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# Toll-like receptor 6 in Alzheimer's disease

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BOSTON UNIVERSITY  
SCHOOL OF MEDICINE

Thesis

**TOLL-LIKE RECEPTOR 6 IN ALZHEIMER'S DISEASE**

by

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B.S., Temple University, 2011

Submitted in partial fulfillment of the  
requirements for the degree of  
Master of Arts  
2013

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## **ABSTRACT**

Increasing evidence suggests that much of the neurotoxicity seen in Alzheimer's disease is due to activation of pro-inflammatory cascades of microglia upon binding to Amyloid- $\beta$  peptide ( $A\beta$ ). Such cascade also promotes  $A\beta$  production and reduces  $A\beta$  clearance leading to increased  $A\beta$  accumulation. As these self-damaging processes participate in the progression of Alzheimer's, research has been aimed at understanding how such activation occurs. Previous studies have shown that different receptors of the innate immune system bind to  $A\beta$  and activate distinctive signaling cascades in microglia. One way by which microglia recognize  $A\beta$  is through a heterodimer of three cell-surface receptors, Toll Like Receptor 4 (TLR4), Toll Like Receptor 6 (TLR6) and the class B scavenger receptor type II. In this study, we tested the hypothesis that TLR6 deficiency is associated with reduced  $A\beta$  accumulation. For this purpose, we quantified fibrillar  $A\beta$  deposits (plaques) in the 5xFAD mouse model of Alzheimer's disease in comparison to 5xFAD mice with partial deficiency in TLR6 at the age of 5

months. Our data show that 5xFAD mice heterozygous for TLR6, exhibit a statistically significant reduction in the total number of plaques and in the surface area covered by such plaques in the cortex. Additional research is needed to confirm these results and provide a mechanistic explanation for these findings.

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## List of ABBREVIATIONS

ACh	Acetylcholine
AD	Alzheimer's Disease
A $\beta$	Amyloid $\beta$
APC	Antigen Presenting Cell
APP	Amyloid Precursor Protein
BBB	Blood Brain Barrier
CD36	Class B Scavenger Receptor Type II
CNS	Central Nervous System
EOFAD	Early Onset Familial Alzheimer's Disease
IL-1 $\beta$	Interleukin-1 $\beta$
LOAD	Late Onset Alzheimer's Disease
LRR	Leucine Rice Repeat
MAL	MyD88 Adaptor Like Protein
MyD88	Myeloid Differentiation Factor 88
NF- $\kappa$ B	Nuclear Factor $\kappa$ Light Chain Enhancer of Activated B Cells
NO	Nitrogen Monoxide
PAMP	Pathogen Associated Molecular Pattern
PRR	Pattern Recognition Receptor
PS	Presenilin
ROS	Reactive Oxygen Species

SARM	Sterile $\alpha$ - and Armadillo-Motif-Containing Protein
SR-A	Class A Scavenger Receptor
TF	Transcription Factor
TIR	Toll-Interleukin 1 Receptor
TLR	Toll Like Receptor
TNF $\alpha$	Tumor Necrosis Factor $\alpha$
TRAM	Toll Receptor Associated Molecule
TRIF	TIR-Domain-Containing Adapter-Inducing Interferon $\beta$

## Introduction

Of the vast number of diseases affecting the elderly, none have impacted as many as Alzheimer's disease (AD), which affected nearly 36 million people in 2010 (Honjo, 2012). Patients suffering from AD, a neurodegenerative disorder, exhibit progressively worsening dementia including memory loss, impaired judgment, physical orientation and language (Candore, 2007). As modern medicine improves, the average life expectancy increases. As this happens, the prevalence of AD will also increase. In 2000, the Baltimore Longitudinal Study of Aging patients, which includes 1236 volunteers, revealed that 155 individuals had some form of dementia, of which 114 was AD (Kawas, 2000). This study also showed that incidence rate of AD rose from .08% per year in the 60-65 year old age group to approximately 6.5% in the 85+ group (Kawas, 2000). A prospective cohort study of 2581 patients (10,591 person-years) demonstrated that incidence rates triple between the ages of 75 and 79 as well as between 80 and 84, and then increases at a much smaller rate (Kukull, 2002). Additionally, an inverse relationship was seen between baseline cognitive level, and total years of education, and the risk and age of onset of AD (Kukull, 2002). Current studies project that by 2030, 66 million people worldwide will be living with dementia, and by 2050, that number will be 115 million (Bettens, 2013). In 2010, the cost of dementia treatment was an estimated \$604 billion, of which 70% was in North America and Western Europe (Alzheimer's Disease International). Several

retrospective studies suggest that anti-inflammatory drugs can reduce the risk of getting AD and delayed onset (Tahara, 2006; McGeer, 1990; Breitner, 1994; Breitner, 1995; Stewart, 1997; Szekely, 2004).

Neuropathologically, patients exhibit an accumulation of extracellular  $\beta$ -Amyloid protein ( $A\beta$ ) senile plaques, intracellular neurofibrillary tangles composed of  $\tau$  protein, activated immune cells including microglia and astrocytes, and neuronal degeneration, primarily in the hippocampus and frontal cortex (Wang, 2011). As such,  $A\beta$  senile plaques and microglial activation by  $A\beta$  is a pathological hallmark of AD (Hickman, 2008). Chemical studies of brains of patients suffering from AD revealed remarkably low levels of the neurotransmitter Acetylcholine (ACh) due to depletion of the enzymes responsible for its formation (Davies, 1976). This evidence suggested that the pathological alterations in AD occur primarily in the hippocampus and the frontal cortex of the brain, two areas that rely heavily on ACh (Selkoe, 2002). In fact, it has been shown that  $A\beta$  activates the Mitogen-activated protein kinase cascade, a pro-inflammatory cellular response, in the hippocampus (Dineley, 2001). Recent evidence also demonstrated elevated oxidative stress, a result of increased inflammation, in the hippocampus and frontal cortex as compared to other brain areas (Venkateshappa, 2012). Of note, research has shown that AD pathologically involves neuronal loss as well as synaptic loss (Horn, 1996; Ruppin, 1995). Furthermore, the loss of neurons does not fully account for the synaptic failure,

suggesting that the loss of synapses in itself plays a major pathological role in AD progression (Small, 2001).

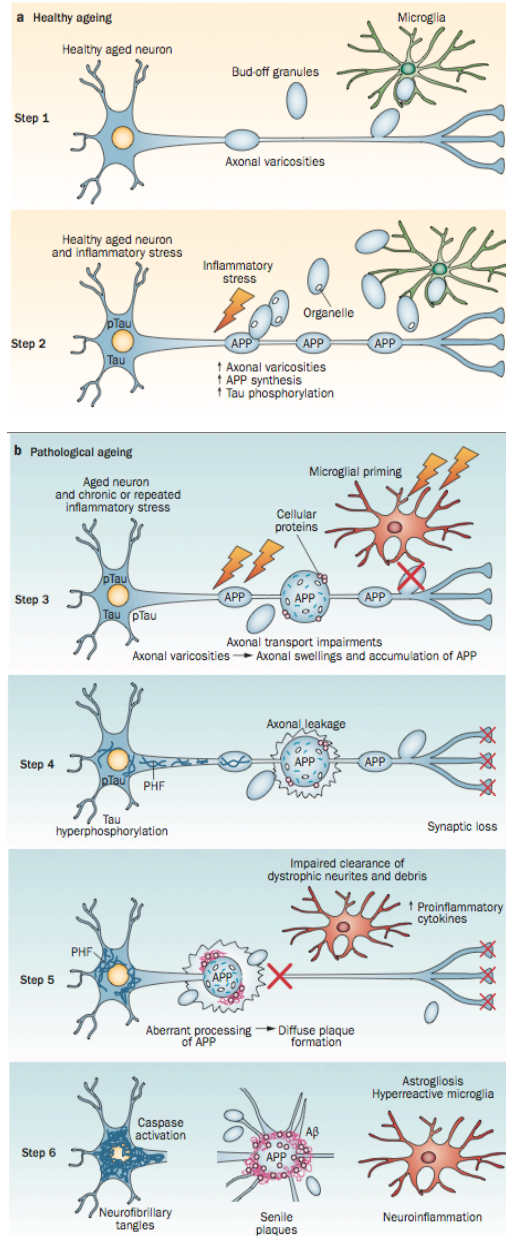
While A $\beta$ -induced activation of microglia causes phagocytic clearing of A $\beta$ , it also stimulates the microglia to release the respiratory burst molecules, resulting in negative neurotoxicity. One method of microglial activation is through the Toll Like Receptor (TLR), an innate immune Pattern Recognition Receptor (PRR) protein that recognizes ligands of various pathogens. Studies have shown that TLR4, as well as the possibility of TLR2, is involved in the neuroinflammation that accompanies AD (Walter, 2007).

### **Pathophysiology**

Completely understanding the pathology of AD in humans is difficult, as the tissue can only be analyzed upon autopsy. Among the known triggers for AD, one is a dysfunctional gene producing A $\beta$  (Wenk, 2003). Although the function of Amyloid  $\beta$  Precursor protein, a transmembrane protein expressed in large numbers in synapses of neurons in the brain, is unknown, it is suspected to be involved in cell-cell communication, cell adhesion, platelet aggregation and a vast range of neuronal actions including neuroprotection, memory formation and synaptic transmission (So, 2013; Aydin, 2012; Bush, 1990; Soba, 2005).

During post-translational processing, Amyloid Precursor Protein (APP) can be proteolytically cleaved by several proteases in two different pathways. In one pathway, APP is cleaved by  $\beta$ -secretase followed by the  $\gamma$ -secretase protein complex, forming A $\beta$ , a 40-42 amino acid long peptide (Portelius, 2011). In AD,

elevated production of this protein, as well as misfolding of A $\beta$ , eventually forms dense, insoluble plaques, with an A $\beta$  core surrounded by neuronal processes, including microglia, around neurons. A second driving force for AD involves the phosphorylation of a  $\tau$  protein complex, which stabilizes microtubules and thus cytoskeleton. This hyperphosphorylation results in a formation of  $\tau$  protein tangles. The build-up of A $\beta$  leads to hindered synaptic transmission and, eventually, apoptosis of cells through the innate immune system. This immune response in turn causes inflammation, which will result in further neuronal death. As is seen in Figure 1a, normal ageing involves some amount of elevated  $\tau$  phosphorylation, APP production and inflammatory response as neurons age and undergo apoptosis. However, Figure 1b demonstrates that in patients suffering from AD, decreased clearance of A $\beta$  results in elevated expression of this protein, which, along with chronic inflammation, results in microglia activation, further intensifying the neuroinflammation, and defective axonal movement, resulting in hindered synaptic transmission. All the while,  $\tau$  protein is continually being hyperphosphorylated further aggravating the axonal transport. Eventually, the hyperphosphorylation of  $\tau$  and the uncontrolled production and processing of APP results in intracellular neurofibrillary tangles, composed of hyperphosphorylated  $\tau$ , and extracellular senile plaques composed of A $\beta$ , microglia, neurons and cellular debris that cannot be properly removed by the impaired microglia.



**Figure 1: Healthy versus Alzheimer's Disease Ageing.** Pathological Ageing involves the same processes seen in healthy patients in an uncontrolled manner, which results in the inflammatory response.  $A\beta$  accumulation from APP processing is one major pathological alteration that results in plaques, and  $\tau$  hyperphosphorylation results in neurofibrillary tangles hindering cellular function. Figure taken from Krstic, 2013.

AD arises in two forms, familial and sporadic AD (Bettens, 2013). In Early Onset Familial AD (EOFAD), genetic mutations in the APP gene can result in intensified production of all forms of the protein, or just  $A\beta_{42}$ , results in early-onset of dementia and AD before the age of 65 (So, 2013; Selkoe, 2004). The most common variation of these mutations, about 90% of early-onset AD, is a missense mutation in the transmembrane protein Presenilin 1 (PS1), and PS2 to a lesser extent. PS1 is part of  $\gamma$ -secretase and works with  $\beta$ -secretase to create  $A\beta_{42}$  (Braga-Neto, 2013). EOFAD comprises 5% of AD cases in hospitals and includes about 230 different mutations in genes coding proteins involved in APP synthesis and cleavage (Wu, 2012; Cruts, 2012; Lleó). Interestingly, increasing research suggests that although the Amyloid cascade is responsible for EOFAD, another mechanism is responsible for sporadic, late-onset AD (LOAD) (Krstic, 2012; Herrup, 2010). One factor that strongly implicated in the cause of sporadic LOAD is the presence of the  $\epsilon 4$  allele of Apolipoprotein E (Krstic, 2013; Corder, 1993).

### **Innate Immune System**

The innate immune system is one of two lines of defense in the body, and protects the host by differentiating between molecular structures that are, and are not, present when healthy. To detect these changes, the innate immune system relies on PRRs on various immune cells circulating in the body. One of these cells, monocytes, differentiates in different body tissues into specialized cells. In the brain and the rest of the central nervous system (CNS), disease or insult will

cause monocytes to develop into microglia and act as the main immune defense. Because the Blood Brain Barrier (BBB) separates the brain from the rest of the body, most infections cannot reach the CNS. As such, immune cells of the CNS are much more specialized, and scavenge for plaques and damaged neurons. Additionally, microglia must find antigens, engulf them, and use cytokines to act as antigen presenting cells (APCs) for T lymphocytes, the cytotoxic part of the adaptive immune system, since most antibodies are not small enough to cross the BBB. Once a microglia is activated it will begin the respiratory burst, releasing the reactive oxidative species (ROS) hydrogen peroxide, superoxide radicals and nitrogen monoxide (NO), pro-inflammatory cytokines and lysing enzymes (Khanna, 2001). While this burst is often mandatory for damage repair and antigen removal, it can paradoxically cause neuronal cytotoxicity and death.

### **Alzheimer's Disease and Immune Response**

In AD, microglia detect and bind to A $\beta$  deposits, phagocytize the protein and activate the microglia resulting in a release of respiratory burst molecules and lytic enzymes. While some of these pathways are beneficial, the release of ROS, NO and lytic enzymes has been linked to the neurotoxic effects of AD. One activated molecule, Interleukin-1 $\beta$  (IL-1 $\beta$ ), is critical in the inflammatory response of microglia to A $\beta$ , and is often seen elevated in brains of containing A $\beta$  (Halle, 2008; Heneka, 2007). Microglia are found in large numbers in plaques of late AD patients and models (Hickman, 2008). Data show that decreased levels of microglia in the early stages of AD development leads to elevated A $\beta$  protein

levels, increased plaque numbers and early mortality (El Khoury, 2007). This data supports the hypothesis that microglia provide a neuroprotective role in AD.

Interestingly, some data suggest that as mice age, microglia become deficient in expression of A $\beta$  receptors and A $\beta$ -degrading enzymes, but maintain production of pro-inflammatory cytokines, thus shifting to a pathology-inducing state (Hickman, 2008). To detect plaques, microglia express receptors such as class A scavenger receptor (SR-A) and class B scavenger receptor type II, or cluster of differentiation 36 (CD36), two PRRs that, when bound to an antigen such as A $\beta$ , activate different pathways. SR-A stimulates microglial phagocytosis of A $\beta$  without inducing microglial activation. In contrast, evidence suggests that CD36 is responsible for microglial activation upon A $\beta$  binding, resulting in pathogenesis (El Khoury, 2003). Indeed, El Khoury et al. showed that CD36 deficient microglia exhibit greatly reduced levels of some pro-inflammatory cytokines, including Tumor Necrosis Factor  $\alpha$  (TNF $\alpha$ ) and Interleukin 1 $\beta$  IL-1 $\beta$  (El Khoury, 2003).

CD36 is a PRR that can bind multiple ligands, including from pathogen or host. CD36 has been implicated in the *Staphylococcus aureus*-induced activation of TLR2/6 signaling (Stuart, 2005). By studying CD36<sup>+/-</sup> *Drosophila melanogaster*, Stuart et al. demonstrated that phagocytosis of *S. aureus* requires CD36 to activate the TLR2/6 heterodimer signaling pathway, and that the absence of CD36 in *S. aureus*-positive flies led to extreme bacteremia. Of note, recent evidence has shown that CD36 forms another dimer with TLRs 4 and 6

(Stewart, 2010). In this study, Stewart et al. studied the role of CD36, TLR4 and TLR6 in A $\beta$ -induced microglia activation of Nuclear Factor  $\kappa$  Light Chain Enhancer of Activated B Cells (NF- $\kappa$ B), a protein responsible for the production of many pro-inflammatory cytokines such as IL-1 $\beta$  (Mori, 1996; Libermann, 1990). Stewart et al. discovered that macrophages deficient in CD36, or a CD6-TLR4/6 dimer failed to activate NF- $\kappa$ B, whereas an increase in CD36 expression in cells caused a two-to-three-fold increase of TLR4/6 signaling. Additionally, it was seen that concurrent expression of all three components, TLR 4, TLR6 and CD36, was required for maximal immune response to A $\beta$  (Stewart, 2010), suggesting that a signal complex exists between the three, and it regulates the response of the innate immune system to endogenous ligands, whereas the CD36-induced activation of TLR2/6 signaling was triggered by exogenous bacterial ligands. Therefore, Stewart et al. generated mice deficient in CD36, TLR4, TLR6 or TLR2 and observed the inflammatory response to A $\beta$ . As hypothesized, mice lacking CD36, TLR4 or TLR6 presented a reduced inflammatory response, including reduced release of the respiratory burst, in response to A $\beta$ , whereas TLR2<sup>+/-</sup> mice exhibited an inflammatory response equal to wild-type mice, providing further evidence that a CD36-TLR4-TLR6 complex mediates the innate immune response to host ligands. Interestingly, Stewart also found hindered levels of IL-1 $\beta$  in TLR4<sup>+/-</sup>, TLR6<sup>+/-</sup>, and CD36<sup>+/-</sup> mice, implicating this signaling complex in this cytokine's activation and release.

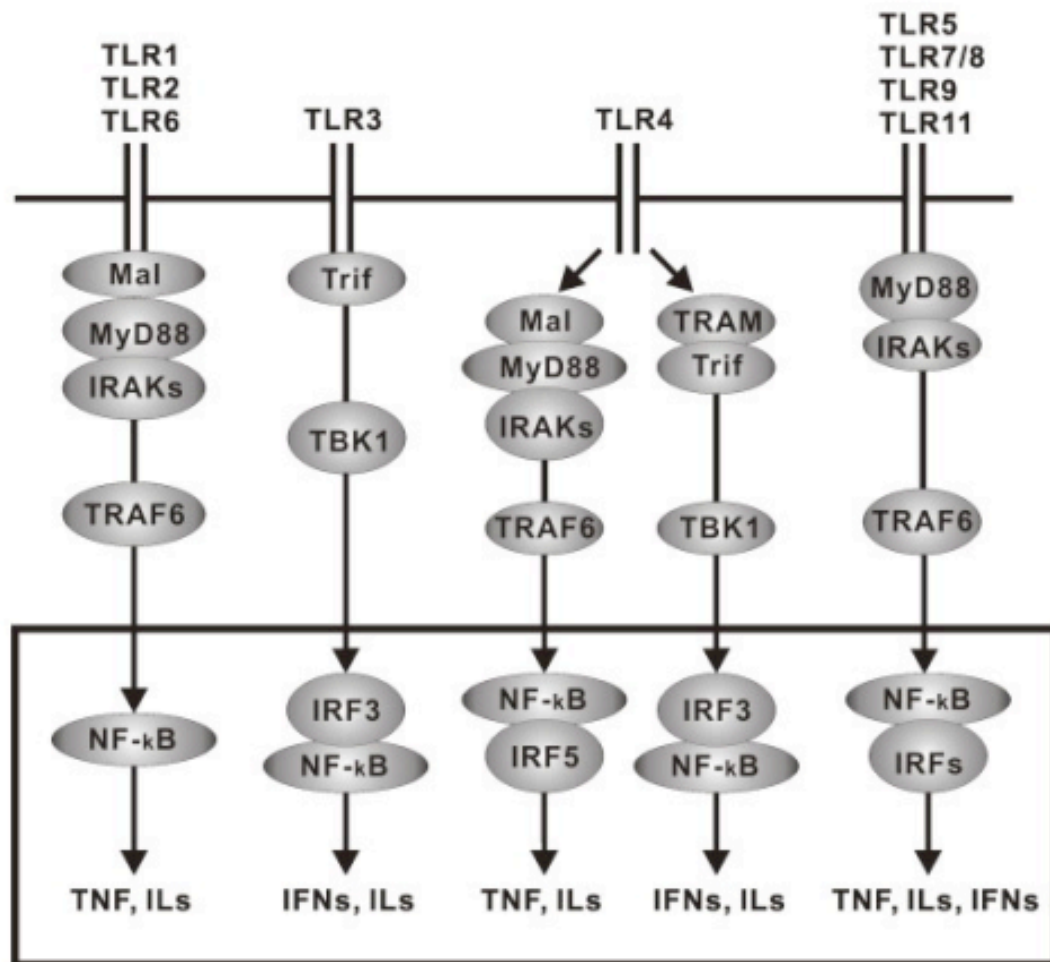
## **Toll-Like Receptor 6**

Microglia rely on TLRs, another set of PRRs, to aid in detection of pathogen-associated molecular patterns (PAMPs) including bacteria, fungi, viruses and even some host molecules (Bell, 2003). After the discovery of the first TLR (Nomura, 1994), studies have demonstrated the vital role they play in regulation of genes critical for host defense. Currently, there are 10 known human TLRs, all containing an extracellular leucine-rich-repeat (LRR) structural motif and cytoplasmic receptor, Toll-Interleukin-1 receptor (TIR), responsible for signal initiation (Stewart, 2010). Due to their prominent role in innate immunity, it is no surprise that high levels of TLRs can be found in organs regularly involved in immune response, such as the lung and spleen (Downes, 2010). Recent evidence suggests that although the brain is considered immune privileged, messenger RNA can be found for TLRs 1-10 in the microglia of mouse models. This allows microglia to quickly respond to endogenous and exogenous substances (Downes, 2010).

It has been demonstrated that a TLR6-TLR2 heterodimer binds diacyl lipopeptides of mycoplasma, bacteria that lack a cell wall, whereas the TLR1/TLR2 heterodimer recognized triacyl lipopeptides, suggesting that TLR6 is involved in the distinguishing between the fine subtleties of bacterial lipopeptides (Takeda, 2002).

Once TIR domains initiate a signal, four proteins carry the signal to proteins and transcription factors (TF) that activate an inflammatory response.

These proteins are Myeloid Differentiation Factor 88 (MyD88), MyD88 Adaptor Like Protein (MAL), Toll Receptor Associated Activator of Interferon (TRIF) and Toll Receptor Associated Molecule (TRAM) (Beutler, 2004). A fifth domain, sterile  $\alpha$ - and armadillo-motif-containing protein(SARM), regulates TLR signaling (Downes, 2010). MyD88, which is used by all TLRs but TLR3, is composed of three subunits. One domain binds to TIR on the TLR, and one, phosphorylates Interleukin Receptor-associated Kinase (IRAK) 1 and 4, which will then activate downstream proteins, and result in elevated transcription of pro-inflammatory genes (Downes, 2010). Due to its vital role, TLR signaling is termed MyD88 dependent or independent. Figure 2 shows the proteins involved in the inflammatory response of each TLR-induced pathway. Upon activation, TLR6 and TLR4 complex induces MyD88 to phosphorylate downstream proteins, causing the activation of NF- $\kappa$ B, eventually resulting in the release of pro-inflammatory cytokines and the respiratory burst molecules (Stewart, 2010).



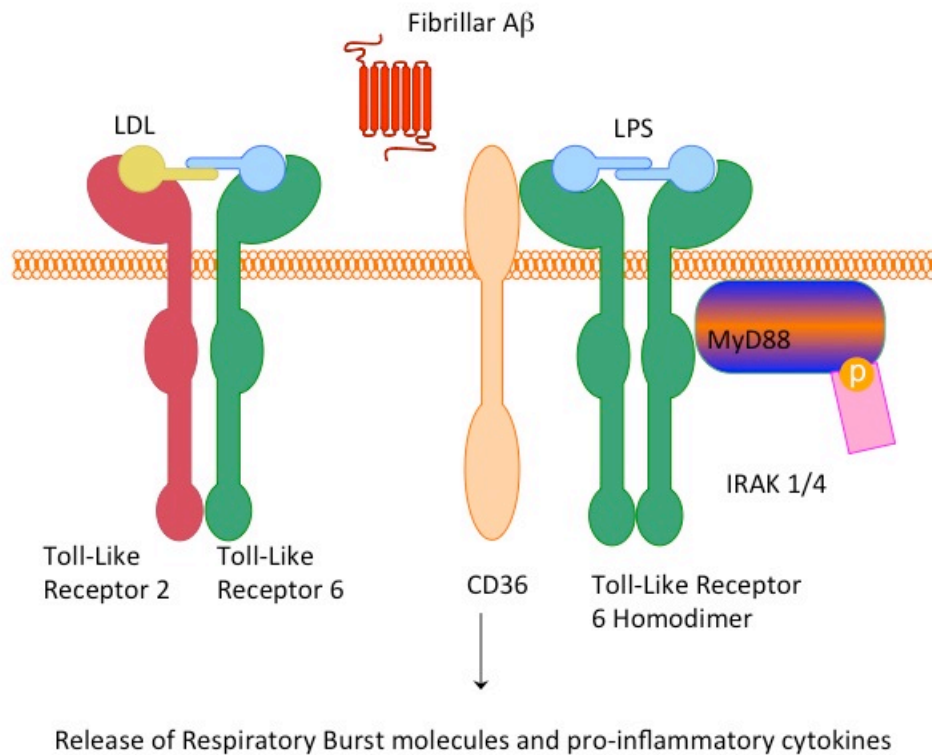
**Figure 2: Signal Cascade of TLR Proteins.** Many Toll Like Receptors share their signaling cascades and result in pro-inflammation. Seen here, TLR6 shares its mechanism with TLR2 and with most of the TLR4 cascade, providing insight into how these receptors may interact. Figure taken from Wang, 2011.

TLRs may also play a major role in AD pathology. Research has already suggested that TLR2 binds to A $\beta$  and activates neuroinflammation, inhibition of TLR2 resulted in a lesser inflammatory response and elevated plaque clearance by microglia (Liu 2012), and that the expression of some TLRs, specifically TLR2 and 4, is elevated in blood of AD patients (Zhang, 2012). However, other studies

have shown that TLR2 acts as a receptor to clear A $\beta$  plaques, and a deficit of TLR2 would lead to elevated A $\beta$  plaque levels and exacerbated memory loss (Hanke, 2011; Richard, 2008). Interestingly, studies have shown that a lack of TLR4 leads to elevated A $\beta$  plaque levels, suggesting that TLR4 is involved in clearance of plaques (Arroyo, 2011; Tahara, 2006). TLR9 is believed to increase A $\beta$  clearance, protect against toxicity by plaques, reduce plaque formation, and improve mental ability loss (Drouin-Ouellet, 2010; Iribarren, 2005; Doi, 2009; Scholtzova, 2009).

As can be seen in Figure 3, evidence suggests that TLRs increase recognition of A $\beta$  plaques by interacting with other cell surface receptors such as CD36, CD47 and SR-A (Arroyo, 2011; Bamberger, 2003; Reed-Geaghan, 2009). TLRs can also form heterodimers with themselves, allowing detection of even more ligands. In one such heterodimer, TLR2/6, it was seen that TLR6 inhibited a A $\beta$ -triggered TLR2-mediated inflammatory response (Liu, 2012). Another dimer is CD36, which acts as a TLR4/TLR6 co-receptor. In fact, research has shown that the cell surface receptor for A $\beta$  is composed of a collection of different proteins, including SR-A, CD36, TLR2, TLR4, TLR6 and TLR9 (Bamberger, 2003; Paresce, 1996; Reed-Geaghan, 2009; Stewart, 2010). TLR4<sup>+</sup> and TLR6<sup>+</sup> microglia deficient in CD36 did not activate pro-inflammatory cytokines in response to oxidized low-density lipoprotein (LDL), a major TLR ligand (Stewart, 2010). Furthermore, it has been shown that CD36 is required for A $\beta$ -induced microglia activation (El Khoury, 2003). Once one of the TLRs is activated, a cell-

signaling cascade is activated including phosphorylation of other proteins. This cascade ends in elevated levels of pro-inflammatory gene transcription through TFs and the respiratory burst of ROS and pro-inflammatory cytokines. In agreement with their detrimental role, it has been seen that TLR levels negatively correlate with brain function (Zhang, 2012). Due to their important role, some have suspected that TLRs may play a genetic role in AD, although results have differed. While some studies have showed a TLR4 polymorphism to be associated with AD in the Italian population (Minoretti, 2006; Balistreri, 2008), this same polymorphism was not seen in the Chinese population (Wang, 2011).



**Figure 3: Role of Toll Like Receptors in Aβ Plaque Activation of Microglia.** TLR2 and TLR6 recognize extracellular Aβ plaques, sometimes by forming heterodimers with other cell surface proteins, including CD36. Once bound to Aβ, TLRs activate downstream effectors and cause transcription of pro-inflammatory genes and the release of neurotoxic molecules. Furthermore, these heterodimers allow the TLR complex to bind multiple different ligands. Upon binding to a ligand, most TLRs will activate MyD88 which will phosphorylate downstream proteins, including IRAK 1 or 4.

## **Toll Like Receptors and Aging**

Evidence suggests that the functions of the innate immune system, and particularly those induced by TLRs, undergo drastic changes in late life (Kollmann, 2012; McElhaney, 2009; Panda, 2009; Panda, 2010; Shaw, 2010). Whereas TLR2 levels in monocytes and conventional dendritic cells and TLR9 in plasmacytoid dendritic cells do not decrease with age, expression of TLR1 and 4 in monocytes, and TLR1, TLR7 and TLR8 in both dendritic cells is decreased in older people (Panda, 2010; van Duin, 2007; Jing, 2009; Shaw, 2010). Furthermore, the innate immune system of the elderly is commonly found in a state of chronic inflammation, including elevated IL-6 and TNF- $\alpha$  (Kollmann, 2012; Franceschi, 2007; Panda, 2010; Qian, 2012). This elevation in TLRs as well as pro-inflammatory cytokines may provide further reasoning behind the worsening of AD pathology with age, and a worsened neurotoxicity resulting from these elevations.

Although little is known about TLR6 apart from its ability to form heterodimers with TLRs 2 and 4, it has been shown that activation of this TLR will up-regulate pro-inflammatory molecules, including IL-1 $\beta$ , Protein Kinase C and Phosphatidylinositide-3-Kinase (PI3K) (Won, 2012). Recent evidence also suggests that mRNA of TLRs 2 and 6 is down-regulated in mice with a high fat diet, suggesting a role for adipose in immunity regulation (Betanzos-Cabrera, 2012). Additionally, an assay of 24 single nucleotide polymorphisms (SNP) revealed that a SNP in TLR6, SNP rs5743827, was associated with decreased

risk of Bronchopulmonary Dysplasia (Winters, 2013), a lung disorder in children born prematurely characterized by chronic inflammation in the lungs (Mosca, 2011). Another TLR6 SNP, SNP in Ser249Pro, was correlated with a decreased risk for asthma, as well as a reduced inflammatory response in hypertensive women (Tantisira, 2004; Sales, 2010). In sequencing full exons, Ma et al. showed that people with a TLR6-249S SNP had considerably elevated risk of tuberculosis (Ma, 2007).

### **Hypothesis of Current Study**

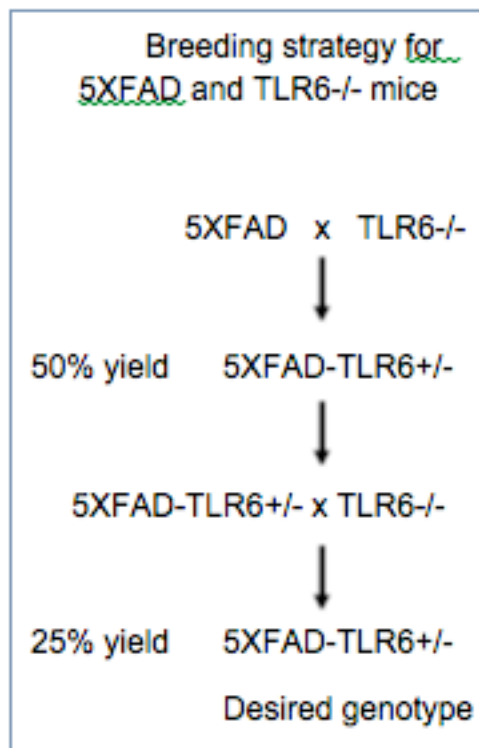
In this study, we aim to prove that TLR6 plays a neurotoxic and plaque-protecting role in AD. We hypothesize that, when activated by A $\beta$ , TLR6 will activate down-stream effectors that result in cytotoxicity of neurons and other cells of the CNS. Due to the large assembly of proteins that bind A $\beta$  on the surface of microglia, we predict that the absence of TLR6 will result in a reduced level of pro-inflammation, allowing other receptors, such as SR-A, to activate A $\beta$  clearance, rather than pro-inflammation, leading to reduced plaque count in TLR6 deficient mice in comparison with 5XFAD mice. As it has been shown that the absence of TLR2 and 4 result in elevated A $\beta$  aggregates, we believe that TLR6 works through its CD36 co-receptor to bind A $\beta$ , and, rather than phagocytize the plaque, activate the respiratory burst, which will result in neurotoxicity and neuronal cell death seen in late-stage AD.

## Methods

### Mouse

Protocols for this study were approved by the Massachusetts General Hospital Institutional Animal Care and Use Committee and met the guidelines for the humane care of animals of the United States National Institutes of Health (NIH publication no. 5377-3 1996). For this study, heterozygous TLR6<sup>+/-</sup> Alzheimer's mice were compared to control heterozygous 5XFAD Alzheimer's mice with normal TLR6 levels. Figure 4 details the breeding strategy used to produce the desired mice. Homozygous TLR6 knockout mice on the 5XFAD<sup>+/-</sup> background were bred in house. The 5XFAD mice, considered generation F<sub>0</sub>, were purchased from Taconic Labs. The F<sub>0</sub> 5XFAD were bred with the TLR6<sup>+/-</sup> mice to generate 5XFAD-TLR6<sup>+/-</sup> heterozygotes and used as test subjects at the age of five months, as it has been demonstrated that 5XFAD mice at this age exhibit a large amount of A $\beta$  plaques. Five TLR6<sup>+/-</sup> were used as the experimental group and seven 5XFAD with normal TLR6 levels were used as controls. Mice were sacrificed using a Carbon Monoxide gas. Blood was then drawn from the heart using the anti-coagulant heparin and centrifuged at 1000g for five minutes to separate immune cells from erythrocytes. The supernatant, composed of white blood cells, was then drawn and stored at -80°C for further analysis. Next, one hemisphere of the brain was extracted and stored in 2% paraformaldehyde (PFA) for twenty-four hours and then transferred to sucrose until used for analysis. PFA is prepared by diluting 10ml 16% PFA with 70ml

PBS. For the sucrose, we dissolved 150g of sucrose in a volume of 500ml H<sub>2</sub>O. This solution is then filtered and aliquotted into 50ml Falcon tubes for storage. The second hemisphere was also acquired and flash frozen in dry ice and stored in -80°C for further analysis. Lastly, 2 sections of each tail were acquired and stored for genetic assessment as needed.



**Figure 4: Breeding Strategy for TLR6+/- mice.** To produce the desired genotype of TLR6 heterozygotes, TLR6 homozygous knockouts were mated with a 5XFAD background and then with the resulting heterozygote TLR6-5XFAD.

## Sectioning & Staining

After a period of at least twenty-four hours in sucrose, brains were sectioned at 12 $\mu$ m using a cryostat at -25°C. 10 sections were taken from each subject and were stored at -80°C for a period of no less than twenty-four hours. From the ten sections, three were then stained with Thioflavin-S and held at 4°C. This provided us with 15 sections of TLR6<sup>+/-</sup> mice to compare to 21 sections from 5XFAD mice, to provide more accurate results. No less than three sections could be analyzed per subject as the statistical significance would be too small. For the stain, .05% Thioflavin-S solution was prepared by adding .1g Thioflavin-S to 20mL distilled water and vacuuming out particulates. To prepare the 70% ethanol (EtOH), 15mL distilled water and 35mL 100% EtOH were combined. Lastly, 4',6-diamidino-2-phenylindole (DAPI) was prepared using 2 $\mu$ L DAPI and 10mL distilled water.

For the staining, slides were removed from -80°C and stored in room temperature for five minutes. Distilled water was then added to the slide for a couple seconds and aspirated. .05% Thioflavin-S was then added to the slide for five minutes in the dark. Thioflavin-S was aspirated and 70% EtOH was added for another five minutes in the dark. EtOH was aspirated and distilled water was added for three thirty-second intervals. After this wash, slides were kept at room temperature for five minutes to allow the tissue to dry and adhere to the slide. Once dry, DAPI was added for one minute in the dark and aspirated. Slides were

washed another three times at thirty-second intervals and dried. Vectashield was then added to reduce the possibility of air bubbles and slide covers placed. Slides were sealed with quick-dry nail polish, in order to prevent movement of the slide cover that could insult the tissue and to prevent particulates from entering, and stored at 4°C until use. Sections were scanned for the Thioflavin-S and DAPI fluorescent stains using TissueFax by Tissuegnostics and analyzed for plaques using the free software ImageJ.

### **Statistical Analysis**

Due to its prominent presence in the hippocampus and frontal cortex, A $\beta$  plaques these areas alone were considered, and compared in 5XFAD versus TLR6<sup>+/-</sup> mice. Using the 15 experimental sections from the TLR6<sup>+/-</sup> mice and the 21 sections from 7 5XFAD mice, the two brain structures were analyzed for the absolute number of plaques, the total surface area in micrometers that the plaques occupied, and the percent area of the brain this constituted. Statistical Analysis was performed using Student's *t*-test using Microsoft's Excel statistical software.  $P < 0.05$  was considered significant.

## Results

### Decreased A $\beta$ levels in TLR6 deficient AD mice

The first step in understanding the role of Toll-Like Receptor 6 on Alzheimer's disease is to understand the role it plays in clearing A $\beta$ . To assess this involvement, we analyzed the plaque count of five mice positive for AD but lacking TLR6 and compared the results to seven mice of the same strain with normal TLR6 levels. To most accurately gauge the results, we looked at the percent of the brain area inhabited by A $\beta$  plaques in order to prevent influence by size of mice or plaques. Table 1 provides the amount of plaques and their area in both test subjects. The seven 5XFAD mice presented with an average of 1,197 plaques per cortex, whereas the five TLR6<sup>+/-</sup> mice exhibited 584, a 61% decrease (Table 2, Figure 6). Furthermore, the area covered with plaques in the 5XFAD was 14,486 $\mu\text{m}^2$ , while plaques occupied only 8,248 $\mu\text{m}^2$  in the TLR<sup>+/-</sup> experimental mice, a 43% reduction from the 5XFAD (Table 3, Figure 5). Due to its smaller size, absolute variations in the hippocampus were smaller. However, as there is already little space to contain plaques, the 1,013 $\mu\text{m}^2$  reduction in occupied area represents a 31% decrease. Similarly, total plaque count in the 5 TLR6<sup>+/-</sup> mice was an average of 91 plaques less than the average of the 7 5XFAD mice, which signifies a 36% reduction in TLR6<sup>+/-</sup> mice.

As is seen in Table 4, 5XFAD mice all presented with approximately 15% of the frontal cortex covered in A $\beta$  plaques with no outliers, while there was a greater variation in the hippocampus, with an average of 21% covered in plaques

and a 13% difference between the highest and the lowest plaque counts. In the TLR6 deficient mice, however, there was a much greater variation in both the hippocampus and the frontal cortex. In the cortex, an average of 12% is seen, with one outlier at 20%, 6% more plaque area than the next highest.

Interestingly, the hippocampus showed a high level of plaque concentration, with an average of 22% if the drastic outlier of 2% is disregarded. Figures 5, 6 and 7 compare the averages of area (Figure 5), plaque count (Figure 6) and percent area occupied by plaques (Figure 7) between the 5XFAD and experimental mice. These graphs demonstrate the large differences in the number of plaques and the surface area they cover between TLR6 deficient and TLR6<sup>+</sup>. In scanning the whole surface of the brain (Figure 8), the difference between the 5XFAD and the TLR6<sup>+/-</sup> mice is quite evident, whereas scans of the hippocampus (Figure 9) reveal that a smaller change occurred in this brain structure.

Using the Student's *t*-test, we noticed that the decrease in the total number of plaques and the surface area they occupied in the cortex were both of significant difference ( $p=0.006$  and  $0.02$  respectively). However, none of the findings in the hippocampus, nor the percentage of area occupied, were of significant difference, as  $p > 0.05$  in all cases.

**Table 1. A $\beta$  Plaque Count and Surface Area.**

	Cortex		Hippocampus	
	5XFAD	TLR6 <sup>+/-</sup>	5XFAD	TLR6 <sup>+/-</sup>
Plaque Count	1197.52	584.87	252.14	161.21
Total Plaque Area ( $\mu\text{m}$ )	14486.73	8247.8	3253.43	2240.33
Percent Area	15.27%	12.47%	21.11%	20.00%

**Table 2. A $\beta$  Plaque Numbers in Hippocampus and Cortex**

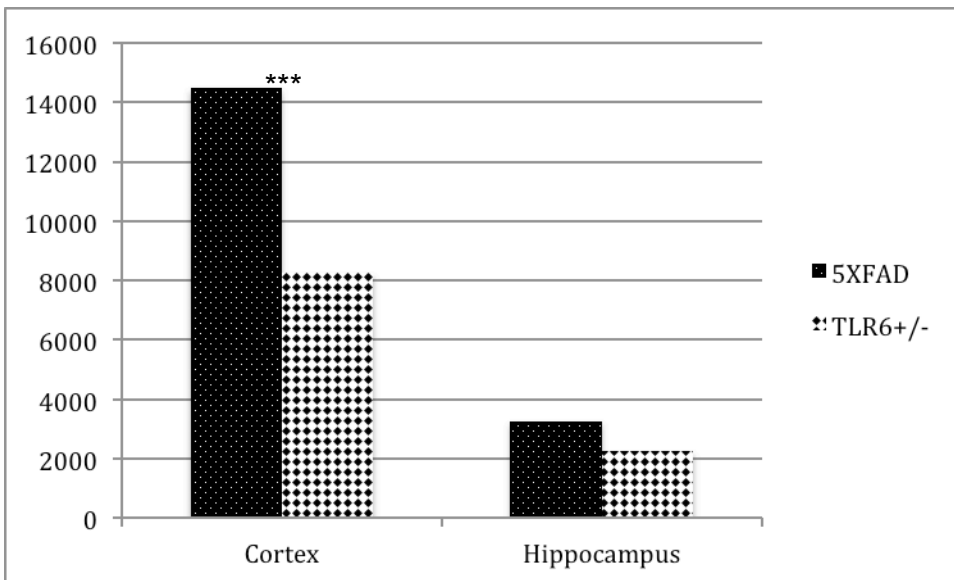
	Cortex		Hippocampus	
	5XFAD	TLR6 <sup>+/-</sup>	5XFAD	TLR6 <sup>+/-</sup>
	1232.67	342	258	202.67
	1116	914.33	282.33	226.33
	748.67	378.67	250.33	3.5
	810	436.33	105.33	187.67
	1496.67	853	383.33	133.33
	1506		183.67	
	1472.67		302	
Average	1197.5	584.87	252.14	161.21

**Table 3: Total Area in Brain Structures Occupied by A $\beta$  Plaques.**

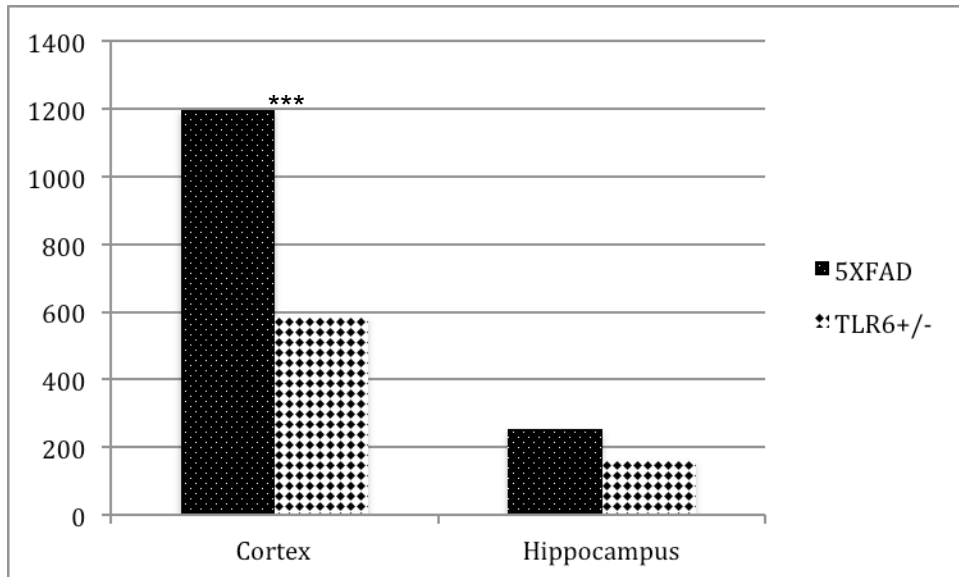
	Cortex		Hippocampus	
	5XFAD	TLR6 <sup>+/-</sup>	5XFAD	TLR6 <sup>+/-</sup>
	12068.77	4593.93	2325.13	2511.44
	11108.39	11482.29	2552.85	2905.02
	10617.73	6466.55	2556.10	68.05
	12208.05	5137.42	1689.39	2630.34
	16507.95	13558.83	5170.81	2362.72
	20318.94		3621.73	
	18577.28		4857.98	
Average	14486.73	8247.80	3253.43	2240.33

**Table 4: Fraction of Brain Structures Covered by A $\beta$  Plaques.**

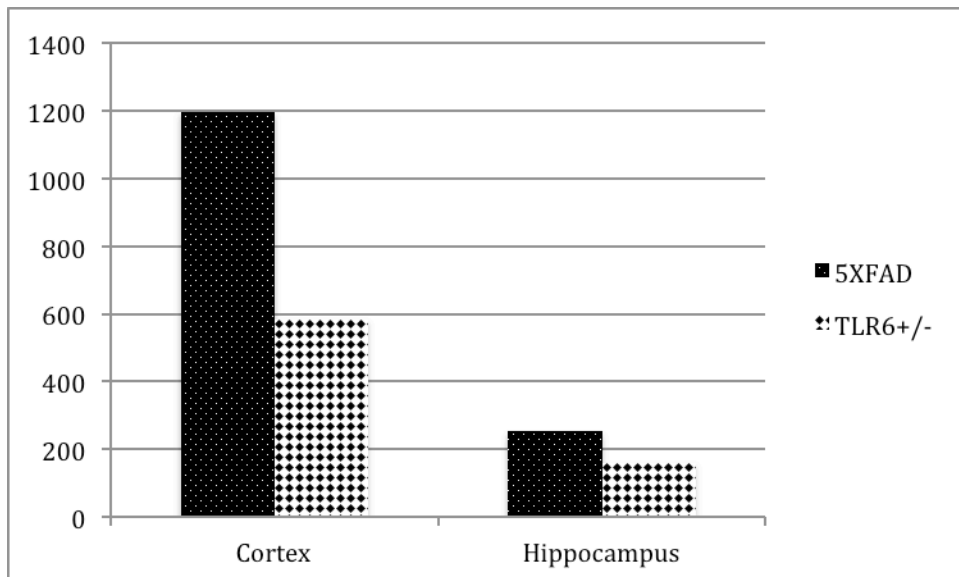
	Cortex		Hippocampus	
	5XFAD	TLR6 <sup>+/-</sup>	5XFAD	TLR6 <sup>+/-</sup>
	11.78%	7.97%	17.97%	17.77%
	11.46%	13.58%	16.67%	22.70%
	13.86%	12.58%	19.46%	2.21%
	15.82%	7.30%	14.83%	27.46%
	16.85%	20.91%	27.84%	23.90%
	19.44%		24.15%	
	17.72%		26.87%	
Average	15.27%	12.47%	21.11%	20.00%



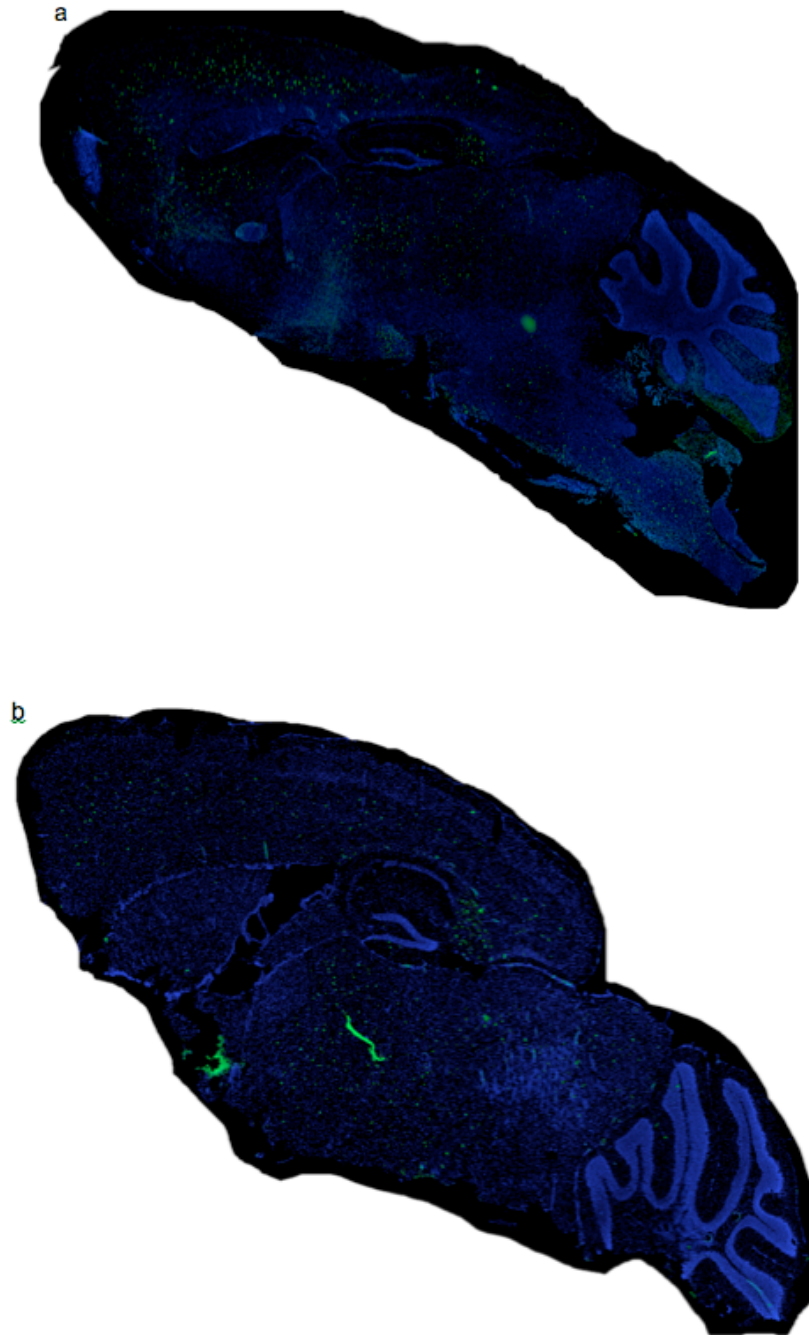
**Figure 5: Average Area Containing A $\beta$  Plaques.** In analyzing the plaque levels in each brain structure, a much larger area was occupied by A $\beta$  in both the cortex (left) and hippocampus (Right) in the 5XFAD mice (dark) than in the experimental mice (light). (cortex  $p=.0227$ , hippocampus  $p=.1487$ ).



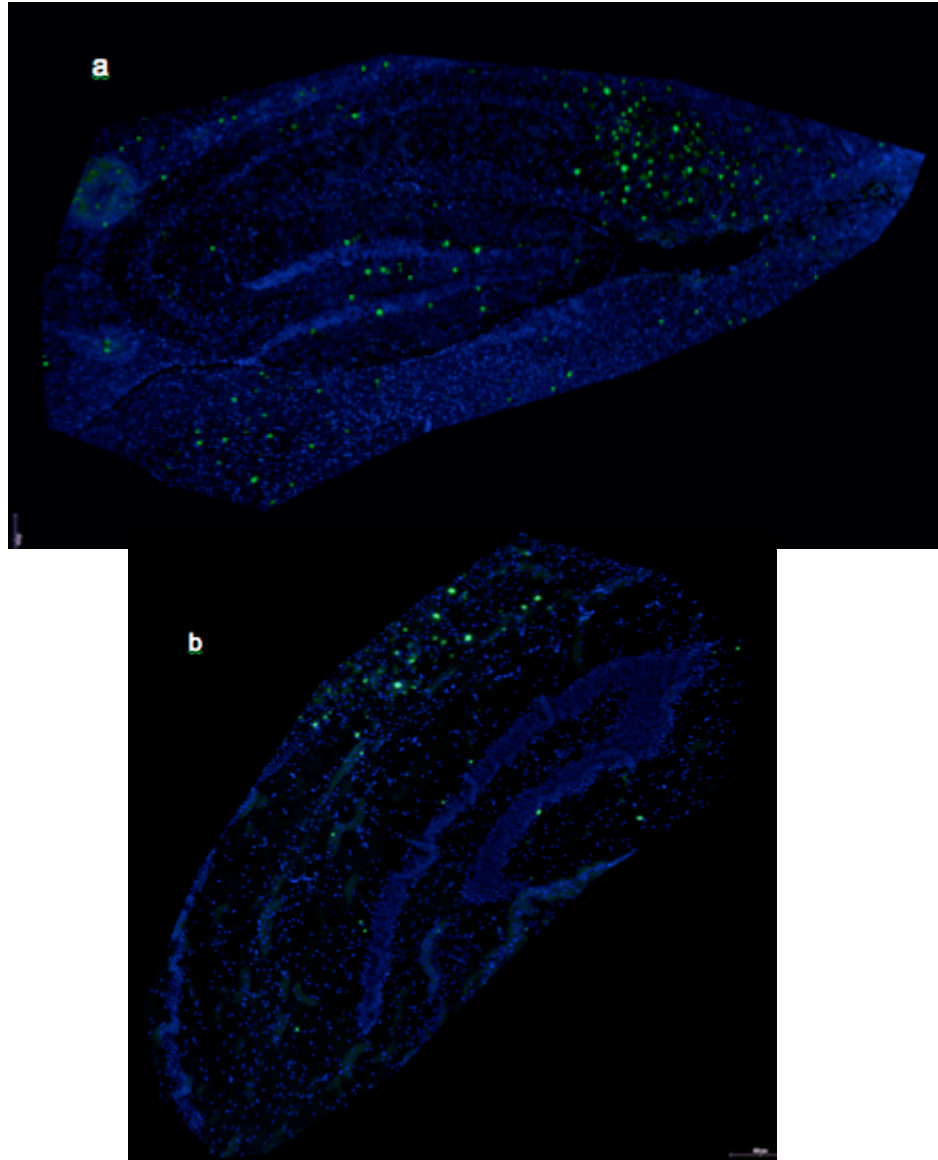
**Figure 6: Average Plaque Count in Brain Structures.** The brain of 5XFAD mice exhibited many more plaques than that of the TLR6<sup>+/-</sup> mice. (cortex p=.0063, hippocampus p=.0792).



**Figure 7: Average Percent Area of AD Brain Covered with A $\beta$  Plaques.** Our results revealed a larger fraction of the brain covered with A $\beta$  plaques in the brains of 5XFAD (white) over TLR6<sup>+/-</sup> mice (dark). A larger difference was seen in the frontal cortex (left) than in the hippocampus (right). (cortex p=.2776, hippocampus p=.6081).



**Figure 8: A $\beta$  TLR6 absence and A $\beta$  Accumulation.** 5XFAD AD mouse (a) exhibits elevated levels of A $\beta$  (green) throughout the brain as compared to TLR6<sup>+/-</sup> mouse (b).



**Figure 9: A $\beta$  Accumulation in TLR6 Deficient Hippocampus.** The hippocampus of 5XFAD AD mouse (a) presents with a large amount of A $\beta$  (green) in comparison to the hippocampus of a TLR6<sup>+/-</sup> mouse (b).

## Discussion

Alzheimer's disease is pathological ageing characterized by a markedly elevated level of microglia and Amyloid  $\beta$  protein congregates in the brain. Being an age-dependent disease, as the mean age of survival of the population rises, so too does the incidence of LOAD among the elderly. Symptoms of AD involve drastic mental changes, including confusion, memory loss and difficulty with higher-level thinking such as solving problems.

The leading cause of dementia, AD affects many millions of people worldwide, especially over the age of 65. Because most research suggests  $A\beta$  and hyperphosphorylated  $\tau$  as the causative agents, most biopharmaceutical drug development has been geared to treating these to mechanisms, mostly by reducing  $A\beta$  accumulation (DuBoff, 2012; Palop, 2010; Schenk, 2012; Lee, 2011; Mullane, 2013). Unfortunately, over 100 compounds attempting to treat AD failed between the years of 1998-2011 (Mullane, 2013; Pharmaceutical Research and Manufacturers of America). Therefore, current treatment options aim at pain and symptom management, such as Acetylcholinesterase Inhibitors and donepezil, and often show little efficacy (Kaduszkiewicz, 2005; Raina, 2008; Mullane, 2013).

During normal post-translational modifications of the APP protein, an excess production of  $A\beta$  may occur.  $A\beta$  first accumulates in the hippocampus and the frontal cortex. As  $A\beta$  accumulates, microglia clear the protein through phagocytosis by binding  $A\beta$ . However, as  $A\beta$  becomes pathologically produced,

microglia cannot properly clear all the A $\beta$ , and thus get stuck in aggregates of the protein. This accumulation of microglia and A $\beta$  results in the pathological A $\beta$  plaque, which induces the release of microglial respiratory burst elements, including NO and ROS, and pro-inflammatory cytokines. These elements then act in a neurotoxic manner and damage neurons and cause cell death. Aided by  $\tau$  hyperphosphorylation, the AD senile plaque begins to hinder proper cellular function and synaptic transmission, resulting in some of the symptoms seen in AD.

Due to their large variety of functions, microglia express a wide range of receptors. Research has shown that of these, TLRs are some of the most important. TLRs can bind both endogenous ligands and exogenous ligands including bacterial LPS and viral double stranded RNA. TLRs can recognize a large span of molecules by creating homo- and hetero-dimers. Once recognized, most TLRs induce a MyD88-dependent signaling cascade that results in inflammation through pro-inflammatory cytokines and molecules, such as IL-1 $\beta$ .

In this experiment, we studied the function of the TLR6 receptor protein in the immune system recognition of A $\beta$  and the receptor's contribution to AD pathology. We hypothesized that mice deficient in TLR would exhibit a less severe pathology, as shown by reduced number of A $\beta$  plaques. Though not conclusive, our data suggest that the absence of TLR6 allowed mice to more thoroughly clear A $\beta$  protein in the frontal cortex, as 61% fewer plaques were

found in the frontal cortex of the TLR6<sup>+/-</sup> mice as compared to 5XFAD mice suffering from AD, with a statistical significance ( $p < 0.05$ ). However, the TLR6 deficiency did not support clearance of A $\beta$  from the hippocampus, as the results were not statistically significant ( $p > 0.05$ ). We also theorized that TLR6 works through a complex with the cell surface receptor CD36. As was previously shown, CD36 works in conjuncture with TLRs 4 and 6 to aid in the clearing of A $\beta$  protein, and that these three proteins, which can form a heterotrimer, regulate the activation and release of pro-inflammatory cytokines and molecules of the respiratory burst previously shown to have deleterious and pathological effects on the host (Stewart, 2010). Of interest, it has been previously demonstrated that mice deficient in CD36 demonstrate diminished levels of the pro-inflammatory cytokine IL-1 $\beta$  (Janabi, 2000; El Khoury, 2003) and a decreased inflammatory response. As TLR6 is an activator for IL-1 $\beta$  expression, this finding further suggests a connection in the mechanism of action of TLR6 and CD36. The discovery of TLRs, and their critical role in host recognition of antigens, has provided vast information on the capabilities of the innate immune system and its ability to recognize and eliminate foreign pathogens. Unfortunately, the mechanism by which TLRs act is profoundly unknown.

Although the analysis of our data revealed a decrease in overall A $\beta$  accumulation in the brains of mice lacking TLR6, the results are skewed. While we witnessed large differences in the number of plaques and the total amount of

room they acquired, the percent of area covered in A $\beta$  plaques was very small, under a 3% difference in the frontal cortex and 2% in the hippocampus, with no statistical significance. However, there was a significant standard deviation. In our test subjects, the percent of the hippocampus covered by plaques was at 20%, but there was a standard deviation of 50%, suggesting the presence of a negative outlier, which when removed, resulted in a percent coverage greater than of the 5XFAD. Conversely, when the positive outlier was removed from the TLR6<sup>+/-</sup> cortex data, the average percent covered was under 10% almost 6% lower than the 5XFAD. We believe that this was caused by a problem in the analysis of total surface area of the brains, as visual comparison reveals that these percents do not match the visible change. We are working on discovering what this error was in order to correct the calculations.

Alternatively, there was almost a 50% decrease in the actual number of plaques in the cortex of the TLR6 deficient mice as the 5XFAD mice (8,247 versus 14,486,  $p < 0.05$ ) and a 33% decrease in the hippocampus, 2,240 in TLR6<sup>+/-</sup> mice compared to 3,253. Similarly, an overall decrease in the area containing A $\beta$  plaques was seen. In the cortex, the 5XFAD specimens had an average surface area of 1,200 $\mu\text{m}^2$  containing plaques whereas the experimental mice had only 585 $\mu\text{m}^2$ , with statistical significance ( $p < 0.05$ ). In the hippocampus, a decrease of approximately 33% was seen again, as 5XFAD mice had an average of 252 $\mu\text{m}^2$  of brain tissue containing plaques whereas the

experimental mice lacking TLR6 had only  $161\mu\text{m}^2$ .

Our results suggest that TLR6 is involved in the response to  $\text{A}\beta$  accumulation, and therefore contributes to AD pathology. Based on work by Stewart et al., our findings propose that it is possible that TLR6 works in conjunction with CD36 and TLR4 to activate a pro-inflammatory response that somehow hinders the clearance of  $\text{A}\beta$  protein, resulting in elevated protein aggregates, and  $\text{A}\beta$  plaques. By releasing pro-inflammatory cytokines and neurotoxic elements like NO and ROS may cause neuronal apoptosis that then release cellular debris. As this debris accumulates, it further aggravates the already building  $\text{A}\beta$  aggregates, resulting in plaques that prevent clearance of  $\text{A}\beta$ . As this occurs, microglia will remain in a constant state of pro-inflammation, which will further damage neurons.

### **Fallbacks of the Study and Future Research**

As our results complement each other in two of the three analysis scales, it is assumed that the percent of the tissue containing  $\text{A}\beta$  plaques was distorted. This could be because the tissue was smaller in the experimental mice. This disagreement between the results represents a major problem with this study, which is the little amount of information available. Firstly, there is only one time point. This instant in time of five months may not provide the full picture, as the lack of TLR6 may delay the development of plaques rather than reduce it totally.

Furthermore, as few mice were used as test subjects, our results could have been very skewed by the outliers we encountered. This could distort the results and suggest a link that is not actually present. In order to remedy this, we are analyzing data from three and four months of age as well in order to construct a progression of the pathology. Additionally, this study may not correctly show the full scheme, as only one fluorescent stain was used and it may not react with all A $\beta$ . Therefore, we will be analyzing the data with more stains, including mouse anti-CD11b antibody, which recognizes CD11b on macrophages, and will therefore provide information on the number of microglia activated in both the 5XFAD and the TLR6<sup>+/-</sup> mice.

Although this data suggests a correlation between TLR6 levels and the progression of AD pathology, more research is required to verify the theory that TLR6 and CD36 work in conjuncture to activate pro-inflammatory cytokines and further the neurotoxicity seen in patients suffering from AD. A study of the animals' behavior would be beneficial in seeing if this altered pathology impacts the mice and their actions, including survival, mating pattern, feeding pattern and social interaction. Although our results are promising, much research will be needed to show how TLR6 differs from the other cell surface receptors in its mechanism, as a deficiency in the other receptors results in reduced microglial phagocytosis, elevated A $\beta$  aggregation, and worsened AD pathology. These results may also suggest a novel mechanism of action for TLR6 separate of TLR4 cooperation. To test this, a study should be done to see the effect of

eliminating various cell surface receptors associated with TLRs, as a similar result to ours would give more insight into the TLR6-related plaque protection we witnessed.

### **Future Implications: From Discovery to Drug**

While our results suggest a novel action cascade for microglia in the presence of A $\beta$ , much more research is needed to validate and explain these results. After these pre-clinical studies are able to determine the cause of these results, it will be possible to develop drugs to solely disable this cascade while allowing microglia to continue clearing A $\beta$ . Such a drug would be able to reduce the neurotoxic effects seen in AD pathology and prevent worsening of the condition. After finding a molecule that properly disables TLR6-mediated inflammation, the safety of such a drug would be assessed, first in animal models and then in humans. This would be very important, especially in a drug targeting TLR6 itself, because of the vast array of effects the TLR receptors have. As such, it is possible that a drug that inhibits TLR6 function in the brain will have a similar effect in the rest of the tissue, making the host more susceptible to secondary infection or disease. Assuming the drug passes these safety measures, large-scale studies would be needed to assess the efficacy of this drug. Although much more work is required to possibly bring this drug to market, the benefits far exceed the cost, as there is currently no drug that effectively prevents the worsening of AD, making this a novel possibility.

## **Conclusion**

Using a small-scale mouse study, we demonstrated with statistical significance that the absence of TLR6 supports clearance of A $\beta$  in the frontal cortex by 61% ( $p < 0.05$ ), but not in the hippocampus ( $p > 0.05$ ). Upon analysis, we deduce that A $\beta$  binding to TLR6 activates a previously unknown cascade that activates a pro-inflammatory response without supporting microglial clearance of A $\beta$ . This discovery provides novel possibilities for drug targeting, and offers a new advantage in battling one of the most troublesome disorders to afflict the elderly worldwide.

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