthermore, the aggregation species in the supernatant was recognized by OC antibody (data not shown). This antibody recognizes fibrils and fibrillar oligomeric species, which are described as small soluble aggregates that are arranged in a similar conformation as in fibrils (Kayed et al., 2007). Taken together, these additional results further confirm that a soluble fibrillar precursor species is the proinflammatory form of Aβ. This project was done in collaboration with Deepa Ajit.

Several other biophysical methods were utilized for characterization of our bioactive Aβ species and these additional data are presented in her dissertation.

Although numerous investigations have suggested that large fibrillar forms of Aβ can kill neurons (Kowall et al., 1991; Pike et al., 1993; Lorenzo and Yankner, 1994; Geula et al., 1998), accumulating evidence in vitro now demonstrate that the soluble assembly forms of Aβ are the key neurotoxic effectors in AD (Lambert et al., 1998; Walsh et al., 1999; Klein et al., 2004). The presence of soluble oligomeric Aβ assemblies have also been observed from the supernates of AD brain and extracts of amyloid plaques (Roher et al., 1996; Enya et al., 1999; McLean et al., 1999), which suggest that soluble
Aβ could be the earliest mediators of neuronal dysfunction. Aβ is capable of upregulating cytokine and chemokine expression by microglia and monocytes/macrophages (Meda et al., 1995; Yan et al., 1996; Klegeris et al., 1997); however, there are still conflicting discussions and debates as to whether it is the oligomers or fibrils that are more potent neurotoxins. Recent report by Sondag and colleagues (Sondag et al., 2009) showed that proinflammatory cytokine IL-6 production was significantly higher when microglial cells were stimulated with Aβ oligomers than with fibrils. However, their results also showed that Aβ fibrils are more potent in inducing expression of proinflammatory chemokine keratinocyte chemoattractant (KC, a mouse homologue of chemokine IL-8) than the soluble aggregates. These findings demonstrate the ability of Aβ to act as a proinflammatory stimulus in microglia, but more importantly, these results suggest that the expression and release of proinflammatory products may depend on the specific conformation of Aβ. Our present data reveal that cells of the monocytic origin respond to an intermediate soluble yet fibrillar form of Aβ, and not the later more mature fibrils, by secreting the proinflammatory cytokine, TNFα. Taken together, these evidences further accentuate the intricacy involved in studying the association of Aβ aggregation with the proinflammatory response in AD brain.
3.4. Bibliography


4.1 Introduction

The brains of individuals with Alzheimer’s disease contain reactive microglia and these immune cells cluster at sites of Aβ deposition (Akiyama et al., 2000; Perry et al., 2003). Microglial activation is always associated with production of inflammatory products and mediators, which include complement proteins, cytokines and chemokines (Das and Potter, 1995; Yates et al., 2000). Extensive and compelling evidence shows that these activated microglia surround Aβ plaques (Miyazono et al., 1991; Frautschy et al., 1998; Apelt and Schliebs, 2001; Wegiel et al., 2001) and in vitro activation of microglia by Aβ results in the production and secretion of proinflammatory molecules such as reactive oxygen species, cytokines and neurotoxins (Griffin et al., 1989; Venters et al., 1999; Akiyama et al., 2000; Yates et al., 2000; Combs et al., 2001). However, it was difficult to ascertain whether Aβ-induced inflammation contributes to or causes AD. Several investigators have reported that the use of anti-inflammatory agents or non-steroidal anti-inflammatory drugs (NSAIDs) significantly reduced the risk for AD (McGeer et al., 1996; Stewart et al., 1997; Rogers, 2008). These evidences strongly support the concept that chronic inflammatory process contributes to AD progression.

Inflammation is considered to be a double-edged sword: it may be useful when
controlled, but deadly when it is not (Akiyama et al., 2000; Lai et al., 2006). Over the years, investigators have tried to answer the mechanism by which A\(\beta\) causes heightened expression of proinflammatory products in microglia. Some groups reported several inflammation-related receptors present in the microglia as key players in A\(\beta\)-induced microglial activation and inflammatory response. These include receptors for advanced glycosylated end-products (RAGE) (Yan et al., 1996), scavenger receptor class A (El Khoury et al., 1996; Paresce et al., 1996), B-class scavenger receptor CD36, intergrin associated protein/CD47 and \(\alpha_6\beta_1\)-integrin receptor complex (Bamberger et al., 2003), as well as calcium-, protein kinase C-, and tyrosine kinase- dependent second messenger pathways (Klegeris et al., 1997; Combs et al., 1999; Yates et al., 2000). Recent studies by Fassbender and colleagues demonstrated that fibrillar A\(\beta\) interacts and binds with the bacterial lipopolysaccharide (LPS) receptor, CD14 (Fassbender et al., 2004). Moreover, Bate et al reported that the subsequent killing of A\(\beta\)-damaged neurons by microglia is a CD14 dependent process (Bate et al., 2004; Heneka and O'Banion, 2007). Furthermore, Liu et al demonstrated a direct role of CD14 in fibrillar A\(\beta\)(1-42) phagocytosis, and an observed elevation of CD14 immunostaining in AD brains compared with controls (Liu et al., 2005). These evidences connecting CD14 with A\(\beta\) strongly suggest that innate immunity is related to AD pathology.

A wealth of data now indicates that CD14 interacts with TLR4 and TLR2 (Chapter 1 review). In this study, we aspired to investigate and identify the transmembrane TLR(s) that may be involved in the induction of innate immune response by A\(\beta\)(1-42). For this investigation, we utilized cell systems including THP-1 monocytes as a model of primary microglia (Chapter 3 review), as well as human embryonic kidney
(HEK293) cells. We propose that TLR4 and TLR2 are highly involved in Aβ(1-42)-induced proinflammatory cytokine production in these mammalian cell model systems.

4.2 Results

4.2.1 Mammalian cell model system: THP-1 monocytes

4.2.1.1 Toll-like receptor ligands activate the proinflammatory response in THP-1 monocytes

The interaction of LPS with TLR4 is the best studied model of innate immunity (Aderem and Ulevitch, 2000). Several groups have extensively analyzed LPS-mediated TLR4 downstream signaling for induction of proinflammatory response (Poltorak et al., 1998; Hoshino et al., 1999; Qureshi et al., 1999). Aside from TLR4, numerous studies also focused on TLR2 due to its capability to recognize a broad range of ligands (Chapter 1 review; (Albiger et al., 2007)). We started our investigation by first testing whether THP-1 monocytes produce TNFα upon induction with known TLR agonists LPS (TLR4), synthetic tripalmytoyl cysteinyll seryl tetralysine Pam3CSK4 (TLR2/1) and synthetic diacylated lipopeptide FSL (TLR2/6). THP-1 monocytes were treated with increasing concentration of the agonists, and incubated for 6 hours, as discussed in Methods. Supernatants were collected after incubation, and secreted TNFα was measured by ELISA. TNFα measurements revealed a concentration-dependent response for all TLR agonists (Figure 4.1). Fitting the data to a sigmoidal three-parameter equation produced
EC$_{50}$ values of 5 ng/ml for ultrapure K12 LPS, 1 ng/ml for Pam$_3$CSK$_4$ and 7 ng/ml FSL-1. For succeeding experiments, we decided to utilize the following concentrations: 10 ng/ml LPS, 1 ng/ml Pam$_3$CSK$_4$ and 1 or 3 ng/ml FSL-1. We further extended our investigation by determining the best condition for maximal TNF$\alpha$ production by our TLR agonists. We found highest TNF$\alpha$ response for LPS, Pam$_3$CSK$_4$ and FSL-1 when cells were exposed to these agonists for 6 hours (Figure 3.3, FSL data not shown). These data confirmed the responsiveness of THP-1 monocytes to known TLR ligands, and is particularly useful in our investigation of A$\beta$-TLR interaction since these agonists can be used in later experiments as positive controls.

4.2.1.2 Amyloid beta(1-42) is devoid of any contamination

We have previously shown that soluble fibrillar A$\beta$(1-42) activates THP-1 monocytes for proinflammatory response (Chapter 3). In the previous report, we have demonstrated that the maximal TNF$\alpha$ response was achieved when 100 $\mu$mol/L A$\beta$(1-42) was allowed to aggregate at 4$^\circ$C between 48 and 96 hours. Because of this result, the same conditions were utilized for the A$\beta$ samples in this study. For every analysis, corresponding AFM images and height analyses were performed. Height measurements were in accord with the soluble fibrillar A$\beta$(1-42), discussed in chapter 3.

Bacterial LPS utilizes TLR4 for activation of TLR downstream signaling that culminates in production of proinflammatory cytokines and chemokines such as TNF$\alpha$. Because our main objective is to identify the TLR that plays a functional role in A$\beta$-
Figure 4.1. Known TLR agonists induce TNFα production in a dose-dependent manner. THP-1 monocytes were stimulated with increasing concentration of ultrapure K12 LPS (circles), synthetic Pam₃CSK₄ (triangles) or synthetic FSL-1 (diamonds) for 6 hours, as described in Methods. The same volume of water was added to the cells as a control in the absence of agonist. After stimulation, secreted TNFα in the supernatants was analyzed by ELISA. Error bars represent standard error for three measurements. Data was fit to sigmoidal three-parameter equation using SigmaPlot graphing program.
induced inflammatory response, we have to ensure that our Aβ(1-42) is devoid of any contaminating bacterial lipopolysaccharide that might interfere with the proper interpretation of our results. We also wanted to confirm that the secreted TNFα observed in THP-1 monocytes was due to Aβ as a proinflammatory stimulus, and not due to traces of contaminating bacterial LPS. To rule out the presence of traces of LPS in our Aβ(1-42) samples, we tested our Aβ preparation using the compound Polymyxin-B sulfate (PMX-B). PMX-B is a cationic decapeptide that binds to lipid A moiety of LPS and neutralizes its pathogenicity and prevents LPS-induced cytokine production (Pristovsek and Kidric, 1999). To determine if PMX-B can neutralize TNFα secretion by 10 ng/ml of ultrapure K12 LPS, THP-1 monocytes were pretreated with medium alone, or with increasing concentration of PMX-B for 30 minutes. After preincubation, cells were stimulated with 10 ng/ml LPS for 6 hours prior to TNFα measurement. PMX-B effectively neutralized the proinflammatory effect of LPS in a dose-dependent manner, with a 2-fold reduction of LPS signal for as low as 10 ng/ml of PMX-B (Figure 4.2a). This data implies that PMX-B is an effective tool in neutralizing LPS response and thus, can be used to test for LPS contamination in our Aβ samples. Using XTT proliferation assay, it was found that PMX-B alone did not have any toxic effect on our THP-1 monocytes (data not shown).

Next, we pretreated the THP-1 cells with 0.1 μg/ml of PMX-B for 30 minutes, as described previously, prior to THP-1 stimulation with 15 μmol/L of Aβ(1-42). Figure 4.2b shows different profiles for PMX-pretreated Aβ and PMX-pretreated LPS (Udan et al., 2008). PMX-B almost completely abrogated the K12 LPS signal (LPS/PMX % response of 1.85 ± 0.84, as compared to 100 ± 1.1 % response by LPS alone), but with little or no effect on Aβ signal (Aβ/PMX % response of 95.7 ± 3.25 compared to 100 ±
Figure 4.2. PMX-B is a powerful tool for ruling out the presence of small traces of contaminating bacterial LPS. (A) The LPS proinflammatory response was neutralized by PMX-B in a dose-dependent manner. THP-1 were preincubated with increasing concentrations of PMX-B prior to stimulation with 10 ng/ml LPS, as described in Methods. Error bars represent standard error for three experiments. (B) PMX-B does not have an effect on Aβ(1-42) proinflammatory response. THP-1 cells were treated with 15 μM Aβ or 10 ng/ml LPS in the presence or absence of 0.1 μg/ml of PMX-B. Results are presented as % TNFα of the Aβ or K12 LPS without PMX-B. Error bars represent standard errors for 15 trials in five separate experiments. Actual TNFα levels averaged 328 pg/ml for Aβ and 859 pg/ml for K12 LPS.
0.43% response by Aβ without PMX-B). This signifies that our Aβ preparations are devoid of contaminating LPS. Moreover, the results imply that TNFα production in THP-1 is mainly due to Aβ as a proinflammatory stimulus, and not because of possible traces of LPS in the Aβ preparation. For subsequent experiments involving Aβ, Aβ/PMX-B and LPS/PMX-B samples were included for continual monitoring of trace contamination. Some results showed greater than 10% reduction in Aβ-induced TNFα response when pretreated with PMX-B, although XTT cell proliferation experiments indicated that our Aβ samples are devoid of any bacterial contamination (data not shown). For accurate interpretation of results, those experiments were not included in the study of Aβ-TLR interaction.

4.2.1.3 Toll-like receptor antibody neutralization assay was effective in blocking the activity of known TLR agonists bacterial LPS and synthetic Pam3CSK4

We have developed a TLR antibody neutralization assay to aid us in investigating which transmembrane TLR mediates Aβ-induced immune response. We initially tested the effectiveness of this assay on our known TLR ligands, bacterial LPS (TLR4) and synthetic Pam3CSK4 (TLR2). THP-1 cells were preincubated with 10 μg/ml TLR antibodies prior to addition of either 10 ng/ml E. coli 026:B6 LPS or 1 ng/ml Pam3CSK4. TLR4 neutralization lowered the E. coli 026:B6 LPS response by almost 79% (% response of 29.3 ± 1.56 compared to 100 ± 2.9 of LPS alone) (Figure 4.3, gray bars). Surprisingly, TLR2 also had a significant effect on our LPS response. Antibody blockade of TLR2 considerably decreased the % LPS response to 33.2 ± 0.25. This result implies
Figure 4.3. Toll-like receptor (TLR) neutralization of bacterial lipopolysaccharide. THP-1 cells were pre-incubated with 10 μg/ml of TLR antibodies (eBioscience) or mouse isotype controls (IgG2 isotype control for TLR2 and TLR4 antibodies, IgG1 isotype control for CD14) for 1 hour prior to addition of 10 ng/ml LPS, as described in methods. Experiments done several times, and representative graph is shown. Pure LPS (E. coli 026.B6) (gray bars) utilized both TLR4 and TLR2 in secretion of TNFα. TLR2 conferred sensitivity to LPS may be due to contaminating bacterial lipoproteins. Repurified LPS preparation (E. coli K12) abolished the effect of TLR2 on LPS-induced TNFα response. Error bars are SE of three measurements.
that TLR2 lowered the TNFα response by almost 70%, similar to the effect of TLR4 neutralization. Because substantial evidences show that LPS utilizes TLR4 for downstream activation of innate immunity, this finding was unexpected. Several investigators reported that TLR2-conferred sensitivity to LPS may be due to contaminating lipoproteins (TLR2 agonists) in commercially available LPS preparations (Kielian, 2006), and repurifying the LPS preparations and removal of trace amounts of TLR2 agonists abated the effect of TLR2 in LPS activation (Hirschfeld et al., 2000). Because of this possibility, we repeated the TLR neutralization using a commercially available repurified, ultrapure K12 LPS. This LPS preparation has undergone stringent repurification by double phenol extraction of a 0.2% triethylamine/0.5% deoxycholate aqueous phase which ensures removal of contaminating lipoproteins that signal through TLR2 ((Hirschfeld et al., 2000), InvivoGen.com). Our result (Figure 4.3, black bars) demonstrated a TLR4-, but not TLR2-, dependent LPS-induced TNFα production (% TNFα response of 13.0 ± 3.4 for TLR4, 87.9 ± 1.4 for TLR2). The TLR2 and TLR4 isotype control IgG2 had 10% inhibitory effect (% response of 90.0 ± 2.4) on LPS response; this denotes that the 10% inhibitory effect observed on K12 LPS response when TLR2 was neutralized was not statistically significant.

Pam3CSK4 utilizes TLR2 in activation of the innate immune response. This was clearly demonstrated by an almost complete eradication of Pam3CSK4- induced secreted TNFα when TLR2 was blocked by 10 μg/ml of the TLR2 antibody (Figure 4.4, gray bar; % response of 4.2 ± 0.76) (Udan et al., 2008). As expected, TLR4 is not being utilized by Pam3CSK4 for TNFα secretion (80.9 ± 1.4 % TNFα response). Although there is a 20% inhibitory effect of TLR4 neutralization in Pam3CSK4 response, the value was not
Figure 4.4 TLR antibody neutralization of bacterial lipopolysaccharide and Pam₃CSK₄. THP-1 monocytes were pre-incubated with 10 μg/ml of TLR antibodies (eBioscience) or isotype controls (IgG2 for TLR2 and TLR4 antibodies; IgG1 for CD14) as described in Methods, prior to treatment with either 10 ng/ml ultrapure *E. coli* K12 LPS (black bars) or 1 ng/ml Pam₃CSK₄ (gray bars). Secreted TNFα was measured by ELISA. Results are presented as % TNFα of LPS or Pam₃CSK₄ pre-incubated with phosphate-buffered saline (PBS) medium. Actual TNFα levels were 216 pg/ml for LPS and 257 pg/ml for Pam₃CSK₄. Error bars for LPS data correspond to SE for six trials (2 experiments), and three trials, one experiment for Pam₃CSK₄ data. A Student’s t-test was used to calculate the significance between individual TLR and their respective isotype IgG controls ( *p < 0.001 and #p < 0.0025)
This experiment also demonstrated the necessity of the accessory protein CD14 for ultrapure LPS- and Pam3CSK4- induced activation of downstream TLR signaling. K12 LPS response was significantly attenuated by CD14 antibody, as shown by 75% inhibition. This result was significantly different from the CD14 isotype control IgG1 which inhibited only 6% of the K12 LPS response. Likewise, neutralization with the CD14 antibody blocked 90% of Pam3CSK4 signal. The data presented here indicates that the developed TLR neutralization assay is sensitive in specific recognition of TLR agonists and may be a tool for investigating Aβ-TLR interaction.

4.2.1.4 TLR2 and TLR4 play a role in fibrillar Aβ(1-42)-induced TNFα response in THP-1 monocytes

TLR neutralization was performed to clarify which TLR is being utilized by fibrillar Aβ for TNFα production. As described in methods, THP-1 cells were incubated with 10 μg/ml of TLR antibodies or isotype control for 1 hour prior to stimulation with 15 μmol/L of Aβ(1-42). Our results demonstrated the importance of TLRs and TLR accessory protein CD14 for Aβ-induced activation of innate immune response. As shown in Figure 4.5, CD14 neutralization significantly reduced Aβ response by 62%, relative to its isotype control IgG1 (Udan et al., 2008). This result suggests that Aβ utilizes CD14 for TNFα production, which was in accord with the previous report (Fassbender et al., 2004). Surprisingly, both TLR2 and TLR4 neutralization also diminished Aβ-induced
TNFα response. TLR4 blockade decreased the TNFα response by 35% (% response of 65.45 ± 1.87). More surprisingly, TLR2 antibody was more effective in neutralizing the Aβ response, as evidenced by 50% attenuation of TNFα signal (% response of 50.62 ± 3.26). Using Student’s t-test, these results were significantly different from IgG control (p < 0.001).

The TLR2 and TLR4 antibodies (InvivoGen) that were used in this experiment were different from the ones used for TLR neutralization of LPS and Pam3CSK4. This is due to a consistent significant attenuation of Aβ response by IgG2 control used in LPS and Pam3CSK4 studies. Nevertheless, this different set of TLR antibodies was still effective in blocking TLR. Moreover, a consistent 20 – 30% increase in Aβ response was observed when THP-1 monocytes were pre-treated with 10 to 20 μg/ml of CD14 isotype control, IgG1 (Figure 4.5 and 4.6b). This consistent stimulation may have cancelled out some of the effectiveness of anti-CD14 neutralization. Because of the IgG effect, it was not possible to compare the differences between TLR2, TLR4 and CD14 antibody neutralizing ability of Aβ response.

The Aβ-induced TNFα response was neutralized by TLR antibodies in a dose-dependent manner (Figure 4.6 a and b) (Udan et al., 2008). The effectiveness of TLR antibodies in neutralizing Aβ response was clearly demonstrated when THP-1 cells were pre-treated with as low as 5 μg/ml TLR antibodies. About 29% reduction of TNFα response was observed when THP-1 cells were treated with 2.5 μg/ml of TLR2 antibody, and 15% reduction of TNFα response for TLR neutralization. Increasing the concentration of the TLR antibodies augmented the effectiveness of neutralization, as depicted by a more dramatic decrease in TNFα response. A boost of TLR2 or TLR4
Figure 4.5. TLR2, TLR4 and CD14 play an active role in Aβ-induced innate immune response activation. THP-1 monocytes were pre-incubated with 10 μg/ml of TLR2, TLR4 (InvivoGen), CD14 (eBioscience) antibodies, or IgG isotype controls, as described in Methods. Isotype controls were rat IgG (Sigma) for TLR2 and TLR4, and mouse IgG1 (eBioscience) for CD14. After incubation, THP-1 cells were stimulated with 15 μM of Aβ(1-42) for 6 hours. TNFα was measured using ELISA. Results are expressed as % TNFα of Aβ response treated with phosphate buffered saline (PBS) medium. 20-30% stimulation of Aβ response was consistently observed for IgG1. Actual TNFα levels induced by Aβ alone averaged 328 pg/ml. Error bars correspond to SE for 12 trials (four separate experiments) for TLR2, TLR4 and rat IgG, and six trials (2 experiments) for CD14 and mouse IgG1. A Student’s t-test was used to calculate the significance between individual TLR and their respective isotype IgG controls ( *p < 0.0001). rIgG; rat IgG
Figure 4.6. Dose-dependent neutralizing ability of TLR antibodies against Aβ(1-42). THP-1 cells were pre-incubated for 1 hour with increasing concentrations of TLR antibodies or their isotype controls, as described in the Methods, prior to stimulation with 15 μM of Aβ(1-42). After 6 hours post-stimulation, TNFα was measured using ELISA. Results are expressed as % TNFα of Aβ response treated with phosphate buffered saline (PBS) medium. (A) TLR2 (circles) and TLR4 (triangles) antibodies (InvivoGen) blocked Aβ response in a dose-dependent manner. Rat IgG (rIgG, squares) is the isotype control for TLR2 and TLR4. The 10 μg/ml TLR and IgG result are reproduced from Figure 4.5. Standard errors are SE for n = 3 trials (5 μg/ml) and n = 6 trials (20 μg/ml). A Student’s t-test was used to calculate the significance between individual TLR and their respective isotype rat IgG (*p < 0.0001). Actual TNFα was the same as Figure 4.5. (B) CD14 (circles) antibody (eBioscience) also efficiently blocked Aβ response in a dose-dependent manner. Error bars are SE of three trials. Actual TNFα of Aβ alone averaged 170 pg/ml.
concentration from 10 μg/ml to 20 μg/ml further lowered the TNFα response by 10%. On the other hand, the neutralizing effect of CD14 antibody on Aβ appeared to plateau at ~45 ± 3.0% starting at 5 μg/ml. The TLR2 and TLR4 isotype control rat IgG did not have an effect on Aβ response. However, a consistent stimulation of the response was observed for CD14 isotype control mouse IgG1. Moreover, similar with the results for neutralization using 10 μg/ml TLR antibodies, TLR2 antibody was more effective in blocking Aβ response than TLR4. Nevertheless, the results presented here suggest that Aβ is utilizing multiple toll receptors for the activation of innate immune response. More importantly, TLR2 and TLR4 play an active role in Aβ-induced production of proinflammatory products. Noticeably, a complete abrogation of the Aβ-induced immune response was not observed when higher concentrations of TLR antibodies were used. Individual TLR neutralization using 20 μg/ml antibodies caused only 50-70% inhibition. A number of reasons may explain this result. First, previous reports suggested that Aβ fibrils also interact with other receptors for cellular activation and neurotoxicity like scavenger receptors, RAGE, CD11b/CD18receptor, CD36/αβ1-integrin/CD47 multireceptor complex or complement factor C1 (Bamberger et al., 2003; Fassbender et al., 2004). Thus, to explain the remaining unblocked activity, it is possible that Aβ utilized these other receptors when TLRs were made unavailable.

A second possibility may be that since TLR2 and TLR4 play a role in Aβ response, one receptor may compensate for the other when one is inaccessible. We tested this hypothesis by using a combination of TLR2, TLR4 and CD14 antibodies to investigate whether neutralizing multiple receptors will further enhance the reduction of TNFα response as compared to individual receptor blockade. For this study, we lowered
our TLR antibody concentration to 5 μg/ml to better observe the effect of combination antibody treatment. Moreover, we also supplemented each cell treatment with up to 10 μg/ml of rat IgG or 5 μg/ml of mouse IgG1 to match the triple combination TLR2/TLR4/CD14 neutralization with isotype control amounts. Since we have used a lower antibody concentration, and individual TLR neutralization was supplemented with IgG isotype controls, it is not possible to directly compare the results that we obtained in this experiment with that in Figure 4.5 and Figure 4.6. This experiment was performed numerous times and the results are shown in Figure 4.7 (Udan et al., 2008). As shown, the Aβ-induced TNFα response was only slightly reduced when individual TLRs were blocked with 5 μg/ml of the antibody (% response of 92.6 ± 0.72, 85.2 ± 3.73 and 98.52 ± 1.57 for TLR2, TLR4 and CD14, respectively). These individual TLR neutralization results were not statistically different from that of the mouse IgG1/rat IgG control (% response of 95.2 ± 2.7). As discussed earlier (Figures 4.5 and 4.6), a consistent enhancement of Aβ response was observed when cells were treated with mouse IgG1 control. Since the individual TLR2 and TLR4 (and all other samples that do not contain CD14) were supplemented with mouse IgG1 to match the concentration of the triple combination TLR2/TLR4/CD14, this may have masked some of the TLR blocking activity. A double combination of TLR2/CD14 and TLR4/CD14 antibody neutralization was better in lowering the Aβ response compared to the individual TLR blockade (34.4% and 35.5% reduction, respectively). The neutralizing activity of TLR2/CD14 and TLR4/CD14 compared to that of mouse IgG1/ rat IgG control was significantly different (p<0.001). These comparable results of TLR2/CD14 or TLR4/CD14 blockade suggest that an overlap to some extent of Aβ interaction with both TLRs and CD14 may be
Figure 4.7. Combination TLR antibody neutralization of Aβ(1-42)-induced TNF α response. THP-1 monocytes were pre-incubated with 5 μg/ml of TLR2, TLR4 (InvivoGen), CD14 (eBioscience) antibodies, or a combination of 5 μg/ml mouse IgG1 and 10 μg/ml rat IgG isotype control, as described in Methods. Individual cell treatments were also supplemented with one or both of the mentioned IgGs to match the concentration of the IgG controls. After pre-treatment, THP-1 cells were stimulated with 15 μM of Aβ(1-42) for 6 hours. TNFα was measured using ELISA. Results are presented as % TNFα of Aβ response treated with phosphate buffered saline (PBS) medium. Actual TNFα levels induced by Aβ alone averaged 487 pg/ml. Error bars correspond to SE for n = 3 trials. A Student’s t-test was used to calculate the significance between individual TLR and the mouse IgG1/rat IgG isotype control ( *p < 0.001). Less significant differences were observed for individual anti-TLR2 (p< 0.25), anti-TLR4 (p< 0.05) and anti-CD14 (p< 0.20) antibody treatments. rIgG; rat IgG, mlgG1; mouse IgG1
occurring. Blocking both TLR2 and TLR4 simultaneously (TLR2/TLR4) was much more effective in neutralizing the response (60% reduction). The most effective reduction of Aβ response was observed when all TLR2, TLR4 and CD14 (TLR2/TLR4/CD14 triple combination) were neutralized (71.3% reduction, compared with the mouse IgG1/rat IgG isotype control).

4.2.1.5 TLR1 and TLR6 may also be involved in fibrillar Aβ(1-42)-induced activation of the innate immune response

We have so far demonstrated that TLR2 plays an active role in Aβ-induced initiation of innate immune response. TLR2 forms a TLR2/TLR6 or TLR2/TLR1 heterodimer to recognize diacylated and triacylated lipopeptides (LPT), respectively (Nakata et al., 2006). To assess if TLR2 also requires complex formation with either TLR1 or TLR6 for recognition of fibrillar Aβ, we have included TLR1 and TLR6 antibodies in our TLR neutralization analysis.

To test the effectivity of the added antibodies, we performed neutralization assay using the known TLR2 agonists, Pam3CSK4 (for TLR2/1) or FSL-1 (for TLR2/6) (Figure 4.8). Unlike the previous neutralization experiment for TLR ligands (Figure 4.3 and 4.4), we lowered our TLR antibody concentration to 1 μg/ml in this study to better assess the effect of the antibody neutralization, since using 10 μg/ml of the TLR1 or TLR6 antibody completely eradicated the agonist signals (data not shown). As described in Methods, THP-1 cells were pre-incubated with TLR antibodies or isotype control for 1 hour prior to 6-hour cellular stimulation with Aβ(1-42). Results clearly showed that triacylated
Figure 4.8. TLR2 complex antibody neutralization of known TLR2 agonists, synthetic diacylated or triacylated lipopeptides. THP-1 monocytes were pre-incubated with 1 μg/ml of TLR antibodies (InvivoGen) or its isotype control rat IgG (Sigma) for 1 hour, prior to 6-hour cellular stimulation with either 10 ng/ml of FSL-1 (black bars) or 1 ng/ml of Pam3CSK4 (gray bars). Secreted TNFα was measured by ELISA. Results are presented as % TNFα of FSL-1 or Pam3CSK4 pre-incubated with phosphate-buffered saline (PBS) medium. Error bars represent SE for nine trials. A Student’s t-test was used to calculate the significance between individual TLR and their respective isotype IgG controls ( *p < 0.001)
synthetic Pam$_3$CSK$_4$ activates downstream immune signaling via TLR2/TLR1 heterodimers. A significant 60% and 75% reduction in TNFα response were observed when TLR2 and TLR1 were neutralized, respectively (p< 0.001). Antibody blockade of TLR6 lowered the Pam$_3$CSK$_4$-induced secretion of TNFα by 13% (% response of 86.9 ± 1.9); however, this result was not significantly different from that of its isotype control rat IgG (% response of 87.65 ± 2.4). Likewise, diacylated synthetic FSL-1 utilizes TLR2/TLR6 complex for activation of downstream signaling, as depicted by 71% and 91% reduction in TNFα response when TLR2 and TLR6 were blocked with 1 μg of the antibody, respectively.

Next, the TLR antibody neutralization experiment was utilized to answer the question: is TLR2/TLR1 or TLR2/TLR6 complex formation required for Aβ-induced activation of the innate immune response, or does Aβ interact with TLR2 alone? For this study, we have reverted back to using 10 μg/ml of the TLR antibodies. For the purpose of investigating the importance of TLR2 complex formation, we have included combination neutralization of TLR2/TLR1 and TLR2/TLR6 in our neutralization experiment. The cell treatments with individually blocked TLRs were supplemented with 10 μg/ml of the isotype rat IgG control to match the concentration of double combination TLR2/TLR1 or TLR2/TLR6 with isotype control amounts. Our preliminary results suggest that TLR1 and/or TLR6 may also have an active role in Aβ response (Figure 4.9). Neutralizing TLR2 with 10 μg/ml of the antibody decreased the Aβ-induced %TNFα response to 39.9 ± 2.6 (70% reduction). Surprisingly, blocking either TLR1 or TLR6 also significantly lowered the TNFα response, compared to the isotype rate IgG (p< 0.001). TLR1 was much more effective in neutralizing the Aβ signal than TLR6, as shown by 57%
Figure 4.9. Combination TLR2 complex antibody neutralization of Aβ(1-42)-induced TNFα response. THP-1 monocytes were pre-incubated with 10 μg/ml of TLR2, TLR1, TLR6 (InvivoGen) antibodies, or 20 μg/ml rat IgG isotype control for 1 hour, as described in Methods. Individual cell treatments were also supplemented with 10 μg/ml of rat IgG to match the concentration of the 20 μg/ml rat IgG control. After pre-treatment, THP-1 cells were stimulated with 15 μM of Aβ(1-42) for 6 hours. TNFα was measured using ELISA. Results are presented as % TNFα of Aβ response treated with phosphate buffered saline (PBS) medium. Error bars represent SE for n= 18 trials (6 separate experiments) for TLR2, TLR1, TLR6, and n = 6 trials (2 separate experiments) for TLR2/TLR1 and TLR2/TLR6. A Student’s t-test was used to calculate the significance between individual TLR and the rat IgG isotype control ( *p < 0.001). rIgG; rat IgG
reduction for TLR1 neutralization compared to 43% for TLR6 blockade. The Aβ signal was further decreased when the combination of TLR2/TLR1 or TLR2/TLR6 were blocked. Neutralization of TLR2/TLR1 complex decreased the % response to $24.7 \pm 2.43$, while TLR2/TLR6 complex blockade lowered the % response to $33.77 \pm 2.04$. A slight enhancement of Aβ response was observed for cells pre-incubated with $20 \mu g/ml$ of TLR isotype rat IgG control. Overall, the presented results demonstrated that TLR2 and TLR4 have an active role in Aβ-induced activation of the innate immune response. Additionally, our recent data also suggests a possible involvement of TLR1 and TLR6 as well for Aβ activation of downstream TLR signaling.

4.2.2. Mammalian Cell System: Human Embryonic Kidney (HEK) cells

4.2.2.1 Induction of proinflammatory IL-8 production in transfected HEK 293 cells with known TLR agonists

The main purpose of the study is to determine which TLR is responsible for Aβ-dependent inflammatory response. Using THP-1 monocytes, we have identified TLR2 and TLR4 to have a role in Aβ activation of the innate immune response. Numerous investigators whose focus is to study the mechanisms involved in TLR recognition and signaling utilize model cell lines such as Human embryonic kidney (HEK) cells (Bauer; Chen et al., 2006; Walter et al., 2007; Goodridge and Underhill, 2008). We have started utilizing HEK293 cells to further investigate the role of TLR2 and its complex (TLR2/1 or TLR2/6) in Aβ proinflammatory response. This mammalian cell line does not express
TLR2 and other TLRs (Razonable et al., 2006), although recent studies have shown that HEK293 cells express low amounts of TLR1 and TLR6 (InvivoGen, 2006). We have acquired human HEK293 cells transfected with TLR2 (herein referred to as HEK 293hTLR2) to substantiate the role of TLR2 in Aβ response. For proper interpretation of results, we have also acquired non-transfected HEK293 cells (herein referred to as null HEK293).

We began our investigation by first testing the ability of our known TLR agonists to induce IL-8 production in our HEK293 cell lines (Figure 4.10). HEK 293hTLR2 cells were strongly stimulated to produce IL-8 in the presence of 1 ng/ml of Pam₃CSK₄ (TLR2/1 ligand) or 1 ng/ml of FSL-1 (TLR2/6 ligand) (Figure 4.10b). Expectedly, our ultrapure LPS (TLR4 ligand) failed to stimulate our TLR2 transfected cells in producing IL-8. Null HEK293 cells, on the other hand, were not stimulated by same concentration of the known TLR agonists (Figure 4.10a). The data presented here signify that the acquired TLR2-transfected HEK 293 cells are devoid of any traces of TLR4, and our Null HEK293 cells may be used for succeeding experiments as control cells.

We wanted to know if our TLR neutralization assay can also be applied to our HEK 293hTLR2. HEK 293TLR2 cells were grown, as described in Methods. Cells were pre-treated with 10 μg/ml of the TLR antibodies or isotype rat IgG control for 1 hour prior to 24-hour stimulation with either 1 ng/ml of Pam₃CSK₄ or 1 ng/ml of FSL-1. After post-stimulation, IL-8 levels were measured by ELISA. Our results confirmed the effectiveness of the TLR neutralization assay on this cell line. Antibody blockade of TLR2 or TLR1 decreased the Pam₃CSK₄-induced IL-8 response to 30.9 ± 0.84 and 20.9 ± 1.34, respectively (Figure 4.11). These signify a 48% reduction for TLR2, and 58%
Figure 4.10. PAMP activity on HEK 293 cells. (A) Activity of PAMPs on Null HEK293 cells. Cells were grown, as described in Methods, prior to 24-hour stimulation with known TLR PAMPs. Concentration of the agonists are *E. coli* K12 LPS (10 ng/ml), Pam3CSK4 (1 ng/ml), and FSL-1 (1 ng/ml). (B) Stimulation of TLR2-transfected HEK 293 cells (HEK 293hTLR2) with known TLR agonists. Concentration of agonists is the same as in A. For both instances, secreted IL-8 was measured by ELISA. These results represent 1 representative experiment of 4. Error bars represent SE of n= 3 trials (1 experiment).
Figure 4.11. TLR2 complex antibody neutralization of TLR2 ligands. HEK 293hTLR2 cells were pre-incubated with 10 μg/ml of TLR2, TLR1, TLR6 (InvivoGen) antibodies, or 20 μg/ml rat IgG isotype control for 1 hour, as described in Methods. After pre-treatment, HEK 293hTLR2 cells were stimulated with 1 ng/ml of either Pam3CSK4 or FSL-1 for 24 hours. IL-8 was measured using ELISA. Results are presented as % IL-8 of Pam3CSK4 or FSL response treated with phosphate buffered saline (PBS) medium. Error bars represent SE for n= 6 trials (2 separate experiments) for Pam3CSK4 and n = 3 trials (1 experiment) for FSL-1. rIgG; rat IgG
reduction for TLR1, compared to the rat IgG isotype control. TLR6 blockade evoked a 28% attenuation of Pam3CSK4 response. However, this attenuation was comparable to that of the rat IgG isotype control (22% reduction). Antibody neutralization of the TLR2/TLR6 agonist FSL-1 was clear. TLR2 neutralization almost completely abrogated FSL-1-induced IL-8 response (85% reduction) while TLR6 blockade lowered the FSL-1 response by 57% (% response of 42.8 ± 3.13). TLR1 neutralization did not have an effect on the FSL-1 response. For all neutralization experiments, we have included stimulation of null HEK293 cells with the TLR agonists. Similar to Figure 4.10, 1 ng/ml of Pam3CSK4 or FSL-1 did not induce IL-8 production in null HEK 293 cells.

4.2.2.2 Induction of proinflammatory IL-8 production in transfected HEK 293 cells with fibrillar Aβ(1-42)

To understand the contribution of TLR2 in Aβ-induced immune response, and to assess the responsiveness of the HEK cells to Aβ, we next stimulated our HEK 293 cells with 15 μmol/L of Aβ(1-42). Null HEK 293 cells and HEK 293hTLR2 cells were grown, as described in Methods. After 4 hours of allowing the cells to adhere in the cell culture plate, cells were stimulated with 15 μmol/L of Aβ(1-42) for 24 hours. The IL-8 level was measured post-stimulation by ELISA. As seen in Figure 4.12, TLR2-transfected HEK cells produced a significant amount of IL-8 (81 pg/ml/10⁶ cells ± 1.8), while null HEK cells failed to produce IL-8 upon Aβ stimulation. This result further substantiates our finding that TLR2 plays a role for Aβ-induced proinflammatory production.

We carried out the TLR antibody neutralization to further verify the role of TLR2,
as well as TLR2 complex, in Aβ response. We pre-treated our Null HEK cells and HEK 293hTLR2 cells with 10 μg/ml of TLR antibodies (InvivoGen) or rat IgG isotype control (Sigma) for 1 hour prior to 24-hour cell stimulation with 15 μmol/L of Aβ(1-42). As expected, Aβ treatment did not stimulate our null HEK cells in producing IL-8. Our initial results with the HEK 293TLR2 cells (data not shown) revealed a big reduction in Aβ-IL-8 response when TLR1 was neutralized (70% reduction). A 30% reduction was also observed when TLR2 and TLR6 were blocked. However, there was a substantial attenuation of Aβ response when cells were pre-treated with the isotype IgG control. This issue of reduced Aβ response with IgG control made it impossible to interpret our TLR neutralization results properly. The TLR neutralization of Aβ-induced IL-8 response in HEK 293TLR2 is an ongoing investigation in our lab and the effect of isotype IgG control needs further analysis.

4.3. Discussion

The data that we have presented in this study emphasize the role of toll-like receptors in Aβ-induced activation of the innate immune response. By utilizing THP-1 monocytes which naturally express TLRs ((Faure et al., 2000), we have identified TLR4 and TLR2 to be involved in proinflammatory response initiated by fibrillar Aβ(1-42). These results were consistent with that of Fassbender and colleagues (Fassbender et al., 2004). Using an antibody neutralization assay, they reported a CD14-dependent microglial activation by fibrillar Aβ. Moreover, they presented Aβ activation by nuclear
Figure 4.12. Fibrillar Aβ(1-42) activity on HEK 293 cells. Null HEK293 cells and TLR-2 transfected HEK293 cells (HEK 293hTLR2) were grown, as described in Methods, prior to 24-hour stimulation with 15 μM of Aβ aggregated at 216 hours. Secreted IL-8 was measured by ELISA. This result represent 1 representative experiment of 3. Error bars represent SE of n= 3 trials (1 experiment)
translocation of NFκB in CD14-transfected Chinese hamster ovary (CHO-K1) cells. Since the CHO cells lack a functional TLR2, they suggested that TLR4 may be responsible for Aβ signal transduction.

Our TLR antibody neutralization in THP-1 monocytes clearly demonstrated the importance of TLR4 in Aβ-induced immune response (Figure 4.5, 4.6b, 4.7). A consistent 50-60% reduction of the Aβ-induced TNFα response was observed when cells were pre-incubated with TLR4 antibody. Additionally, we identified TLR2 to be equally responsible for fibrillar Aβ response. In fact, our results showed a more significant reduction of Aβ response from TLR2 antibodies than from TLR4 antibodies (Figure 4.5, 4.6b). We have further substantiated our finding that TLR2 is involved in fibrillar Aβ-induced immune response by using a TLR2-transfected cell line. HEK cells do not express TLR2 (Razonable et al., 2006), thus it is a model system in further clarifying the importance of TLR in agonist signaling. Stimulation of HEK 293hTLR2 with 15 μmol/L of Aβ resulted in the production of proinflammatory IL-8, while the same concentration of Aβ failed to stimulate the Null HEK293 cells for IL-8 production (Figure 4.12). This result implies that the transfected TLR2 gene was responsible for Aβ-induced IL-8 response.

Our results have also indicated that TLR2 and TLR4 may compensate for each other for transduction of Aβ signal when necessary (Figure 4.7). Moreover, we have further demonstrated the importance of CD14 on the Aβ-induced activation of downstream signaling (Figure 4.5, 4.6a, 4.7). One difficulty that we encountered using TLR neutralization of Aβ response is a consistent stimulation of TNFα response for CD14 isotype control, IgG1 (Figure 4.5, 4.6a). This 20-30% increase in signal may have
offset the effectiveness of our anti-CD14 neutralization. These issues emphasize the importance of including the isotype controls in the experiment for proper and accurate interpretation of results.

Several groups have started to focus on Toll-like receptors and its accessory proteins to better understand the mechanism of Aβ inflammatory response or clearance. Using cultured CD14-positive microglia and microglia derived from CD14-deficient mice, Liu et al emphasized the importance of CD14 in Aβ(1-42) phagocytosis by microglia (Liu et al., 2005). In vivo studies using Mo/Hu APPswe PSIdE9 mice deficient in TLR4 showed a decreased clearance of diffuse and fibrillar Aβ deposits, demonstrating the significance of TLR4 signaling pathway in Aβ load and clearance in AD brain (Tahara et al., 2006). Furthermore, using immunohistochemical staining, Liu et al. reported a strong expression of CD14 on brain sections of AD patients as compared to that of control subjects (Liu et al., 2005). Recently, Fassbender’s group also released their findings that TLR4-deficient mice C3H/HeJ strongly inhibited monocytic and microglial activation by aggregated Aβ(1-42) as demonstrated by a significant decrease in the secretion of IL-6, TNFα and nitric oxide compared to the wildtype C3H/HeN (expressing TLR4) mice (Walter et al., 2007). This result further supported our findings of TLR4-mediated Aβ proinflammatory response. Similarly, findings by Jin, et al. showed that TLR4 was also implicated in the upregulation of proinflammatory (TNFα, IL-1β, IL-10, IL-17) products in the brains of TLR4 wildtype AD mice as compared to TLR4 wildtype non-transgenic mice (Jin et al., 2008). Recently, Rivest and colleagues have also published reports implicating TLR2 an endogenous receptor that is involved in clearance of Aβ(1-42) (Richard et al., 2008). Overall, our findings, together with these other
studies, contributed to growing evidences linking neurodegenerative diseases with innate immune response.

Investigating the ligand-TLR interaction means that utmost care should be taken to ensure that the agonists are free of contamination that might falsely activate certain TLRs (Kielian, 2006). Over the years, the purity of TLR agonists has been an ongoing issue in investigating agonist-TLR interaction (Hirschfeld et al., 2000; Gao et al., 2001; Lee et al., 2002). We have carefully prepared our Aβ(1-42) samples and made sure that our preparations are free of contaminating traces of LPS. Although the commercial Aβ lots were already endotoxin-tested prior to shipment (0.35 EU/mg, which corresponds into an effective LPS concentration of 8 pg/ml) (Gao and Tsan, 2003), we still have continuously monitored our Aβ preparations using PMX-B. PMX-B is often used in cell culture systems to test for LPS contamination (Weaver et al., 2007). Our Aβ preparations were devoid of any traces of bacterial LPS contamination, as depicted by Figure 4.2b. XTT proliferation assay was also done routinely to verify the purity of our Aβ preparations. Moreover, the possibility of contamination in our Aβ preparation was invalidated by our Aβ aggregation data (Figure 3.1) which demonstrated a steady increase of TNFα production when Aβ was aggregated at 4°C for up to 96 hours, followed by a decline of the TNFα signal to baseline level when Aβ was incubated for longer period of time (216 hours). This trend would not be observed if traces of contaminating TLR ligands are present in our Aβ preparation. Degradation of the contaminants is also not expected, thus, if there is a presence of any traces of bacterial contaminants, the Aβ-induced TNFα signal should remain at 216 hours.

Our preliminary results with THP-1 monocytes (Figure 4.9) also suggest a
possible role for TLR1 and TLR6 in Aβ activation of the innate immune response. Our laboratory, and others, have demonstrated the importance of TLR2 in Aβ-induced immune response (Jana et al., 2008; Richard et al., 2008; Udan et al., 2008). TLR2 is known to form a heterodimeric complex with either TLR1 or TLR6 for recognition of a wide spectrum of ligands (Schroder et al., 2004; Manukyan et al., 2005; Kielian, 2006). Our TLR neutralization revealed a significantly decreased TNFα response for Aβ when TLR1 and TLR6 were blocked. Interestingly, TLR1 seems to have more effect in Aβ response than TLR6, as demonstrated by greater inhibition with 10 μg/ml of TLR1 antibody as compared to TLR6. We wanted to further investigate the possibility of TLR2 complex formation for recognition of Aβ by doing antibody neutralization on TLR2-transfected HEK293 cell line. We are on our preliminary stage of investigation using HEK 293 cells, however, rat IgG effects on the Aβ response have been encountered and are being carefully assessed. Nevertheless, understanding the role of TLR1 and TLR6 in Aβ interaction with TLR2 will give us further insight on Aβ-TLR2 recognition.

Numerous investigations about structures of TLR-ligand complexes have continuously emerged over the years to better understand their activation mechanisms. Previous report about the crystal structure of TLR1-TLR2-Pam₃CSK₄ emphasizes the importance of the lipid chains of Pam₃CSK₄ in its interaction with the TLR2/TLR1 heterodimers (Jin et al., 2007). The crystal structure showed the interaction of two of the three lipid chains of Pam₃CSK₄ with TLR2 pocket, and the last lipid chain is inserted into a hydrophobic channel of TLR1. Likewise, structure studies of LPS-TLR4-MD2 binding were done using LPS antagonist Eritoran. LPS is an amphipathic macromolecule composed of hydrophobic lipid A, composed of four to seven acyl chains and
phosphorylated di-glucosamine, core and O-antigen (Chapter 1, Figure 1.5). Eritoran, on the other hand, is a structural mimic of lipid A of LPS, with four acyl chains and a phosphorylated glucosamine backbone (Mullarkey et al., 2003; Rossignol and Lynn, 2005). Structural studies of Eritoran-MD2-TLR4 shows binding of Eritoran to the hydrophobic pocket of MD-2 via its acyl chains (Kim et al., 2007). Moreover, a crystal structure of mouse CD14 shows a large hydrophobic pocket on the N-terminal which was identified to be the main component of the LPS-binding site (Kim et al., 2005). A structural model of Aβ fibrils by NMR spectroscopy revealed a double-layered β sheet structure with a hydrophobic core and a hydrophobic face. It is thus possible that hydrophobicity plays a big role in ligand-TLR recognition, and the hydrophobicity possessed by Aβ may also be responsible for its recognition by CD14, TLR4 and TLR2.

The association of Aβ with inflammation is ongoing research in the field of AD. It remains to be determined whether inflammatory response is advantageous or detrimental to neuron survival. The identification of several receptors on microglia and monocytes that recognize fibrillar Aβ has opened new venues for understanding the mechanism of Aβ and inflammatory response. The inclusion of TLRs in the list of receptors that recognize Aβ contributes to the role of innate immunity in the pathogenesis of AD, and therefore may be a powerful tool for identification of therapeutic targets that slow the progression of AD.
4.4. Bibliography


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5 THE ROLE OF MONOCYTE MATURATION AND ITS RELATIONSHIP TO AMYLOID BETA AND INFLAMMATION

5.1 Introduction

The presence of activated microglia surrounding neuritic plaques in the AD brain strongly suggests a specific interaction between Aβ and microglia. Moreover, numerous evidence now shows that microglia may be activated by Aβ, leading to initiation of inflammation (Frautschy et al., 1992; Barger and Harmon, 1997; Akama et al., 1998; Hu and Van Eldik, 1999). A plethora of inflammatory products in the brain upon microglial activation may encourage the transmigration of monocytes from the circulation across the blood-brain barrier. Thus, the peripheral monocytes, which differentiate into macrophages during the infective process, may also be present as infiltrated phagocytes along with the resident microglia surrounding the senile plaques (Fiala et al., 1998). Previous reports have shown that Aβ modulates monocyte adhesion (Yan et al., 1996) and differentiation to macrophages (Fiala et al., 1998). Our recent finding demonstrating oligomeric Aβ(1-42)-induced THP-1 monocyte maturation and adhesion was consistent with these previous reports (Crouse et al., 2009). Furthermore, additional studies have documented monocyte/macrophage infiltration to sites of brain Aβ accumulation (Simard et al., 2006; El Khoury et al., 2007). Macrophages play an important role in inflammation through production of proinflammatory cytokines and chemokines, cell adhesion molecules and nitric oxide (NO), among others (Kim et al.,
Thus, the presence of macrophages in the brain may also contribute to the exacerbation of inflammation induced by Aβ.

To study the contribution of macrophages in Aβ-induced immune response, we have modeled the macrophages present in the brain by differentiating our THP-1 monocytes using phorbol myristate acetate (PMA) (Tsuchiya et al., 1982). We have chosen TNFα production as the outcome variable for studying effect of macrophages in Aβ-induced proinflammatory production. In this investigation, we report that differentiation of THP-1 monocytes to macrophages significantly enhanced TNFα production by fibrillar Aβ(1-42), and may further contribute to inflammation in the diseased brain.

5.2. Results

5.2.1 TNFα production induced by known TLR agonists in differentiated and undifferentiated THP-1 cells

For our initial investigation, we examined the proinflammatory response of PMA-differentiated THP-1 macrophages to known TLR agonists. THP-1 monocytes grow in suspension and they do not adhere to the surfaces of the cell culture plates. For the induction of differentiation to macrophage-like cells, THP-1 monocytes were treated with 10 ng/ml of PMA for 24 hours, as described in Methods. After incubation with PMA, THP-1 cells became adherent and developed morphological changes characteristics of differentiation to macrophages. The extent of cell adherence was
measured by direct counting of the adherent cells and dividing it by the total number of plated cells. Cells that were used for subsequent experiments have % adherence values of 75% and above. Cells that were poorly adherent were not used. Once the % adherence was measured, cells were treated with either ultrapure K12 LPS (TLR4 agonist) or Pam3CSK4 for 6 hours. Figure 5.1 shows the comparison of TNFα secretion for THP-1 monocytes or PMA-induced macrophages that were stimulated with 10 ng/ml of ultrapure K12 LPS. Although undifferentiated THP-1 cells produced TNFα upon stimulation with ultrapure LPS, differentiation of monocytes to macrophages substantially elevated the LPS-induced TNFα response five-fold. Moreover, PMA-differentiated THP-1 macrophages responded to LPS and Pam3CSK4 (TLR2 agonist) stimulation in a dose-dependent manner (Figure 5.2). Our data is consistent with that of Takashiba (Takashiba et al., 1999). The extent of TNFα secretion varies from one experiment to another. This explains the different levels of TNFα when PMA-differentiated THP-1 monocytes were stimulated with 10 ng/ml LPS (Figure 5.1 and 5.2).

5.2.2 Aβ-induced TNFα production is augmented in PMA-derived THP-1 macrophages

We next compared the proinflammatory response of Aβ(1-42) in PMA-differentiated THP-1 cells to that of undifferentiated THP-1 cells. We incubated our THP-1 monocytes and PMA-differentiated THP-1 cells with 15 μmol/L of Aβ(1-42) aggregated at 4°C for 48 hours. At various incubation times, secreted TNFα was
Figure 5.1. LPS-induced TNFα production from differentiated and undifferentiated THP-1 cells. THP-1 monocytes were differentiated for 24 hours using phorbol-myristate acetate (PMA), as described in the Methods. After incubation, undifferentiated cells (THP-1 monocytes, black bars) and PMA-differentiated cells (macrophages, gray bars) were stimulated with 10 ng/ml of LPS for 6 hours. TNFα was measured in the supernatant after stimulation by ELISA. This result is a representative experiment of 3. Error bars denote n = 3 trials (1 experiment)
Figure 5.2. Dose response of TNFα production by known TLR agonists. THP-1 monocytes were differentiated using 10 ng/ml PMA, as described in Methods. After differentiation, PMA-differentiated cells were treated with increasing concentration of (A) K12 LPS (TLR4 agonist) or (B) Pam3CSK4 (TLR2 agonist) for 6 hours. TNFα was measured using ELISA. This result represents 1 representative experiment of 2. Error bars represent n = 3 trial (1 experiment).
measured. Figure 5.3a demonstrates that Aβ(1-42) stimulated THP-1 monocytes (circle) for TNFα production as early as 4 hours incubation (18.9 ± 2.9 pg/ml), with maximal response observed at 10 hours (also shown in Figure 3.3a). In comparison, PMA-differentiated THP-1 (triangles) cells began producing considerable TNFα at 6 hours of incubation (27 ± 0.53 pg/ml) and the TNFα continued to rise even at 24 hours post-stimulation. At this time (24 hours), Aβ-induced TNFα production is three-fold higher in PMA-differentiated cells than undifferentiated cells. Like the response of differentiated THP-1 cells to known TLR agonists, PMA-differentiated THP-1 cells were also stimulated by Aβ(1-42) in a dose-dependent manner (Figure 5.3b). These results demonstrate a more enhanced production of proinflammatory products when macrophages are stimulated by fibrillar Aβ(1-42).

5.3 Discussion

Differentiation of THP-1 monocytes to macrophages with phorbol esters have been well-characterized (Tsuchiya et al., 1982; Auwerx, 1991; Takashiba et al., 1999; Traore et al., 2005). One of the most widely-used differentiating agent is 4α-phorbol-12-myristate-13-acetate (PMA) (Chong et al., 2003; Lai et al., 2006). In this study we have analyzed the contribution of macrophages in Aβ-induced proinflammatory production. We have shown in this preliminary investigation that TNFα production is considerably increased when PMA-derived THP-1 cells were stimulated with known TLR agonists LPS(TLR4) and Pam3CSK4 (TLR2) as compared to undifferentiated cells. Similar results were observed when PMA-differentiated cells were stimulated with 15µM of Aβ(1-42).
Figure 5.3. Fibrillar Aβ(1-42)-induced TNFα secretion from differentiated and undifferentiated THP-1 cells. (A) THP-1 monocytes (circles) or PMA-differentiated THP-1 macrophages (triangles) were stimulated for 0, 2, 4,6,10 and 24 hours with 15 μM Aβ(1-42). After post-stimulation, TNFα was measured using ELISA. (B) Dose response of TNFα secretion. THP-1 monocytes were differentiated with 10 ng/ml PMA, as described in Methods. PMA-derived THP-1 macrophages were stimulated with increasing concentration of Aβ(1-42) for 24 hours. TNFα level was measured by ELISA. For both experiments, error bars represent n = 3 trials (1 experiment).
Takashiba et al investigated the relationship between THP-1 cell maturation and mechanism of LPS stimulation. They revealed the novel role for NF-κB in the maturation process. They found that differentiation of THP-1 monocytes to macrophages results in accumulation of NF-κB in the cytoplasm, which is mainly responsible for the enhanced ability of the cell to respond to LPS stimulation (Takashiba et al., 1999). This suggests that accumulation of NF-κB in the cytoplasm upon maturation of monocytes to macrophages primes the cells for increased responsiveness to LPS and in turn, leads to rapid secretion of inflammatory mediators.

Recent reports showed that cytokine gene transcription by Aβ requires stimulation of NF-κB pathway (Combs et al., 2001). The accumulation of NF-κB in the cytoplasm during the differentiation process may thus be correlated to enhanced production of TNFα upon Aβ stimulation. Another possibility for elevated TNFα production in macrophages is the constitutive expression of TLRs. Although the level of TLR2 in macrophages that were derived in vitro is similar to that of monocytes, TLR4 expression was significantly increased by about 300% in macrophages when compared to monocytes (O'Mahony et al., 2008). We have previously demonstrated the role of TLR2 and TLR4 in Aβ-induced inflammatory production (Udan et al., 2008) (Chapter 4). A combination of elevated TLR and NF-κB expression in macrophages may be responsible for enhanced proinflammatory production by Aβ.

Several studies have shown a correlation between Aβ accumulation and infiltration of peripheral blood monocytes/macrophages in senile plaques (Fiala et al., 1998; Simard et al., 2006; El Khoury et al., 2007). Oligomeric Aβ(1-42) has likewise been reported to have chemotactic activity (Giri et al., 2000; Le et al., 2001). Moreover,
our lab has reported that oligomeric Aβ(1-42) aggregates induce THP-1 monocyte differentiation to macrophages (Crouse et al., 2009). These evidences suggest that oligomeric Aβ(1-42) may also induce recruitment of blood-derived macrophages to the site of inflammation.

The correlation between Aβ and inflammation in AD brain is an active focus of investigation. Our preliminary data suggests that Aβ-induced heightened inflammation may be mediated by TLRs from resident activated microglia as well as infiltrating macrophages that may also be present surrounding the senile plaque. Further investigations need to be performed to further clarify the mechanism of Aβ-induced immune response and to better understand the contribution of immune cells in AD pathogenesis.
5.4 Bibliography


6 CONCLUSION

In these studies, we successfully demonstrated that Aβ(1-42) invoked proinflammatory response in human THP-1 monocytes/ macrophages, a mammalian cell model system for human microglia. Moreover, the data presented suggest that the ability of Aβ to induce TNFα production is dependent on its aggregation conformation. Using a combination of AFM and cellular studies, along with high speed centrifugation of Aβ samples and employing conformation-specific antibodies, we reported that the soluble yet fibrillar Aβ species are the bioactive Aβ. These Aβ species exist prior to formation of longer, more mature fibrils, and are thus called fibrillar precursors.

Studying Aβ fibrillogenesis is a very challenging task. Although microscopy (AFM and EM) are valuable tools in studying the morphology of the formed Aβ species, this technique alone could not provide detailed information of the fibril size for different aggregation species nor is appropriate for real time analysis. In order to have a better understanding of the bioactive Aβ(1-42) species, the use of other biophysical techniques that will further provide vital information such as molecular weight and conformation, along with the microscopy studies, may aid in elucidating the Aβ(1-42) species that can activate our THP-1 cells.

We also showed the correlation between Aβ and the innate immune response by identifying the involvement of toll-like receptors, particularly TLR4 and TLR2, in Aβ-induced response. Our findings further suggest that TLR4 and TLR2 may compensate for
one another for Aβ-induced activation of TLR downstream signaling. Moreover, the possibility of Aβ utilizing TLR2/TLR1 or TLR2/TLR6 complex was also suggested based on a significant neutralizing effect of the TLR1 and TLR6 antibodies on Aβ response. The possible involvement of the TLR2/1 or TLR2/6 in Aβ-induced immune response is a good area to follow up on for the information that can be obtained will give us further insight on Aβ-TLR2 recognition. Aside from the TLR neutralization assay, the role of TLRs in Aβ-induced inflammatory response may be confirmed by other methods such as using cell lines transfected with TLR as well as utilizing TLR knockout mice. These are valuable tools for studying the ligand-TLR interaction.

Our results also showed that Aβ-induced TNFα production for PMA-induced THP-1 macrophages is undoubtedly much higher compared to that of THP-1 monocytes. This suggests that infiltrating macrophages that may be present surrounding the senile plaque may also contribute to heightened inflammation that is observed in the AD brain.

Overall, we presented evidences suggesting that inflammation in the AD brain is induced by soluble yet fibrillar species of Aβ(1-42), and the heightened inflammation observed in microglia surrounding the AD senile plaques may in part be due to the contribution of Aβ interaction with TLR2 and TLR4 that are expressed on microglia, as well as the infiltrating macrophages that may be present in the site of injury. This study has thus opened new venues for understanding the mechanism of Aβ-induced inflammatory response and may be a new therapeutic target for AD.
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