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Novel Mice for Investigating Triggering Receptor Expressed on Myeloid Cells-2 (TREM2), an Alzheimer's Disease-Linked Immune Gene That Alters Microglia Responses to Neurodegeneration

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A dissertation submitted in partial satisfaction of

the requirements for the degree Doctor of Philosophy

in Neuroscience

by

Anthony Daggett

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ABSTRACT OF THE DISSERTATION

Novel Mice for Investigating
Triggering Receptor Expressed on Myeloid Cells-2 (TREM2),
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That Alters Microglia Responses to Neurodegeneration

by

Anthony Daggett
Doctor of Philosophy in Neuroscience
University of California, Los Angeles, 2016
Professor Xiangdong William Yang, Chair

Triggering Receptor Expressed on Myeloid cells-2 (TREM2) is an immunoreceptor with genetic links to human neurodegenerative disorders. TREM2 is thought to alter the microglial response to neurodegeneration in a protective way. This dissertation describes the generation of two separate lines of Bacterial Artificial Chromosome (BAC) transgenic animals, BAC-TREM2 and BAC-TREM2-R47H mice, useful in better understanding the role of TREM2 *in vivo*. BAC-TREM2 is designed to express human TREM2, increase TREM2 gene dosage, and enhance TREM2 signaling, creating mice with efficient microglia primed to handle a damaged and degenerating brain. BAC-TREM2-R47H is designed to express the Alzheimer's disease-associated R47H allele in order to determine if R47H TREM2 has lost TREM2's protective functions or gains new toxic functions.

We find that BAC-TREM2 mice correctly express human TREM2. In addition, cultured microglia demonstrate increased Ca^{2+} signaling when stimulated with the TREM2 ligand phosphatidylserine and have suppressed pro-inflammatory cytokine secretion when stimulated with lipopolysaccharide, both signs of increased TREM2 signaling.

To test the effect of increased TREM2 gene dosage *in vivo,* we crossed BAC-TREM2 mice with two mouse amyloidosis models of Alzheimer's disease, APPswe/PS1ΔE9 and 5xFAD mice. We found BAC-TREM2 significantly reduced microgliosis around amyloid-beta plaques in both models without reducing astrogliosis. This changed in microglia activation was accompanied by a significant improvement in a contextual fear conditioning learning task that is disrupted in APP/PS1 mice, but no change in hyperactivity found in these mice. Altogether, this study suggests BAC-TREM and BAC-TREM2-R47H will useful tools for the study of TREM2 function in vivo, and that TREM2 signaling is a promising target for manipulating microglia in order to improve the innate immune response to neurodegeneration.

The dissertation of Anthony Daggett is approved.

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Figure 1.1 is reprinted from Soto C. Unfolding the role of protein misfolding in neurodegenerative diseases. Nat Rev Neurosci. 2003 Jan;4(1):49–60. Copyright (2003), courtesy of Nature Publishing Group.

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PUBLICATIONS

1. Williams ME, Wilke SA, ***Daggett A***, Davis E, Otto S, Ripley B, Bushong EA, Ellisman M, Klein G, and Ghosh A. Cadherin-9 regulates input-specific synaptic differentiation in the developing hippocampus. Neuron. 71:640-655, 2011. PMCID: PMC3272880

ABSTRACTS & POSTER PRESENTATIONS

1. ***Daggett A***, Cantle J, Cui Y, Gu X, Yang XW. Testing striatopallidal-specific gene Gpr6 as a genetic modifier of Huntington's disease in BACHD mice. CHDI Conference. February 29, 2012.

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3. ***Daggett A,*** Lee CYD, Murillo K, Yang XW. Overexpression of Alzheimer's disease risk gene TREM2 to improve the immune response to neurodegeneration. Nanosymposium. Society for Neuroscience Annual Conference. October 20, 2015.

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Chapter 1

Introduction

Triggering Receptor Expressed on Myeloid Cells-2 (TREM2) and the Microglia Immune Response to Neurodegeneration

1.1 Prologue

Neurodegenerative diseases constitute a major class of late-onset brain disorders with relentless progression, severe morbidity and early mortality. Neurodegeneration is defined as the progressive loss of neuronal structure and neuronal death in the central nervous system (Przedborski 2003). The anatomical regions and cell types affected by neurodegeneration, and the manifested neurological symptoms, define the specific disease, though degeneration may become less selective with progression. Two broad categories of neurodegenerative disease affect primarily cognitive function (i.e Alzheimer's Disease (AD)) or primarily motor function (i.e Parkinson's Disease (PD) and Huntington's disease (HD)), but many of these disorders affect multiple domains of motor and cognitive function. Roughly speaking, the degeneration of the cerebral cortical neurons (and those in the hippocampus) often characterizes AD and other forms of dementia. Neurodegenerative diseases that cause motor symptoms primarily affect the basal ganglia and associated structures (PD and HD), the cerebellum and its connecting tracts (cerebellar ataxias), or motor neurons in the brainstem and spinal cord (i.e. amyotrophic lateral sclerosis (ALS)).

Two key, tragic aspects of neurodegenerative diseases are their relentless progression and the lack of effective therapeutics. Neurodegeneration often begins before symptoms manifest and continue throughout the course of the disease. It is age-dependent, and while the onset of

neurological symptoms can occur during childhood, neurodegeneration inevitably worsens with age. Though several drugs and surgical approaches can effectively lessen a portion of the symptoms of neurodegenerative disorders, these are temporary balms, as no interventions so far have been shown to prevent the onset or alter the course of these diseases (Lleó 2006, Smith 2012). Thus, better understanding the mechanisms of disease pathogenesis, including at disease onset and with progression, is key to a rational path towards developing new disease-modifying therapies for the millions afflicted with these devastating disorders.

Besides region-specific and progressive neuronal loss, there are two other common pathological findings in most neurodegenerative diseases that provide insight into disease pathogenesis. First is the accumulation of abnormal protein species, such as Aβ-containing amyloid plaques in AD, mutant huntingtin inclusions in HD, α-synuclein-containing Parkinson's Lewy bodies in PD, and superoxide dismutase plaques in ALS (Soto 2003, Fig 1.1). This abnormal protein aggregation and accumulation has been the focus of pathogenic study and therapeutic development during the past few decades (Karran 2011, Lashuel 2012). It is a promising avenue of exploration towards understanding the cause of these devastating diseases, though no effective therapeutics compounds have yet to emerge. In addition, each disease will likely require a unique strategy to influence the specific toxic protein species that is altered.

A second common pathological finding in neurodegenerative diseases is chronic inflammation in the regions undergoing degeneration. This is defined by increased inflammatory mediators, such as complement factors and cytokines, and changes in the morphological and functional state of glia cells, such as astrocytes and brain resident innate immune cells, the microglia (Amor 2010). The inflammatory response is well suited to manage a wide variety of acute

insults, such as viral or bacterial infections, but the chronic inflammation found in neurodegenerative diseases has a more uncertain, and likely more complex, overall impact on disease pathogenesis. The subject of intense current interest, the role of chronic inflammation in neurodegenerative diseases, is relatively understudied compared to abnormal protein accumulation. Therapeutics aimed at modifying inflammation in the nervous system (i.e. neuroinflammation), if effective, may be generalizable to treat a wide variety of neurodegenerative diseases and even disorders of chronic intermittent neuronal insults (e.g. chronic ischemia, chronic traumatic encephalopathy, autoimmune diseases of the brain).

1.2 The Inflammatory Response in Neurodegenerative Diseases

The role of the immune system in late-onset neurodegeneration was first suspected in the 1980's, when complement proteins, circulating effectors of innate immunity, were found in senile plaques in the brains of AD patients (Eikelenboom & Stam 1982, Ishii & Haga 1984). Later pathological studies demonstrated high expression of pro-inflammatory cytokines, such as Il-1β and TNFα, by microglia-like cells, suggesting microglia involvement in this inflammatory reaction (McGeer 1988, Rogers 1988, Griffin 1989, Dickson 1993). Shortly after, microglia activation was recognized as a common response in a variety of neurodegenerative diseases, including ALS, PD and HD, with activation occurring in locations specific to the vulnerable brain regions for each disease (McGeer 1988). This led to many questions, including what is the role of neuroinflammation in the pathogenesis of these diverse diseases. Is it beneficial, detrimental, a combination of both, or an insignificant epiphenomenon, merely a consequence of the primary neuronal dysfunction and degeneration and inconsequential to the course of disease? Evidence for the cytotoxic potential of microglia towards neurons led to the 'inflammatory hypothesis of AD', which posited that the cause of widespread neuronal damage is not the fundamental pathology of AD (such as Aβ plaques), but rather the destructive inflammatory response after

initial insults (McGeer & McGeer 1995). This is similar to peripheral diseases, such as atherosclerosis, where the evolution from relatively harmless cholesterol-rich lipid streaks by macrophages (a resident myeloid cells similar to microglia) in the vascular wall to destructive sclerotic plaques involves aberrant immune reactions (Libby 2002).

While brain-region specific activation of microglia has become an established correlate with neurodegeneration, its detrimental effects are much less straight-forward to demonstrate. Early *in vitro* experiments demonstrated that microglia stimulated with Aβ, the primary component of senile plaques in AD, elicited a response that included secretion of molecules cytotoxic to cultured neurons (Meda 1995, Giulian 1996, Combs 1999). The possible mediators of this cytotoxicity include Nitrous Oxide (NO), Reactive Oxygen Species (ROS), and certain pro-inflammatory proteins including cytokines (Ii 1996, McDonald 1997). Together, this early evidence suggested microglia activation can contribute to the pathogenesis of AD.

In addition to directly harming neurons, these pro-inflammatory cytokines recruit surrounding microglia and astrocytes, as well as infiltrating monocytes from the blood stream. The subsequent astrocytosis can amplify neurotoxicity,. This may involve the loss of normal astrocyte functions in regulating cerebral blood flow, metabolic homeostasis and excitatory synaptic transmission (Maragakis 2006). In addition, activated astrocytes can form glial scars that permanently alter the extracellular environment and can inhibit regeneration (Silver & Miller 2004), though scars have been shown to aid regeneration in specific contexts (Anderson 2016). The recruitment and activation of additional microglia and monocyte immune cells can lead to a positive feedback loop, whereby initial neuronal dysfunction and degeneration is amplified by the inflammatory response, which in turn induces further neuronal damage and even more damaging inflammation. Although mechanistically appealing, the demonstration of inflammation

inhibition as clinically beneficial has been lacking. Several clinical trials attempting to use nonsteroidal anti-inflammatory drugs (NSAIDS) to inhibit the inflammatory response have failed to influence the progression of Alzheimer's Disease (Aisen 2003), even though epidemiological studies have found decreased risk for AD and PD with chronic NSAID use (in t' Veld 2001, Chen 2003).

There is also evidence suggesting microglia activation may be beneficial in neurodegenerative diseases. Evidence in transgenic mouse models of neurodegenerative diseases demonstrate that microglia phagocytose neuronal debris, aggregated proteins, and damaged myelin, which may limit their toxicity and facilitate tissue repair (Simard 2006, Neher 2012). More recent studies have suggested 'activated' microglia found in AD brains may actually be dystrophic or degenerating, supporting an idea that loss of beneficial microglia function could be an important step during neurodegeneration in AD (Streit 2009). One such function of microglia is the maintenance of synaptic plasticity through active synaptic pruning and the release of neurotrophic factors such as BDNF (Schafer 2012, Parkhurst 2013).

Whatever the effects of microglia activation and neuroinflammation on neurodegeneration, several lines of compelling evidence provided by mouse and human genetics support the idea that neuroinflammation is more than an epiphenomenon in neurodegenerative disorders. One early, convincing animal study found manipulating microglial mutant SOD1, an ALS-causing gene, can non-cell-autonomously ameliorate motor neuron degeneration in a mouse model of familial ALS, and delay disease progression (Boillee 2006). Subsequent studies in AD-related models knocking out an important microglial receptor, CX3CR1, demonstrate the ability of altered microglia to influence the course of neurodegeneration (Bhaskar 2010, Lee 2010). Finally, and most crucial, are studies of genetic risk factors in human patients for multiple

neurodegenerative diseases, which show variants in immune system genes are linked to a change in the risk of AD, PD, and ALS (See Section 1.3). In total, is highly likely that microglia are more than insignificant bystanders to neurodegeneration.

1.3 Genetics of Late-Onset Neurodegenerative Diseases and Inflammation: The Emergence of TREM2

As summarized in Table 1, studies of genetic risk factors of Alzheimer's disease have found significant genome wide association (GWAS) of genetic variants in or near genes functioning in the innate immune system, including Complement Receptor 1 (CR1), CD33, and Triggering Receptor Expressed on Myeloid cells 2 (TREM2) (Lambert 2009, Hollingworth 2011a, Guerreiro 2013, Jonsson 2013, see Table 1). Inflammatory gene variants, such as IL-1β, have also been shown to be associated with PD and ALS (Hirsch & Hunot 2009, Cady 2014, see Table 1).

One microglial signaling pathway closely associated with neurodegeneration involves the TREM2 protein (Fig 1.2). TREM2 is a single-transmembrane protein closely associated with its signaling adaptor DAP12 through positively charged lysine residues in its transmembrane domain and negatively charged DAP12 residues. Activation of the TREM2 receptor leads to phosphorylation of tyrosine residues on DAP12's immunoreceptor tyrosine-based activation motif (ITAM), recruitment of signaling kinases such as Syk, and phosphorylation of further downstream molecules involved in Phospholipase C, Erk, and Ca signaling (McVicar 1998, Takahashi 2005, Peng 2013, Fig 1.2). Cleavage of TREM2 into a soluble and a C-Terminal fragment may also be relevant to its signaling (Wunderlich 2013, Kleinberger 2014, Zhong 2015).

Dysfunction in the TREM2 signaling pathway has been linked to neurodegeneration for more than a decade. Recessive, loss-of-function mutations in either TREM2 or its signaling adaptor DAP12 were found to cause an identical early-onset neurodegenerative disease with bone cysts called polycystic lipomembranous osteodysplasia with sclerosing leukoencephalopathy (PLOSL) or Nasu-Hakola disease (Hakola 1972, Nasu 1973, Paloneva 2000, Paloneva 2002). Patients first present clinically with pathological fractures, bone fractures from mild impacts, in their twenties. X-ray imaging then reveals widespread bone cysts. These bone cysts are attributable to dysfunctional osteoclasts, myeloid-lineage cells similar to macrophages and microglia that express TREM2 and resorb bone during remodeling. Patients then develop neurological symptoms over the next decade, including frontal lobe symptoms (e.g. loss of executive function, poor concentration, and euphoria), memory disturbances, seizures, and involuntary choreiform movements (Hakola 1972, Nasu 1973, Paloneva 2001). Eventually this leads to complete loss of mobility, dysphagia, and death often caused by aspiration pneumonia. These neurologic symptoms are associated with widespread neuronal and white matter degeneration, likely due to loss of TREM2 or DAP12 in the resident myeloid cells of the brain, the microglia.

Two recent human genetic studies have suggested that *heterozygous* TREM2 variants influence the risk of late onset AD (LOAD). Using two separate methods, an unbiased GWAS and a candidate approach, two labs independently discovered that the same heterozygous TREM2 missense variant, R47H, is strongly linked to an increased risk of Alzheimer's Disease (Jonnson 2013, Guerreiro 2013). The odds ratio they found (between 2.9-4.6) makes TREM2 the second strongest genetic risk factor for LOAD (Hollingworth 2011b). The highest genetic risk factor for LOAD is the E4 variant of APOE, which has been shown to influence microglia function in Aβ clearance (Laskowitz 1997). As summarized in Table 2, the association between TREM2 variants and LOAD has since been confirmed by multiple studies in independent populations

(Borroni 2014, Ruiz 2014, Slatterly 2014, Hooli 2014, see Table 2). In addition, a recent genomic analyses of the gene networks dysregulated in AD patient brains discovered TREM2's binding partner DAP12 was the highest ranked causal regulator of a CNS-dysregulated immune-related gene module in AD (Zhang 2013). These studies underscore the potential roles of TREM2-DAP12 signaling and an innate immune cell, microglia, in AD pathogenesis.

TREM2's association with neurodegeneration has quickly expanded beyond PLOSL and AD. Recent studies have found TREM2 mutations are significantly associated with increased risk of additional neurodegenerative diseases, such as ALS, frontotemporal dementia (FTD), and PD (Rayaprolu 2013, Cady 2014, Borroni 2014), as summarized in Table 2. This suggests that TREM2 (and DAP12) may play a critical role in the microglial response to chronic neurodegeneration and that targeting this signaling pathway in microglia may affect pathogenesis in a variety of neurodegenerative disorders.

1.4 TREM2 Signaling: Beneficial Priming of Microglia for Neurodegeneration

An essential yet poorly understood question is how TREM2 signaling influences microglia function in neuroinflammation and neurodegeneration *in vivo*? Detectable levels of TREM2 and DAP12 in the brain are restricted to microglia, though bone osteoclasts and peripheral leukocytes also show high levels of expression (Daws 2001, Schmid 2002, Roumier 2004, Kiialainen 2005, Chertoff 2013). PLOSL brain pathology includes white matter loss, ventricular enlargement, basal ganglia degeneration and, importantly, widespread microglial activation (Hakola 1972, Nasu 1973, Paloneva 2001).

In vitro studies of TREM2 have shown that TREM2/DAP12 signaling reduces the inflammatory response of microglia and macrophages to LPS and apoptotic neurons, while increasing their

phagocytic capacity, particularly of apoptotic cells (Takahashi 2005, Hamerman 2005/2006, Turnbull 2006, Hsieh 2009, Zhong 2015). The cultured microglia or macrophages from DAP12 or TREM2 knockout (KO) mice have consistently shown an elevated pro-inflammatory cytokine (i.e. IL-1β, IL-6, TNFα) response after LPS stimluation (Hamerman 2005/2006, Turnbull 2006), and TREM2/DAP12 knockdown leads to a similar results (Takahashi 2005, Hsieh 2009, Zhong 2015). In addition, lentiviral-mediated overexpression of TREM2 in cultured microglia has the opposite effect, suppressing pro-inflammatory cytokine release (Takahashi 2005, Hsieh 2009, Zhong 2015), possibly through phosphorylation of the DOK3 adaptor that binds activated DAP12 (Peng 2013).

While TREM2 signaling suppresses the pro-inflammatory response, it promotes another important and possibly beneficial function of microglia, phagocytosis, especially of apoptotic cells (Fig 1.2; Takahashi 2005, Hsieh 2009). The importance of TREM2-induced phagocytosis is further supported by the TREM2 ligands so far reported. The first clues to a possible ligand came from the finding that TREM2-FC hybrid proteins bind cultured apoptotic neurons (Hsieh 2009). Moreover, the molecular chaperone Hsp60, which was found on the surface of the apoptotic neurons, was shown to be a ligand that binds and activates TREM2 (Lin 2007, Stefano 2009). Recent studies have found alternative apoptosis-associated TREM2 ligands, including phosphatidylserine, which interestingly can bind to wildtype TREM2 but not the R47H variant (Wang 2015). Additional lipids, such as those associated with the Aβ on the lipid membrane (i.e. phosphatidylcholine) and lipids released by damaged myelin (i.e. sphingomyelin) also elicit TREM2 signaling (Wang 2015). Excitingly, genes with the two major late-onset AD risk variants, ApoE and TREM2, have been linked together, as ApoE binds TREM2 with high affinity and increases apoptotic cell phagocytosis in a TREM2-dependent manner (Atagi 2015, Bailey 2015).

A final possible function of TREM2 signaling is to promote age-dependent microglia survival, which depends on the activation of CSF-1R by its two ligands, CSF1 or IL-34 (Fig 1.2; Otero 2009, Wang 2015, Poliani 2015). In conditions in which CSF-1R signaling is dampened, microglia survival appears to be dependent on TREM2 signaling. TREM2 KO microglia were found to be more vulnerable to apoptosis, especially with CSF1R withdrawal, and TREM2 KO microglia have increased apoptosis in the context of an AD model brain (Wang 2015). Hence, prolonging microglia survival and their beneficial functions in the brain (e.g. synaptic plasticity) may be an alternative role for TREM2 in age-dependent neuronal function and in the pathogenesis of AD.

Combined with the link between dysfunctional TREM2 signaling and neurodegeneration, these data lead to the following hypothesis (Fig 1.2): TREM2 signaling primes microglia for an overall neuroprotective response in the context of neurodegeneration, including reduced neuronal-toxic activities (e.g. pro-inflammatory cytokine release), increased phagocytic capacity (e.g. of abnormal protein species, dying neurons, and damaged myelin), maintained ability to support synaptic plasticity, and prolonged microglia survival. With impaired TREM2 signaling, microglia may overreact to neuronal dysfunction or neuronal loss by releasing cytotoxic agents, impairing neuronal repair or neuroplasticity, and shortening microglia survival. Distinct deficits in TREM2 signaling deficits may lead to distinct clinical outcomes, such as the widespread degeneration in PLOSL (with no TREM2) or increased risk of late-onset neurodegenerative diseases such as AD.

1.5 Alzheimer's Disease: The Most Prevalent Neurodegenerative Disease

AD is the most common cause of neurodegeneration (Mayeux & Stern 2012), affecting over 20 million patients worldwide (Mayeux & Stern 2012). AD prevalence is expected to double every 20 years due to population growth and aging (Ferri 2005), leading to hundreds of billions of dollars in economic cost and untold burden to patients and their family (Wimo 2010). Clinically, there are three recognized stages of AD progression that exist on a continuum (Sperling 2011). First is an asymptomatic preclinical phase whereby the neuropathological processes of AD described below are detectable using biomarkers, but there is not yet significant cognitive impairment. This may last up to a decade before the second stage, Mild Cognitive Impairment (MCI). MCI, the early symptomatic phase of AD, is diagnosed based on age-inappropriate decline in cognitive domains such as memory and attention without dementia (Albert 2011). The final stage of AD is frank dementia, when chronic cognitive impairment leads to a loss of independence and significant social or occupational impairment not explainable by other factors (McKhann 2011).

Currently approved treatments are not disease-modifying and focus mainly on symptomatic improvement. There are two classes of compounds: cholinesterase inhibitors aimed to augment cholinergic neurotransmitter circuits and partial inhibition of NMDA receptors (Anand 2014). Cholinergic projection neurons in the basal forebrain innervate brain regions critical to learning, memory, and attention, such as the cerebral cortex and hippocampus. These basal forebrain neurons degenerate over the course of Alzheimer's disease, reducing acetylcholine transmission and contributing to cognitive decline, which can be partially slowed by cholinesterase inhibitors (Schliebs & Arendt 2006). The low-affinity, noncompetitive NMDA antagonist memantine is designed to reduce the Ca^{2+} mediated excitoxicity seen in AD (Hynd 2004). Through a variety of proposed mechanisms, NMDA and glutamatergic dysfunctions in AD increase Ca^{2+} influx through the Ca^{2+}-permeable NMDA receptors and damages neuronal cells.

Memantine mildly, though statistically significantly, improved dementia symptoms in AD (Reisberg 2003). None of these current treatments prevent neuronal loss or progressive cognitive decline in AD. Therefore, it is critical to better understand the early pathological processes of AD in order to develop more effective preventative or disease-modifying treatments.

1.6 The Complex Neuropathology of Alzheimer's Disease

The two classic neuropathological findings of AD were reported when AD was first described over a century ago (Alzheimer 1904, 1911): extracellular neuritic amyloid plaques and intracellular neurofibrillary tangles throughout affected areas of the brain (Fig 1.1). Amyloid plaques additionally accumulate in the walls of cerebral blood vessels and may contribute to vascular pathology. Another key pathologic finding is synaptic loss, which is thought to precede frank neuronal cell loss (Masliah 1989, DeKosky & Scheff 1990, Terry 1991). Finally, AD brains are characterized by gliosis, including astrogliosis in the cerebral cortex (Duffy 1980, Schechter 1981) and plaque-associated microgliosis (Rogers 1988, Styren 1990).

Amyloid plaques were found decades after their discovery to consist primarily of the amyloid-β protein (Aβ) (Glenner & Wong 1984, Masters 1985). Aβ forms through a series of proteolytic steps from the membrane-spanning amyloid precursor protein (APP). APP is first cleaved by the β-secretase enzyme and then to its final Aβ form by the γ-secretase enzyme. The α-secretase pathway is an alternative cleavage pathway to β-secretase and precludes Aβ formation. The final product is either a 40 ($A\beta_{40}$) or 42 ($A\beta_{42}$) residue peptide, depending on the site of γ-secretase cleavage. $A\beta_{42}$ is the major form in amyloid plaques and deposits first during plaque formation, as it contains two additional hydrophobic residues that increase its amyloidgenic properties (Kang 1987, Iwatsubo 1994). In addition to large plaques, Aβ coalesces into smaller

oligomeric species that can have direct toxic effects without formation of amyloid plaques (Lambert 1998, Walsh 2002, Lacor 2007). These species may explain why amyloid plaques do not correlate well with cognitive decline in humans or AD mouse models, as changes in Aβ oligomer levels are more strongly associated with cognitive decline in AD (McLean 1999, Mc Donald 2010).

Though the role of amyloid plaques is unclear, human genetic evidence strongly supports the amyloid cascade hypothesis of AD pathogenesis (Hardy & Higgins 1992). Familial forms of Alzheimer's disease (FAD), which have similar pathological findings, but an earlier age of onset (Selkoe 2002), are caused by dominant mutations in proteins involved in Aβ production. Missense mutations in APP were the first mutations found to cause FAD (Goate 1991, Murrell 1991, Chartier-Harlan 1991), and to date 20 different mutations have been described. Many of these mutations are found near cleavage sites, where they increase Aβ production, increase the Aβ42/Aβ40 ratio, or increase Aβ's propensity to fibrillize (Citron 1992, Suzuki 1994, Nilsberth 2001). APP is located on chromosome 21, which is duplicated in Down's Syndrome, leading to increased Aβ production and explaining the strong association between Down's Syndrome and early Alzheimer's first noted over a century ago (Frazer & Mitchell 1887). Recently, duplications of normal APP have been found to be another cause of autosomal dominant early-onset AD (Rovelet-Lecrux 2006). In addition to APP mutations, FAD can be caused by mutations in Presenilin 1 and 2 (PS1 and PS2), which are key components of the γ-secretase complex (Sherrington 1995, Levy-Lahad 1995, Rogaev 1995). These FAD-associated PS mutations increase the Aβ42/Aβ40 ratio through unclear mechanisms (Citron 1997). Recently, new PS1 mutations were found to be associated with neurofibrillary tangles, without amyloid plaques, raising the possibility of additional disease-relevant roles of PS1 beyond APP processing (Dermaut 2004), such as altering TREM2 cleavage (Wunderlich 2013). Providing further support

for the amyloid cascade hypothesis, a recent genetic study uncovered an APP mutation that is protective against AD and reduces Aβ production (Jonsson 2012). Overall, these lines of genetic evidence strongly support a causal role for Aβ in AD.

Beyond neuritic plaques in the brain parenchyma, AD patients commonly have Aβ amyloid deposits in the wall of cerebral blood vessels in a condition known as cerebral amyloid angiopathy (CAA) (Greenberg 2004). CAA may be related to a reduction in microvasculature function in AD (Hassler 1965, Buée 1994), as well as AD's association with micro- and macro-infarcts. CAA with or without AD pathology is associated with cognitive decline (Greenberg 1993, Natte 2001). Aβ vasculature toxicity may often act in concert with direct neuronal toxicity and neuroinflammation to cause neurodegeneration.

The second characteristic pathological finding in AD brains is intracellular neurofibrillary tangles. Hyperphosphorylated tau was found to be the major component of these tangles (Grundke-Iqbal 1986a, 1986b, Kosik 1986, Lee 1991). Tau, expressed highly in neurons, is a microtubule-associated protein that promotes microtubule assembly and stabilization. Tau exists in multiple alternatively-spliced isoforms (Goedert 1989). Neurofibrillary tangles with hyperphosphorylated tau are also found in many other neurodegenerative diseases, including frontotemporal dementias such as progressive supranuclear palsy and Pick's disease (Lee 2001). These diseases do not result in Aβ plaques, however, and the tau isoform composition and filament morphologies of these tangles differ between specific diseases.

Considering their appearance in a wide variety of neurodegenerative diseases and the possibility they are a general reaction to neurodegeneration, the relevance of these neurofibrillary tangles to disease pathogenesis was initially unclear. Tau mutations can lead to

reduced microtubule-mediated axonal transport, perhaps due to loss of axon volume due with high fibril load (Vossel 2010), though other mechanisms have been explored (Ittner 2010). A tight correlation between neuronal degeneration and tau pathology was the first clue to tau's importance (Braak & Braak 1991). In addition, in mouse models of AD, reducing levels of endogenous mouse tau reduces Aβ-mediated toxicity (Roberson 2007, Ittner 2010). The most convincing evidence, however, again arose from human genetics, as mutations in tau were found to cause a variety of frontotemporal dementias with neurofibrillary tangles (Hutton 1998, Poorkaj 1998, Spillantini 1998). To date, almost 100 disease-associated mutations have been found in tau. Most mutations either interfere with tau's ability to promote microtubule assembly (Hasegawa 1998, Hong 1998), increase tau's propensity to fibrillize (Barghorn 2000, Bergen 2001), or promote tau phosphorylation (Alonso 2004). Clearly tau dysfunction is associated with neurodegeneration and likely involved in AD pathogenesis.

Synapse loss is now regarded as a key step in the pathogenesis in AD. In late-stage patients, decreases in synaptic density of 25-35% have been reported in the frontal cortex of AD brains (Davies 1987, Masliah 1989). This synaptic loss is the strongest pathological correlate with cognitive decline and disease (DeKosky 1990, Terry 1991), even in early stages of AD and MCI (Scheff 2006). The cause of synaptic loss is unclear, though Aβ oligomers have been demonstrated to be directly toxic to the synapses (LaCor 2007). Since synaptic loss precedes significant neuronal loss, plaque deposition, and the appearance of neurofibrillary tangles in animal models of AD (Lanz 2003), it is thought to be crucial to the initial stages of disease pathogenesis and early memory loss.

Additional cells types beyond neurons are altered in the AD brain. Gliosis, glial cell proliferation and activation, of both astrocytes and microglia is found in degenerating brain regions. Signs of

astrocytosis were initially found in the cerebral cortex of AD patients, particularly near amyloid plaques (Duffy 1980, Schechter 1981). This reaction may be due to factors released by damaged or dying neurons, signals released by activated microglia, or extracellular Aβ. The importance of astrogliosis in AD is unknown, though a variety of proposed beneficial and detrimental mechanisms have been explored in animal models of AD, including containment of neuronal dysfunction, inhibition of axon regrowth, Aβ clearance, and loss of their primary functions in cerebral blood flow regulation, metabolic homeostasis, and synaptic maintenance (Maragakis 2006).

As discussed above (see Section 1.2), microglia are also highly reactive in the brains of AD patients. They densely surround and infiltrate amyloid plaques (Itagaki 1989), even in patients with Aβ plaques but no dementia (Sasaki 1997, Vehmas 2003), suggesting microgliosis occurs early in pathogenesis. Microglia imaging using PET supports early microgliosis in AD patients, which is correlated with cognitive scores, despite amyloid plaque load showing no such correlation (Cagnin 2001, Edison 2008). The details of microgliosis have been best explored in cultured microglia and in mouse models of AD, many of which demonstrate the progressive deposition of amyloid plaques with plaque-associated microgliosis seen in AD patients (see Chapter 3).

1.7 Summary

Neurodegenerative diseases such as AD are devastating diseases with no effective treatments. While the characteristic Aβ plaques and neurofibrillary tangles have been modeled in animals and thoroughly studied, exploring other aspects of disease pathogenesis may provide additional promising therapeutic targets. One key candidate pathway is the microglia inflammatory response that is common to variety of chronic neurodegenerative diseases. This pathway has

been associated through genome-wide association studies with increased risk of neurodegenerative diseases, with the TREM2 R47H variant being the most strongly associated microglia gene (Tables 1 & 2). This supports the relevance of this inflammatory reaction to disease processes, with TREM2 signaling being the most promising target pathway.

Insights into the possible roles of the inflammatory reaction in the pathogenesis of disease are well illustrated by TREM2's well described functions in cell culture (Fig 1.2). First, TREM2 suppresses of pro-inflammatory factor secretion. These factors can be directly toxic to neurons and activate additional microglia and astrocytes, furthering the inflammatory cycle. Second, TREM2 promotes microglia phagocytosis, especially of apoptotic neurons. This may help clear dysfunctional neurons before they release damaging intracellular molecules, but also may promote phagocytosis and degradation of toxic proteins like Aβ. Finally, TREM2 improves microglia survival. This may support beneficial microglia actions, such as promoting synaptic plasticity, by preventing their loss with aging and disease.

Though the genetics of TREM2 in AD and PLOSL are clear, and *in vitro* studies support several intriguing mechanisms for TREM2's possible protective function, there is still uncertainty to the nature of TREM2s influence on neurodegeneration. TREM2 is actually upregulated in AD patient hippocampi and cortices (Lue 2014, Martiskainen 2015, Celarain 2016), as higher TREM2 expression is linked with a CD33 AD-risk allele in patient monocytes, leading some to suggest TREM2 may play a possible-pathogenic role (Chan 2015). In addition, two important initial studies using TREM2 knockout (KO) mice to abolish TREM2 signaling in mouse models of AD have produced some conflicting results (Jay 2015, Wang 2015). While both studies noted significant reduction in microgliosis surrounding the Aβ plaques, the effects on other signs of inflammation and AD pathogenesis were inconsistent. Jay et al. reported overall improvement,

with a reduction in pro-inflammatory cytokines, astrocytosis, and soluble Aβ/amyloid plaque load further questioning the protective nature of TREM2 signaling. Wang et al., on the other hand, reported exacerbation of several disease phenotypes including increased plaque and soluble Aβ load, accelerated neuronal loss, and increased pro-inflammatory cytokine expression (Wang 2015). These two papers seem to draw opposite conclusion to the role of reduced microgliosis with TREM2 inhibition in AD mice. Unraveling the exact role of TREM2 in AD will require new approaches.

Exploring TREM2 functions further *in vivo* will allow a more complete understanding of TREM2's activity in microglia in a normal and diseased brains. To date TREM2 KO mice and viral overexpression of TREM2 are the primary methods for manipulating TREM2 *in vivo*. Each of these methods has its own limitations for studying TREM2 in the context of chronic degeneration and assessing its therapeutic potential (see Section 2.1). The present study aims to address the need for new tools to further study TREM2 *in vivo*. To this end, we have developed BAC-TREM2 mice to genetically overexpress human TREM2 and increase TREM2 signaling (Chapter 2). We have crossed these mice with two different animal models of Alzheimer's disease to explore the effect of increased TREM2 signaling in a diseased brain (Chapter 3). These studies will provide insight into the function of TREM2 and microglia in neurodegeneration and help resolve controversies about the protective nature of TREM2. Furthermore, these studies can test the viability of the targeting the TREM2 pathway therapeutically. We have also developed BAC-TREM2-R47H mice with the disease associated TREM2 R47H variant to explore how it is different from normal TREM2 and may contribute to increased risk of neurodegeneration (Chapter 2).

Determining TREM2's protective potential is important, but BAC-TREM2 mice also will allow us to unravel the mechanisms of TREM2 action *in vivo.* This, in turn, may clarify the currently opaque nature of microglia's role in neurodegeneration as beneficial, detrimental or some combination. Multiple possible roles have been described to TREM2 signaling. Understanding which of these mechanisms is critical for TREM2's protective function will provide insight into the specific roles of microglia in neurodegeneration and provide downstream targets for future therapeutic exploration.

Overall, we propose that enhanced expression of human TREM2 with BAC-TREM2 will promote TREM2 signaling in microglia in vivo, leading to suppression of pro-inflammatory activation and promotion of phagocytosis. We further expect these altered microglia phenotypes will be beneficial in mouse models of AD, confirming the potential of TREM2 and microglia-focused treatment strategies in chronic neurodegeneration.

Figure 1.1

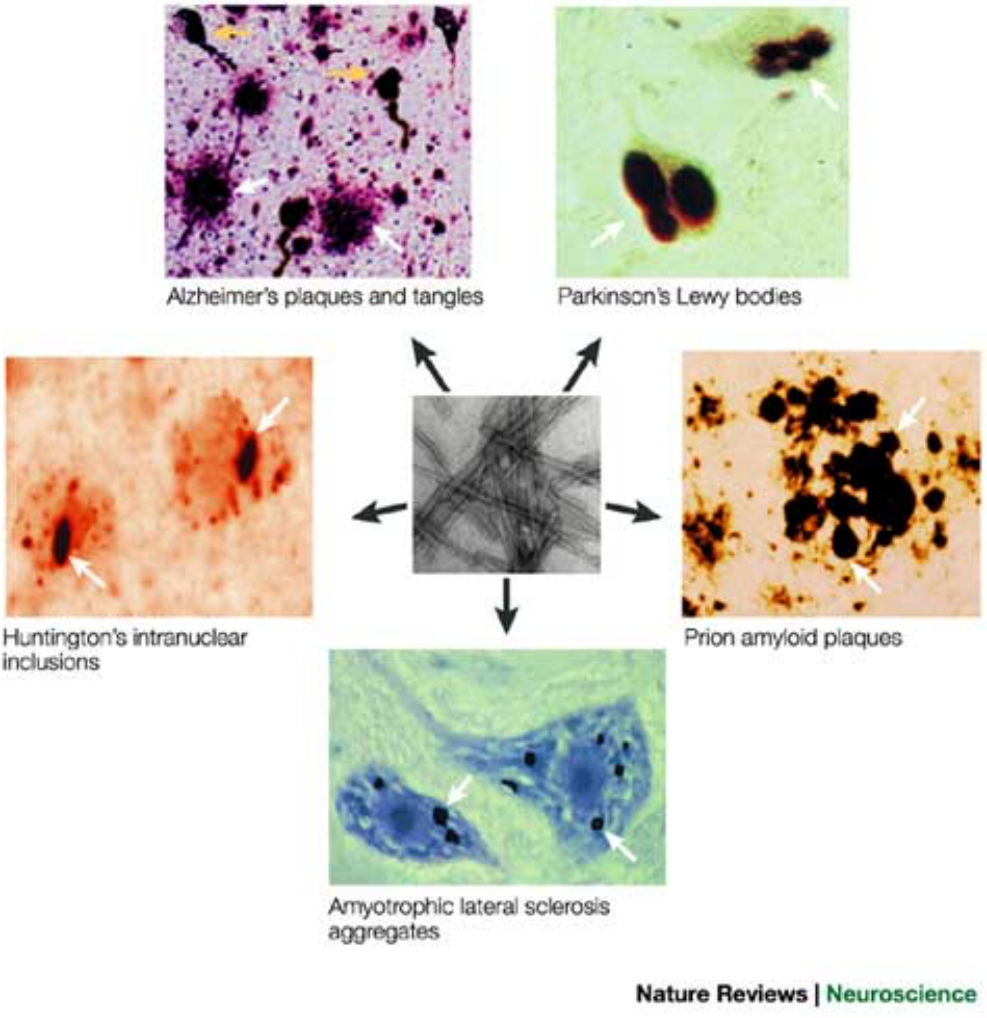

Fig 1.1 Accumulation of abnormal proteins in various neurodegenerative diseases. AD pathology is associated with intracellular tau-containing neurofibrillary tangles (white arrows) and extracellular Aβ-containing amyloid plaques (yellow arrows) (See Section 3.1.1). Some cases of prion diseases like spongiform encephalopathy also have prion protein-containing amyloid plaques. Other neurodegenerative diseases have there own characteristic abnormal protein species, including cytoplasmic α-synuclein containing Lewy bodies in Parkinson's disease and superoxide dismutase aggregates in ALS. HD pathology involves unique intranuclear mutant huntingtin inclusions. Despite the varied appearance on light microscopy and differing protein content, the electron microscopic ultrastructures of these protein accumulations often share a fibrillar conformation (center panel). Reprinted from Soto et al. 2003.

Table 1

Gene	Reference	Disease	OR*	Immune Function
IL-1α	Rainero et al., 2004	AD	**1.49	Cytokine
CD33	Naj et al., 2011	AD	0.91	Immune Cell Receptor
	Hollingsworth et al., 2011		0.91	
SHIP1	Lambert et al., 2013	AD	1.08	Intracellular Signaling
CR1	Lambert et al., 2009	AD	1.21	Complement Receptor
	Naj et al., 2011		1.16	
	Hollingsworth et al., 2011		1.18	
ABCA7	Naj et al., 2011	AD	1.15	Phagocytosis
	Hollingsworth et al., 2011		1.23	
APOE	Corder et al., 1993	AD	***4.39	Suppress Inflammation
	Corder et al., 1994		***0.25	
CLU	Lambert et al., 2009	AD	0.86	Suppress Inflammation
	Naj et al., 2011		0.89	
EPHA1	Naj et al., 2011	AD	0.90	Immune Cell Receptor
	Hollingsworth et al., 2011		0.87	
LRRK2	Simón-Sánchez et al., 2009	PD	1.14	Intracellular Signaling
HLA-DRA	Hamza et al., 2010	PD	1.26	Antigen Presentation
BST1	Satake et al., 2009	PD	1.24	Immune Cell Receptor
	Nalls et al., 2014	PD	1.13	
DDRGK1	Nalls et al., 2014	PD	1.11	Intracellular Signalingg
IL-1β	McGeer et al., 2002	PD	1.13	Cytokine

Table 1 Summary of immune-related genes with disease-associated variants found in large genetic studies. Suggested immune functions are described.
*OR = Odds Ratio
**Based on homozygous state for disease-associated allele
***Two different alleles of APOE were found to have opposing effects on AD-risk, either increasing (APOE4 allele) or decreasing (APOE2 allele) risk

Table 2

Reference	AD	PD	ALS	FTD
Guerrero et al., 2013	1.4/4.5			
Jonsson et al., 2013	2.9	NS		
Rayaprolu et al., 2013		2.7	NS	5.1
Ruiz et al., 2013	4.1			NS
Benitez & Cruchaga 2013		23.5*		
Reitz & Mayeux 2013	1.2			
Cuyvers et al., 2013	**			**
Borroni et al., 2014	2.5			4.0
Cady et al., 2014			2.4	
Slatterly et al., 2014	2.2			NS
Hooli et al., 2014	1.7			
Lill et al., 2015	2.7	NS	NS	NS
Liu et al., 2015		3.9/1.2		
Korvatska et al., 2015	**			
Rosenthal et al., 2015	7.4			
Mengel et al., 2016		NS		

Table 2 Summary of large genetic studies associating TREM2 variants with neurodegenerative diseases Alzheimer's disease (AD), Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS), & frontotemporal dementia (FTD). Numbers represent reported odds ratio from each study.

* = Odds ratio estimated from provided data

** = Significant association, but nature of analysis precluded traditional odds ratio calculation.

NS = Association tested, but no significance found

Figure 1.2

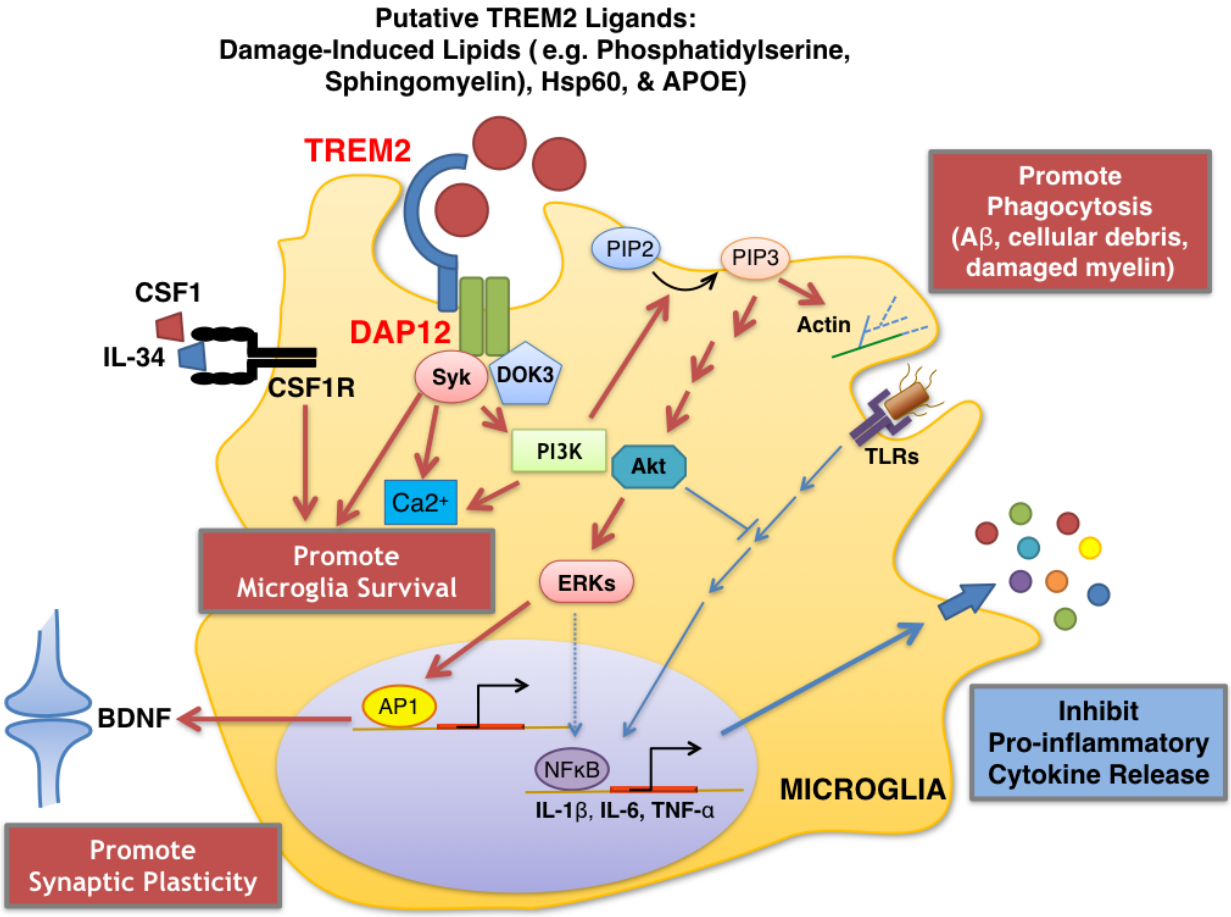

Figure 1.2 Schematic of TREM2 signaling and proposed protective functions in neurodegeneration. Putative TREM2 ligands, such as phosphatidylserine, Hsp60, and ApoE initiate TREM2 signaling. As TREM2 lacks an intracellular signaling domain, clustering of the TREM2-associated DAP12 signaling adaptor and subsequent phosphorylation of DAP12's immunoreceptor tyrosine-based activation motif (ITAM) by Srk-family kinases creates phosphorylated tyrosines (phosphotyrosines). Phosphotyrosines serves as docking sites for downstream signaling molecules such as Syk family kinases and DOK3, which initiate downstream signaling such as the PI3K pathway. TREM2 signaling leads to 4 proposed effects: 1) Increased actin mobilization which increases phagocytic capacity, aiding microglia in clearance of pathological proteins like Aβ, apoptotic cells, and damaged myelin that can inhibit neural repair. 2) Inhibition of TLR signaling and pro-inflammatory cytokine release in a DOK3 dependent manner. 3) Promotion of microglia survival, interacting with the CSF-1R signaling pathway. 4) Promotion of trophic factor secretion that supports synaptic health and plasticity.

Chapter 2

Generation and Characterization of BAC-TREM2 and BAC-TREM2 R47H Mice

2.1 Introduction

The genetics of PLOSL and TREM2's R47H variant, as well as *in vitro* investigations of TREM2/DAP12 function have led to the hypothesis that TREM2 signaling is beneficial in the context of neurodegeneration. Increased phagocytosis of dying neurons may prevent their necrotic death and the spread of inflammatory cell contents, while increased phagocytosis of toxic protein species such as Aβ may curb their toxicity. TREM2 mediated inhibition of NO and pro-inflammatory cytokine release may also reduce the more harmful parts of the inflammatory response. Two early *in vivo* studies using the experimental autoimmune encephalitis (EAE) mouse model support this idea, as inhibiting TREM2 signaling with an anti-TREM2 antibody exacerbated EAE, while augmenting TREM2 using viral overexpression in myeloid precursor cells ameliorated behavioral deficits (Piccio 2007, Takahashi 2007). Viral-mediated, high-level overexpression in transplanted peripheral myeloid cells, however, cannot address TREM2s function in microglia.

Recent studies have investigated TREM2's role in the inflammatory response to chronic neurodegeneration using two separate strategies. First is to genetically abolish TREM2 function using TREM2 knockout (KO) mice. TREM2 KO mice have been used in the context of mouse models of AD (Ulrich 2014, Jay 2015, Wang 2015), Traumatic Brain Injury (Saber 2016), prion-infection (Zhu 2015), experimental stroke (Sieber 2013, Kawabori 2015), and drug-induced demyelination (Cantoni 2014, Poliani 2015). The second major strategy has been to use a lentivirus based overexpression with a CD11b, myeloid lineage-specific promoter. This has been

used in animal models of AD (Jiang 2014, Jiang 2016a) and FTD (Jiang 2016b). However, studies using this method have serious caveats, including lack of confirmation for microglia-specific expression, exogenous promoter that do not confer proper spatial and temporal patterns of TREM2 expression, high levels of TREM2 overexpression, and putative toxicity with viral infection that may lead to neuronal cell loss over time (Dodart 2005, Jiang 2015).

A major goal of the thesis is to test the hypothesis that enhancing TREM2 levels or its signaling under endogenous genomic regulation may elicit overall beneficial microglia responses in the context of neurodegeneration that may modify the onset or progression of neurodegenerative diseases such as AD. To this end, we adopt a strategy to increase TREM2 gene dosage by generating bacterial artificial chromosome (BAC) transgenic mouse that modestly overexpresses human wildtype TREM2 under its endogenous genomic regulation (BAC-TREM2 mice). Based on the fact BAC transgenes contain large genomic DNA segments and often can confer endogenous-like transgene expression in vivo (Heintz 2001), we expect BAC-TREM2 mice will increase TREM2 expression levels in the endogenous TREM2-expressing myeloid cells including microglia. Moreover, BAC-TREM2 microglia are likely to show elevated TREM2-DAP12 signaling based on prior in vitro experiments demonstrating increased TREM2 signaling and a decreased pro-inflammatory response in cultured lentiviral-mediated TREM2 overexpressing microglia (Takahashi 2005, Hamerman 2006, Zhong 2015). This strategy has the added theoretical benefit of not indiscriminately activating TREM2, as it should maintain the native patterns of TREM2 activation, increasing its magnitude only when the natural ligands are present.

In addition to creating BAC-TREM2 mice, with microglia primed to effectively respond to chronic neurodegeneration, we generated BAC-TREM2-R47H mice in order to overexpress the

disease-associated R47H variant. The effect of this manipulation will be compared with the effects of BAC-TREM2 to understand the functional differences and *in vivo* consequences of the R47H variant. *In vitro* experiments in cultured cells have suggested a variety of differences between wild type and R47H TREM2, including reduced lipid ligand binding (Wang 2015), reduced ApoE binding (Atagi 2015, Bailey 2015), and reduced protein maturation and ectodomain shedding (Kleinberger 2014). All of these studies suggest a loss of function mechanism for the R47H variant.

Despite the *in vitro* evidence suggesting R47H is a loss-of-function variant, human genetic studies have discovered a few people homozygous for the R47H variant. If this was a full loss-of-function variant, these subjects should have manifested PLOSL (e.g. bone cysts with early dementia). However, all four subjects discovered lived into their fifties and sixties, well past the age of onset and eventual death in PLOSL patients (Jonnson 2013). In addition, several known full loss-of-function mutations in TREM2 have been examined for disease association, but have been shown no or smaller effects on AD risk (Guerreiro 2013, Cuyvers 2014). This suggests that R47H may have either a novel gain-of-function toxic effect, or partial loss-of-function. We reason that by overexpressing TREM2-R47H in an endogenous expression pattern, the BAC-TREM2-R47H mice may help directly test the gain-of-function toxicity hypothesis. Additionally, this model may also help to ascertain the partial loss-of-function hypothesis in the following scenario. If the BAC-TREM2 show a neuroprotective effect of overexpressing WT TREM2 in a neurodegenerative disease model *in vivo* (e.g. AD mice), a partial loss-of-function or TREM2-R47H would predict the BAC-TREM2-R47H should exhibit diminished, yet still neuroprotective effects, in the same disease model.

There are several rationales for our choice to overexpress human TREM2, but not murine Trem2 in these BAC transgenic mice. First, we expect mouse and human TREM2 have an evolutionarily conserved core function in microglia and other myeloid cells. By choosing human TREM2 to overexpress in the mouse, we will be able to elevate specifically this core function of TREM2 in microglia. This rationale is not unlike the method used to develop mouse models of neurodegenerative disorders, in which human version of the causal AD (e.g. APP, PS1) or HD genes are overexpressed in the mice. Second, assuming our overexpression enhances the core conserved functions of TREM2, using human alleles will allow us to test in mice small molecules and reagents that engage human TREM2 protein. Third, perhaps one of the most important reason to study human TREM2, is the AD-associated R47H variant confer disease risk in the context of the human TREM2 gene. Notwithstanding the potential transcriptional and RNA processing differences, the human TREM2 protein share only 69% of their amino acid sequence with the murine Trem2. There is non-conservative substitutions at 51 of the 226 residues between the two orthologues (NCBI Protein Blast). Thus, we reason that the study of the R47H variant in the context of human TREM2 genomic, mRNA and protein context may be the best strategy to test the potential dominant pathogenic toxicity hypothesis, similar to the modeling of other dominant neurodegenerative disorders with human BAC transgenics (Johnson 2011, Lee 2013). Indeed, recent genetic studies suggests that human pathogenic variants may act in synergy with cis-, human-specific protein context to elicit diseases (Jordan 2015). Therefore, we believe the study of human and murine variants of TREM2 will be complementary in understanding TREM2 biology and its role in AD, and our human BAC TREM2 models may have distinct advantages of uncovering the aspects of biology and disease related to human TREM2.

To conclude, this study will genetically overexpress human TREM2 in mice using bacterial artificial chromosome (BAC) transgenics for use with existing mouse models of chronic neurodegeneration. This is expected to increase TREM2 signaling, with expected effects on the inflammatory response and phagocytic capacity of cultured microglia.

2.2 Results

2.2.1 Generation of BAC-TREM2 Mice

We used the BAC transgenic approach to develop a mouse model of increased TREM2 signaling (Yang 1997, Gong 2002). BACs contain large regions of genomic DNA that carry several advantages over traditional transgenes used in mice. First, BAC's have been used in genomic sequencing, so BAC libraries encompassing most of the human and mouse genome are readily available. Second, the large size of BACs (~50-300kb) allows them to not only carry the entire genomic locus encoding the transcribed exons and introns of the TREM2 gene, but also the flanking genomic regulatory elements (i.e. promoter, enhancers, suppressors, & locus control regions). The BAC transgenes are more likely to faithfully reproduce the endogenous expression patterns of TREM2, avoiding any potential effects of less-finely regulated non-genomic transgenes (Heintz 2001). The BAC we chose for this study, RP11-237K15, is a 165 kb BAC with the entire TREM2 genomic locus as well as 50 kb 5' and 80 kb 3' of surrounding regulatory elements (see Fig 2.1), including a nearby Histone 3 Lysine 27 acetylation (H3L27Ac) modification, an epigenetic marker of active enhancer element (Creyghton 2010).

The third advantage of BAC transgenesis is that BAC transgenes can be readily engineered using homologous recombination in the bacteria. This was critical for our experiments, as TREM2 is located in the middle of the TREM locus, and RP11-237K15 BAC contained 3 other genes for TREM-like (TREML) proteins: TREML1, TREML2, & TREML4, which are thought to

function in immune system (Klesney-Tait 2006). To avoid overexpression of multiple TREML molecules in our BAC-TREM2 or BAC-TREM2-R47H mice, which would complicate the interpretation of the role of TREM2 itself in our functional study, we applied iterative steps of BAC modification with homologous recombination to eliminate critical exons of all three TREM-like genes on the BAC, while leaving TREM2 and the H3L27Ac site near TREML2 intact (Fig 2.1). The process of homologous recombination involves a shuttle vector designed with two homology arms (A and B arms) surrounding the region to be modified (Yang 1997, Gong 2002, Gong & Yang 2005, Fig 2.2). The shuttle vector also contains 3 important genes: RecA, SacB, and Amp. RecA enzymatically induces homologous recombination between the shuttle vector and the BAC, creating a 'co-integrate' with the entire shuttle vector integrated in the BAC. Co-integrates are positively-selected for based on the Ampicillin resistance provided by the Amp gene. The final, modified BAC is produced when the co-integrate 'resolves', eliminating the remnants of the shuttle vector and leaving only the desired modification. This is accomplished based on negative selection by sucrose, which is turned into a toxic metabolite in the presence of a SacB-encoded enzyme.

After all three surrounding TREM-like genes were deleted successively via this method, it was crucial to confirm the absence of gross rearrangements or deletions in the TREM2 BAC. This is accomplished via rare cut-site restriction fragment mapping with the pulsed-field gel electrophoresis (Schwartz and Cantor 1984). Using this technique, the modified TREM2 BAC was compared to the parental unmodified BAC (Fig 2.3a). NotI digest revealed identical fragments. Furthermore, a SalI digest reveled predicted restriction fragment length polymorphisms (RFLP), i.e. four additional SalI restriction sites added with each step of homologous recombination.

Intact TREM2 BAC was then purified using a cesium gradient ultracentrifugation. This requires careful handling and confirmation of no significant nicking or shearing during purification. Pulsed-field gel electrophoresis was used to analyze this purified BAC compared to unpurified BAC DNA, which is primarily supercoiled due to minimal handling. Similar to the unpurified BAC, the purified TREM2 BAC showed a strong upper band (Fig 2.3b), representing supercoiled DNA, which migrates more slowly when DNA is very large (Beverly 1988). In addition, there is no smear below the upper band, indicative of no significant shearing of the large BAC. After confirmation of intact structure, the TREM2 BAC was linearized with PS-SceI at a unique site found in the BAC backbone and transferred to an injection buffer using dialysis. This linearized BAC ran to the expected ~165 Kb site (Fig 2.3b).

This purified, linearized BAC (Fig 2.3b) was microinjected in the pronuclei of single-cell mouse zygotes in the FvB/N inbred background. The FvB/N background has the advantage of larger, more easily visualized pronuclei for more effective microinjections (Taketo 1991). In addition, they exhibit tremendous fecundity, with consistently larger litter sizes than other commonly used inbred strains such as C57Bl/6, SJL, and 129. Finally, they have low levels of aggression and reduced anxiety with constant handling (Taketo 1991). In addition, the FvB/N strain is especially vulnerable to neuronal insults, heightening the phenotype of animal models of neurodegeneration (Hsiao 1995, Royle 1999). There are downsides however, as the FvB/N background is prone to seizures and is limited in certain cognitive tests due to visual deficits (Pugh 2004). This injection produced two independent founder transgenic mice, which gave rise to germline transmission and generated BAC-TREM2 lines A and B.

2.2.2 Generation of BAC-TREM2-R47H Mice

As an additional tool to study the disease-associated R47H TREM2 variant, we made another mouse model overexpressing this AD-associated variant. The modified TREM2 BAC transgene used in BAC-TREM2 was further engineered using homologous recombination to introduce the R47H variant, which is caused by single nucleotide polymorphism (SNP) (guanine to adenine). Due to the difficulty in screening for modifications with a single base pair difference, a two-step strategy was employed (Fig 2.4). In the first step, exon 2 of TREM2, which encodes the R47 residue, was deleted and replaced with 'junk' DNA from an unrelated vector. In the second step this 'junk' DNA was replaced by Exon 2 with the R47H G to A mutation, producing an identical transgene construct to BAC-TREM2 except for this single SNP (Fig 2.4).

After several positively-screen colonies were found, the correct insertion of R47H was confirmed by both Sanger sequencing and restriction analysis (Fig 2.5). The G to A mutation abolishes a restriction cut site for HhaI and a PCR fragment containing this region can no longer be cut (Fig 2.5b). This newly modified BAC was purified as explained above for BAC-TREM2 (Section 2.2.1) and microinjected into the pronucei of one-cell FvB/N zygotes. Five independent BAC-TREM2-R47H founder mice were obtained (Lines A-E). 3 lines, Lines A, C, & D, demonstrated germline transmission and were further analyzed.

2.2.3 Transgene Integration and mRNA Expression

The relative number of TREM2 BAC transgenes inserted in all BAC-TREM2 and BAC-TREM2-R47H lines was compared using real-time polymerase chain reaction (RT-PCR) of genomic DNA samples (Fig 2.6). BAC-TREM2 Line A and BAC-TREM2-R47H Line A have an estimated copy number of 3.5-4 times that of BAC-TREM2 Line B. Due to their higher transgene levels and comparable levels of transgene insertion compared to each other, Line A of both BAC-

TREM2 and BAC-TREM2-R47H mice were chosen for further analysis. BAC-TREM2 and BAC-TREM2-R47H will refer to Line A for the remainder of this dissertation unless otherwise noted.

Expression of human TREM2 in BAC-TREM2 mice was analyzed in mRNA isolated from cultured primary neonatal microglia and mouse brain tissue. Consistent with the copy number data, Line A expressed significantly higher levels of human TREM2 than Line B in cultured microglia (Fig 2.7). Human TREM2 is not detected in WT mice (Fig 2.8a). A time course of human TREM2 expression was compared to endogenous mouse Trem2 after high dose LPS stimulation (100ng/ml) in microglia from the BAC-TREM2 Line A (Fig 2.8b). In both wild type (WT) and BAC-TREM2 microglia, mouse Trem2 exhibits an initial spike in expression at 2 hours, followed by a slow decline to below the original levels by 24 hours. Human TREM2 follows a similar pattern in BAC-TREM2 microglia (Fib 2.8b). Like mouse Trem2, human TREM2 is significantly reduced after LPS treatment (Fig 2.8a)

In vivo expression of human TREM2 was assayed by RT-PCR in cDNA from 6-month forebrain tissue in BAC-TREM2 and WT mice. Human TREM2 expression was detected in BAC-TREM2, but not WT mice (Fig 2.9).

2.5 Pro-inflammatory Response of Primary BAC-TREM2 Microglia

Knowing that human TREM2 is in fact expressed in TREM2 microglia and brain tissue, the next question was whether this expression increases TREM2 signaling and alters the immune response of BAC-TREM2 microglia. A major finding in macrophages, dendritic cells, and microglia is that TREM2 signaling suppresses LPS-induced pro-inflammatory cytokine (such as Il-1β & TNFα), but not anti-inflammatory cytokine (such as TGFβ) release (Takahashi 2005, Hamerman 2005/2006, Turnbull 2006). To assay the inflammatory response in BAC-TREM2

microglia, primary microglia cultures were stimulated with low-dose LPS (0.2 ng/ml) for 24 hours and cytokine mRNA expression was quantified using qPCR (Fig 2.10). Consistent with increased TREM2 activity, the pro-inflammatory cytokine Il-1β demonstrated significantly reduced expression after LPS stimulation, while expression of anti-inflammatory cytokine TGFβ was not changed in BAC-TREM2 microglia.

A known lipid TREM2 ligand, phosphatidylserine (PS), has been shown to result in Ca^{2+} influx in HEK cells ectopically expressing TREM2 (Wang 2015). Using this ligand, we directly stimulated TREM2 in BAC-TREM2 microglia and recorded the Ca^{2+} response with a Ca^{2+}-sensitive dye. Minutes after PS application, increased cellular Ca levels are seen in both WT and BAC-TREM2 microglia (Fig 2.11). However, BAC-TREM2 microglia demonstrate a higher peak and a significantly more sustained Ca-signaling response. Together these data suggest human TREM2 overexpression in BAC-TREM2 mice leads to increased TREM2 signaling with the expected corresponding inhibition of LPS-induced pro-inflammatory cytokine release.

Figure 2.1

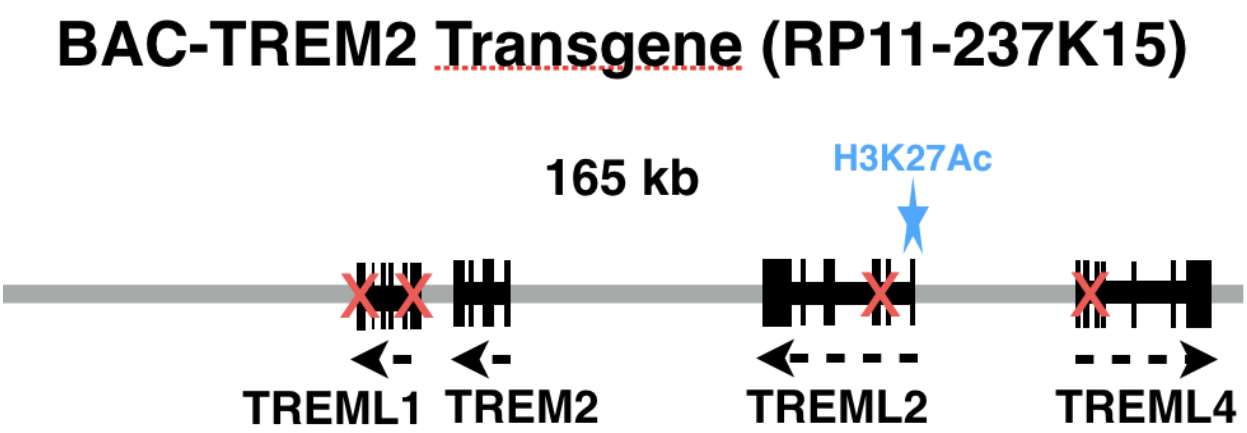

Fig 2.1 Schematic of BAC-TREM2 construct and engineering strategy. RP11-237K15 BAC contains 165 kb of human genomic DNA that includes TREM2 and 3 TREM-like (TREML) genes. Vertical bars represent mRNA exons and arrows represent the direction of transcription. Deletion of three TREML genes was engineered via homologous recombination (red X) to prevent overexpression of non-TREM2 genes. Exons 1-3 were deleted from TREML4, destroying a majority of the protein coding sequence. After deletion of exon 1 and 2 from TREML1, a downstream in frame ATG site was still present, so exons 5/6 were also deleted, abolishing 80% of TREML1's protein-coding sequences. TREML2's exon 1 was preserved, as it contains a region of high Histone 3-K27 Acetylation (blue star), suggesting an important transcriptional regulatory region for the TREM locus. Exon 2/3 were removed, frame-shifting the remainder of the mRNA and causing an early stop site in exon 4. This should lead to nonsense mediated decay of the truncated TREML2 mRNA (Hentze & Kulozik 2001), a strategy successfully used in mouse genetics in the design of knockout lines (Skarnes 2011).

Figure 2.2

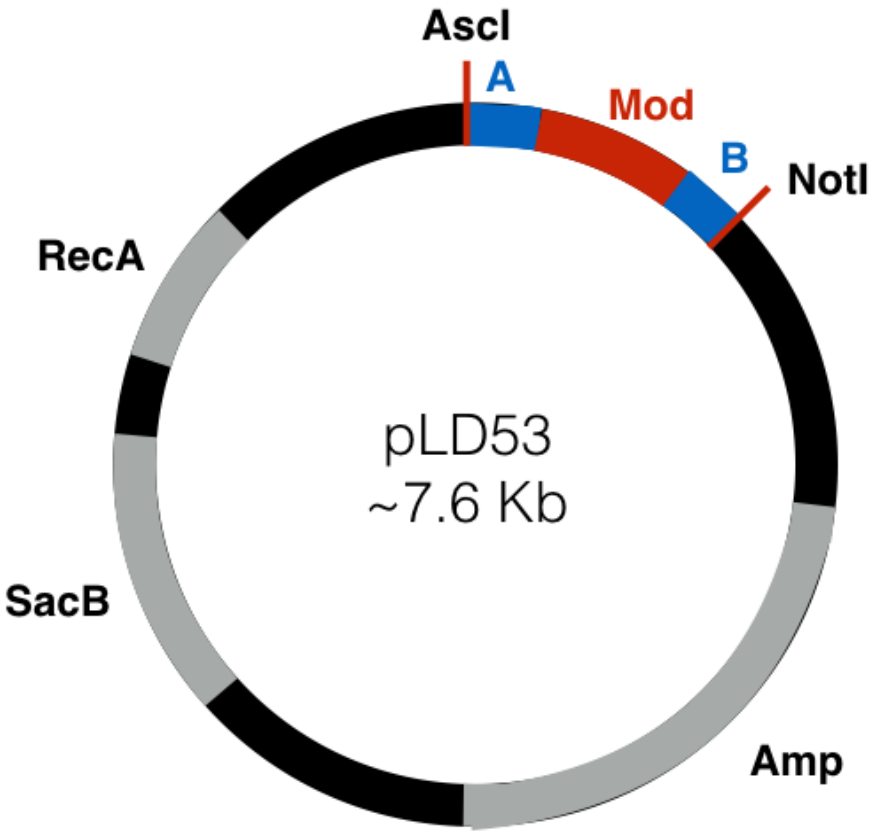

Fig 2.2 Map of pLD53 shuttle vector used for BAC engineering. Contains a recombination cassette consisting of homologous A and B arms (A & B respectively) surrounding the modification that will replace the original sequence (Mod). The RecA gene helps catalyzes homologous recombination of the recombination cassette with the original BAC DNA. Amp is used for positive selection with the antibiotic ampicillin of BAC: shuttle vector cointegrates, while SacB supports subsequent negative selection with sucrose for the final engineered BAC after excision of shuttle vector backbone.

Figure 2.3

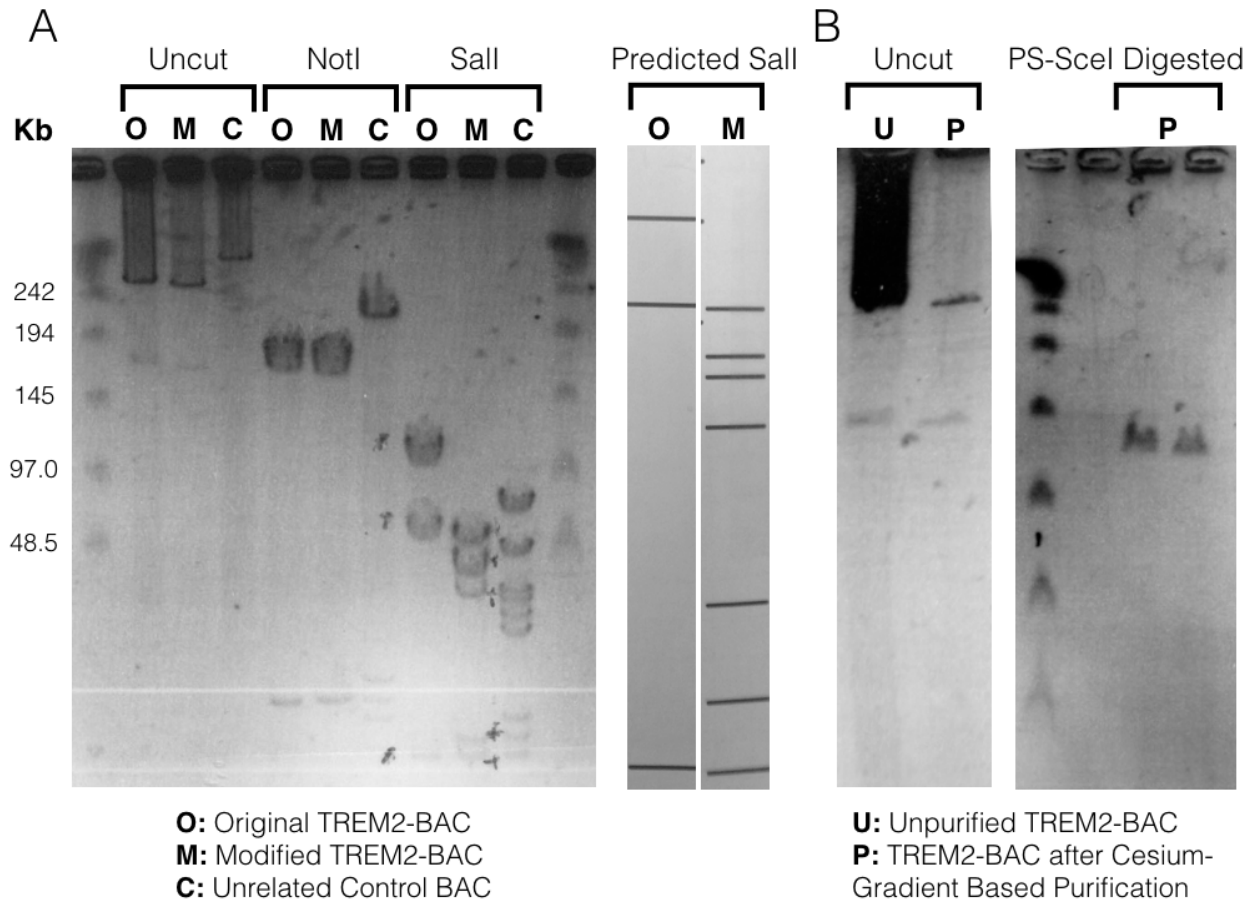

Fig 2.3 Confirmation of BAC-TREM2 BAC DNA Integrity. **A)** Pulse-field Gel Electrophoresis (PFGE) of low-frequency cutting site NotI and SalI BAC-TREM2 restriction digests to ensure no gross abnormalities after multiple rounds of homologous combination. Modified BAC-TREM2 (M) has the same pattern as the Original RP11-237K15 BAC (O) after NotI digestion. SalI digested modified BAC-TREM2 demonstrated predictable differences based on the introduction of SalI sites after each of the 4 modifications. **B)** Purified TREM2 BAC (P) was ran using PFGE to ensure no significant shearing (seen as smears below the top supercoiled band and a strong, intact supercoiled band (top). After linearization BAC-TREM2 ran to its expected size 165 kb size

Figure 2.4

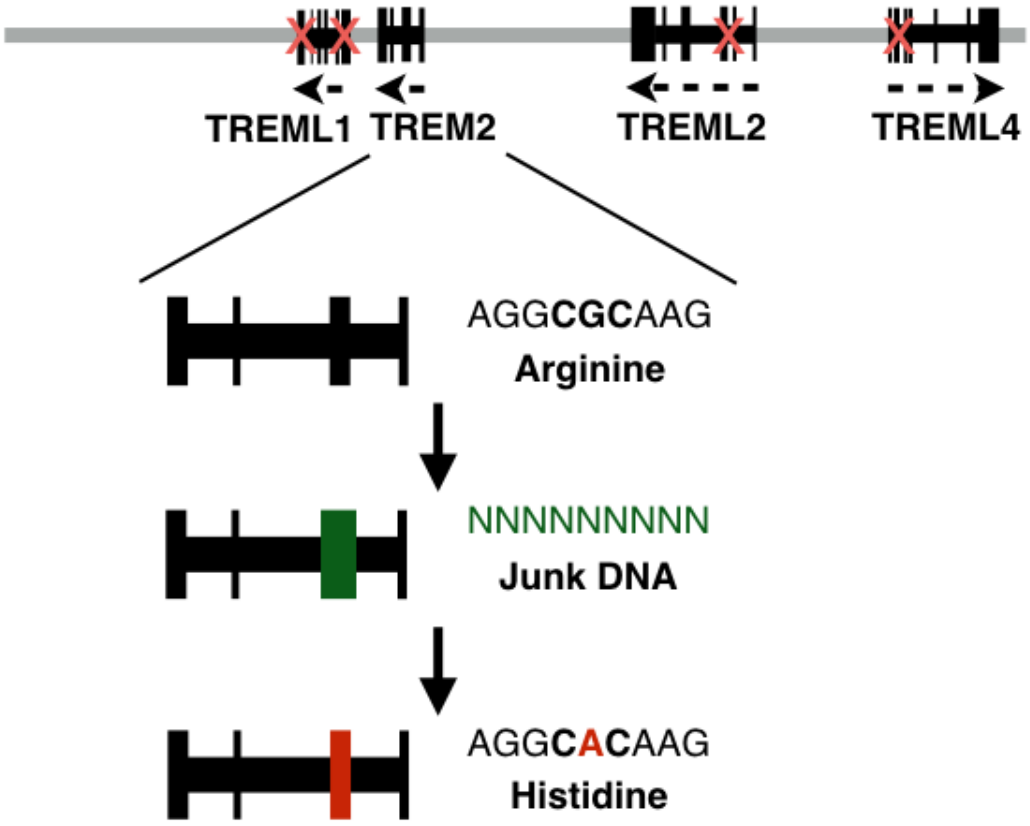

Fig 2.4 Strategy for introducing AD-associated R47H mutation into BAC-TREM2 BAC using two steps of homologous recombination. In order to aid screening for correctly recombined BACs, R47 encoding Exon 2 was first replaced with unrelated 'Junk DNA' (DNA). Then Exon 2 containing the R47H encoding SNP was re-inserted in its place to create the almost identical BAC-TREM2 R47H transgene with only a single base pair change.

Figure 2.5

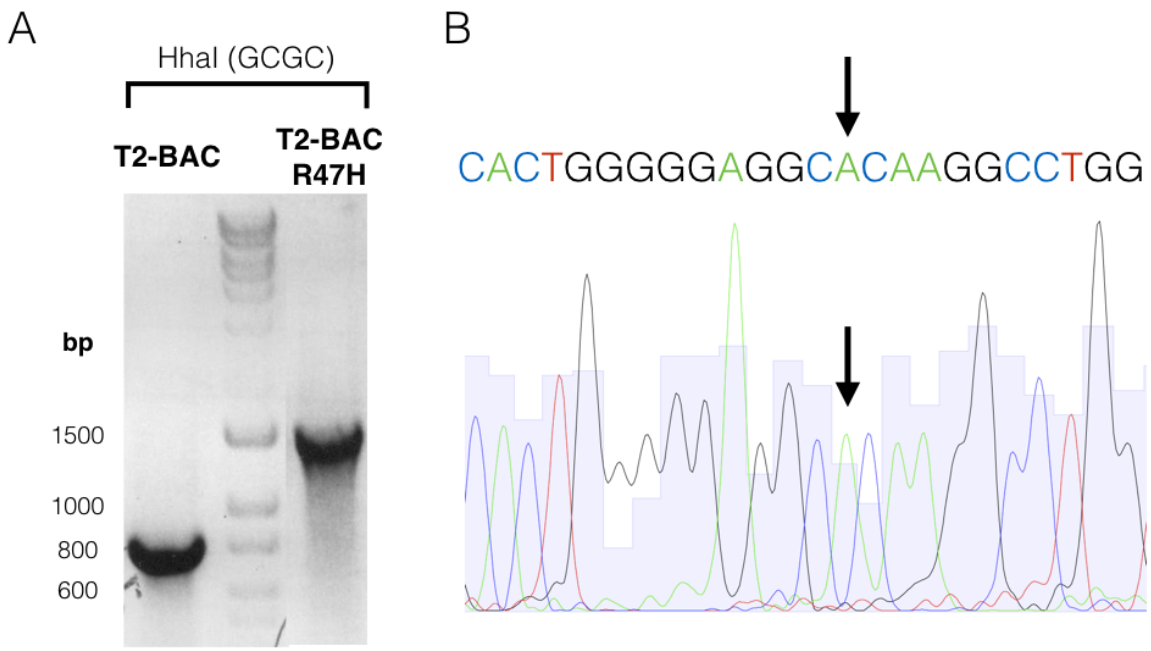

Figure 2.5 Confirmation of successful introduction of R47H SNP into BAC-TREM-R47H. **A)** HhaI restriction digest cuts PCR product from normal T2 BAC at GCGC sequence, creating two equal ~750 bp fragments. The R47H G to A SNP abolishes this sites and HhaI fails to cut it. **B)** Sanger sequencing confirms adenine nucleotide where normally a guanine would reside with no additional mutations (data not shown).

Figure 2.6

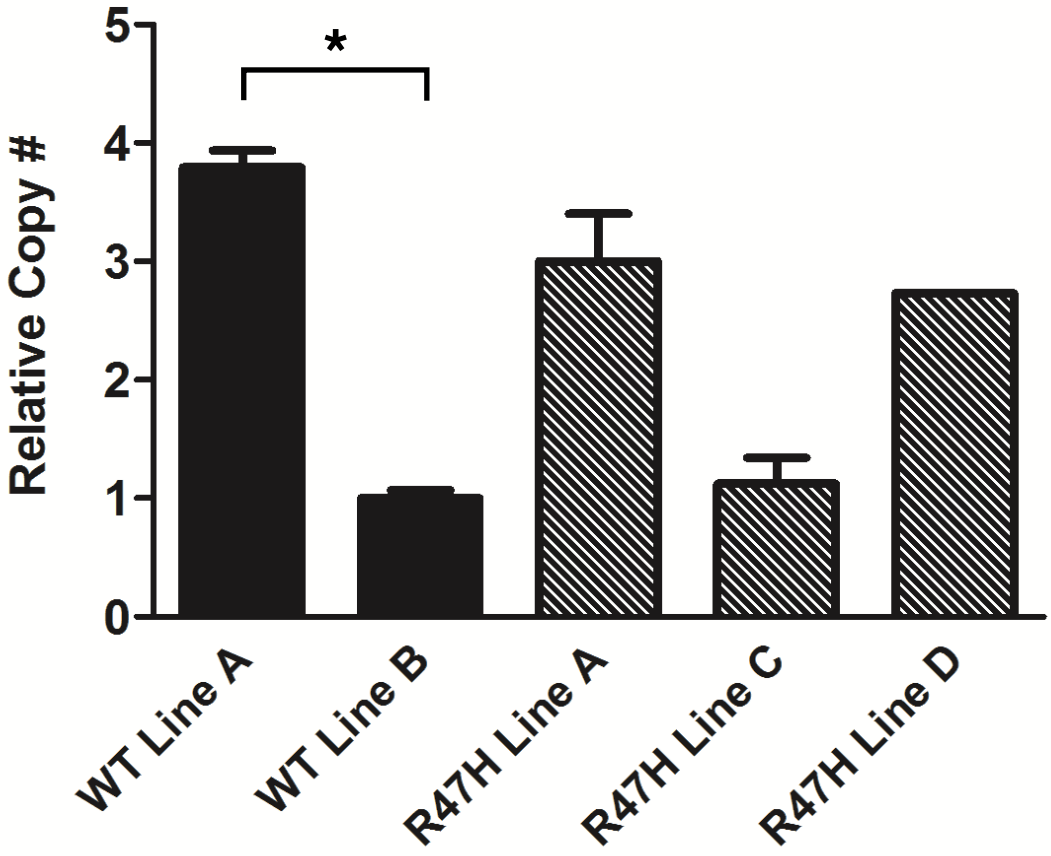

Fig 2.6 Analysis of copy number of BAC-TREM2 and BAC-TREM2-R47H transgenes in 5 different lines. Relative copy # was determined by RT-PCR of genomic DNA with BAC-TREM2 specific primers. Each value was normalized to β-actin and reported relative to BAC-TREM2 (WT) line B. A significant difference between BAC-TREM2 Lines A and B was found (p=.029, Mann-Whittney), but not between R47H line A and C, though there was a trend (p=.100, Mann-Whittney). Copy number for BAC-TREM2 Line A is approximately 3-4x Line B. BAC-TREM2-R47H (R47H) Line A copy number is approximately 3x that of Line C. n=4/genotype for BAC-TREM2 lines A/B, 3/genotype for BAC-TREM2-R47H lines A/C, and 1/genotype (founder) for line D. Error bars = SEM

Figure 2.7

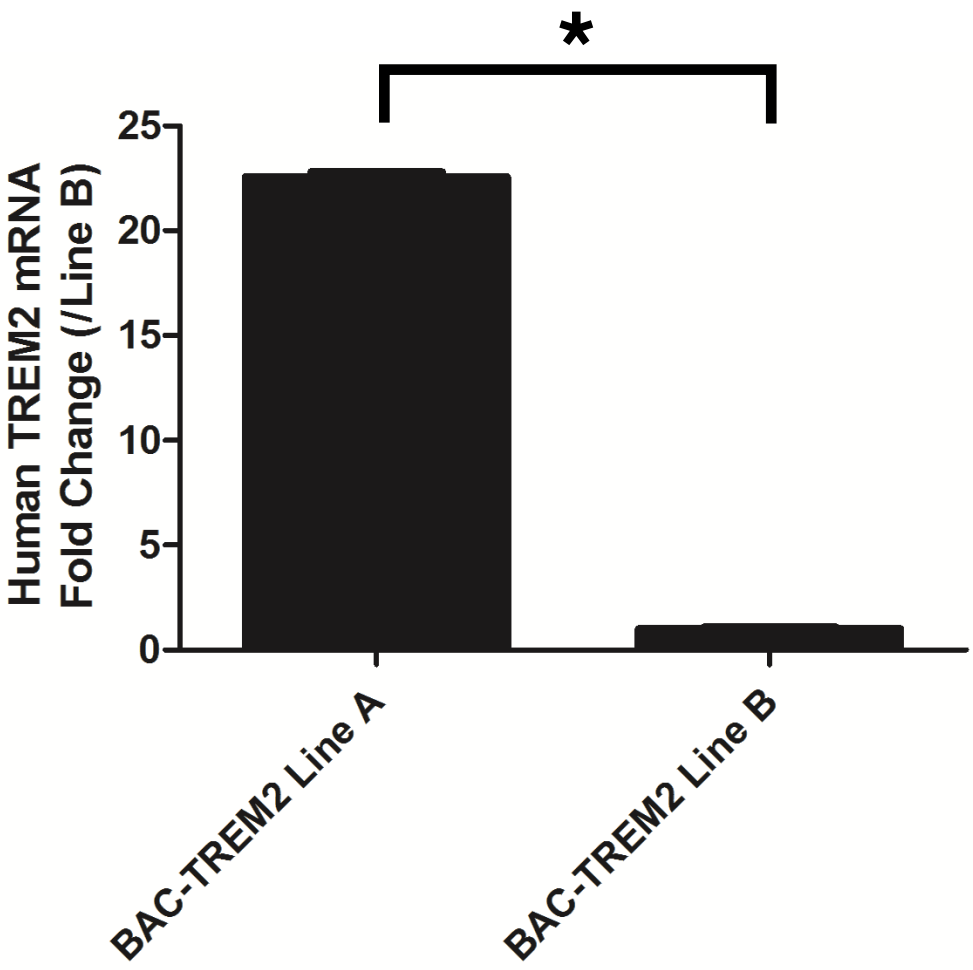

Figure 2.7 TREM2 expression in primary microglia cultures. RT-PCR analysis demonstrated that human TREM2 is expressed in unstimulated microglia in both BAC-TREM line A and B microglia. Each value was normalized to GAPDH expression and reported relative to BAC-TREM2 line B. Comparison of microglia from lines A and B showed significantly increased levels of expression in BAC-TREM2 line A ($p<.0001$, unpaired t test). The level of upregulation is estimated to be ~20 fold. n=2/genotype. Error bars = SEM

Figure 2.8

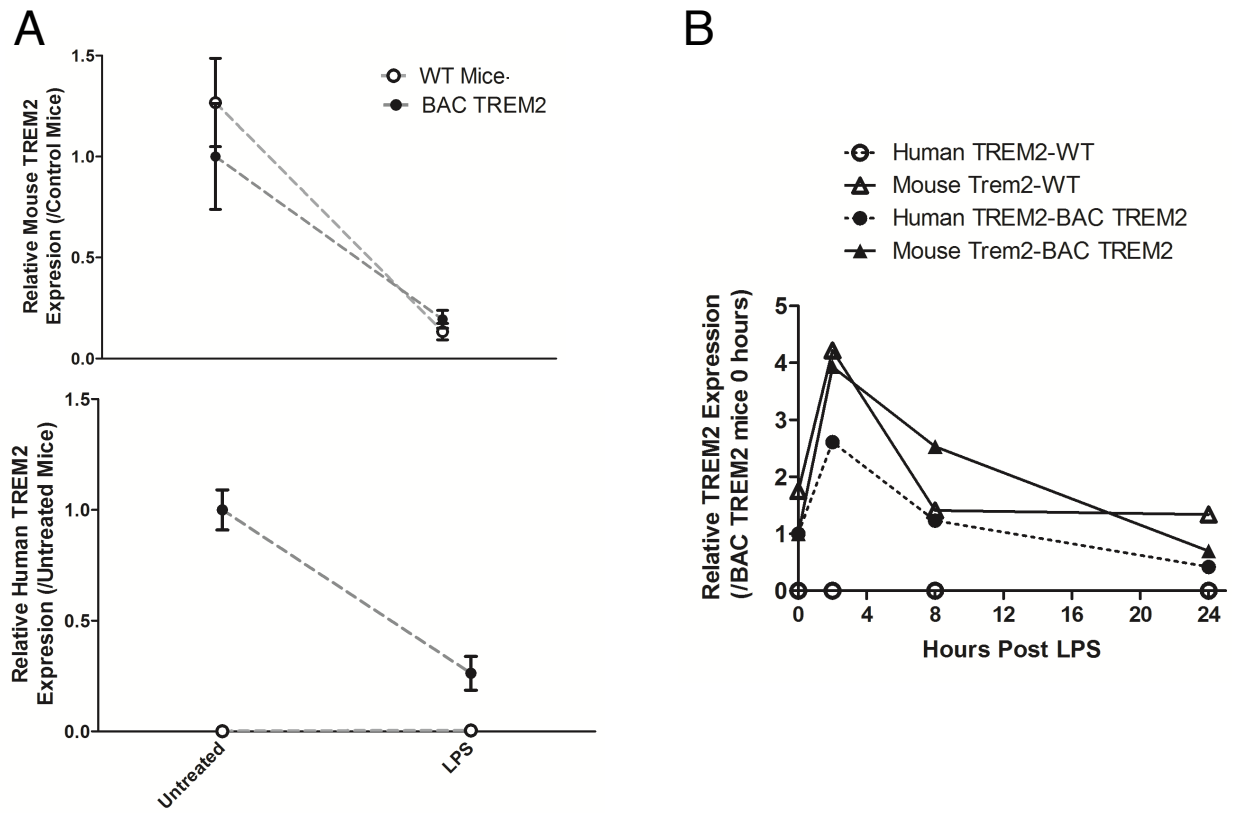

Fig 2.8 Intact regulation of human TREM2 transgene and endogenous mouse Trem2 after LPS stimulation in primary microglia cultures. **A)** RT-PCR analysis demonstrates low-dose LPS treatment (.2 ng/ml) induced a significant decrease in both mouse Trem2 (top panel) and human TREM2 (bottom panel) expression (2-way ANOVA, main effect on human TREM2 for LPS factor, p=.0004, F(1,10) = 27.61, main effect on mouse Trem2 for LPS factor, p=.005, F(1,4)=31.40). For human TREM2 analysis n=3/genotype for WT & 4/genotype for BAC-TREM2 microglia. For mouse Trem2 n=2/genotype. Values are mean +/- SEM normalized to GAPDH and reported relative to untreated BAC-TREM2 microglia. **B)** Mouse and Human TREM2 expression time course after high dose LPS stimulation (100ng/ml) shows similar pattern of early TREM2 induction followed by reduction at later time points. n=1/genotype. Human TREM2 was not detected in microglia from wild type mice in either analysis.

Figure 2.9

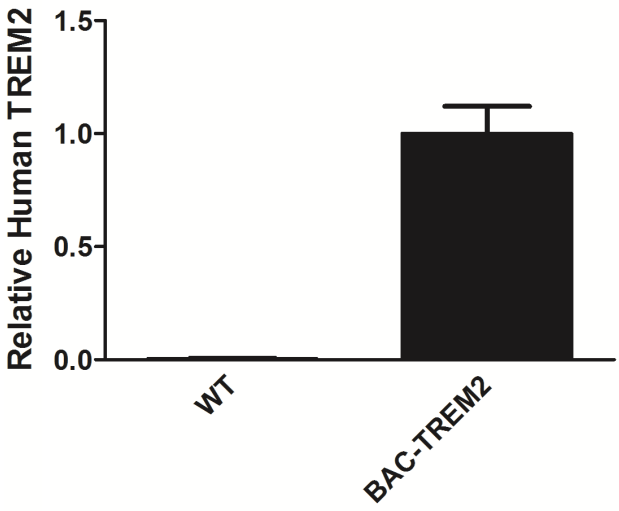

Fig 2.9 Human TREM2 transgene expression in 6 month BAC-TREM2 mice. RT-PCR analysis of human TREM2 expression demonstrated BAC-TREM2 transgene was detectable in BAC-TREM2, but not WT mice forebrains. n=2 mice/genotype. Values normalized to BAC-TREM2 expression. Errors bars = SEM.

Figure 2.10

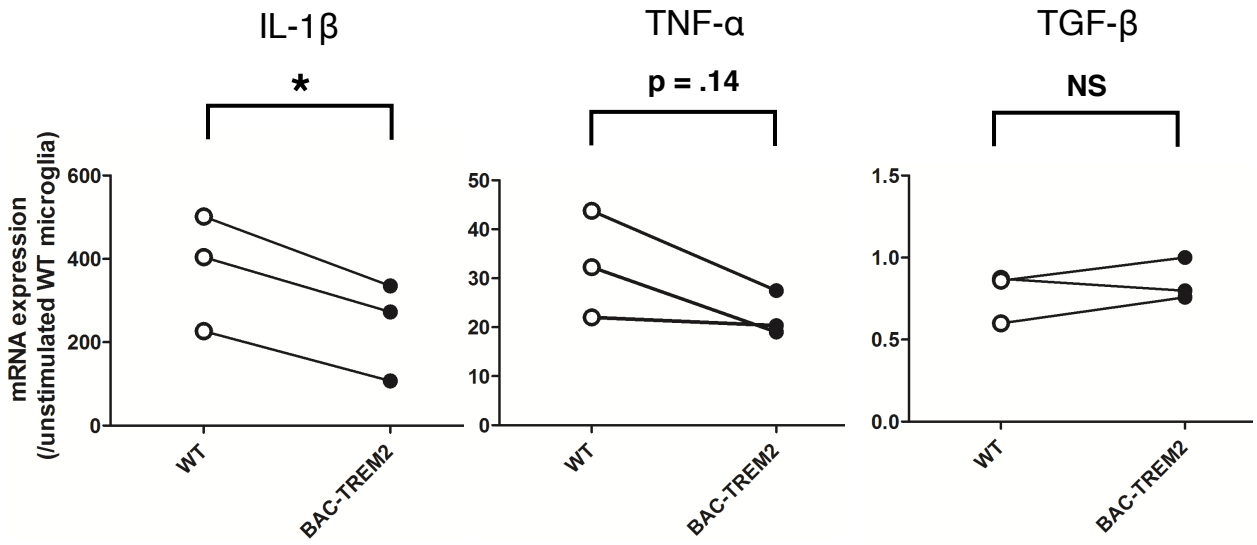

Fig 2.10 BAC-TREM2 suppresses pro-inflammatory cytokine response in primary microglia cultures. RT-PCR analysis after low-dose LPS (.2ng/ml) stimulation demonstrates BAC-TREM2 microglia have significantly reduced LPS-induced pro-inflammatory cytokine IL-1β expression (p=.01, paired T test), non-significant reduction in TNF-α (p=.14, paired T test), and no change in TGF-β (p=.4133, paired T test). Values represent individual cultures paired based on the time of culture and littermate origin. n=3 individual cultures/genotype. NS = not significant.

Figure 2.11

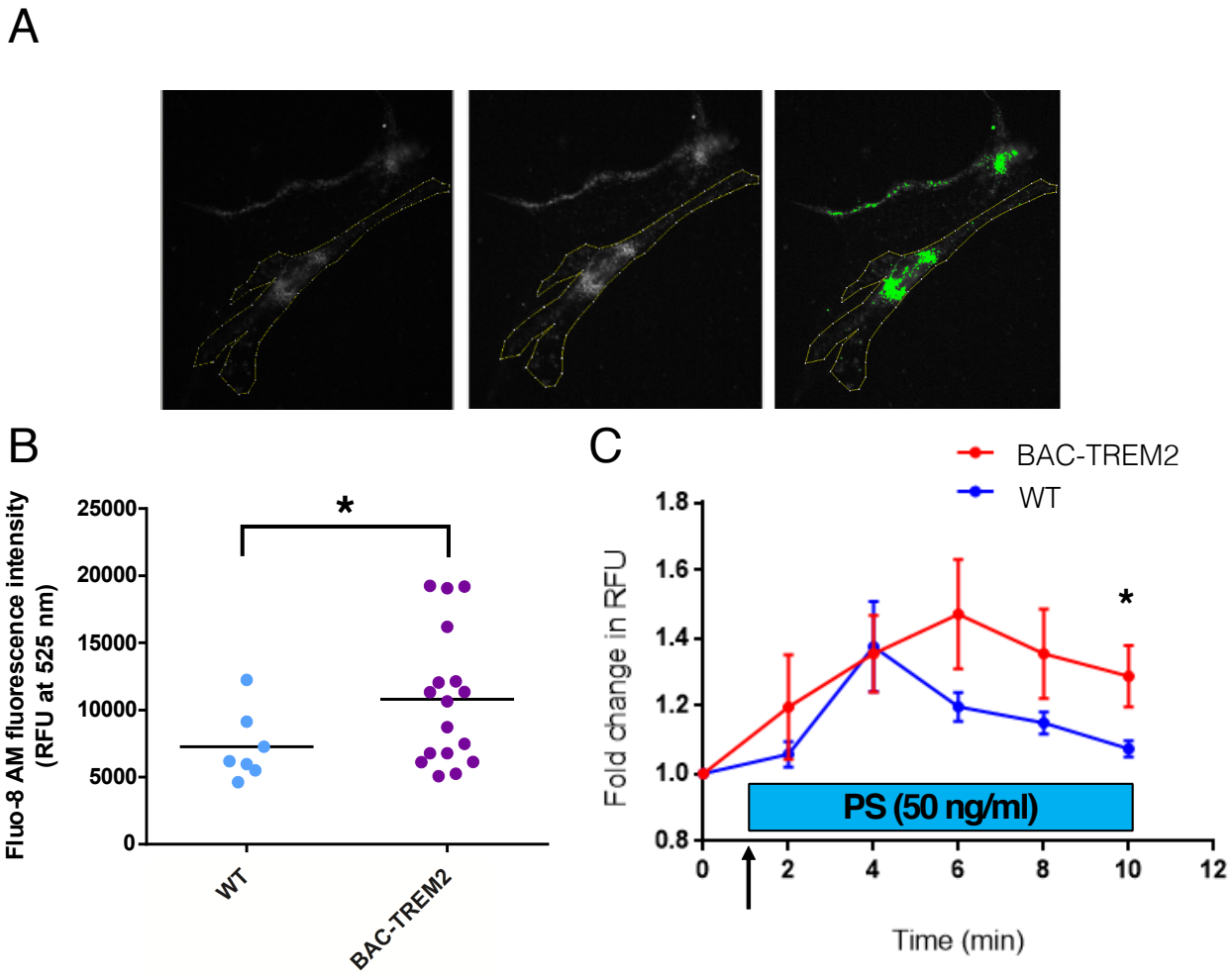

Fig 2.11 Increased Calcium (Ca2+) signaling in BAC-TREM2 microglia. **A)** Representative Images of Ca2+ Signal. Pseudocolored image (third panel) represents difference between PS-induced maximal Ca2+ fluorescence (Fmax, second panel) and the unstimulated minimal Ca2+ fluorescence (Fmin, first panel). **B)** Baseline Ca2+ flux before stimulation was quantified from WT (light blue) and BAC-TREM2 microglia (purple). n=7 for WT & 17 for BAC-TREM2 **C)** Time course of Ca2+ flux after phosphatidylserine (PS) stimulation (50ng/ml; arrow) demonstrates extended Ca2+ flux in BAC-TREM2 microglia (red), which is statistically significant at 10 minutes ($p<.05$, unpaired T test). n=4/genotype. {<.05. Values represent mean +/- SEM. Unpublished data; Bodhinathan K and Xu H.

2.3 Discussion

2.3.1 BAC-TREM2 Mice: Novel Tool to Study TREM2

We have generated two new transgenic mouse lines, BAC-TREM2 and BAC-TREM2-R47H, novel tools for the study of TREM2 biology and its influence on mouse models of neurodegeneration *in vivo*. We have increased human TREM2 or disease-associated TREM2-R47H allele gene dosage under its endogenous genomic promoter. Finally, we have demonstrated that this alters TREM2 signaling and the microglial pro-inflammatory response in primary culture.

These mice extend our ability to manipulate TREM2 *in vivo.* Previous strategies have used KO mice to inhibit TREM2 (Wang 2015) and viral overexpression to stimulate TREM2 (Jiang 2014). KO mice are valuable in genetic modifier studies of disease. They can test the viability of therapeutic inhibition of a given pathway *in vivo*. As a large portion of therapeutic compounds are designed to antagonize known pathogenic pathways, KO mice, especially in the heterozygous state that may provide a proxy for partial inhibition, serve as an effective initial test to the viability and safety of blocking a target gene's function. However, inhibition still may not have the expected effect, as compensatory responses and similar genes can mask the effect of inhibiting a single gene (Son 1996, Gingrich & Hen 2000).

For TREM2, inhibition is not the therapeutic goal, as TREM2 signaling is hypothesized to be protective based on the association of TREM2 loss-of-function with neurodegeneration in human genetic studies (Paloneva 2002, Guerreiro 2013, Jonsson 2013). In this situation, KO mice are not optimal for testing the therapeutic potential of TREM2, as TREM2 KO is expected to worsen disease phenotypes in mouse models. This does not reliably signify TREM2 activation will improve disease phenotypes, especially considering the relative lack of specificity

of the phenotypes in most mouse models of neurodegeneration. General motor and memory behavioral tests used to evaluate mouse models (like accelerating rotarod and fear conditioning assays) can decline for a variety of reasons unrelated to specific disease pathogenesis, especially when multiple unrelated insults are combined together (Norden 2015). For this reason, BAC-TREM2 genetic activation of the TREM2 pathway is better suited to test the therapeutic potential and function of TREM2 in neurodegeneration models.

Viral overexpression of TREM2 is potentially an attractive alternative method to testing the benefit of TREM2 signaling in neurodegenerative disease, but has its own set of strengths and weaknesses. As compared to the months to years it currently takes to engineer, breed and characterize a transgenic mouse line, viral constructs can be cloned and packaged for injection within weeks. In addition, maintaining virus constructs and infective particles requires much fewer resources than maintaining lines of transgenic mice in a vivarium. Together these advantages allow viral studies and follow-up experiments to occur at quicker pace with minimal initial investment. The second major advantage of a viral approach is it provides more temporal and anatomical control of TREM2 expression by controlling the timing and location of stereotactic injection. However, the efficiency of transduction drops with distance from the injection site and the spread pattern and exact location differ between animals, increasing experimental variability. Finally, viral overexpression is more directly transferable to human disease, as gene therapy using viral vectors is a valid therapeutic approach being thoroughly explored to treat neurological disease (LeWitt 2011, Simonato 2013)).

Viral TREM2 expression has major limitations, especially in the context of studying gene function and pathogenesis in the microglia. Despite the use of a myeloid-lineage specific CD11b + promoter (Jiang 2014, Jiang 2015a), there is currently no reproducible evidence for a viral

based approach that consistently, efficiently, and specifically target microglia in the mouse brain. Moreover, the expression levels and spatial and temporal specificity of an exogenous promoters will not faithfully mimic the endogenous TREM2 genomic regulation. For example, CD11b+ promoter in a transgene drives ubiquitous microglia expression, but endogenous TREM2 appear to express at low levels in quiescent microglia but at high levels upon neuronal insults and around amyloid plaques. Another major hurdle with lentiviral overexpression of TREM2 is the neuronal injury introduced by stereotactic injection and toxicity associated with chronic viral infection (Dodart 2005, Jiang 2014). Finally, unlike AAV, lentivirus does not spread very well from the site of injections, therefore at best the lentiviral approach can only infect microglia surround an injection sites. Therefore, the use of viral approach introduce many aspects of confounding factors that prevent unambiguous interpretation of the findings in the study of TREM2 *in vivo*.

Genetic overexpression of TREM2 in BAC-TREM2 addresses many limitations of these current *in vivo* techniques. BAC-TREM2 is expected to improve disease outcomes, which better evaluates the therapeutic benefit of TREM2 signaling than TREM2 KO mice. The downstream effects of TREM2 can be more readily assayed and may provide additional therapeutic targets. Unlike lentiviral TREM2 overexpression, BAC-TREM2 can increase TREM2 signaling for the entire lifetime of the mouse without overt toxic effects (see Chapter 3). Genetic overexpression in BAC-TREM2 also provides valuable consistency to the pattern and levels of overexpression between experimental animals, as every cell contains the same amount of the transgenic construct, unlike with viral overexpression. In addition, TREM2 levels are clearly regulated with respect to development and microglia activation (Fig 2.8, Cherthoff 2013). This is recapitulated more faithfully in BAC transgenic using large (~100 kb) endogenous genomic regulatory regions

that drive a more natural pattern of expression than the small, limited promoters used in viruses (1-5 kb).

2.3.2 BAC-TREM2 Mice: Potential Uses

BAC-TREM2 mice will allow us to investigate the potential beneficial effects of increased TREM2 gene dosage in microglia and other peripheral myeloid cells in chronic neurodegeneration. By analyzing BAC-TREM2's effect on mouse models of neurodegenerative disease, such AD and HD, the extent of any benefit on disease behavioral and pathological phenotypes will be apparent (see Chapter 3). This would validate the protective nature of TREM2 signaling suggested by human genetic studies and help determine the limitations to that benefit. Will TREM2 prevent disease from progressing or just slow it down? Will it improve all phenotypes or only a subset? Second, BAC-TREM2 mice help validate a general approach to treating neurodegeneration: targeting microglia and the immune response. Though recognized as a correlate to neurodegeneration early, microglia reactivity has been understudied and under-appreciated before the past decade, often dismissed as an epiphenomenon. Recent human and mouse genetic studies however, have accented the importance of the microglia reaction in disease progression. Any benefit of BAC-TREM2 will further support the critical importance of understanding the role of microglia and neuroinflammation in neurodegenerative disease.

Importantly, BAC-TREM2, as well as BAC-TREM2-R47H, has multiple lines available. With all transgenic lines generated by random insertion, the confounding effect of insertional mutagenesis must be considered. Though chances of disrupting the sparse protein coding regions of the mouse genome are relatively low, it can happen (Cases 1994), and insertion of

the transgene can also disrupt regulatory elements and less-understood non-coding RNAs (Picciotto 1998). For this reason, it is appropriate to test key phenotypes of any transgenic mice in at least two lines, such as BAC-TREM2 Lines A and B. (Yang & Gong 2005)

Finally, BAC-TREM2 mice will allow us to further explore the mechanisms of TREM2 action *in vivo.* Understanding which of the several beneficial consequences of TREM2 signaling (i.e. pro-inflammatory suppression, increased phagocytosis, microglia survival) are important to TREM2's protective function *in vivo,* will illuminate specific functions of microglia activation in neurodegeneration and provide downstream targets for future investigation.

2.3.3 BAC-TREM2-R47H Mice

Recent interest in TREM2 and microglia function in neurodegeneration has been driven by numerous studies linking TREM2 variants, particularly the R47H variant, to Alzheimer's disease (Guerreiro 2013, Jonnson 2013). BAC-TREM2-R47H mice provide an excellent opportunity to study the altered function of this variant *in vivo.* Studies to date on the mechanism of R47H have all been performed in cultured cells. They have found multiple deficits in R47H, including protein maturation and ectodomain shedding (Kleinberger 2014), ApoE binding (Atagi 2015, Bailey 2015), and lipid sensing, though binding of certain lipids is not affected (Wang 2015). Human genetics, however, suggests this variant is not simply a complete loss-of-function mutation, as homozygous R47H subjects do not exhibit signs of early dementia and mortality seen in PLOSL, the genetic disease caused by homozygous loss-of-function mutations in TREM2 or its signaling adaptor DAP12. Furthermore, R47H has an especially strong association with AD risk compared to other variants, including those known to cause PLOSL (Guerreiro 2013, Cuyvers 2014), suggesting additional effects beyond haploinsufficiency.

BAC-TREM2-R47H mice can help clarify these questions. If R47H is simply a loss-of-function mutation, crossing BAC-TREM2-R47H to mouse models of neurodegeneration should provide no benefit, unlike the expected effects of BAC-TREM2 mice (see Chapter 3). If R47H is a partial loss of function, then improvement may be seen, but will reduced when compared to BAC-TREM2. One important caveat is that overexpression of a mild, partial loss-of-function variant may still increase TREM2 signaling, leading to similarities between the effects of BAC-TREM2 and BAC-TREM2 R47H. Analyzing the multiple lines available with differing levels of expression (Fig 2.6) may help address this issue.

BAC-TREM2-R47H mice are ideally suited to test the possibility of dominant, gain-of-function effects. The R47H variant is associated with a significant increased risk of AD, but it is nowhere near the effect of familial AD genes, like PS1 mutations. This means a simple R47H knock-in with normal mouse levels of expression may have subtle, difficult to detect effects. Overexpressing TREM2 R47H will increase the size of any gain-of-function effect and our ability to detect it. In addition, the R47H allele may have different effects in human compared to mouse TREM2. Studying R47H in the context of the human protein in BAC-TREM2-R47H mice addresses this concern.

If the R47H variant of TREM2 does in fact have a dominant effect, crossing BAC-TREM2-R47H mice with models of neurodegeneration will worsen disease phenotypes. No mechanism has been suggested, but a dominant-negative effect is a likely possibility. By occupying TREM2's signaling adaptor, but with a reduced ability to detect TREM2 ligands, the R47H variant may crowd out wild type mouse Trem2. Additionally it could interfere with its surface traffic (Kleinberger 2014). In this case, the effect of BAC-TREM2-R47H should be similar to heterozygous TREM2 KO mice (Wang et al 2015). A dominant-negative mechanism would also

explain why homozygous R47H does not cause PLOSL if enough residual TREM2 function remains.

Finally, at the very least, BAC-TREM2-R47H mice serve as in ideal control for BAC-TREM2. Since we are interested in human disease relevant effects of TREM2, any improvement induced by BAC-TREM2 should not appear or at least be reduced with BAC-TREM2-R47H. If a subset of BAC-TREM2 effects also occur with BAC-TREM2-R47H, those effects are less likely to be disease relevant than effects that only occur with BAC-TREM2. Also, although the three major TREM-like genes on the original TREM2 BAC were deleted from the BAC-TREM2 and BAC-TREM2-R47H transgene (Fig 2.1), this BAC may contain pseudogenes and functional unannotated non-coding RNAs. It is important to consider the possibility that any effects seen in these mice may be due to the presence of these other factors, and not TREM2. BAC-TREM2-R47H controls for this, as its effects are expected to be reduced, non-existent, or opposing that of BAC-TREM2, despite the facts the BAC transgene is identical aside from the single R47H SNP. If improvements seen in BAC-TREM2 mice are not seen in BAC-TREM2-R47H, then off-target effects of other pseudogenes and non-coding RNA are likely not responsible..

From a BAC engineering perspective, a two-step process was used to introduce the R47H SNP into the TREM2 BAC via homologous recombination. This strategy first introduced sham DNA for TREM2 exon 2 and then replaced it back with R47H TREM2 (Fig 2.4), aiding screening and ensuring homogeneity of the alteration. Additionally, the R47H SNP abolishes a restriction site for HhaI, a simple genotyping method with PCR followed by a short digest can differentiate between BAC-TREM2 and BAC-TREM2-R47H transgenes (Fig 2.5), even if they are combined together in the same mouse.

BAC-TREM2-R47H mice offer a novel tool to investigate this disease-associated variant and normal human TREM2 signaling *in vivo.*

2.3.4 BAC-TREM2 and BAC-TREM2 R47H mice: Transgene Expression

Both BAC-TREM2 (2 lines) and BAC-TREM2-R47H (3 lines) mice were analyzed for transgene copy number (Fig 2.6). For each, there were lines with high (BAC-TREM2 Line A and R47H Lines A & D) and low (BAC-TREM2 Line B, R47H Line C) copy number. As this matched well with RNA levels in primary BAC-TREM2 microglia (Fig 2.7), both BAC-TREM2 and BAC-TREM2-R47H are likely to have high and low expressing lines available. This will permit further exploration of a dose-dependent effect of human TREM2 expression. Further confirmation of TREM2 expression at the protein level is needed (see Chapter 4).

2.3.5 Influence of Human TREM2 Overexpression on Primary BAC-TREM2 Microglia

Since this is the first time a stable transgene was used to genetically overexpress TREM2, it was important to confirm this led to increased TREM2 signaling, as has been shown with viral overexpression. Using primary microglia cultures, we demonstrated a major known effect of increased TREM2 signaling: decreased inflammatory cytokine release in response to stimulation (Fig 2.8). We also demonstrated that a known TREM2 ligand, phosphatidylserine, elicited increased Ca^{2+} signaling activity in BAC-TREM2 microglia compared to WT microglia (Fig 2.9). Furthermore, this confirms human TREM2 can be effective in mouse cells with mouse signaling proteins.

2.3.6 Summary

We developed novel genetic tools to increase gene dosage of TREM2 (TREM2-BAC mice) and explore the function of an important disease-associated variant (TREM2-BAC-R47H mice).

BAC-TREM2 mice express the human TREM2 transgene in cultured microglia and the aged brain, and transgene expression is associated with increased TREM2 activity and reduced pro-inflammatory cytokine release in cultured microglia. Through upregulation of this neurodegeneration-linked TREM2 signaling pathway, TREM2-BAC mice allow us to test our hypothesis that TREM2 signaling primes microglia for a more effective, beneficial response to chronic neurodegeneration (see Chapter 3).

Chapter 3

Genetic Evidence that Increased Human TREM2 Gene Dosage Alters Microglia Function and Ameliorates AD-related Phenotypes in Mouse Models.

3.1 Introduction

In the experiments described below, we aim to investigate the possible benefit of enhanced TREM2 signaling in microglia of mouse models of AD. First we will introduce the various considerations for mouse models of AD, how they have been used to investigate the role of microglia in AD, and the limited current data on the influence of TREM2 in these models.

3.1.1: Mouse Models of AD

Transgenic mouse models are the most commonly used animal models of Alzheimer's disease. There are dozens of widely available models, each based around our knowledge of Familial Alzheimer's disease (FAD)/frontotemporal dementia (FTD)-associated mutations and reproducing the two characteristic pathological findings in AD: Aβ amyloid plaques and neurofibrillary tangles (NFT) with hyperphosphorylated tau (Ashe & Zahs 2010). It is important to note that no one model encompasses the entire pathological gamut of AD, and the cognitive symptoms tend to precede plaques and NFT formation, which inverts the order seen in patients. However, these mice are useful for exploring specific pathological pathways implicated in AD in the context of a live and aging brain. Understanding the general strengths and weaknesses of the various models is crucial for interpreting data gleaned from them.

The first major class of AD transgenic mice are based around Aβ amyloidosis and express various combinations of FAD mutations found in APP or PS1 genes. Though these genetic

mutations are responsible for only a small percentage of total AD cases, they are known to have significant dominant effects suitable to mouse modeling with transgenic overexpression. Driving these human mutant APP/PS1 transgenes is usually 1 of 3 promoters: PrP, PDGF-β, and Thy-1. The prion protein (PrP) promoter, drives the highest levels of expression, but is not specific to neurons, as transgene expression also appears in glial cells and non-nervous system tissues (Baybutt 1997). The platelet derived growth factor-β (PDGF-β) promoter drives neuron specific expression throughout development and adulthood (Sasahara 1991). Finally, thymocyte differentiation antigen 1 (Thy1) promoters drives neuron specific expression beginning several days after birth and avoid potential developmental effects of APP/PS1 overexpression (Caroni 1997).

The original amyloidosis models expressed only human APP mutations. This begun with PDAPP mice, which expressed the V717F Indiana mutation in the γ-secretase site under the PDGF-β promoter (Games 1995). Most subsequent AD mouse models express the K670N/ M671L Swedish mutation (APPswe) in the β-secretase site with or without additional mutations. This includes several commonly used lines such as Tg2576, which expresses APPswe under the hamster PrP promoter (Hsiao 1996), APP23, which expresses APPswe under the Thy1 promotor (Sturchler-Pierrat 1997), and the J20/TgCRND8 lines, which express APPswe with the Indiana mutation under the PDGF-β/PrP promoter respectively (Mucke 2000, Chishti 2001).

Amyloidosis models share many common properties, including the formation of amyloid plaques and AD-like microgliosis around these plaques (Games 1995, Stalder 1999). In addition, they seem to model Aβ-induced neuronal dysfunction seen as synaptic loss and deficits in cognitive behaviors like contextual fear conditioning and Morris Water Maze tasks (Hsaio 1996, Dodart 1999, Mucke 2000, Westerman 2002). On the other hand, there are no neurofibrillary tangles

and no consistent reports of the widespread frank neuronal loss that is seen in patients. In addition, behavioral deficits can be detected prior to plaque formation, which is not the case in AD patients whose behavioral symptoms appear after significant Aβ accumulation (Wang 1999). Thus, APP-based mouse models are effective for studying Aβ accumulation and its pathogenic sequelae: synapse loss, cognitive deficits, and gliosis, even though they do not represent the full spectrum of AD-pathology.

Additional amyloidosis mouse models express mutant forms of human APP and PS1 together. Mutant PS1 expression alone, without human APP, whose processing favors Aβ formation, is not enough to cause AD-like pathology, even with mouse APP overexpression (Qian 1998, Jankowsky 2007). However, crossing a mouse expressing transgenic human PS1 M146L with human APPswe Tg2576 mice to create the PSAPP double transgenic model quickens progression of amyloid pathology and increases the $A\beta_{42}/A\beta_{40}$ ratio (Holcomb 1998). Currently, the two most widespread APP/PS1 mice integrated APP and PS1 expression vectors together so they breed as a single transgene. These mice are the APPswe/PS1ΔE9 (termed APP/PS1 mice for the remainder of this dissertation) and 5xFAD mice. APP/PS1 mice express both a human/mouse APP hybrid with the Swedish mutation and human PS1ΔE9 mutation under a mouse PrP promoter (Jankowsky 2001). They develop amyloid plaques and behavioral deficits beginning around 6 months (Jankowsky 2004). 5xFAD mice contain transgenes with 5 separate mutations, 3 in APP (Swedish, London (V717I), and Florida (I716V)) and 2 in PS1 (M146L and L286V) both expressed under the Thy1 promoter. These mice exhibit an accelerated progression and have been reported to demonstrate significant neuron loss, a rarity in these amyloidosis models (Oakley 2006). There is also a genomic Yeast Artificial Chromosome transgenic model that expresses APPswe under its endogenous human promoter with or

without PS1 mutations (Lamb 1997, 1999), as well as a new knock-in model that replace endogenous murine App with human APPswe with additional familial mutations (Saito 2014).

In order to model neurofibrillary tangles found in AD patients, but not APP-based models, transgenic mouse models have been created to express mutated human tau. Though tau mutations do not cause AD, but rather frontotemporal dementias, the combination of APP and tau transgene expression can create a pathological picture similar to AD. The first such model, the double transgenic TAPP mice combined expression of the most-common FTD-associated tau mutation, P301L, under a mouse PrP promoter with APPswe-expressing Tg2576 line (Lewis 2000, 2001). While Aβ pathology is unchanged from Tg2576 mice alone, tau pathology is worsened when compared to P301L expression alone (Lewis 2001). This model is limited by significant motor deficits that interfere with cognitive behavioral tests (Lewis 2000). A subsequent combined APP/tau model is the influential 'triple transgenic' (3xTg) mouse, which expresses APPswe and P301L tau under Thy1 promoters along with M146V PS1 expressed as a homozygous knock-in (Oddo 2003). This model exhibits Aβ plaques followed by NFTs, as seen in AD patients (Oddo 2004). A final significant tau model expresses normal human tau in place of mouse tau. That alone, without FTD-associated mutations, is sufficient to cause tau hyperphosphorylation and fibrillary tau formation (Andorfer 2003).

APP/Tau models are best suited to study of the interaction between Aβ and tau pathology *in vivo* and these models can show significant neuronal loss. However, this does not discount the role of mouse tau in APP amyloidosis models. Despite the lack of neurofibrillary tangle formation, removing endogenous mouse tau prevented cognitive deficits and synaptic loss caused by Aβ in the J20 APP model (Roberson 2007), suggesting tau is a key component of Aβ

toxicity independent of fibril formation. In addition, tau hyperphosphorylation can be detected in the APP models (Jay 2015).

For these experiment, due to our primary concern with microglia in AD, as well as consideration for the ease of single transgene breeding when combined with BAC-TREM2, we chose to focus on two APP/PS1 models: APPswe/PS1ΔE9 and 5xFAD. Both of these models exhibit amyloid plaque pathology and plaque associated microgliosis similar to AD patients, as well as synaptic loss and cognitive deficits. These models are widely used and represent two different rates of progression, as 5xFAD mice have more severe phenotypes with plaques appearing as early as 2 months, while APP/PS1 mice have a more gradual course.

3.1.2 Microgliosis in Alzheimer's Disease

The details of microgliosis in AD have been best explored using mainly mouse amyloidosis models of AD, as well as cultured microglia. Several lines of research have investigated the cause of microgliosis in AD. While microglia have been shown to react to a wide variety of damage associated molecular patterns (DAMPS), which can be released by damaged cells to signal tissue dysfunction (Perry 2010), the main activator studied in AD is Aβ itself. Fibrillar Aβ has been shown to directly activate microglia (McDonald 1997, Combs 1999) at least partially through Toll-like Receptor (TLR) pathways involving TLR 2 and 4, which induce pro-inflammatory responses in microglia (Reed-Geaghan 2009). Aβ may not be particularly unique in possessing this ability as other proteins with a similar fibrillar structure also can activate microglia (Miyazono 1991).

The dynamics of microglia activation was recently observed by imaging plaque formation in the live brain of a mouse AD model along with the reaction of labeled microglia (Bolmont 2008,

Meyer-Luehmann 2008). Visible plaque formation occurs very quickly within days to weeks and is followed shortly after by activation and recruitment of surrounding microglia. This supports a role for large Aβ plaques in stimulating the microglia response. Microglia have the ability to phagocytose Aβ and contribute to Aβ clearance, and this response can be inhibited by inflammatory cytokine stimulation (Koenigsknecht-Talboo & Landreth 2005). Microglia also demonstrate changes with aging, such as reduced motility, reduced Aβ phagocytic capacity, and a shift to a more pro-inflammatory state (Hickman 2008, Jimenez 2008, Meyer-Luehmann 2008, Streit 2009). Together, these later microglia changes seen in AD may lead to decreased ability for the brain to handle its amyloid load, increased Aβ toxicity, and an overall shift of microglia function from neuroprotective to neurotoxic.

Although Aβ-stimulated cultured microglia have been shown to release neurotoxic factors (Meda 1995, Giulian 1996), attempts to manipulate microglia in order to alter the course of disease pathogenesis in mouse models of AD have produced mixed results. Complete elimination of transgenic thymidine-kinase-expressing microglia using ganciclovir had no effect on amyloid plaque load or neuritic dystrophy after 1 month of microglia depletion (Grathwohl 2009), suggesting the microglia response is not relevant to these phenotypes.

Other attempts to manipulate microglia in AD models have used pharmacological or gene knock out approaches. Treatment of a rat model of AD with a synthetic cannabinoid improved disease phenotypes by reducing microglia activation to Aβ (Ramirez 2005). However, others have shown *increased* pro-inflammatory activation of microglia with chronic Il-1β expression also can improve plaque pathology (Shaftel 2007, Matousek 2012). An additional approach to manipulate the microglia response is based on the Cx3cr1 receptor. The microglial inflammatory response is chronically suppressed by an interaction between microglial Cx3cr1 and the Cx3cl1 ligand

located on neurons (Cardona 2006). Deletion of murine Cx3cr1 in multiple models of AD led to increased pro-inflammatory microgliosis, as expected, but surprisingly was also associated with a decreased amyloid load (Lee 2010, Cho 2011). The importance of this finding is called into question by reports that Cx3cr1 deletion increases tau phosphorylation and worsens cognitive decline in AD mice, despite a reduced plaque load (Cho 2011). In a different AD mouse model, 5xFAD, *in vivo* imaging visualized neuronal loss that was actually rescued by Cx3cr1 deficiency (Fuhrmann 2010). Whether these disparate results may be related to differences in the animal models used or the variety of experimental assays awaits resolution.

A reason for confusion with these microglia-based manipulations may be that they don't account for the heterogeneous nature of microglia responses. As discussed above (See Section 1.2), microglia serve different roles in different regions and different times of disease pathogenesis. Some of these may be beneficial while others are harmful. Extreme manipulations like the complete depletion of microglia (Grathwohl 2009), may yield difficult to interpret results as it eliminates all microglia functions, both good and bad. Increasing TREM2 expression with BAC-TREM2 should be more a specific manipulation that shifts the balance of microglia activity in favor of TREM2 signaling when TREM2 ligands are present, suppressing inflammation, and increasing phagocytosis, all of which are thought to helpful in the context of chronic neurodegeneration.

3.1.3 TREM2 in Mouse Models of AD

The importance of TREM2 signaling and microglial function in AD has been highlighted by recent genetic studies linking the rare R47H variant of TREM2, a microglia and myeloid-lineage specific protein, to increased risk of late-onset AD (Jonsson 2013, Guerreiro 2013). In addition, TREM2 expression has been found to increase in AD patient hippocampi and cortices (Lue

2014, Martiskainen 2015, Celarain 2016), which is reproduced in mouse models of AD (Melchior 2010, Jay 2015, Fig 3.1). Finally, network analysis of gene expression changes in AD patient brain tissue found a key immune-related network module filled with phagocytosis-related genes that is upregulated in AD brains. The key regulatory node identified by this analysis was DAP12, the signaling adaptor protein of TREM2 (Zhang 2013). Together these lines of evidence support the relevance of studying TREM2 in further detail in animal models of AD.

To date, two main TREM2 manipulations have been used in AD mouse models: lentiviral overexpression of TREM2 and genetic KO of mouse Trem2. Lentiviral overexpression of TREM2 has shown benefits in the APP/PS1 model of AD, but these experiments have many caveats (see Section 2.1).

As mentioned in Section 1.9, TREM2 knockout mice have been crossed with 5xFAD and APP/PS1 mice, with disparate results (Jay 2015, Wang 2015). While microgliosis was decreased with Trem2 deficiency in both studies, the effect on many other pathological outcomes was reversed. This may be due to the use of two different models or the relatively early age in which Jay et al analyzed their APP/PS1 mice, especially considering it is a more slowly-progressing model than 5xFAD.

Whatever the reason, these data raise an important controversy that must be addressed: Is TREM2 signaling actually protective in AD mouse models, and how does it accomplish this protection? These experiment aim to answer this question by using BAC-TREM2 to increase TREM2 signaling in two separate models of AD: 5xFAD and APP/PS1 mice.

3.2 Results

3.2.1 TREM2-BAC Transgene Expression Increases in Aged 5xFAD Mice

BAC-TREM2 mice in the FvB/N background were crossed with both 5xFAD and APP/PS1 mice in the C57Bl6 background to create all desired genotypes on the genetically homogenous FvB/N; C57Bl6 F1 background. Expression of the human BAC-TREM2 transgene in forebrain tissue was determined along with mouse Trem2 in 6 month old BAC-TREM2 mice with and without 5xFAD driven amyloidosis (Fig 3.1). As reported previously (Melchior 2010, Jay 2015), mouse Trem2 was increased in this AD mouse model as compared to WT mice. Furthermore, the proportional upregulation of human TREM2 in 5xFAD from WT levels was similar to mouse Trem2 (~3.5 fold)), suggesting human TREM2 is regulated in an endogenous fashion (Fig 3.1).

3.2.2 Increased TREM2 Gene Dosage Reduces Plaque-Associated Microgliosis in Two Separate Mouse Models of AD Amyloidosis

The effect of BAC-TREM2 with increased TREM2 gene dosage, and preliminary evidence of enhanced TREM2 signaling (Fig. 2.10), on microglia responses in AD-related amyloidosis was analyzed in both 5xFAD mice (6 months of age; Fig 3.2) and APP/PS1 mice (10 months of age; Fig 3.3). In WT mice (both at 6m and 10m), microglia throughout the cortex and hippocampus were uniformly “quiescent” and demonstrated a ramified morphology with modest Iba1 staining.. Increased TREM2 signaling in BAC-TREM2 mice caused no gross difference in these aged mice. In contrast, the typical picture in amyloidosis models of AD is very different. Microglia cluster around plaques and exhibit “activated” morphology with an increased Iba1 staining and a less ramified, more ameboid, morphology. This was reproduced in our study with the AD mice in our FvB/C57Bl6 F1 background (Fig 3.2-3). There is a high density of microglia coincident with Aβ containing plaques in both 5xFAD and APP/PS1 (Fig 3.2, Fig 3.3a-b). Overexpression of human TREM2 led to a drastic change in this microglia morphology, especially those surrounding the Aβ plaque (Fig 3.2b, Fig 3.3c-d). There was a reduction in microglia number

associated each plaque, corrected for plaque size (Fig 3.3c), as well as a significant reduction in IbaI intensity from microglia in the area of plaques (Fig 3.3d) While normally microglia infiltrate the center of plaques with thick processes and cell somas, TREM2 overexpression prevented this phenomenon (Fig 3.2b, Fig 3.3b). In total, BAC-TREM2 significantly altered plaque-associated microglia activation.

3.2.3 Preliminary Studies Showed Increased TREM2 Gene Dosage Did Not Significantly Alter Soluble and Insoluble Aβ Load.

A major sign of Aβ accumulation is appearance of Aβ containing amyloid plaques (3.2/3). These plaques consist of Aß, which can be assay in either the soluble or insoluble (mostly found in plaques) forms. Sensitive ELISA biochemistry of soluble and insoluble Aβ showed no clear reduction in either $A\beta_{40}$ or the more pathogenic $A\beta_{42}$ in the forebrain of 5xFAD mice (Fig 3.4a-da-j). No significant change in soluble or insoluble Aβ was detected in forebrain extracts of 5xFAD mice as well, though there may be a trend towards Aβ reduction with BAC-TREM2 (Fig 3.4b/d). Aß from the cortex and hippocampus of 10 month APP/PS1 mice was also assayed. These studies are still at an early stage due to the small number of mice analyzed thus far, and we clearly will need to study more mice to obtain more definitive result. Together, our preliminary data indicate increased TREM2 gene dosage in BAC-TREM2 mice did not appear to alter Aβ load, thus its functional effects may be manifested on other aspects of microglia function.

3.2.5 Reduction in Microgliosis in BAC-TREM2/AD MIce Was Not Accompanied by Reduction in Astrogliosis

Astrocytosis near plaques is a common inflammatory reaction that accompanies microgliosis in AD mouse models. To determine if reduced microglia activation led to subsequent reduction in astrocytosis, the cortex of 10 month APP/PS1 brains was stained for GFAP, a common

astrocytic markers, whose expression increases with astrocyte activation, similar to Iba1 in microglia. Cortical GFAP staining is sparse in WT and BAC-TREM2 mice (Fig 3.5a). In APP/PS1 mice, however, there a striking increase in cortical GFAP staining (Fig 3.5a). Unlike the drastic change in microglia activation in the BAC-TREM2 and APP/PS1 double transgenic mice, there was no significant reduction in astrocytosis with TREM2 overexpression (Fig 3.5c-d), though there may be a moderate increase in these mice in our preliminary study (Fig 3.5c).

3.2.5 Influence of TREM2 Gene Dosage on Memory Deficits in the AD mice

A major impairment in AD is the gradual loss of patients' ability to reason, remember, and learn. This can be modeled in mouse models as impaired performance on learning and memory tasks, such as Morris Water Maze and contextual fear conditioning. Contextual fear conditioning is a hippocampus-dependent learning task this is impaired in several different mouse models of AD, likely due to Aβ and synaptic pathology in the hippocampus and cortex (Corcoran 2002, Dineley 2002, Gerlai 2002, Nagahara 2009, Kilgore 2010). Can increased TREM2 signaling and the altered microglia response improve these behavioral symptoms?

Male mice on in our FvB/N; Bl6 F1 background showed robust contextual fear conditioning, demonstrating the ability to remember a single shock from the previous day, as measured by freezing behavior (Fig 3.7). This was unchanged in BAC-TREM2 mice, suggesting TREM2 overexpression alone did not alter this memory behavior. APP/PS1 mice, however, demonstrated a significant impairment that was, remarkably, rescued in BAC-TREM2; APP/PS1 double transgenic mice. Notwithstanding the limitation of our preliminary study with a relatively small number of mice, it demonstrates a potential cognitive benefit of increased TREM2 gene dosage and altered microglia response in an AD mouse model.

Open field activity was also assayed in these mice. Hyperactivity has been reported in APP/PS1 models (Lalonde 2005), and this was reproduced in our F1 background (Fig 3.8). TREM2 overexpression had no apparent impact on this phenotype, as APP/PS1; BAC-TREM2 mice demonstrated a similar level of hyperactivity as APP/PS1 mice without BAC-TREM2. Importantly, BAC-TREM2 alone caused no overt deficits in open field behaviors. Therefore, it appears that TREM2 gene-dosage increase more selectively affects cognitive behaviors, and not motor behaviors, in the context of an AD mouse model.

Figure 3.1

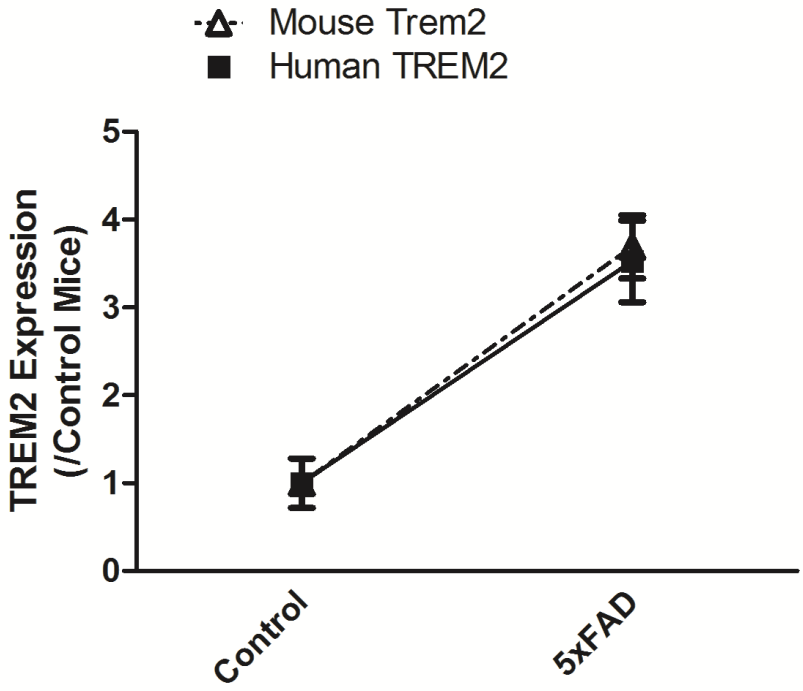

Fig 3.1 Upregulation of both human and mouse TREM2 in 6 month 5xFAD mouse forebrain. RT-PCR analysis in BAC-TREM2 mice demonstrates significant upregulation of both human and mouse TREM2 (2 way ANOVA, Main effect for 5xFAD, $p<.001$, $F(1,4)=62.00$). There is no significant difference between the degree of this upregulation between mouse and human TREM2 (interaction between 5xFAD and TREM2 species, $p=.81$, $F(1,4)=.06$). Values represent mean +/- SEM. values for mouse and human TREM2 normalized separately to control mice (=1). n=2 mice/genotype.

Figure 3.2

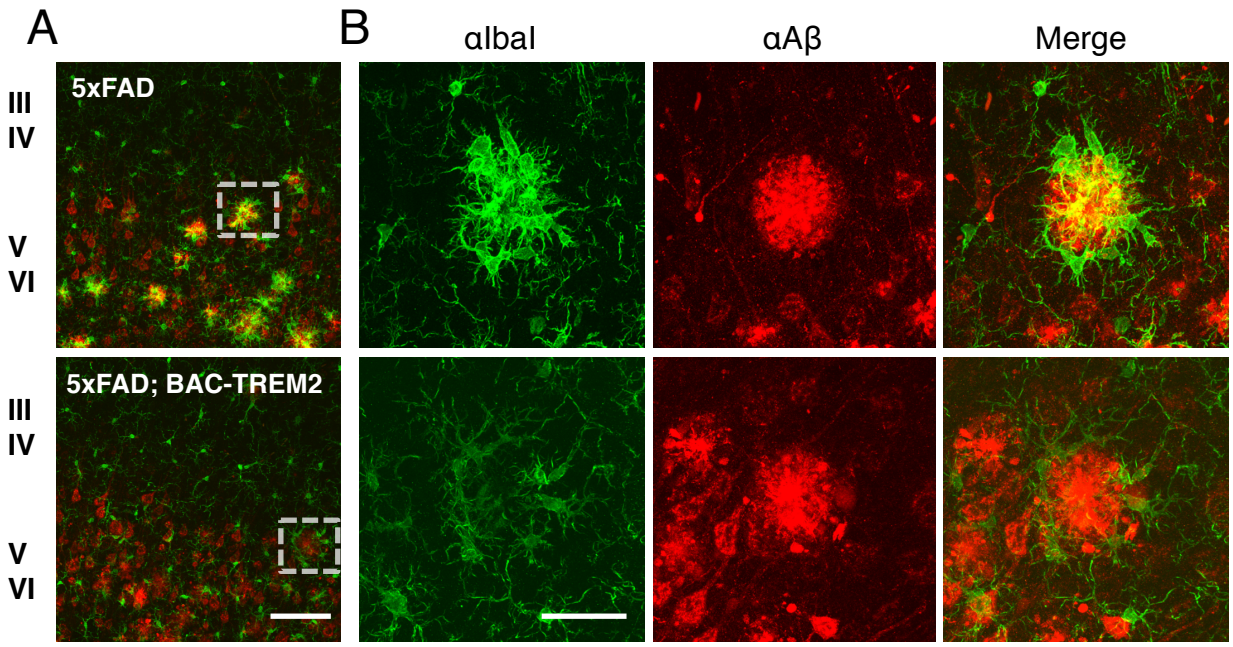

Fig 3.2 Decreased plaque-associated microgliosis in 6 month 5xFAD mice with BAC-TREM2 human TREM2 overexpression. **A)** 20x confocal images of 5xFAD(top) and 5xFAD; BAC-TREM2 (bottom) show reduced Iba1+ microglia staining (green) near Aβ-containing plaques (red) in lower layers of the cortex. Roman numerals represent cortical layers. Dotted boxes represent areas of zoom for **B).** White bar = 100 µm. **B)** 63x images of individual, similarly sized plaques show cell bodies and thick IbaI+ microglia processes densely penetrating the center of the plaque in 5xFAD mice (top). This is not seen in in 5xFAD mice with BAC-TREM2 (bottom) as microglia stay more to the plaque periphery and express less IbaI. Scale bar = 50 µm.

Figure 3.3

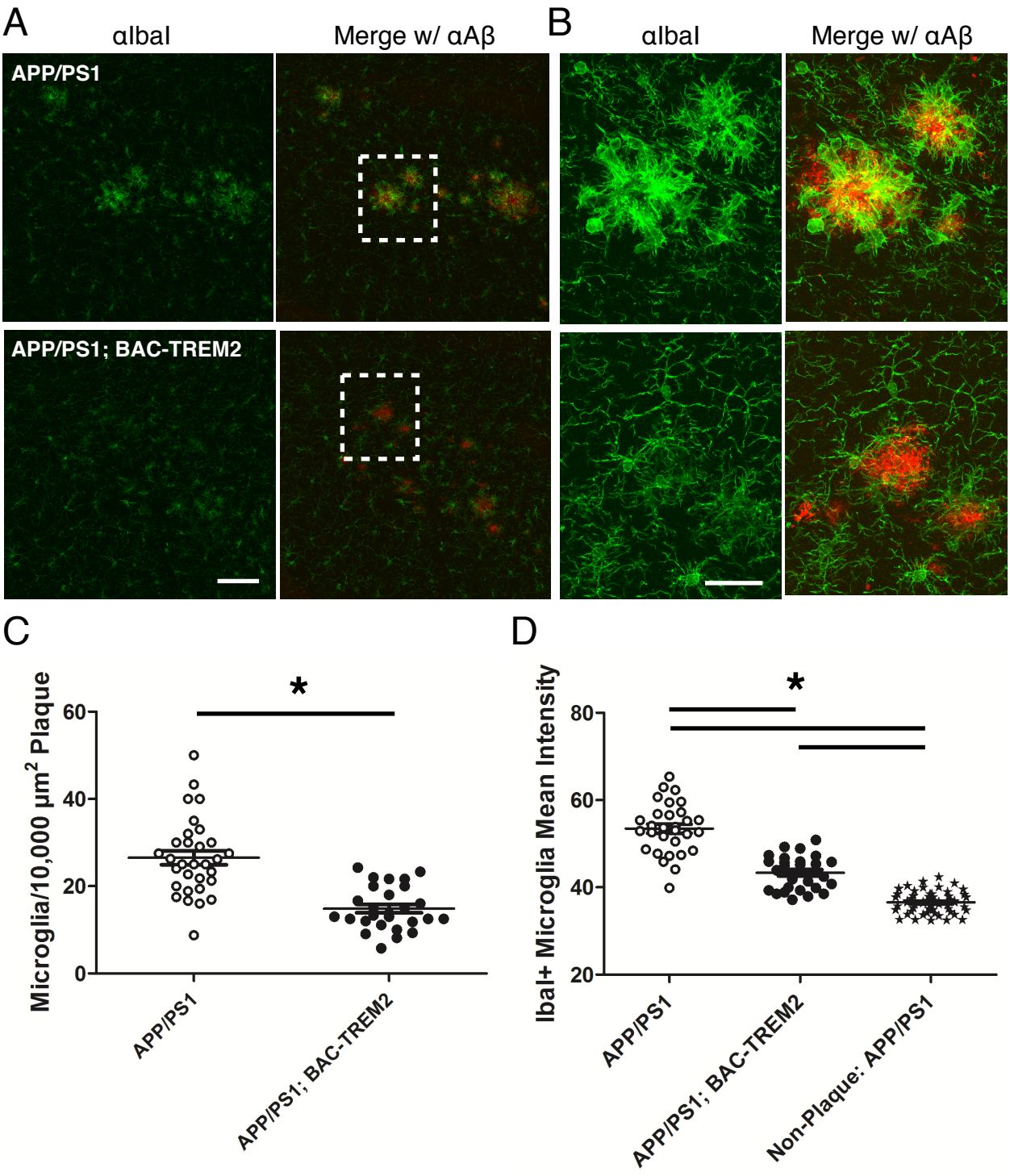
A
αIbaI
Merge w/ αAβ
APP/PS1
APP/PS1; BAC-TREM2
B
αIbaI
Merge w/ αAβ
C
Microglia/10,000 µm² Plaque
60
40
20
0
*
APP/PS1
APP/PS1; BAC-TREM2
D
Iba1+ Microglia Mean Intensity
80
60
40
20
*
APP/PS1
APP/PS1; BAC-TREM2
Non-Plaque: APP/PS1

Fig 3.3 Decreased plaque-associated microgliosis in 10 month APP/PS1 mice with increased human TREM2 gene dosage. **A)** 20x confocal images of APP/PS1 (top) and APP/PS1; BAC-TREM2 (bottom) show reduced Iba1+ microglia staining (green) near Aβ-containing plaques (red) in the hippocampus. White bar = 100 μm. **B)** 63x images of individual, similarly sized plaques show similar features as in 5xFAD mice, cell bodies and thick IbaI+ microglia processes densely penetrating the center of the plaque in APP/PS1 mice (top). Again, this is not seen in the presence of BAC-TREM2 (bottom). Scale bar = 50 μm. **C)** Count of the number of microglia per area plaque (average size of plaques counted = 7,880 μm^2) significantly reduced microgliosis ($p<.0001$, Mann Whitney test). **D)** Quantification of IbaI intensity reveals a significant reduction in APP/PS1; BAC-TREM2 mice compared to APP/PS1 mice ($p<.0001$, Kruskal-Wallis, $p<.01$, Dunn's Multiple Comparison test between APP/PS1 & APP/PS1; BAC-TREM2). Both mice have significantly increased IbaI staining at plaques as compared to non-plaque areas in APP/PS1 ($p<.001$, Dunn's Multiple Comparison test). Non-plaque area intensity in APP/PS1; BAC-TREM2 mice had identical means and SEM (36.5 +/-.3) as APP/PS1 (data not shown). For all analysis n=31 plaques in 5 slices from 2 mice for APP/PS1, while n= 27 plaques from 6 different slices from 2 mice in APP/PS1; BAC-TREM2. n=57 regions of interest across 5 slices from 2 mice for non-plaque IbaI intensity.

Figure 3.4

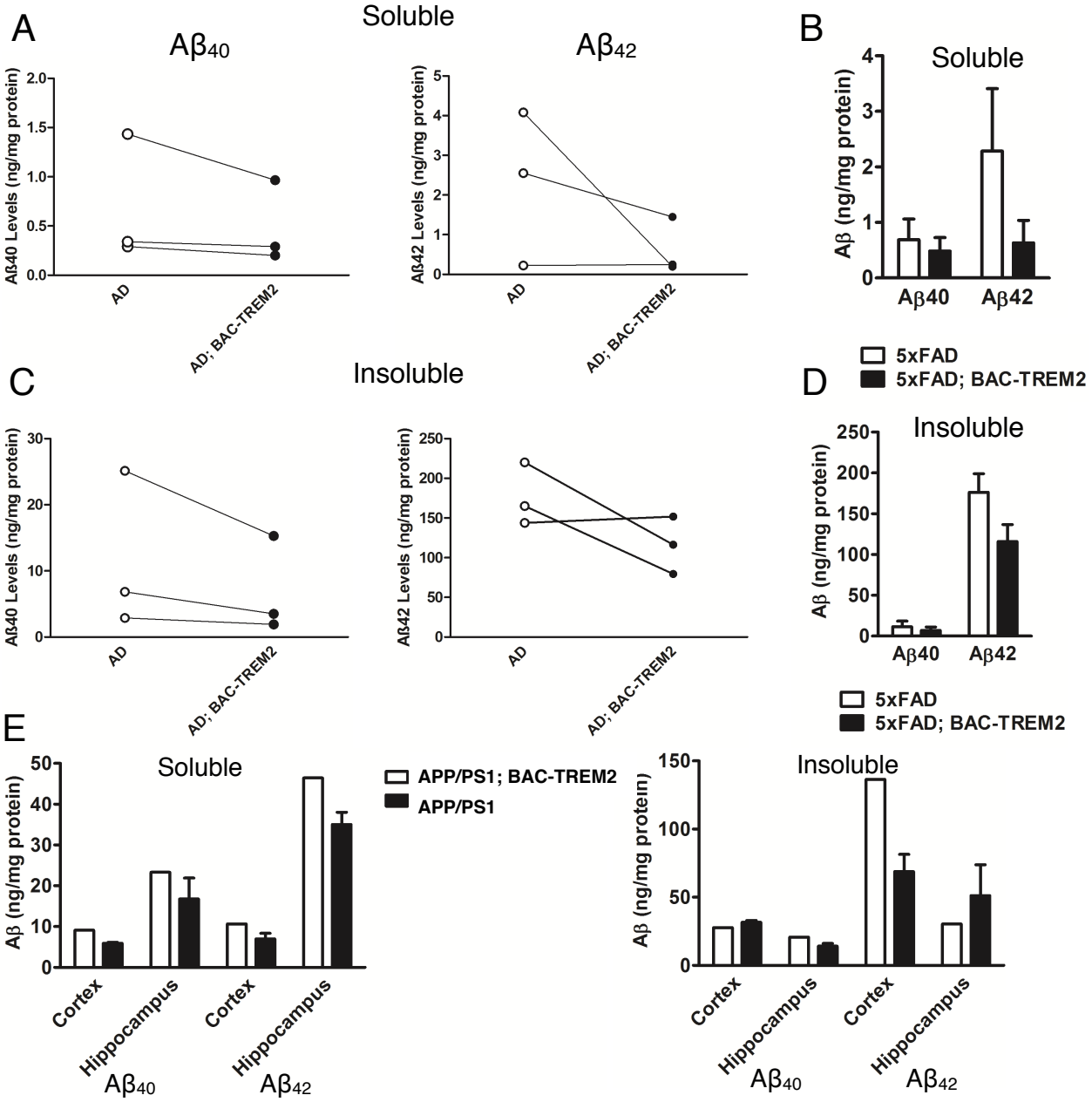

Fig 3.5 No significant change in soluble or insoluble Aβ load in AD mice with BAC-TREM2 **A & B)** No significant change on ELISA in soluble DEA-extracted $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$ in forebrain of 5-6 month old 5xFAD mice with BAC-TREM2 (p>.2, unpaired T test).**. C & D)** No significant change in insoluble formic acid extracted Aβ in 5-6 month old 5xFAD mice with BAC TREM2 (p>.2, unpaired T test). Littermates of the same sex were paired in **A/C)** and summarized with Mean + SEM in **B/D)**. n=3/genotype for all experiments. **E)** Preliminary analysis of soluble and insoluble Aβ in the cortex and hippocampus of APP/PS1 mice with (n=2) and without (n=1) BAC-TREM2. **E)** Summary of initial ELISA analysis of soluble and insoluble Aβ in the hippocampus and cortex of 10 month old APP/PS1 mice (n=1 for APP/PS1 & 2 for APP/PS1; BAC-TREM2).

Figure 3.5

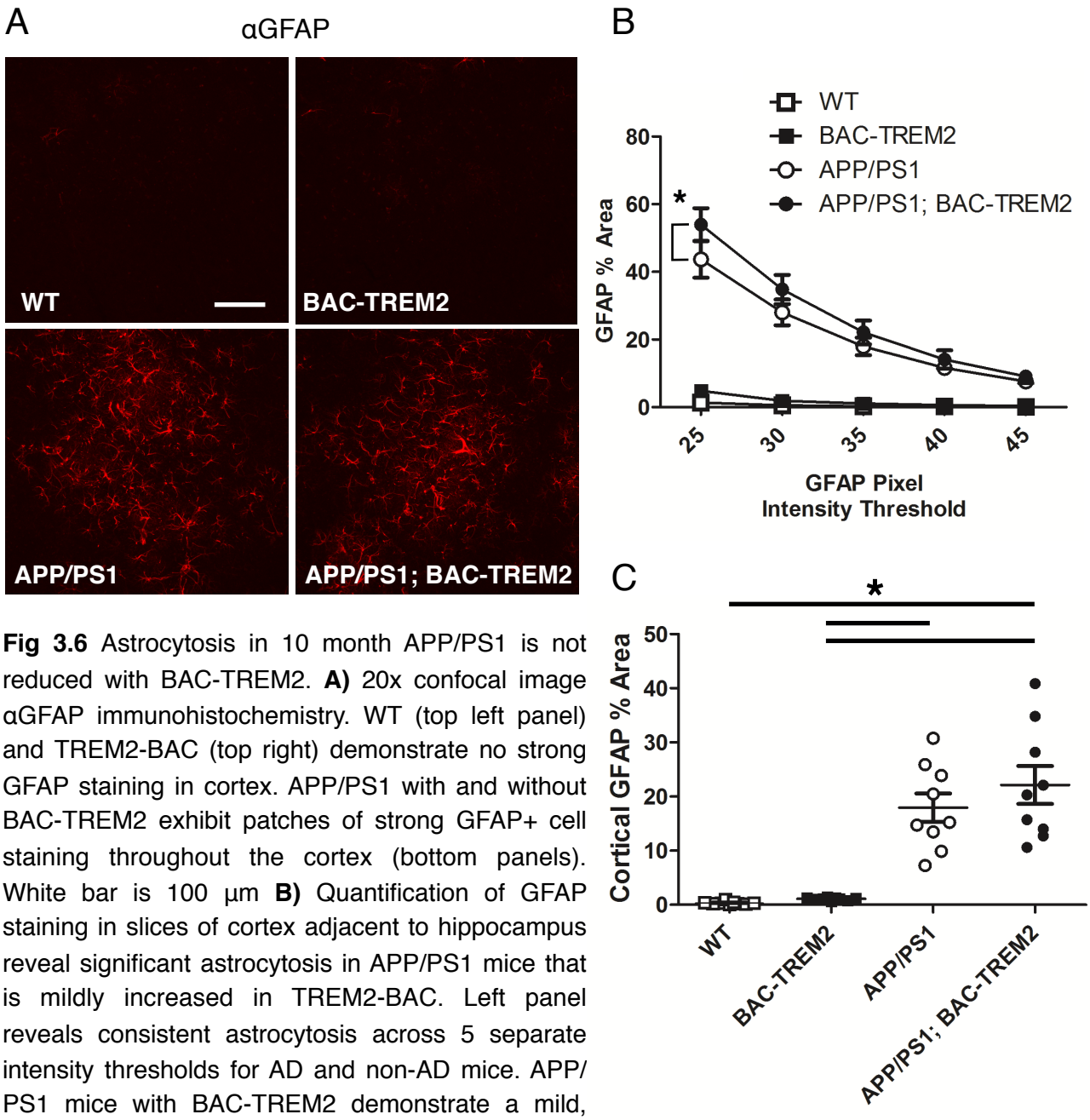

Fig 3.6 Astrocytosis in 10 month APP/PS1 is not reduced with BAC-TREM2. **A)** 20x confocal image αGFAP immunohistochemistry. WT (top left panel) and TREM2-BAC (top right) demonstrate no strong GFAP staining in cortex. APP/PS1 with and without BAC-TREM2 exhibit patches of strong GFAP+ cell staining throughout the cortex (bottom panels). White bar is 100 μm **B)** Quantification of GFAP staining in slices of cortex adjacent to hippocampus reveal significant astrocytosis in APP/PS1 mice that is mildly increased in TREM2-BAC. Left panel reveals consistent astrocytosis across 5 separate intensity thresholds for AD and non-AD mice. APP/PS1 mice with BAC-TREM2 demonstrate a mild, significant increase in astrocytosis at the lowest threshold (2 way repeated measures ANOVA, main effect for genotype p<.0001, F(3,32)=36.81, p<.05, Bonferroni post-test between APP/PS1 & APP/PS1; BAC-TREM2 at threshold of 25. Not significant difference between the two at any other thresholds). Values are mean +/- SEM Right panel: Scatterplot of GFAP % area at threshold of 35 reveals significant astrocytosis in APP/PS1 mice with and without BAC-TREM2 (p<.05, Kruskal-Wallis test, Dunn's Multiple Comparison Test shows significant differences (P<.05) between WT & APP/PS1, WT & APP/PS1; TREM2, and BAC-TREM2 & APP/PS1; BAC-TREM2. n=3 slices/mouse, 3 mice/genotype.

Figure 3.6

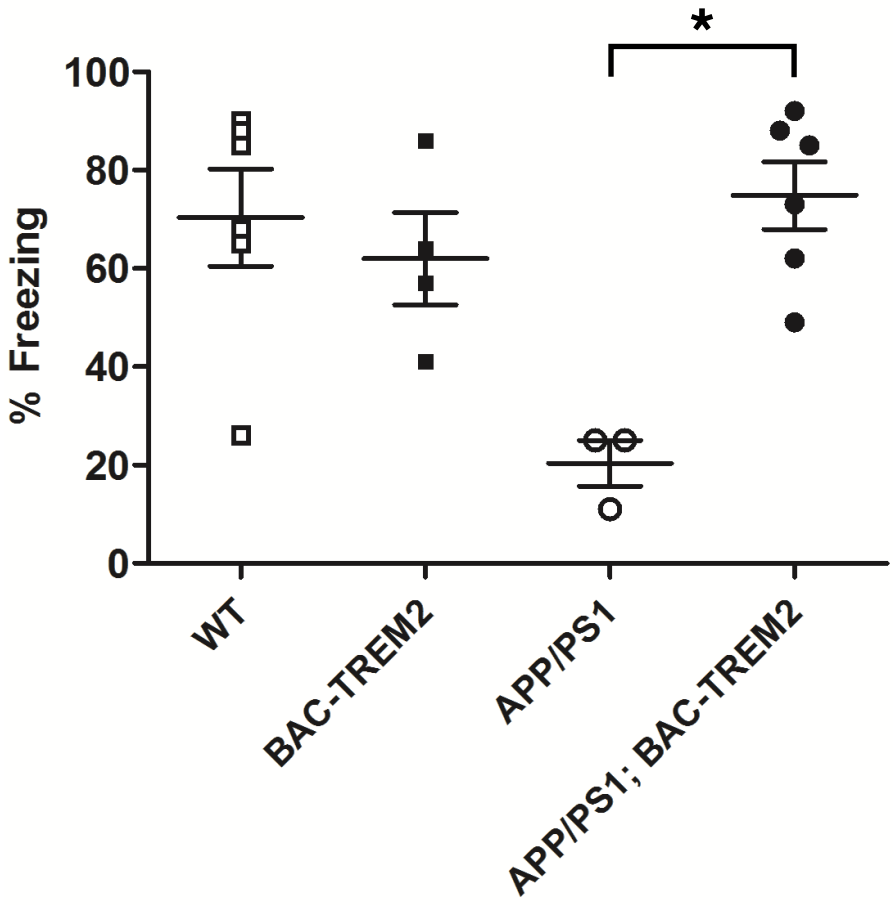

Fig 3.6 Deficit in contextual fear conditioning freezing behavior in 10 month APP/PS1 mice rescued by BAC-TREM2. A single shock was delivered to mice in a novel context. Freezing behavior was analyzed the following day immediately after being placed in the same context for 6 minutes. APP/PS1 mice demonstrate impaired performance that was significantly improved by BAC-TREM2 ($p<.05$, Kruskal-Wallis test, Dunn's Multiple Comparison test is significant ($p<.05$) between APP/PS1 and APP/PS1; BAC-TREM2 mice). n=6, 4, 3, and 6 for WT, BAC-TREM2, APP/PS1, and APP/PS1; BAC-TREM2 respectively. Values are means +/- SEM

Figure 3.7

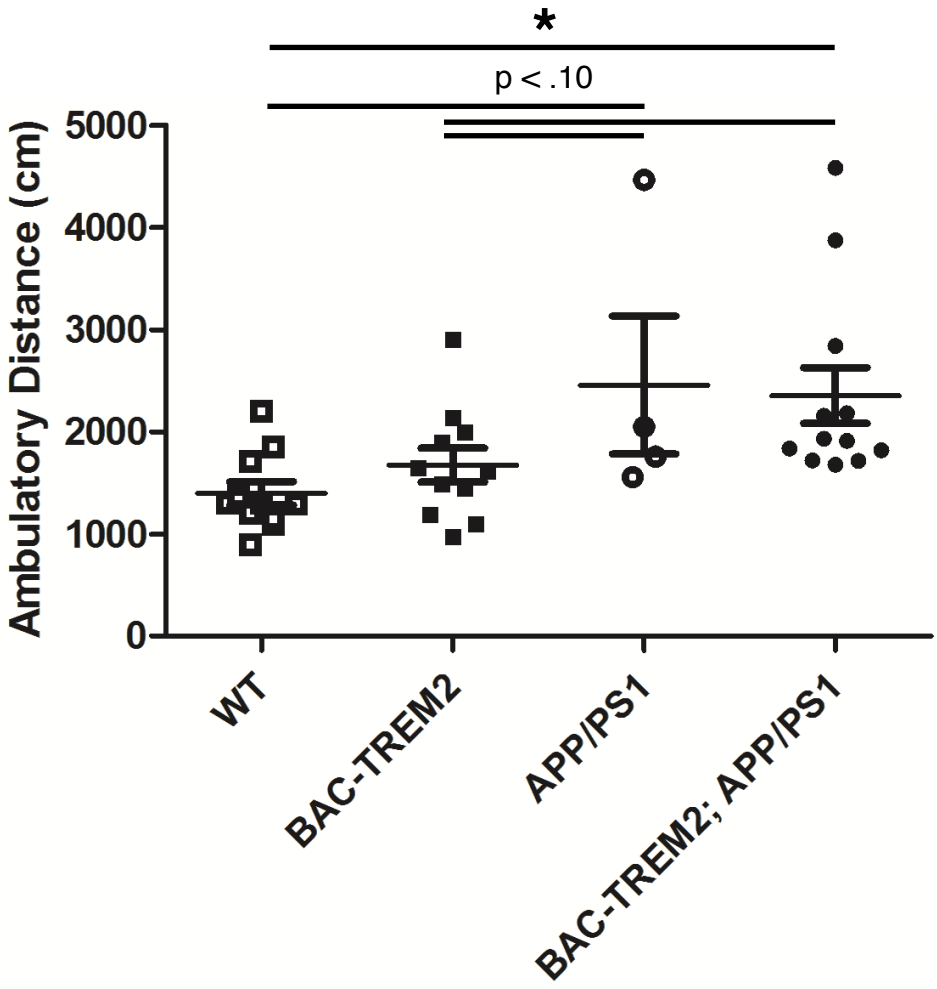

Fig 3.7 Hyperactivity in 10 month APP/PS1 mice is not altered by BAC-TREM2. Open field analysis measured exploratory behavior for 10 mins. APP/PS1 mice demonstrate hyperactivity that is not affected by BAC-TREM2 (p=.0055, Kruskal-Wallis test, Dunn's Multiple Comparison test shows significance (p<.05) between WT & APP/PS1; BAC-TREM2 mice, and approaching significance (p<.1) for WT & APP/PS1, TREM2-BAC & APP/PS1, & TREM2-BAC & APP/PS1; TREM2 BAC mice). n=11, 11, 4, and 12 for WT, BAC-TREM2, APP/PS1, and APP/PS1; BAC-TREM2 respectively. Values are means +/- SEM

3.3 Discussion

3.3.1 TREM2-BAC Transgene Expression Increases in Aged 5xFAD Mice

Similar to previous studies in Human AD patients and other AD mouse models (Lue 2014, Martiskainen 2015, Melchior 2010, Jay 2015), both human and mouse TREM2 expression increased in the brains of 5xFAD mice. This raises an important question: if TREM2 is in fact protective, as is suggested by data from human genetic studies, microglia culture experiments, and this dissertation, why is increased TREM2 associated with disease? The most obvious hypothesis is that TREM2 upregulation may represent a homeostatic response to better handle increasing neuronal debris or Aβ. However, it seems insufficient to overcome the pro-inflammatory stimulation of Aβ stimulation and the associated neuronal dysfunction. So why does BAC-TREM2 overcome this limitation?

Three possible explanations could coexist to account for differences between the effectiveness of BAC-TREM2's TREM2 overexpression compared to endogenous upregulation in suppressing microglia activation in AD: the timing, degree, and cellular pattern of overexpression. Endogenous TREM2 upregulation may occur too late in disease. The pro-inflammatory cascade can be a self-propelling cycle, with increased pro-inflammatory cytokines stimulating more activation and more cytokine release. If TREM2 expression is too low early in this process, later upregulation may not be sufficient to stop the inflammatory cascade. BAC-TREM2 expresses high levels of human TREM2 earlier when TREM2 signaling may be more effective. Aside from timing, the degree of endogenous TREM2 upregulation may not be enough to overcome pro-inflammatory stimuli, which BAC-TREM2 overcomes by increasing TREM2 receptor levels several fold (Fig 2.8).

The final explanation for BAC-TREM2's effectiveness compared to endogenous TREM2 upregulation is the pattern of TREM2 upregulation may not be appropriate to control the overall microglia response. Endogenous TREM2 upregulation in AD occurs in microglia associated with amyloid plaques (Lue 2014, Melchior, 2010, Jay 2015), but not in surrounding microglia that may be affected by, and in turn regulate, the changing inflammatory environment. BAC-TREM2 may increase TREM2 signaling in these non-plaque associated microglia as well and may not only suppress possible toxic pro-inflammatory reactions, but prevent microglia senescence associated with aging and disease (Streit 2009). This loss of normal microglia function can contribute to AD progression, as microglia performs several critical functions including maintaining synaptic plasticity (Parkhurst 2013, Schaffer 2013). Furthermore, a significant described function of TREM2 signaling is to interact with the CSF-1 trophic factor signaling pathway to enhance the survival of myeloid-lineage cells (Otero 2009, Poliani 2015, Wang 2015).

Additionally, the increased human BAC-TREM2 in 5xFAD, similar to mouse TREM2, is further evidence that regulation of human TREM2 is intact in this transgenic mouse, as was seen in primary microglia culture after LPS stimulation (Fig 2.7b).

3.3.2 Possible Origins of Increased TREM2 Expression in AD

The mechanism of TREM2 upregulation in plaque-associated microglia is unclear. LPS stimulation has actually been shown to suppress TREM2 expression acutely in culture (Zhong 2015, Fig 2.8a), possibly through a NF-κB dependent microRNA, miRNA34a, which binds the TREM2 3' UTR with unusually high affinity and leads to its degradation (Zhao 2013). Despite acute inflammatory stimuli suppressing TREM2 in culture, the effect of chronic stimulation on TREM2 in a live AD brain may be very different. Additionally, TREM2 expression has been

shown to increase *in vivo* in acute stroke models and traumatic brain injury models (Sieber 2013, Sugimoto 2013, Kawabori 2015, Saber 2016), as well as with direct Aβ injection into a mouse brain (Jiang 2014).

A possible clue to the source of TREM2 upregulation, despite its apparent suppression by inflammatory stimulation, is the source of highly-expressing TREM2 cells found in AD.

A major unresolved issue and important consideration in AD microgliosis is whether activated plaque-associated microglia develop solely from migration and/or proliferation of resident microglia or from infiltrating peripheral monocytes from the blood stream. Several studies using bone-marrow transplants in AD mouse models have demonstrated bone-marrow derived myeloid cells can infiltrate the brain and localize to Aβ plaques (Malm 2005, Simard 2006). Inhibition of peripheral monocyte infiltration using CCR2 KO mice, a receptor found on monocytes that is critical for chemotaxis from the blood into the brain, led to increased Aβ load and worsening cognition (El Khoury 2007, Naert & Rivest 2011). Coupled with evidence these bone-marrow derived cells exhibit reduced pro-inflammatory cytokine expression with increased ability to phagocytose Aβ (Simard 2006), there is reason to believe monocytic infiltration serves an important protective function in AD.

The increased phagocytic capacity and decreased pro-inflammatory phenotype of these infiltrating monocytes is reminiscent of the effects of TREM2 signaling, which is especially intriguing considering recent evidence suggesting highly expressing TREM2^{+}, plaque-associated microglia found in the brains of AD mouse models are derived from infiltrating monocytes (Jay 2015). Many of these studies rely on dynamically regulated markers of uncertain reliability to label peripheral cells or whole-body radiation that disrupts normal vascular homeostasis for bone-marrow transplants, which are important caveats when considering their

data. Further elucidation of the role of peripheral vs. central myeloid-lineage cells in the inflammatory reaction is critical (see Chapter 4), as peripheral monocytes are easier to manipulate than resident microglia. This may provide a novel treatment strategy of TREM2 overexpression in transplanted monocytes if peripheral monocytes can, in fact, infiltrate and function in the AD brain.

3.3.3 Increased TREM2 Gene Dosage Reduces Plaque-Associated Microgliosis in Two Separate Mouse Models

A major finding of this study is that BAC-TREM2 human TREM2 expression and chronic increases in *in vivo* TREM2 signaling can significantly alter the microglia phenotype in two separate AD amyloidosis mouse models (Fig 3.2-3). The striking microglia activation morphology normally seen around plaques in these mouse models, as in AD patients, was severely curtailed. While microglia still clustered around Aβ plaques in BAC-TREM2; AD mice, there were fewer, and they demonstrated signs of reduced activation, such as a more ramified morphology and reduced Iba1 staining (Fig 3.2-3), despite no clear reduction in amyloid load (Fig 3.4).

How does increased TREM2 gene dosage in BAC-TREM2 mice lead to these altered phenotypes in AD mice? One possibility is that microglia with elevated TREM2 levels are more efficient in responding to insults elicited by Aβ species, by increasing phagocytosis and increase Aβ clearance. This is consistent with our finding that fewer microglia are found to surround the plaques.

A second possibility is that microglia activation downstream of Aβ toxicity or direct binding is dampened by elevated intracellular TREM2 signaling in BAC-TREM2 mice, as TREM2 signaling

is known to inhibit pro-inflammatory signaling of the TLR pathway, possibly in a DOK3-dependent manner (Hamerman 2005/2006, Turnbull 2006, Peng 2013). While we have not yet determined the inflammatory cytokine profiles in BAC-TREM2/AD mouse brains, we expect it may be reduced. If this is the case, then the influence of TREM2 memory improvement (Fig 3.7), may be due to a reduction of microglia-induced toxicity or improved microglia-mediated repair of neuronal dysfunction downstream of the initial Aβ insult.

Together, this data supports a role for TREM2 signaling in the regulation of the microglia response to Aβ or Aβ induced neuronal dysfunction *in vivo,* though the exact consequences of this altered response remains to be determined.

3.3.4 Reduction in Microgliosis and Altered Microglia State Had no Significant Influence on Aβ Load.

Plaque-associated microglia surround and invest amyloid plaques, suggesting they may be critical for phagocytosis of these large plaques. Microglia can phagocytose Aβ (Weltzien 2000), which may contribute to Aβ clearance and plaque containment. Since the plaque-associated microgliosis is reduced with BAC-TREM2 and increased TREM2 signaling, it is possible that this phagocytosis could be reduced due to fewer phagocytosing microglia being present. This does not seem to be the case however, as Aβ does not increase and in fact shows a trend towards a decrease by ELISA. This clashes with a previous paper that demonstrated a decreased Aβ load with TREM2 inhibition in 4-month APP/PS1 mice (Jay 2015), though it matches the other major TREM2 KO paper in 5xFAD mice (Wang 2015). If plaque-associated microglia are crucial for Aβ phagocytosis and clearance, then altered, less activated microglia from BAC-TREM2 may be more efficient at this job, in agreement with TREM2's published effects on phagocytic capacity in culture (Hammerman 2005, Takahashi 2006, Hsiao 2009). Due to low sample size in these

initial experiments, further investigation is required to draw definitive conclusions on the role of altered Aβ load with BAC-TREM2. If our eventual definitive study reaches the same conclusion that increased TREM2 gene dosage can improve microglia phenotypes and cognitive behaviors without significantly impact astrocytosis or Aβ load in the AD mice, it would be an important support to the idea that targeting TREM2 pathway in microglia may constitute an Aβ-independent strategy to ameliorate AD phenotypes.

3.3.5 Reduction in Microgliosis was Not Accompanied by Reduced Astrogliosis

In APP/PS1 mice, Jay et al. reported a reduction in astrocytosis in TREM2 KO mice, suggesting plaque-associated microglia activity, which is also reduced in KO mice, may reduce astrocytosis. While Jay et al. reported this was associated with improvements in plaque load, previous reports suggest specifically inhibiting astrocyte activation accelerates Aβ pathogenesis (Kraft 2013). Whatever the role of astrocytes, our initial analysis indicated reduced microglia activation had no effect on gross astrocytosis (Fig 3.5). This has three important implications. First, decreased astrocytosis and the possible associated harm or benefits is not the likely cause of improved AD phenotypes with BAC-TREM2. Second, despite the known responsiveness of astrocytes to microglia-secreted factors (Selmaj 1990), astrocytosis is likely independent of microglia-secreted cytokines, as a change in microglia phenotype had no effect on astrocytosis. Aβ itself (Johnstone 1999) or other down-stream effects are likely the main regulators of astrocyte activity in AD models. Future efforts to assay any change in cytokine expression in AD; BAC-TREM2 mice will strengthen this conclusion (See Chapter 4).

Finally, as astrocyte-secreted factors can in turn regulate microglia (Sawada 1989, Bianco 2005), the lack of altered astrogliosis suggests the microglia phenotype is not due to changes in

astrocytes, though molecular changes not detectable by analysis of gross morphology in AD; BAC-TREM2 astrocytes is a distinct possibility.

3.3.7 TREM2 Signaling Improves Memory Loss in APP/PS1 Mice

A key pathologic sequelae of amyloidosis modeled in AD mice is memory deficits. The hippocampus is a key region affected in AD mouse models (Fig 3.3-4). Thus, they are expected to have deficits in hippocampal-dependent contextual fear conditioning as has been previously shown in APP/PS1 mice as early as 6 months (Kilgore 2010). We demonstrated that APP/PS1 in our F1 background have significant deficits in contextual fear conditioning at 10 months. Remarkably, our preliminary study showed that TREM2-BAC completely rescued this deficit. TREM2-BAC most likely improved overall neuronal health through the many possible mechanisms discussed above. However, a recent paper linked fear conditioning deficits in the inflammatory EAE model to increased TNFα that acted on astrocytes to impair their synaptic function (Habbas 2015), linking the pro-inflammatory myeloid-cell response directly to learning deficits. In total fear conditioning data suggest that less reactive, TREM2-conditioned microglia lead to significant cognitive benefits in this AD mouse model. We plan to add more mice in different genotypes to substantiate this potentially important finding in our study.

3.3.9 Relationship to Data Generated by TREM2 KO Mice

TREM2 KO mice are the primary current method for assessing TREM2's function *in vivo.* Unfortunately, previous studies in AD mice in a TREM2 KO background had important conflicts (Jay 2015, Wang 2015). They both found TREM2 KO decreased microglia proliferation and activation around plaques, but one paper reported this had a beneficial effect in APP/PS1 mice (Jay 2015) while the other reported worsening phenotypes in 5xFAD mice, including in neuronal dysfunction (i.e. cell loss; Wang 2015).

We analyzed both of these AD models crossed with BAC-TREM2 to increase TREM2 signaling and found a similar reduction in microgliosis, despite opposite manipulations to TREM2. This raises important questions about what the differences are between BAC-TREM2 and TREM2 KO, despite the seemingly similar effect on microgliosis. First, while TREM2 KO microglia have increased pro-inflammatory cytokine release (Turnbull 2006), BAC-TREM2 microglia exhibit reduced Il-1β secretion (Fig 2.10). In addition, TREM2 KO mice reportedly have significantly decreased AD-induced astrogliosis (Jay 2015), while BAC-TREM2 had no such effect. Finally, while TREM2 inhibition was reported to increase neurodegenerative phenotypes like cell loss (Wang 2015), TREM2-BAC led to improved cognition. Unfortunately, memory deficits were not assessed in these two papers, preventing direct comparison.

Why might a similar change in the plaque-associated microgliosis with TREM2 KO and BAC-TREM2 lead to contrasting downstream consequences? First, the cause of reduced microgliosis may be different. Due to TREM2's known role in microglia survival, TREM2 KO microglia degenerate when challenged and activated by the damaged AD brain (Wang 2015). Increased TREM2 signaling in BAC-TREM2 mice should have a different effect: suppression of the inflammatory response and reduced recruitment of surrounding microglia, without microglia degeneration. This would better maintain basal microglia functions and suppress potentially detrimental changes in their activation state. Careful analysis of microglia outside of plaques in BAC-TREM2 and TREM2 KO mice as well as TUNEL staining of microglia in BAC-TREM2 brains may address this issue. Profiling of BAC-TREM2 microglia from AD mice (See Chapter 4) can also be compared to similar data collected by Wang et al to further understand the differential effects of TREM2 KO and overexpression in AD mouse microglia.

Despite the similar effects on microglia between BAC-TREM2 and TREM2 KO, our study suggests a protective role for TREM2 signaling, in agreement with Wang et al., the human genetic data associating dysfunctional R47H with AD, and the central role of TREM2's absence in PLOSL early-onset neurodegeneration. This is at odds with most of the findings in Jay et al, who found TREM2 KO was protective, though they looked at very young APP/PS1 mice (4 months as opposed to 10 months in the current study). There may be a differential effect of TREM2 signaling early versus late in pathogenesis. Assaying BAC-TREM2's effect on AD microgliosis, synaptic loss, and Aβ load at earlier time points would help better elucidate the reason for Jay et al. conflicting results.

3.3.11 Conclusion

BAC-TREM2 and increased TREM2 signaling led to remarkable changes in the microglia response in two separate models of Alzheimer's disease. This alteration was associated with significant cognitive improvement, supporting the idea that TREM2 and microglia-centric treatment strategies may be effective in the prevention of neurodegenerative disease. Further analysis of AD; BAC-TREM2 mice will provide new insights into the mechanism of TREM2's protective action and the role of microglia in chronic neurodegenerative diseases.

Chapter 4

Conclusions and Future Directions

4.1 Overview

Here I have described two new mouse models, BAC-TREM2 and BAC-TREM2-R47H, novel tools for the study of TREM2 signaling *in vivo.* TREM2 is a critical microglia and peripheral myeloid cell specific gene whose dysfunction is strongly linked to neurodegenerative disease through human genetics, pathological analysis of patient tissue, and mechanistic studies in cultured cells and mice. BAC-TREM2's increased TREM2 gene dosage will further our ability to study the effect of augmenting this central microglial signaling pathway in mice, and help to validate increasing TREM2 expression or agonizing TREM2 signaling as a novel therapeutic strategy for a variety of neurodegenerative diseases including AD. BAC-TREM2-R47H provides an additional tool to study the in vivo biology of this GWAS-significant disease risk allele of TREM2 and to compare with its wildtype counterpart in BAC-TREM2.

Our goal was to increase TREM2 signaling through increased TREM2 gene dosage in mice. We have demonstrated human TREM2 expression in BAC-TREM2 mice that is dynamically regulated in a manner similar to endogenous murine Trem2. We have found that increased human TREM2 dosage in BAC-TREM2 mice is linked to prolonged TREM2 Ca^{2+} signaling and suppression of pro-inflammatory cytokine expression in cultured microglia. Further characterization is warranted (see below), but our preliminary experiments suggest increased human TREM2 gene dosage does in fact increase TREM2 signaling in microglia.

Finally, we tested the ability of BAC-TREM2 to influence the progression of AD-like phenotypes in two AD amyloidosis models. An important use of BAC-TREM2 will be to assess the therapeutic potential and the mechanisms of any TREM2 benefit in mouse models of neurodegeneration. Our initial findings show that BAC-TREM2 and increased human TREM2 gene dosage does in fact influence the microglia response by suppressing microgliosis associated with amyloid plaques in both AD models, but soluble and insoluble Aβ was not significantly altered, though it may trend towards a reduction. In addition, these changes are associated with preliminary evidence of improved cognition, which would be an important finding that supports the therapeutic relevance of TREM2-mediated changes to microglia and the impetus to further understand these microglial alterations.

4.2 Further Analysis

In addition to the TREM2 mRNA analyses described, there is a need for confirmation of human TREM2 protein expression in BAC-TREM2. Protein and mRNA levels do not always correlate (Gygi 1999). Protein based methods can provide an effective method for comparing levels of human TREM2 overexpression to mouse TREM2. To date, we have not had success with immunohistochemical or western blot approaches with multiple separately published antibodies. Any possible signal we have seen has also been present in TREM2 KO tissue (data not shown), suggesting non-specific effects. Others have successfully used TREM2 antibodies quantitatively with flow-cytometry approaches (Jay 2015), and we are accumulating the mice needed to replicate this in BAC-TREM2 and BAC-TREM2-R47H mice. It is an important next step to fully characterizing the transgene and understanding the cell type specificity and levels of TREM2 overexpression in our models that are associated with the phenotypes we have observed in cell culture and AD mouse models.

Furthermore, additional characterization of the nature of TREM2-induced changes to microglia in culture is ongoing. TREM2 signaling has been shown to not only suppress pro-inflammatory cytokine expression, but also to increase phagocytosis of apoptotic cells (Takahashi 2005, Hsieh 2009), clearance of Aβ, and prolong survival when deprived of growth factors (Wang 2015). Future experiments will confirm if BAC-TREM2 microglia demonstrate these additional reported phenotypes of increased TREM2 activity and provide insight into whether they could be involved in the *in vivo* effects of BAC-TREM2.

Our first major use of BAC-TREM2 is assess the role of TREM2 and altered microglia in AD mouse models. We have several planned experiments to follow up on and confirm our initial findings that BAC-TREM2 suppressed the microglial response to amyloid plaques and improved cognition in these models. Our first line of study is to better understand these microglial changes and how BAC-TREM2 led to them. An important first step is RNA sequencing, which will give us insight into gene expression changes associated with BAC-TREM2. We are currently collecting dissected cortical and hippocampal tissue from the BAC-TREM2 x APP/PS1 cross for this purpose. Assessing whole tissue is promising, as network analyses of gene expression changes of brain tissue from human AD patients have consistently found immune-related modules dysregulated (Miller 2008, Zhang 2013, IGAP 2015). This is also the case in AD-related mouse models, including APP/PS1 mice (Wes 2014, Holtman 2015), and strong overlap between an AD mouse model and AD patients has been found in immune-related genes (Gjoneska 2015). In addition to seeing BAC-TREM2's effect on global immune signatures, we aim to profile gene expression in purified microglia from aged APP/PS1 mice and assay the effect of BAC-TREM2 specifically on molecular changes in microglia.

Additionally, we aim to better understand changes in AD-related phenotypes with BAC-TREM2, which will give us further clues to TREM2's actions in AD. If BAC-TREM2 is acting through TREM2's proposed role in Aβ phagocytosis (Ulrich 2014), we expect to see significantly reduced amyloid load when we add additional mice to our initial studies. We also expect to see improvement in Aβ clearance by cultured BAC-TREM2 microglia. If this is the case, that could be the significant explanation for improved performance in contextual fear conditioning.

There are additional hypotheses we are exploring for TREM2's beneficial effects, especially through the analysis of a key pathological deficit in amyloidosis models, synapse loss. Sensitive assays of synapse number and synapse markers are underway, as are electrophysiology studies to assess any improvement in hippocampal LTP deficits found in APP/PS1 mice (Francis 2009). There are several promising mechanisms that may link TREM2 to synaptic health aside from Aβ clearance, especially in light of an early study in mice lacking DAP12, TREM2's signaling adaptor, that demonstrated synaptic deficits (Roumier 2004). First, microglia secrete trophic factors, such as BDNF, that are critical to synaptic health (Parkhurst 2013). Two of these factors, VEGF and IGF1, are reduced in mouse models of AD when TREM2 is inhibited (Wang 2015). Thus increased secretion of these synaptic trophic factors in BAC-TREM2 may account for any improved synaptic pathology.

A second alternative explanation for any beneficial impact of BAC-TREM2 on synapse loss may involve microglia's known role in synaptic pruning. Microglia-mediated pruning begins with tagging of synapses by classical complement proteins, immune proteins involved with well-defined roles in innate immunity in the periphery (Stevens 2007). Microglia then recognize activated complement on the synapse and engulf them (Schaffer 2012). This process is critical for synaptic pruning during development and may have pathological activation in

neurodegenerative disease that contributes to early synapse loss (Howell 2011). C1q, the initial activating protein of the complement cascade, is secreted by microglia and induced by pro-inflammatory stimulation, and TREM2 signaling has been shown to inhibit Cq1 secretion (Sharif 2014). This provides a possible mechanism for reduced synaptic loss in BAC-TREM2; AD mice: decreased microglial activation and C1q secretion leads to a reduction in synaptic complement tagging and pathological pruning. Further experiments aimed at assaying complement expression, tagging, and synaptic pruning by microglia with immunohistochemistry will help explore this possibility. In addition, gene profiling of purified microglia will be an ideal way to better understand any altered secretion of these various synapse-related factors in BAC-TREM2 microglia (See Chapter 4).

Finally, we are focused on confirming our promising preliminary findings concerning the cognitive benefits of BAC-TREM2 in AD mice. Aside from testing additional mice in contextual fear conditioning, we will add a separate sensitive test of learning and memory, the Morris water maze. As a central purpose of this dissertation and what follows is to determine if augmenting TREM2 is a promising strategy for AD therapeutics, it is crucial we confirm these exciting findings with multiple behavioral tests.

4.3 Future Directions

BAC-TREM2-R47H will allow us to ask important questions about the nature of the disease-associated R47H allele, including answering key questions about whether it has dominant effects or if it is a loss or gain of function allele. In addition, by understanding differential effects of BAC-TREM2-R47H and BAC-TREM2 on AD mouse models, we hope to pinpoint disease relevant changes to microglia and their molecular profile in the context of a degenerating brain.

Following up any BAC-TREM2 phenotypes that are not recapitulated by BAC-TREM2-R47H will be our first priority (see Section 2.3.3).

Several Aβ-independent mechanisms of microglial benefit and toxicity have been proposed to explain the function of TREM2. These may be generalizable to other neurodegenerative diseases, which also exhibit significant microglia activation and neuroinflammation. The TREM2 R47H variant has been associated with many other common neurodegenerative diseases, including Parkinson's disease, ALS, and FTD (Rayaprolu 2013, Cady 2014, Borroni 2014, Table 2), In addition, inhibition of TREM2 signaling using TREM2 KO mice in diverse mouse disease models such as ischemic stroke (Kawabori 2015) and FTD (Jiang 2015) show worsening disease. However, no effect or opposite findings were seen in other studies (Sieber 2013, Saber 2015). By increasing TREM2 signaling, TREM2-BAC mice manipulate TREM2 *in vivo* in a new way, and will be useful tools for resolving these controversies.

We mean to assess this generality of TREM2's benefit for neurodegenerative diseases. The biggest change we see in AD mice crossed with BAC-TREM2 is found in microglia associated with Aβ plaques. As this a pathological factor unique to amyloidosis models and AD, we would need to test BAC-TREM2's function in other models of neurodegeneration. One promising avenue would be non-Aβ based AD-related mouse models, such as tau models. Another avenue being investigated currently is testing the ability of BAC-TREM2 to improve a mouse model of Huntington's disease, BACHD-ΔN17, and the unique pattern of HD-like microgliosis found in that model (Gu 2015). The differing possible roles of microglia in these various diseases and the nature of TREM2's influence on these functions is of intense future interest. These initial studies with BAC-TREM2 could provide impetus to explore TREM2-based

treatment strategies beyond AD, in patient populations more amenable to early interventions that may be required to see significant benefits (Ross & Tabrizi 2011).

We are also developing an additional model of BAC-TREM2 that is conditional, turning on TREM2 expression in a Cre-dependent manner. This model will allow us to address possible concerns of all transgenic mice like BAC-TREM2, that developmental effects are partially responsible for any phenotypes. Even more exciting, we will be able to ask a key question about the cell types that BAC-TREM2 is influencing to alter neuroinflammation: are they resident microglia or infiltrating monocytes? Cre-lines such as Cx3cr1-CRE-ER can induce permanent CRE recombination in long-lived microglia, but spare stem cells for circulating monocytes that are constantly being replaced (Parkhurst 2013). This will allow us to differentiate between increasing TREM2 gene dosage in resident microglia versus peripheral immune cells and determine the differential influences of the two important immune cell types. Knowing if increasing TREM2 in peripheral monocytes can improve AD phenotypes is critical for targeting potential TREM2-based therapies.

As the ultimate goal of any disease research is eventually improved therapeutics for patients, it is important to briefly consider these TREM2-based treatment strategies. Activating TREM2 therapeutically could be done in multiple ways. As a receptor protein, TREM2 can potentially be directly stimulated by therapeutic compounds. However, widespread indiscriminate activation of TREM2 may not be appropriate and may interfere with immune cell regulation, particularly in the peripheral immune system, where TREM2 is known to help immune cells phagocytose and eliminate infectious bacteria (Daws 2003, N'Diaye 2009, Wei 2015, Gawish 2015, Hommes 2015). An alternative approach is similar to TREM2-BAC mice: upregulate TREM2 expression, so as to increase its activation selectively when the correct ligand is present. This may involve

gene therapy with viral vectors, though their limited promoter size may limit finer control over TREM2 expression. Screening for compounds and genes that can increase TREM2 expression is another approach to augment TREM2 signaling (see Chapter 4). One known candidate is inhibition of miRNA34a, a microRNA that strongly inhibits TREM2 expression (Zhao 2013).

Better understanding TREM2 and its role in neurodegeneration could be an important step forward for targeting microglia and neuroinflammation in neurodegenerative diseases. BAC-TREM2 mice provide a new approach for investigating this critical pathway and furthering our appreciation of TREM2's functions *in vivo*.

MATERIALS AND METHODS

BAC Modification and Founder Generation. A 165kb human BAC (RP11-237K15) containing the 5kb *TREM2* gene was purchased from the BACPAC resource center (Oakland Children's Hospital, Oakland). Sequential deletions of critical exon in TREM-like (TREML) genes found on the BAC (see figure 2.1) were performed by sequential steps of homologous recombination using the pLD53 shuttle vector (see Fig 2.2). Exons 1-3 were deleted from TREML4, destroying a majority of the protein coding sequence. After deletion of exon 1 and 2 from TREML1, a downstream in frame ATG site was still present, so exons 5/6 were also deleted, abolishing 80% of TREML1's protein-coding sequences. TREML2's exon 1 was preserved, as it contains a region with high Histone 3-K27 Acetylation (Fig 2.1, blue star), suggesting an important transcriptional regulatory region for the TREM locus. Exon 2/3 were removed, frame-shifting the remainder of the mRNA and causing an early stop site in exon 4. This should lead to nonsense mediated decay of the truncated TREML2 mRNA (Hentze & Kulozik 2001), a strategy successfully used in mouse genetics in the design of knockout lines (Skarnes 2011). Modified BAC was prepared for microinjection with ultracentrifugation using a cesium gradient, according to published protocols (Gong et al., 2002). TREM2-BAC was microinjected at UCLA into fertilized FVB embryos at a concentration of ~1ng/ul. TREM2-BAC-R47H was microinjected at Cedars Sinai Medical Center. Potential founders were screened by PCR using primers specific to new cut sites introduced into the TREM2 BAC during TREML gene deletion, so as to avoid contamination with human DNA.

Transgene Copy Number Relative transgene copy number was determined by real-time PCR (Light Cycler) using genotyping primers from founders. Genomic DNA was purified from all 5 lines of BAC-TREM2 and BAC-TREM2-R47H lines analyzed. SYBR-green 2X reaction mixture (Applied Biosystems) was combined with BAC-TREM2 genotyping primers and 10ng of mouse

gDNA. Samples were normalized using b-actin primers. Fluorescent signals were compared using $\Delta\Delta C_t = (C_{t,BAC\text{-}TREM2} - C_{t,\beta actin}) - C_{t,Control\ (Average\ of\ WT\ B\ Line)}$, making all values relative to BAC-TREM2 Line B. Fold change was calculated by raising 2 to the power of the ΔΔCt value.

Gene Expression and RNA Isolation Gene expression of microglia cultures and mouse forebrain was determined by real-time PCR (Light Cycler) using a combination of newly designed and published primers. For culture experiments, 50-100 thousand microglia were lysed in their culture wells and RNA was isolated using a NucleoSpin II kit (McNally-Nagel). Concentrations were calculated using a NanoDrop spectrometer (Thermo Scientific) and equal amount of RNA (50-500 ng/ml depending on RNA yield for a given set of experiments) were reverse-transcribed into cDNA using a QuantiTect Reverse Transcription kit (Qiagen) with random heaxamers. For mouse forebrain, fresh frozen tissue was passed through a .22 gauge needle into TRIzol reagent (ThermoFisher Scientific) and submitted to a QIAshredder homogenizer (Qiagen) and RNA was isolate using RNeasy Lipid mini, kit. RNA was quantified and converted to cDNA as described above. SYBR-green 2X reaction mixture (Applied Biosystems) was combined with specific genotyping primers and 10-50ng of cDNA. Samples were normalized using GAPDH. Fluorescent signals were compared using $\Delta\Delta C_t = (C_{t,sample\text{-}} - C_{t,GAPDH}) - \Delta C_{t,Control\ group\ average}$, making all values relative to the control group. Fold change was calculated by raising 2 to the power of the ΔΔCt value.

Open Field test. TREM2-BAC, WT, APP/PSI, and APP/PS1;TREM2 BAC mice were submitted to Open Field analysis during their light cycle. It was performed with an Automated Open Field system (Tru Scan 99; Coulbourn Instruments). Analyses were performed 9.5 months of age. All animals were tested for 10 min a week before fear conditioning behaviors were assessed.

Contextual Fear Conditioning. Contextual fear conditioning was performed at the UCLA Animal Behavior Core. Mice were acclimated to the Core vivarium for one week before testing. Mice were also handled regularly and ran through experimental procedures (but not placed in the novel cage) the days before training to reduce anxiety. On the day of training and testing, mice are placed in darkened room for at least 15min to acclimate before they are brought into the cage room. Shock cage context is differentiated from their home cage by distinctive walls, floor grids and a pine scented odor. A shock of .75 mA was delivered for 2 seconds after the animals were in cage for 3 minutes, then the animals were left in the cage an additional minute before being returned to their home cage. The following day freezing behavior was assessed over an 8 minute period, recorded digitally and automatically analyzed using Video Freeze software (Med Associates). % freezing was calculated over the entire testing session, equal to time spent frozen divided by total time.

Immunohistochemistry. Antibodies used include: IbaI (Wako) (1:1000), GFAP (DAKO (1:5000), and Aβ 6E10 antibody (BioLegend). APP/PS1 x BAC-TREM2 mice and their littermate were perfused with filtered .01M Phosphate Buffered Saline. Half brains were obtained. One half was flash frozen in dry ice and stored at -80 for protein and RNA experiments. The other half was dropped fixed in ice-cold 4% paraformaldehyde in .01 phosphate buffer over night before dehydration with 30% sucrose and freezing. Frozen, fixed half brains were cut into 40um sections on a Leica 1800 Cryostat (Deerfield). Sections were washed in .01M PBS, boiled in a pH 6.0 citrate buffer for 20 minutes for antigen retrieval, and blocked using 5% normal goat serum (NGS) (Vectorlabs). Sections were incubated in the appropriate primary antibody in .01M PBS, 5% NGS and .1% Triton X-100 overnight at 4°C on a shaker to gently agitate them. Sections were then incubated with fluorescent dye conjugated goat anti-rabbit or anti-mouse

antibody (Vectorlabs) (1:300), washed, treated with Hoescht for nuclear staining, and mounted in ProLong Gold mounting media (Fisher Scientific) for confocal imaging.

Enzyme-Linked ImmunoSorbent Assay (ELISA). Protein extracts were obtained by homogenizing fresh frozen brain tissue in a buffered protease inhibitor cocktail (Sigma). Soluble Aβ was extracted further with diethylamine (DEA) while insoluble was extracted with a more severe formic acid (FA) treatment. A sandwich ELISA was used to quantify Aβ, with the 6E10 antibody (BioLegend) acting as the plate absorbed antibody. Samples were incubated with plates to bind 6E10. Then either a horseradish peroxidase conjugate $A\beta_{42}$ or $A\beta_{40}$ antibody (Promega) was added. A peroxidase substrate was added and color change was measured with a spectrometer. Every experiment measured a standard panel of Aβ concentrations in order to create a standard curve for our results the amount of Aβ calculated thusly was compared total protein, determined using a BCA assay (ThermoFisher Scientific).

Quantitation of Microglia. Microglia count/plaque area was determined as follows. 10x fluorescent microscopy images of coronal slices of the cortex of APP/PS1 mice and APP/PS1; BAC-TREM2 mice were acquired. Regions with multiple distinct plaques were chosen. Plaques in each image were numbered and measured for diameter. Plaque size was determined by area for a circle formula (pi*r^2), as most plaques were regular circles. After numbering, microglia were hand counted under the microscope at 40x for each individual plaque. Only microglia physically touching the plaque were counted. The total microglia count for each plaque was divided by the plaque area calculated to determine the reported value. Microglia intensity was determined using ImageJ (NIH). Regions of Interest (ROI) were determined based on αAβ 6E10 antibody staining of plaques before looking at images of microglial channel. Intensity of αIbaI staining microglia was determined with the measure function of ImageJ, and values reported were mean pixel intensity (each pixel is numbered from 0 to 255).

Quantitation of Astrocytosis. GFAP stained 40 µm coronal slice slices were imaged at 2.5x. 3 slices were chosen from each mouse at similar anterior-posterior locations starting at the beginning of the dorsal hippocampus, with each slice separated by ~500 µm. Images of GFAP and DAPI were taken for each slice of dorsal cortex adjacent to hippocampus at constant exposure and gain settings. ROIs were drawn using image J in the DAPI channel to encompass the entire dorsal cortex above layer V (where there is only faint, diffuse GFAP staining in WT mice). The GFAP channel was then thresholded to a minimum pixel intensity of 25 (from 0 to 255), which had significant background but still showed a general pattern similar to astrocyte patches seen by eye. % Area was calculated as number of pixels with GFAP intensity above the threshold/total # of pixels in ROI. % Area was measured at 5 different thresholds to demonstrate consistency in the results at whatever threshold chosen. Threshold of 35 was shown in more detail because it did the best job of including GFAP staining seen by eye without significant background staining.

Primary Cell culture. Microglia were isolated from perinatal pups (P1-P3) using established methods (Saura, 2003) In short, brains from WT and TREM2-BAC pups were trypsinized, triturated, and cultured in glial-supportive DMEM-F12 media with 10% FBS. After 2-3 weeks of culture, mixed glia cultures of astrocytes and microglia are obtained. Microglia were harvested after initial mild trypsination separates the astroglial layer from the plate, leaving only microglia. These are cultured separately in 24 well plates in 1% FBS for 1-3 days before being serum-starved 24 hours before LPS stimulation. Stimulation with lipopolysaccharide(LPS, 0.2 or 100 ng/ml) occurred 24 hours before RNA Isolation. Time course samples were harvested for RNA at the given time points (0, 2, 8, & 24) hours after LPS was added to the media.

Statistical Analysis. All statistics were calculated and analyzed using Prism software (GraphPad). Specific statistical tests are reported in figure legends and were chosen with a

preference towards non-parametric tests when applicable. Values are reported as mean +/- SEM, along with a detailed scatter plot when able. Paired values (for cytokine analysis and Aβ ELISA) were individually graphed with their partner as described in figure legends and connected by a line.

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