Title
Studying the Interactions Between Tau, Amyloid, and α-Synuclein in Alzheimer’s Disease Animal and Human Cell Models

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UNIVERSITY OF CALIFORNIA, IRVINE

Studying the Interactions Between Tau, Amyloid, and α-Synuclein in Alzheimer’s Disease Animal and Human Cell Models

DISSETRATION

submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in Biological Sciences

by

Wesley WL Chen

Dissertation Committee:
Assistant Professor Mathew Blurton-Jones, Chair
Professor Leslie Thompson
Professor Peter Donovan

2016
DEDICATION

To my family, friends, and Jenny,

Life is not easy for any of us. But what of that? We must have perseverance and above all confidence in ourselves. We must believe that we are gifted for something and that this thing must be attained.

Marie Curie
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<td>3xTg</td>
<td>triple transgenic</td>
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<td>5xFAD</td>
<td>Thy1-5xFAD</td>
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<td>α-syn</td>
<td>alpha synuclein</td>
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<tr>
<td>aa</td>
<td>amino acid</td>
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<tr>
<td>Aβ</td>
<td>amyloid beta</td>
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<tr>
<td>AD</td>
<td>Alzheimer’s disease</td>
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<td>AD LBD</td>
<td>Alzheimer’s disease with Lewy bodies</td>
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<td>APP</td>
<td>amyloid precursor protein</td>
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<td>APLP2</td>
<td>amyloid precursor like protein 2</td>
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<tr>
<td>B3T</td>
<td>beta-III tubulin</td>
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<td>B-cell</td>
<td>B lymphocyte</td>
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<tr>
<td>BDNF</td>
<td>brain derived neurotrophic factor</td>
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<tr>
<td>BM</td>
<td>bone marrow</td>
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<tr>
<td>CA1</td>
<td>hippocampus CA1</td>
</tr>
<tr>
<td>CA3</td>
<td>hippocampus CA3</td>
</tr>
<tr>
<td>CD</td>
<td>cluster of differentiation</td>
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<tr>
<td>Cdk5</td>
<td>cyclin-dependent kinase 5</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>CsA</td>
<td>cyclosporine A</td>
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<tr>
<td>CSF</td>
<td>cerebral spinal fluid</td>
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<tr>
<td>CTF</td>
<td>C-terminal fragment</td>
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<tr>
<td>DG</td>
<td>dentate gyrus</td>
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<td>DLB</td>
<td>dementia with Lewy bodies</td>
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<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
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<td>EPM</td>
<td>elevated plus maze</td>
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<td>fAD</td>
<td>familial Alzheimer’s disease</td>
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<td>GFAP</td>
<td>glial fibrillary acidic protein</td>
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<td>GFP</td>
<td>green fluorescent protein</td>
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<td>GSK-3β</td>
<td>glycogen synthase kinase 3 beta</td>
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<td>GWAS</td>
<td>genome-wide association studies</td>
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<td>Iba1</td>
<td>ionized calcium-binding adapter molecule 1</td>
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<td>IgG</td>
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<td>immunohistochemistry</td>
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<td>IL</td>
<td>interleukin</td>
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<tr>
<td>IL2rγ</td>
<td>interleukin 2 receptor gamma</td>
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<tr>
<td>GDNF</td>
<td>glial-derived neurotrophic factor</td>
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<tr>
<td>iPSC</td>
<td>induced pluripotent stem cell</td>
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<tr>
<td>iPSC-NSC</td>
<td>induced pluripotent-derived neural stem cell</td>
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<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinases</td>
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<tr>
<td>kDA</td>
<td>kilodalton</td>
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<tr>
<td>Klf4</td>
<td>Kruppel-like factor 4</td>
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2012 N113L: Neuroanatomy Laboratory
2013 N113L: Neuroanatomy Laboratory
2014 Bio 41: Mood Disorders
ABSTRACT OF THE DISSERTATION

Interactions between Tau, Amyloid, and α-Synuclein in Alzheimer’s Disease

By

Wesley WL Chen

Doctor of Philosophy in Biological Sciences

University of California, Irvine, 2016

Assistant Professor Mathew Blurton-Jones, Chair

Alzheimer’s disease (AD) is the leading cause of age related dementia and involves a progressive loss of neurons and synapses, leading to anxiety, cognitive impairment and a diminished quality of life. Pathologically, AD is characterized by extracellular amyloid beta (Aβ) plaque accumulation and the intraneuronal formation of tau-laden neurofibrillary tangles. In up to 70% of AD patients, these two hallmark pathologies are accompanied by a third proteinopathy; the aggregation of α–synuclein into intraneuronal Lewy bodies. The aggregate stress caused by these proteinopathies interacts with the immune system to produce a chronic neuroninflammatory condition that can further exacerbate disease progression.

The goal of my dissertation is investigate how β-amyloid, tau and α-synuclein pathologies interact with each other and to examine the role of the immune system in these interactions. To study how these proteinopathies interact in later stages of AD, I developed a new mouse model of AD termed ‘T5x’ mice, by crossing two existing transgenic lines; 5xFAD and Tau22 mice—aggressive lines that exhibit robust amyloidosis and tauopathy
respectively. During my studies I found that T5x mice exhibit dramatically increased tau hyperphosphorylation, neuroinflammatory response, and microgliosis. However, quite surprisingly I also found that T5x mice exhibit increased microglial Aβ phagocytosis leading to decreased amyloid plaque burden and insoluble Aβ. In subsequent studies I further determine that T5x mice also develop inclusions composed of murine α-synuclein (α-syn) and form Lewy-body like pathologies—the first transgenic AD model to our knowledge to be found to develop Lewy body-like inclusions without an α-synuclein transgene.

To further study the specific effects that the immune system has on the development of tau pathology, I also developed a mouse model of tau pathology lacking the adaptive immune. Thy1-Tau22 mice were crossed with Rag2−/−/Il2rg−/− double knockout mice over multiple generations to create ‘RagTau’ mice. RagTau mice lack T-, B- and NK-cells, yet exhibit significant accumulation and hyperphosphorylation of human tau. Although RagTau mice exhibit increases microglial activation relative to immune-intact Tau22 transgenics, no significant increases in tau pathology were detected in RagTau mice. To further validate the minimal influence of the adaptive immune system on the development of tau pathology, I performed adoptive transfer experiments, transplanting bone marrow cells from strain-matched GFP donors into RagTau mice. Using this approach I examined the potential effects of bone marrow reconstitution on tau pathology and the infiltration of GFP-labeled cells into the brain of tau mice. Unlike recent equivalent studies performed in our lab with Aβ-producing mice, I detected no changes in tau pathology in response to bone marrow transplantation. However, I did find that tau pathology induces a significant increase in T-cell infiltration into the brain parenchyma in comparison to Rag-
wild type recipients. Lastly, I was the first in our lab to establish the paradigm of generating human induced pluripotent stem cells (iPSCs). Using this approach I differentiated iPSCs into neural stem cells (NSCs) and transplanted into RagTau mice to examine potential questions about tau propagation. Interestingly, while iPSC-derived NSCs survived for at least 3 months post-transplantation I found no evidence that tau pathology could spread to these transplanted human cells. Taken together, my thesis research has helped to improve our understanding of the interactions between AD pathologies and the immune system.
INTRODUCTION

A. Alzheimer's Disease.

Alzheimer's Disease (AD) is the leading cause of age-related dementia, affecting over 5 million people in the United States alone [1, 2]. Recent failures in clinical AD immunotherapy trials and the lack of both consistent and effective drug candidates highlight the urgent need to develop novel therapies [3, 4]. With over 13 million projected cases of AD in the US by 2050, there is growing need not only for potential cures but also for treatments that effectively delay the onset and progression of AD and improve quality of life [5]. Alzheimer's disease is the world's most common neurodegenerative disorder at least in part because it is a disease of aging. The vast majority of AD cases are diagnosed as sporadic with no obvious family history or clear cause of disease, and only about 0.1% of all AD cases are familial with autosomal dominant inheritance [5]. There is increasing evidence that although sporadic AD (sAD) has no clear single genetic cause like other neurodegenerative disorders such as Huntington's disease, genetic background plays a large role in the onset and progression of sAD. Genome-wide association studies (GWAS) have identified a growing list of genetic risk factors for AD and twin studies have shown that sAD is up to 79% hereditary [6, 7]. Clinical symptoms of AD manifest in anxiety, social withdrawal, mood swings, changes in personality, difficulty carrying out routine activities, memory loss, and dementia [8-10]. Current treatments for AD are relatively ineffective and mainly consist of cocktails of acetylcholinesterase inhibitors and NMDA receptor antagonists [11, 12]. Yet despite the lack of effective human AD treatments, an abundance of successful therapies in AD transgenic animal models have been reported over the past decade [13]. The failure to successfully translate murine therapies into human patients can
be attributed to the lack of animal models that sufficiently recapitulate the aggressive aspects of AD. While human AD patients have extensive neurodegeneration by the time symptoms manifest, most animal models reporting successful treatment have little or no cell death at the onset of cognitive deficits. It is also unclear how relevant the treatment of behavioral impairment in mice is to the application of addressing human cognitive impairment. Instead, therapeutic development has primarily focused on examination of underlying AD pathology while taking into account changes in murine behavior as a secondary measure.

Alzheimer’s Disease is characterized by two primary pathologies: extracellular amyloid plaques and intraneuronal tau-laden neurofibrillary tangles (NFT). The mechanisms that drive AD are still widely debated, but the ‘amyloid cascade hypothesis’ first proposed by Hardy and Higgins in 1992 has emerged as the most popular theory [14]. This hypothesis posits that cleavage of amyloid precursor protein and accumulation of pathogenic isoforms of Aβ is the driving factor behind the development of AD. Deposition of Aβ results in increased hyperphosphorylation of tau (pτau) and higher levels of pτau and neurofibrillary tangles which in turn are thought to promote neuronal death [15]. Accumulation of extracellular Aβ and tau also appear to disrupt important biological functions such as packaging and transport systems, increase oxidative stress, promote inflammation, and ultimately lead to neuronal death [16, 17]. While significant evidence supports the amyloid cascade hypothesis, it is becoming increasingly clear that Aβ accumulation begins some 10-15 years prior to clinical symptoms [18]. As a result, gaining a better understanding of the interactions between Aβ, tau and other AD pathologies is an important step towards developing effective therapies.
B. Amyloid Production

Figure 1. Amyloid Processing. Amyloid precursor protein (APP) can be cut by multiple enzymes to produce several cleavage products including Aβ.

Amyloid plaques were first identified as a hallmark of AD in 1906 by the psychiatrist and neuropathologist the disease is now named after, Alois Alzheimer [19]. It took another almost 80 years to then isolate and identify Aβ as the principal component in amyloid plaques [20, 21]. Today, we have a much better grasp of amyloid and its processing, although the functions of the various APP cleavage products is still not well understood. Proteolytic processing of amyloid begins with the cleavage of amyloid precursor protein (APP). The APP gene lies on chromosome 21 and codes for a large transmembrane protein
translated up to 770 amino acids in length. Although the endogenous function of APP is not completely understood it has been implicated in a number of physiological functions including neural plasticity, iron transport, and synapse formation [22, 23]. The transmembrane conformation of APP has also suggested that APP may play a role as a cell signaling receptor or cell adhesion molecule [24]. Previous studies have also posited that APP plays a role in neurodevelopment. Combined knockout of APP and its homolog amyloid precursor like protein 2 (APLP2), for example, cause perinatal lethality and cortical dysplasia that closely resembles human lissencephaly [25]. Array studies have also shown that both APP and APLP2 single knockouts exhibit significant dysregulation of neurogenic transcriptional pathways [26]. Roles for APP and its derivatives in neurite outgrowth, adult neurogenesis, NSC differentiation, and human ESC differentiation have also recently been shown [27-31]. While this suggests a potential developmental role for APP in the development of AD, the over 25 APP genetic mutations that cause early onset familial AD (fAD) point to a greater contribution from the proteolytic cleavage of APP and the generation of Aβ [32].

APP is cleaved by one of two enzymes: α- or β-secretase. Enzymatic processing of APP by α-secretase leaves a soluble APP beta (sAPPα) fragment and the C-terminal fragment (CTF) C83. sAPPα has been shown to play a prominent role in learning and memory. sAPPα levels are reduced in AD patients [33] and lower levels of cerebral spinal fluid (CSF) sAPPα correlate with worse memory performance [34]. While cognitive correlation with lower levels of sAPPα may be more indicative of the shift of APP processing from α- to β-secretase, additional studies have shown that depletion of sAPPα in rats alters NMDA receptor function, reduces long term potentiation, and is sufficient to
affect learning and memory [35]. Furthermore, increased sAPPα production has been reported to enhance neuronal plasticity and improve learning and memory [36, 37]. In addition to affecting cognition and synaptic plasticity, sAPPα has also been implicated in axonal transport and cell proliferation [24, 38, 39]. APP cleavage by amyloid’s other main proteolytic enzyme, β-secretase, creates a soluble APP beta (sAPPβ) fragment and the CTF C99. Our lab has previously shown that sAPPβ can play a role in neuronal maturation and application of sAPPβ to human embryonic stem cells drives cells towards neuronal differentiation [29].

CTF fragments produced by either α- or β-secretase are then cleaved by an additional enzyme, γ-secretase. Cleavage by γ-secretase creates an APP intracellular domain (AICD) and small peptide fragment of P3 or Aβ from the C83 or C99 CTF’s respectively. The AICD fragment is highly interactive, binding with over 20 known proteins, and influences a variety of cellular functions [40]. Its binding to developmental proteins such as X11, NUMB, JIP, and G0, suggest that AICD plays a role in regulating neural development and differentiation [41-43]. Binding of AICD with nuclear translocating protein FE65 has also implicated AICD in transcriptional regulation, neurite outgrowth and cytokine remodeling [40, 44-47]. Perhaps most prominently, AICD has been widely reported to induce cell death in transfected cells in vitro and has been implicated in p53 and JNK mediated apoptosis [24, 48]. Although the P3 fragment co-produced alongside AICD from the C83 CTF is considerably less understood in the context of AD development, some studies suggest that P3 may play a role in the formation of oligomeric Aβ species [49] and P3 reactivity is reportedly significantly higher in AD brains [50].
While APP cleavage products originating from $\alpha$-secretase activity contribute in some form to AD, the vast majority of the field has focused on the study of A$\beta$ formation from $\gamma$-secretase cutting of the C99 CTF. Depending on how $\gamma$-secretase cleaves C99, the resulting A$\beta$ peptides can vary from 38, 40 or 42 amino acids in length. There is substantial evidence in the literature that A$\beta$42 is a major if not primary contributor to the onset and progression of AD and the higher the ratio of A$\beta$42 to A$\beta$40, the greater the intensity of the disease [51-54]. Most amyloid transgenic mouse models attempt to recapitulate AD by increasing production of A$\beta$42 through APP mutations that hold a greater propensity for pathogenic A$\beta$ production or by overexpressing presenilin-1 and -2 (PS1, PS2), the catalytic subunits of $\gamma$-secretase [55]. A$\beta$ can exist in a number of different forms that differentially contribute to the disease. When A$\beta$ monomers are initially cleaved by $\gamma$-secretase they exist as soluble peptides. As soluble A$\beta$ monomers accumulate at high levels they undergo a conformational shift into beta-sheets and aggregate as A$\beta$ fibrils and oligomers. While A$\beta$ plaque number has not been shown to be a reliable indicator of AD [56-58], previous reports show that soluble A$\beta$ is a strong correlate for synaptic loss and cognitive impairment in human patients [59]. Increasing soluble A$\beta$42 strongly correlates with the onset of AD, age-related neuronal atrophy, and cognitive impairment [60-62]. Intracellular A$\beta$ accumulation has also previously been reported to block synaptic transmission, disrupt axonal transport, and induce apoptosis [63-65]. In addition, AD patients displaying higher levels of soluble A$\beta$ correlate with greater severity of the disease [66]. It should be noted that while plaque number is not a good correlate for AD and insoluble A$\beta$ presides in the cores of plaques, previous studies have reported that in progressing and late-stage AD
human brains a dramatic shift in soluble to insoluble Aβ occurs concurrently with the shift of Aβ38 to Aβ40/42 [67].

C. Tau Pathology

![Image of Tau Pathology](image)

**Figure 2. Tau pathology.** (A) Tau isoforms express 3-repeat (3R) or 4-repeat (4R) microtubules binding domains. (B) Hyperphosphorylation of tau causes tau to detach from and destabilize microtubules.

While the popularity of the amyloid cascade hypothesis has placed much of the AD research field’s focus on amyloid, failures in Aβ immunotherapy has led to renewed emphasis on the role of tau in AD. Tau’s main physiological function is the stabilization of microtubules (MT) [68]. Alternative splicing of the Tau gene results in 6 main isoforms of Tau characterized by the number of MT-binding domains. Most tau isoforms have 3 or 4 MT-binding domains (3R, 4R) and the ratio of 3R to 4R isoforms has been shown to correlate with the probability of developing frontal lobe dementia [69]. Tau binding is post-transnationally regulated by phosphorylation of serine or threonine residues in its MT
binding domains. Under normal conditions, tau phosphorylation is delicately balanced as tau dynamically binds and unbinds MTs. In AD, tau is hyperphosphorylated resulting in free-floating unbound tau that aggregate to form intracellular neurofibrillary tangles. The excess unbinding of hyperphosphorylated tau results in MT destabilization which in turn disrupts cellular signaling, transport of neurotrophic growth factors, and trafficking of AD related proteins [70]. Sufficient hyperphosphorylation of tau can also lead to misfolding of free-floating tau into pathogenic conformations. Sequential phosphorylation of Serine-199 and -202 followed by Serine-396 and 404- cause tau’s N- and C- termini to fold onto each other—forming a ‘paperclip’ structure that has been previously shown to be one of the earliest pathological modifications of tau [71, 72]. The manifestation of tau pathology is considered the most direct and convincing correlate to neuronal death in the brain [73, 74]. A number of transgenic mice models expressing elevated levels of phospho-tau have been previously reported to exhibit cognitive deficits [75-77]. Studies have shown that reduction of hyperphosphorylated tau even in the presence of high levels of Aβ is sufficient to prevent neurodegeneration and alleviate cognitive deficits [78, 79].

While hyperphosphorylated tau eventually assembles to form insoluble fibrils and produce NFTs, more recent studies have focused on intermediary tau oligomers that may prove to be the most deleterious form of pathogenic tau. In the healthy brain, tau is minimally phosphorylated and preferentially binds to tubulin in stabilized microtubules. In the AD brain, however, hyperphosphorylated tau prioritizes binding to normal tau and form tau oligomers. In human AD patients, the presence of granular oligomeric tau in the frontal cortex is detected in early stages of AD and preclude the formation of NFT’s and clinical symptoms [80]. The shift from phospho-tau monomers to oligomeric formation
grants tau additional pathogenic properties. In a recent studies, application of oligomeric, but not monomeric tau inhibited LTP and memory [81, 82]. Similarly, oligomeric and not monomeric tau disrupt retrograde axonal transport [83]. Tau oligomers have also been shown by a number of studies to cause neuronal toxicity and may be the key component driving AD neurodegeneration [83-86]. Additional studies have recently suggested that tau oligomers released from affected cells may be able to translocate to healthy neighboring cells and drive propagation of tau pathology [87-90].

D. Synuclein in Alzheimer's Disease

Although considerably less well studied in the context of AD, α-synuclein (α-syn) has played a prevalent role in the history of the disease. α-syn was actually first identified in AD patients as the non-amyloid component (NAC) of extracellular plaques [91]. Lewy Bodies (LB), typically known as a hallmark of Parkinson’s Disease, are actually also found in up to 70% of human AD patients [92, 93]. Additionally, in advanced cases of AD α-syn LB’s and formations are detected alongside tau NFT’s in the spinal chord—likely contributing to late stage AD dyskinesia and autonomic dysfunction [94]. α-syn positive LB’s and dystrophic neurites are also detected in the brains of Down Syndrome patients with AD, with α-syn labeling observed in ~50% of amygdala tissue sampled [95]. Interestingly, in AD with LB’s and Lewy body dementia (LBD) with AD pathology, lower CSF α-syn have correlated well with occurrence of cognitive impairment, hallucinations, poor executive function, and CSF phospho-tau levels [96-98]. While correlations between α-syn and the development of AD pathology and symptoms have been drawn, little else has
been explored in respect to the role of α-syn in AD despite its common occurrence in the disease.

E. Interactions between amyloid, tau & synuclein

Amyloid & Tau

The vast majority of studies examining the relationship between amyloid and tau have been examined out in the context of the amyloid cascade hypothesis. To summarize, the relationship between amyloid and tau has been predominantly characterized a unidirectional process by which accumulation of Aβ species and amyloid plaques induces the hyperphosphorylation of tau and formation of NFT’s. While it is generally accepted that accumulation of amyloid elicits tau hyperphosphorylation, the exact species or conformation of amyloid that is responsible has been widely reported to include soluble APP, and Aβ monomers, dimers, oligomers and fibrils [99-102]. It is most likely that multiple amyloid cleavage products contribute to the hyperphosphorylation of tau through the activation of different tau kinases and regulation of relevant phosphatases either directly or indirectly. For example, Aβ oligomers have been previously reported to promote tau phosphorylation by upregulating the tau kinase JNK [101]. Another study suggested that the effects of Aβ oligomers on acute tau phosphorylation are localized and application of Aβ oligomers elicited local, but not global elevation of several other kinases in rat hippocampal neurons including: MARK/par-1, BRSK/SADL, p70S6K, and cdk5 [103]. Aβ peptides on the other hand have been previously reported to activate tau kinases AMPK, GSK-3β and TPK-1 [102, 104, 105], while sAPPβ was shown increase MAPK activity [99]. While amyloid ubiquitously upregulates multiple kinases that can induce tau
phosphorylation, the relationships between phosphatases, tau and amyloid are less uniform. Phosphatases PP1, PP2A, PP2B, and PP5 have been shown to desphosphorylate tau in vitro [106]. In the human brain, PP1, PP2A, PP2B and PP5 account for ~11%, ~71%, ~7% and ~10% of total tau dephosphorylation respectively [106]. In the same study and in others, PP2A and PP5 activity was significantly downregulated while PP2B activity was increased in human AD patients [106]. While PP2A inhibition is generally accepted as the main phosphatase-contributing factor towards increased tau hyperphosphorylation, few studies have reported a direct relationship between amyloid and decreased PP2A activity. One study has reported that caspase Asp664 cleavage of APP leads to a decrease in PP2A activity and thereby increases tau phosphorylation in rat hippocampal neuron cultures [107]. Another study has posited that amyloid decreases PP2A activity through a Zinc-Src dependent pathway [108]. Zinc binds amyloid and has been shown to accumulate in AD patient brains [109, 110]. Zinc then activates Src, which in turn phosphorylates PP2A and inhibits its activity [108]. It is interesting to note that while PP1 dephosphorylates tau in vitro, in AD transgenic animals and the AD brain PP1 is decreased and its inactivation has been implicated in amyloid toxicity [111-113]. Thus, while an abundance of evidence has been accrued on the deleterious effects of amyloid on tau pathology, much less has been made on the potential for tau to affect amyloid pathology.

**α-Synuclein & Amyloid**

The fact that α-syn was first identified in the Aβ plaques of AD patients suggests that synuclein and amyloid should share numerous interactions. In subsequent studies, α-syn is also commonly found in dystrophic neurites surrounding Aβ plaques in DLB patients [114].
Reinforcing the notion that α-syn and Aβ aggregate together in plaques, previous studies have shown fibrillar formation of wildtype and mutant α-syn aggregate analogously to amyloid and closely resemble amyloid beta sheets and filaments [115-117]. In vitro, Aβ has been shown to promote α-syn aggregation [118]. In transgenic mouse models, introduction of an α-syn transgene alongside a amyloid transgene has been previously reported to promote the accumulation of intraneuronal α-syn and PS129-positive α-synuclein pathology as well as the acceleration of amyloid plaque deposition and Aβ40/42 production [118, 119]. Interestingly, one previous study has shown that in rat cortical neurons, elevated levels of α-syn ameliorate Aβ42-induce caspase toxicity [120]. Although the abrogation of Aβ42 toxicity by α-syn is a contrast to increases in Aβ42 seen in transgenic mice, the mutual affinity between Aβ and α-syn is consistent and lends credence to the idea that co-aggregation of amyloid and α-synuclein plaques may initially be a neuroprotective attempt to sequester Aβ but that over extended time (such as seen in animal models, but not in culture) Aβ plaques and Lewy bodies further contribute to neuroinflammation and actually inhibit the clearance of Aβ.

In human patients, amyloid and α-synuclein have displayed a strong correlation with cognitive impairment. In Parkinson’s Disease (PD), the extent of cortical LB and amyloid pathology is associated with onset of dementia milestones [121]. In children and young adults, long-term air pollution has also been previously reported to increase chronic neuroinflammation and both Aβ42 and α-syn accumulation [122]. Furthermore, α-syn and Aβ have been shown to react to oxidative stress similarly and both have a propensity for scavenging reactive oxygen species [117, 123]. These findings suggest that amyloid and α-
synuclein most likely elicit similar neuroinflammatory stresses that may further drive the development of AD pathology.

**α-Synuclein & Tau**

Similarly to amyloid, tau also co-localizes with α-syn in Lewy Bodies [124]. In the same manner synuclein fibrilizes with amyloid, α-syn has also shown the propensity to induce fibrilization when coincubated *in vitro* or coexpressed *in vivo* [125]. The reciprocal conformational inductions between α-syn and tau has also been suggested as a contributing factor to the pathological transformations of these proteins. α-syn oligomers have previously been shown to induce and extend the lifetime of toxic tau oligomers [126]. Different strains of α-syn fibrils have also been reported to display differential efficiency in inducing tau fibrillization and inclusion formations in cultured neurons [127]. The synergistic increase in tau and α-synuclein pathology results in increased neurotoxicity and acceleration of AD cognitive deficits [128, 129]. Previous studies have also reported that treatments decreasing tau phosphorylation rescue cognitive deficits in α-syn mice—suggesting that tau may play a greater role in the onset of cognitive deficits in the presence of both tau and α-synuclein pathology [130].

Human studies have also shown a strong correlation between tau and α-synuclein pathology in both PD and AD patients. In both diseases as well as DLB, synaptic fractions are enriched for PS129-positive pathological α-syn and phospho-tau epitope PS396 [131]. In PD, oligomeric tau and α-synuclein colocalize in the cortex [132] and amygdala [133]. AD patients also have colocalized α-synuclein and tau pathology in the cortex, amygdala, spinal cord and olfactory bulb [93, 94, 131, 134]. The levels of tau and α-synuclein
pathology also significantly correlate in the CSF of AD patients. While α-syn levels in AD CSF are expectedly higher compared to healthy controls, AD CSF α-syn levels are surprisingly higher even compared to PD, DLB and multiple system atrophy patients [135, 136].

The strong correlations between tau and α-synuclein pathology bely shared modulatory mechanisms. Prior studies have identified shared transcriptional regulatory proteins such as TRIM28 that promote stabilization of toxic tau and α-syn conformations through the SUMOylation and nuclear translocation of tau/α-synuclein protein in Drosophila, human cells, and mice brains [137]. α-syn has also been previously reported to directly induce the phosphorylation of tau. In one study, binding of α-syn to tau recruits protein kinase A (PKA) phosphorylation of tau amino acids S262 and S356 [138]. α-synuclein-Tau interactions has also been strongly implicated through tau kinase GSK-3β. Transgenic mice carrying point mutations in the GSK-3β and exhibiting dysregulated GSK-3β activity accumulate pathological phosphorylation of α-synuclein PS129 and tau PS396/404 [139]. Extracellular α-syn has also been shown in a prior study to upregulate GSK-3β activity and tau phosphorylation, and chemical inhibition of GSK-3β ameliorates increases in tau phosphorylation despite the addition of extracellular α-syn [140]. Furthermore, α-syn and PD-implicated protein LRRK2 have been shown to directly interact with GSK-3β to increase tau hyperphosphorylation [141].

**F. Mouse models**

In recent years, the AD community has fielded a plethora of treatment successes in transgenic mouse models. Yet despite the abundance of promising treatments in mice, no
therapy has successfully translated into human AD trials. In a review of the effectiveness of current AD mice in modeling the disease Zahs et al., argue that one of the reasons AD research has failed to translate is because the vast majority of AD mouse models only recapitulate pre-symptomatic stages of AD [13]. Thus, successful treatments in current AD mouse models are more likely to reflect potential preventative therapies than cures for patients with a clinical diagnosis and therefore established disease. While many AD murine lines have effectively reproduced amyloid plaques and/or tau neurofibrillary tangles, most transgenic mice fail to show evidence of perhaps the most important pathology of AD: neuronal death. With every failed clinical trial derived from a successful AD murine treatment the evidence that current AD mice insufficiently model the disease grows. There is therefore a pressing need to develop an animal model of AD that successfully recapitulates the three hallmark pathologies of AD: amyloid plaques, tau tangles, and neurodegeneration. In addition, the potential development of immune deficient mouse models has presented an opportunity to study stem cell transplantation and the role of immunity in the context of AD.

Thy1-5xFAD mice

In 2006 Dr. Robert Vassar published findings on an amyloidogenic mouse model coined 5xFAD mice. 5xFAD mice express three APP mutations (Swedish, Florida, London) and two PS1 mutations (M146L, L286V) that are co-inherited and driven under control of a neuronal specific form of the Thy1 promoter[142]. 5xfAD exhibit intraneuronal Aβ accumulation beginning at 1.5 months, amyloid plaque deposition and gliosis starting at 2 months, synaptic loss by 6 months, and neuronal death beginning at 9 months of age [143].
Amyloid deposition is most prominent in the hippocampus, subiculum, deep cortical layers, and the basal lateral amygdala. As a result, 5xfAD mice show frontal cortex dependent cognitive deficits by 4 months of age, and anxiety and hippocampal-dependent cognitive deficits beginning at 6 months of age [144, 145]. Accumulation of amyloid in the spinal cord does, however, result in motor deficits by 9 months of age, limiting the age at which cognitive tasks can be used [146]. Increased neuroinflammation and microglial activation have also been shown to play an essential role in mediating disease progression in this model [147]. Additionally, increased amyloidosis and C99 CTF production has been previously reported to lead to increased mitochondrial dysfunction [148]. Thus, 5xfAD mice provide an aggressive amyloidogenic model that recapitulate AD-associated plaque pathology, behavioral deficits, and neuroinflammation.

**Thy1-Tau22 mice**

In the same year that Vassar reported the 5xFAD model, Dr. Luc Buée published a paper detailing his newly generated Tau22 mouse line [149]. Tau22 mice express human four repeat tau with two mutations (G272V, P301S) under the Thy1 promoter and develop hyperphosphorylated tau, neurofibrillary tangles, and gliosis [149]. Starting at 6 months of age, Tau22 mice begin to show the first signs of cognitive disruption, exhibiting anxiety deficits, whereas hippocampal dependent cognitive deficits first manifest several months later at about 10 months of age [149]. By this age, medial septal cholinergic neuronal number and NGF uptake from the hippocampus is significantly reduced and by 12 months of age, Tau22 mice have begun to recapitulate other behavioral and psychological symptoms seen in human AD patients including increased depression, aggressive behavior,
and disturbances in nocturnal activity [150, 151]. Studies examining the effects of exercise and caffeine in Tau22 mice suggest important roles for microglia and neuroinflammatory response in the accumulation of tau pathology [152, 153]. The recapitulation of substantial tau pathology, cognitive deficits without motor impairments, and neuroinflammatory response highlight the strengths of Tau22 mice as a model of AD-associated tau accumulation.

*Rag2*/Il2rγ dKO mice

*Rag2*−/−/Il2rγ−/− mice exhibit constitutive knockout of recombinase activating gene 2 (Rag2) and interleukin 2 (IL2) receptor gamma, resulting in complete ablation of B-, T- and natural killer (NK) Cells [154-157]. *Rag2*−/−/Il2rγ−/− mice breed adequately and exhibit normal lifespans when housed in sterile immune privileged housing facilities [158]. By knocking out the adaptive immune system, *Rag2*−/−/Il2rγ−/− mice provide a unique opportunity to study several fields of research in vivo. *Rag2*−/−/Il2rγ−/− mice lack the immune machinery to reject foreign antigens including tissue, making it an ideal model for xenotransplantation. Previous studies have reported successful xenotransplantation of human hepatocytes and other sources of rat and human tissue [158-160]. Cancer studies have also taken advantage of the lack of adaptive immunity study the spread of tumors in *Rag2*−/−/Il2rγ−/− mice [161]. While tumor formation is a concern for any transplantation study in *Rag2*−/−/Il2rγ−/− mice, previous studies have reported successful engraftment of stem cells [162], suggesting that there is utility in studying the potential effects of xenografts transplantation in an immune deficient model of AD. Furthermore, by crossing *Rag2*−/−/Il2rγ−/− mice different models of AD, one can study the relationship between
adaptive immunity and AD pathology. Our own lab has recently reported that mice breed from a cross between Rag2−/−/Il2rγ−/− and Thy1-5xfAD exhibited significant changes in amyloid pathology—elucidating roles for the adaptive immune system in Aβ homeostasis [163]. Other studies have also utilized Rag2−/−/Il2rγ−/− mice to examine the relationship between specific immune cell types in modulating adaptive response [164-166]. Lastly, Rag2−/−/Il2rγ−/− mice also serve as ideal candidates for the creation of chimeric human/mouse models [161, 167, 168].

G. Neuroinflammation & Immunity

The role of neuroinflammation in the development and progression of AD has been studied for several decades [169-171]. It has been suggested that neuroinflammation may even play a causal role in the disease [172, 173]. Previous studies have reported that amyloid and phospho-tau induce release of proinflammatory cytokines which subsequently can lead to increased plaque deposition and NFT formation [174-178]. The perpetual cycle of pathology progression and proinflammatory cytokine release create an environment of chronic neuroinflammation. Previous studies have shown that chronic neuroinflammation via continuous LPS stimulation can recapitulate components of AD pathology [179]. Chronic inflammation has also been reported to cause loss of forebrain cholinergic neurons—a key point of neurodegeneration in AD patients [180-182]. While AD pathology plays an important role in shifting the AD brain into a proinflammatory state, the direct modulators of neuroinflammation consist of two main cell types: astrocytes and microglia.
Astrocytes

Astrocytes are star-shaped glia that function in neural repair, homeostasis, blood brain barrier support, neurotransmitter release and neural development in the healthy brain. In AD brain, astrocytes have long been observed to aggregate around amyloid plaques [183]. Astrogliosis is highly upregulated in AD and numerous studies have shown increased staining of reactive astrocyte marker glial fibrillary acidic protein (GFAP) in the presence of Aβ [184, 185]. Reactive astrocytes have also been implicated in the release of proinflammatory cytokines and development of AD cognitive deficits [186, 187]. The production of APOE, sporadic AD’s (sAD) greatest risk factor, by astrocytes further highlights the importance of astrogliosis in AD. APOE promotes the co-localization astrocytes around amyloid plaques and the phagocytosis of Aβ [188]. Although astrocytes can independently clear Aβ in the brain, they also modulate the function of the primary mediator of Aβ phagocytosis within the brain: microglia.

Microglia

Microglia are the resident macrophages of the brain and are important for defending against infectious agents and foreign antigens. Microglia are also, however, responsible for synaptic pruning and clearance of damaged neurons and cellular debris. While completely necessary for the homeostasis of a healthy brain, these microglial processes are thrown into overdrive in the AD brain, resulting in deleterious activity [189].

Oligomeric Aβ can bind to cell surface receptors such as Toll-like receptors (TLR) and push microglia into a classically-activated M1 state [190, 191]. While microglia have a high propensity for Aβ clearance, microglial Aβ phagocytosis becomes impaired in the
aging AD brain—increasing amyloid burden [192]. Microglia also are one the brain’s major sources for cytokine release and their production of pro-inflammatory cytokines in AD has been shown to contribute to acceleration of amyloid and tau pathology [174, 193-195].

**H. Induced Pluripotent Stem Cells & Alzheimer’s Disease**

*Induced Pluripotent Stem Cells*

The advancement of induced pluripotent stem cells (iPSCs) technology has opened the doors for modeling and potential treatment of neurodegenerative diseases. Dr. Shinya Yamanaka first published his findings for iPSC reprogramming in 2006 [196] and has since gone on to win the 2012 Nobel Prize for the development of iPSC technology. iPSC reprogramming involves the engineering of terminally differentiated cell types such as fibroblasts or peripheral blood mononuclear cells (PBMC) back into a pluripotent stem cell state. The fundamental principles guiding iPSC reprogramming are driven by the overexpression of fur transcriptional and master regulatory genes; Sox2, OCT3/4, c-Myc and KLF4 that regulate cellular pluripotency [197]. Over the past few years better understanding of the biology behind iPSC reprogramming has contributed to numerous protocol reiterations that have increased reprogramming efficiency in multiple cell types and species [198-200]. Delivery vehicles for reprogramming factors have also expanded to include packaging in retrovirus, Sendai virus, modified RNA and small molecules [201-204]. The rapid advancement of iPSC technology has also created a unique opportunity to study the genetics of AD using iPSCs reprogrammed from AD patients.
Induced Pluripotent Stem Cells in Alzheimer’s Disease Research.

Genetic studies of early-onset AD identified mutations in APP, Presenilin 1, and Presenilin 2 as driving the development of familial AD. In contrast, sporadic AD does not result from a single highly penetrant genetic cause. Nevertheless, twin studies reveal that the heritability of sporadic AD is as high as 79% [7]. Polymorphisms in the APOE gene clearly provide the greatest single genetic influence on sporadic AD risk [205, 206]. However, recent genome-wide association studies have identified several other genes that can influence the development of sporadic AD [207, 208]. Thus, genetics clearly plays an important albeit complex role in sporadic AD that could potentially be modeled with induced pluripotent stem cells (iPSCs). Patient-derived iPSCs have been used to model a growing list of human genetic disorders [209]. Human iPSCs have been reprogrammed via a number of different methods including the use of mRNA, siRNA, minicircle vectors, episomal plasmids, and viral transduction [210]. Most recently, three groups have reported the establishment and investigation of AD iPSCs [211-213]. In one study, Yagi et al. generated iPSCs from patients carrying familial mutations in PS1 or PS2 [212]. The patient-derived iPSCs recapitulated an important aspect of familial AD; altered generation of Aβ42 versus Aβ40. This report also gave an example of the potential use of AD iPSCs for testing drug efficacy. In a second study, Israel et al. generated iPSCs from not only fAD patients but also two cases of sporadic AD and two unaffected controls [211]. Interestingly, neurons derived from one of the sporadic AD cases mimicked some of the findings from fAD cases; showing increased Aβ40 generation and tau phosphorylation, activation of glycogen synthase kinase-3β, and accumulation of enlarged early endosomes. Importantly, these disease-associated phenotypes were not detected in fibroblast cultures from this
case, demonstrating the importance of studying these phenotypes in iPSC-derived neurons. In the third study, Kondo et al. reprogrammed iPSC lines from both fAD and sAD patients [213]. Kondo showed that neurons generated from one fAD and sAD line showed increased intraneuronal Aβ and Aβ oligomers, increased levels of oxidative stress, and decreased viability in growth-factor reduced media. Treatment with drugs that reduced oxidative stress and ROS-induced trafficking deficits reversed the observed AD related changes. Taken together, these studies represent critical first steps in assessing the potential of AD iPSCs to model AD. As Israel, Kondo and colleagues point out, generation of many additional sporadic AD iPSC lines will of course be needed to fully establish the ability of this approach to guide drug development and enhance our understanding of AD.

*Neural Stem Cell Transplantation for Alzheimer's Disease*

The generation of neural stem cells (NSCs) from AD patients presents another valuable model to better understand the disease. NSCs are tripotent, proliferating stem cells that have the capacity to differentiate into neurons or glia. Neural stem cells are most abundant during early stages of development during the formation of the brain. In the adult brain, endogenous NSCs are found in a few loci of neurogenesis including the subventricular zone of the lateral ventricles, the subgranular zone of the dentate gyrus in the hippocampus, and the olfactory bulb [214]. NSCs provide several advantages that make it an attractive candidate for treatment of neurodegeneration. It has been shown that both endogenous and transplanted NSCs in the brain migrate to areas of inflammation and can modulate inflammatory responses [215-217]. In addition, NSCs can express high levels of neurotrophins including both brain derived neurotropic factor (BDNF) and nerve growth factor (NGF) [218-220]. Thus, stem cells could provide a means to deliver neurotrophins to
the diseased brain, potentially modulating endogenous synaptic plasticity and enhancing neuronal survival. Indeed a growing number of studies support this notion. Our lab has previously found that NSC transplantation increases hippocampal synaptic density and improves learning and memory in transgenic models of both AD and neuronal loss [218, 221]. This enhancement of synaptic growth was coupled with elevated brain levels of BDNF. In a similar study, Hampton et al. examined NSC transplantation in a transgenic model of neurofibrillary tangle formation, finding that NSCs could reduce neuronal loss and elevate levels of glial-derived neurotrophic factor (GDNF) within the brain [222]. Related to these findings, ex vivo-mediated delivery of NGF has also been shown to improve cognition both in animal models with cholinergic lesions and in a phase I trial in AD patients [223, 224]. Delivery of NGF via NGF-secreting NSCs has also been shown to be effective at reducing cell loss in models of stroke and excitotoxicity, suggesting that stem cell-derived trophic support could be effective for a variety of conditions [225, 226].

Potential transplantation of iPSC derived NSC’s provide a unique opportunity for personalized medicine in the future. Dramatic improvements in iPSC reprogramming and NSC differentiation technology has made patient-specific iPSC based medicine a realistic possibility [227]. iPSCs reprogrammed from a patient and their subsequently redifferentiated cell types are thought to be less immunogenic when transplanted back into the same patient [228-231]. The lessened immune response for iPSC-differentiated tissue transplanted back into the original patient allows for reduced immunosuppressant dosage—a distinct advantage for AD where immunosuppressants have been previously shown to have an abundance of non-specific effects in the brain [232, 233]. Most recently, Dr. Masayo Takahashi in Japan have begun to initiate the first human clinical trials using
iPSCs by transplanted retinal cells back into the immune-privileged eyes of patients suffering from age-related macular degeneration. If successful, the process towards developing iPSC derived neural tissue for the treatment of AD could be accelerated in the not too distant future.
Figure 3. **Neural stem cell transplantation in AD.** Stem cell-based therapies could potentially treat Alzheimer’s disease (AD) by targeting several different stages of disease pathogenesis. The amyloid cascade hypothesis argues that overproduction and/or decreased clearance of Aβ drives all other downstream components of AD including neurofibrillary tangles, neuronal and synaptic loss, and cognitive dysfunction (blue arrows). While many drugs in development only target the initial accumulation of Aβ, stem cell-based therapies could intervene at multiple stages of this cascade. Neural stem cells (NSCs) in particular (green) can modulate synaptic plasticity and provide robust neuroprotective and neurotrophic activity. Likewise, various stem cell populations can promote anti-inflammatory signals, slowing disease progression. Capitalizing on the unique migratory capacity of NSCs, genetic modification of stem cells (purple) could also be used to concurrently target Aβ and tangle pathology or enhance neurotrophic and neuroprotective capacity. Given the complex nature of AD, these kind of combinatorial strategies will likely be needed.
Chapter One: Increased Tauopathy Promotes Microglial-mediated Clearance of Beta-Amyloid

Introduction

Alzheimer disease (AD) is the leading cause of age-related dementia, affecting over 5 million people in the United States alone [1, 2]. AD pathology is characterized by two primary lesions: extracellular amyloid plaques and intraneuronal tau-laden neurofibrillary tangles. The mechanisms that drive AD remain unclear, but the ‘amyloid cascade hypothesis’ first proposed by Hardy and Higgins posits that beta-amyloid (Aβ) is the initiating factor in AD pathogenesis [14, 179, 234]. Increased deposition of Aβ, in turn, is thought to promote the hyperphosphorylation of tau leading to neurofibrillary tangle (NFT) formation [15]. Together, Aβ and tau pathologies disrupt critical biological functions such as axonal transport and synaptic function and promote neuroinflammation, ultimately leading to widespread synaptic and neuronal loss [16, 17].

The role of neuroinflammation in the development and progression of AD has been studied for several decades [169-171]. However, the recent identification of AD risk polymorphisms in several microglial-enriched genes such as TREM2, MS4A, and CD33, has intensified this research area [235-242]. Microglia serve as one of the brain’s mechanisms of Aβ clearance, but also play critical roles in neuronal homeostasis and synaptic plasticity [243-245]. In response to Aβ, microglia increase their expression of pro-inflammatory cytokines, which has been shown to promote tau hyperphosphorylation and NFT pathology [177, 246] and contribute to synaptic and neuronal dysfunction [245, 247, 248].
To better understand the role of microglia in the interactions between Aβ and tau pathologies we crossed two transgenic AD models, 5xfAD and Thy-Tau22 mice, to create a novel bigenic line termed ‘T5x’ mice. 5xfAD mice express three APP mutations (Swedish, Florida, London) and two PS1 mutations (M146L, L286V) that are co-inherited and driven under control of the Thy1 promoter [142], which is highly expressed in neurons. 5xfAD exhibit intraneuronal Aβ accumulation beginning at 1.5 months, amyloid plaque deposition and gliosis starting at 2 months, synaptic loss by 6 months, and neuronal death beginning at 9 months of age [143]. Amyloid deposition in these mice is most prominent in the hippocampus, subiculum, deep cortical layers, and the basal lateral amygdala. Increased neuroinflammation and microglial activation have also been shown to play an essential role in mediating disease progression in this model [147]. Thus, 5xfAD mice provide an aggressive amyloidogenic model that exhibit robust AD-associated plaque pathology and associated microgliosis. In comparison, Thy-Tau22 mice express human four repeat tau with two mutations (G272V, P301S) driven under the Thy1.2 promoter and progressively develop hippocampal hyperphosphorylated tau, neurofibrillary tangles, and gliosis [149]. Studies examining the effects of exercise, caffeine and A2A receptor modulation in Thy-Tau22 mice suggest important roles for microglia and neuroinflammatory responses in the accumulation of tau pathology [152, 153].

Interactions between Aβ and tau in mouse models were first reported in two seminal studies in 2001 that clearly demonstrated that Aβ accumulation could accelerate the development of tau pathology [249, 250]. Subsequently, the 3xTg-AD model was generated and extended our understanding of the influence of Aβ on tau and was used to explore the role of microglial inflammation in this process [251, 252]. Since then several
other bigenic models have been created and studies have continued to investigate the effects of amyloid on tau and suggested that the amyloid cascade follows a unidirectional pathway [253-255]. However, many of these models exhibit far less pathology than occurs in human AD cases and thus more complex interactions that might occur over decades in the human brain or at later stages of advanced disease may not be faithfully recapitulated in many of these models [13]. Furthermore, the potential role of inflammation in these interactions between Ab and tau remains greatly understudied.

In the present study, we combined a model of Aβ accumulation (5xfAD mice) with a progressive model of neurofibrillary tangle pathology (Thy-Tau22 mice). The resulting 'T5x' mouse line was generated to provide insight into the later stages of disease progression akin to that observed in clinically-diagnosed AD patients and the interactions and consequences of advanced amyloid pathology on tau and visa-versa. By comparing the resulting T5x bigenic mice to their single transgenic littermates we have uncovered potential new roles for tau in the modulation of Aβ and neuroinflammatory response. Most notably, we identify and examine how amyloid and tau synergize to alter microglial activity and promote Aβ clearance. Thus, these data provide additional insight into the relationship between AD pathology and neuroinflammatory response and suggest that tau could have reciprocal interactions with amyloid.

Material and Methods

Generation of T5x mice. Thy-Tau22 mice express human 4 repeat tau with two frontotemporal dementia-associated point mutations (G272V and P301S) under control of the neuronal driven promoter Thy1.2 and are maintained on a C57Bl6/J background [149].
The 5xfAD mice used in this study are also maintained on a congenic C57Bl6/J background and were purchased from the Mutant Mouse Resource and Research Center (MMRRC, stock# 034848-JAX). The 5xfAD model co-expresses human amyloid precursor protein (APP695) carrying the Swedish, Florida, and London mutations and a human presenilin-1 (PS1) transgene carrying the M146L and L286V mutations under the Thy-1 promoter. Both APP and PS1 transgenes are co-integrated and thus co-inherited. Heterozygous Thy-Tau22 and 5xfAD mice were crossed to create Thy-Tau22-5xfAD (T5x) mice, as well as Thy-Tau22, 5xfAD, and WT littermates that were genotyped via PCR amplification of human tau, PS1, and APP transgenes. The number of mice from each sex-balanced genotype was: WT (n = 8), 5x (n = 11), Tau (n = 14), and T5x (n = 9). All mice were maintained on a purebred C57Bl/6 background and group housed (2-4 mice/cage) on a 12h/12h light/dark cycle with access to food and water ad libitum. All animal procedures were performed in strict accordance to the National Institutes of Health and University of California Institutional Animal Care and Use Committee.

**Tissue preparation and Neuropathological analysis.** Following behavioral testing, all mice were sedated with euthasol and sacrificed by cardiac perfusion with 0.1M phosphate buffered saline (PBS). Brains were removed and one hemisphere was snap frozen on dry ice while the other hemisphere was postfixed in 4% paraformaldehyde for 48 hours then stored in PBS + 0.05% sodium azide. Fixed half-brains were placed in 30% sucrose for at least 48 hours before being cut in the coronal plane (40 µm sections) using a freezing sliding microtome.
**Immunohistological staining.** Brain sections were rinsed in PBS three times and blocked in PBS+0.05% Triton-X with 5% donkey or goat serum for one hour. Primary antibodies used included: 6E10 (Covance; 1:500) and 82E1 (ABL America; 1:500) against Ab, total Tau (human+mouse; Dako; 1:1000), human tau (HT7, ThermoFisher; 1:1000), phosho-tau epitopes AT8 (Pierce; 1:500) and PHF-1, and conformational tau epitope MC-1 (generously provided by Peter Davies; 1:1000). Analysis of gliosis, phagocytosis, and dendritic architecture utilized CD68 (Abcam; 1:200), IBA1 (Wako; 1:1000), GFAP (Abcam; 1:1000), Beta3Tubulin (Covance; 1:1000). Sections were incubated in primary antibodies at 4 degrees Celsius overnight. Sections were then washed three times with PBS and incubated with appropriate Alexa fluor-conjugated secondary antibodies at room temperature for one hour. Sections were then rinsed three additional times, mounted on slides and coverslipped with Fluoromount-G with DAPI.

**Biochemical Analysis.** Hippocampus and cortex was microdissected from frozen brains and processed to collect both soluble and insoluble extracts. Briefly, microdissected tissue was homogenized in TPER (ThermoFisher) and centrifuged at 12,000 RPM for 15 minutes. Supernatant was collected as the soluble fraction and the pellet was treated with 70% formic acid and spun down at 25,000 rpm. The resulting supernatant was collected as the insoluble fraction. Insoluble protein samples were neutralized for Western blotting and further precipitated with trichloroacetic acid (TCA) when probing for insoluble tau. Protein samples were denatured at 95°C for 15 minutes before being loaded onto 4-20% TGX precast polyacrylamide gels (Bio-rad). Antibodies used for western blotting include: HT7 (1:1000), PS199 (Abcam; 1:1000), PS202 (Abcam; 1:1000), AT100 (ThermoFisher;
1:1000), AT270 (ThermoFisher; 1:1000), PHF1 (1:1000), 6E10 (1:1000), GFAP (1:1000). Mesoscale Discovery immunoassay kits (Mesoscale Diagnostics) were used for cytokine (K15048G) and Aβ38, 40, and 42 (K15199E) quantification of cortical samples followed the manufacturers protocols. The proinflammatory MSD was able to detect levels of that were within the standard curve, whereas brain levels of IFN-γ, IL-12p70, CXCL1 and IL-4 were below the threshold of detection.

**Confocal microscopy and quantification.** Equivalent brain sections were picked and immunofluorescent sections were imaged using Olympus FX1200 confocal microscope. Amyloid plaque burden identified by 82E1 were visualized through a Z-stack image taken through the entire depth of the section at 1 µm intervals. Confocal files were then rendered in 3D and analyzed by a blinded observer using the volume function of IMARIS software (Bitplane). Microglia and astrocyte quantification by confocal microscopy was also analyzed using IMARIS. Microglia number was quantified using IMARIS spot function and process morphology was measured using filament length and branching functions. Astrocyte IMARIS quantification was performed using the volumetric function. Microglia phagocytosis of Aβ was quantified using a combined immunofluorescent staining of IBA1, 82E1, and CD68. High magnification Z-stack images were taken of randomly selected plaques while being blinded to IBA1 microglial staining. Quantification of internalized Aβ was done according to previously described protocols [163, 256]. To account for varying total microglia numbers across images, the internalized Aβ index was normalized to the number of microglia per image. Quantification of neuronal degeneration was performed in equivalent hippocampal brain sections using immunohistochemical labeling of Beta-III
Tubulin (B3T). Four randomly selected square sub-areas were selected in pyramidal, radiatum, or molecular layers of hippocampus CA1 and quantified by optical density using ImageJ.

**Nanostring analysis.** RNA was isolated from microdissected hippocampi of WT, Thy-Tau22, 5xFAD, and T5x mice using RNeasy Plus Universal Mini Kit (Qiagen). RNA samples were run a custom Nanostring panel (Nanostring Technologies) examining mouse AD-linked genes. To evaluate mouse amyloidogenesis, we compared levels of murine RNA for genes APP, BACE1, BACE2, ADAM10, PSEN1, and PSEN2.

**Statistical analysis.** Statistical analysis was performed using StatView software (SAS Institute Inc.). Statistical comparisons between multiple groups were performed using ANOVA followed by Fischer’s PLSD post-hoc tests. Error bars represent the standard error of the mean. Groups were considered statistically significant when \( p<0.05 \) for both ANOVA and posthoc analysis.

**Results**

*Aβ pathology induces robust tau hyperphosphorylation and neurofibrillary tangle formation in T5x mice.*

Brains of WT, Thy-Tau22, 5xFAD, and T5x mice (7 months) were examined by immunocytochemistry to assess accumulation of tau and beta-amyloid. Robust accumulation of tau (green) and Aβ (red) plaques was detected throughout the brain of T5x
mice with extensive extracellular amyloid plaque pathology and intraneuronal tau accumulation observed within the hippocampus, neocortex and amygdala (Figure 4). Next, biochemical approaches were used to quantify changes in tau pathology induced by Aβ accumulation. First, soluble levels of total human tau (HT7 antibody) were quantified in the hippocampus and cortex of 7-month old littermates of each genotype (Figure 5, Figure 6). Human tau (HT7, ~60 kDa) was detectable only in mice that expressed the human tau transgene, Thy-Tau22 and T5x. Interestingly, levels of the human tau species that migrates at 60kDa were quite variable but not significantly different between T5x and Thy-Tau22 littermates in both the hippocampus (p = 0.33) and cortex (p = 0.13). In contrast, HT7 also recognized higher molecular weight tau species (65 kDa) that were marked increased in T5x versus Thy-Tau22 mice in both the hippocampus (p = 0.0007; Figure 5A) and cortex (p = 0.01; Figure 6A). Because the hyperphosphorylation of tau results in an electrophoretic shift in migration, these data provide evidence that Aβ accumulation leads to increased soluble hyperphosphorylated tau in T5x mice.

To further characterize how Aβ alters tau hyperphosphorylation, soluble hippocampal and cortical lysates were probed for multiple pathological tau epitopes i.e. AT100, AT270, PHF-1. For many of the phospho-epitopes, two bands were often detected, likely representing different hyperphosphorylated tau species. Quantification of these phospho-epitopes within both the hippocampus and cortex, demonstrate that many exhibit a two-three fold elevation in T5x mice compared to Thy-Tau22 littermates (Figure 5, Figure 6, p<0.001). As tau becomes hyperphosphorylated, it aggregates and becomes increasing insoluble. Therefore, we examined whether insoluble tau within hippocampus and cortex was increased in T5x mice. While total levels of insoluble human tau were
unchanged between T5x and Thy-Tau22 mice, T5x mice exhibited a dramatic increase in several hyperphosphorylated insoluble tau species, as AT100, AT270, and PHF1 levels were elevated 5-8 fold in T5x mice compared to Thy-Tau22 littermates (Figure 5, Figure 6, p<0.001).

Increased tau hyperphosphorylation within T5x brain was further confirmed immunohistochemically. The brains of T5x mice exhibited increased numbers and density of AT8, AT100, and PHF1 immunoreactive neurons within both the hippocampus and cortex when compared to Thy-Tau22 (Figures 5C-D, Figures 6C-D). In AD, it is hypothesized that tau misfolds prior to hyperphosphorylation and this change in tau conformation can be detected with the conformation-specific antibody MC-1 [257]. Indeed, MC-1 immunoreactivity was more prevalent in the brains of T5x when compared to Thy-Tau22. The quantification of MC-1 immunoreactive neurons reveal a more than four-fold increase in T5x versus Thy-Tau22 (Figures 5E-F, Figures 6E-F and Figure 7). Thus, consistent with previous reports, it appears that the presence of beta-amyloid pathology accelerates the accumulation, misfolding, and hyperphosphorylation of tau in T5x mice.
Figure 4. T5x mice exhibit robust accumulation of both beta-amyloid and tau pathologies within the hippocampus, cortex, and amygdala. Tau (Green; Dako total tau) and beta-amyloid (Red; 6E10) were examined by immunofluorescence and confocal microscopy in half brain coronal sections, revealing appropriate labeling of Aβ plaques and/or tau-laden neurofibrillary tangles in the hippocampus and overlying cortex of each genotype; WT (A), 5xfAD (B), Thy-Tau22 (C), T5x (D). Higher power images of the CA1 region of the hippocampus and amygdala further demonstrate a lack of both pathologies in WT controls (E, I), Aβ pathology in 5xfAD mice (F, J), tau pathology in Thy-Tau22 mice (G, K), a combination of amyloid and tau pathology in T5x (H, L). Scale Bar = 300 mm in A-D, 50 mm in E-G, and 100 mm in I-K.
Figure 5. Hippocampal tau hyperphosphorylation is greatly increased by concurrent beta-amyloid pathology. Western blot and immunofluorescent analysis was used to examine the effects of concurrent Aβ and tau pathology on tau accumulation, phosphorylation, and solubility. Examination of the soluble fraction of microdissected hippocampi (A), revealed an electrophoretic shift in total human tau (HT7) to produce a second band at 65kDa, likely representing hyperphosphorylation of tau (p=0.0007), but no significant change in the unphosphorylated band at 60 kDa (p = 0.71). While phosphorylation of tau at Ser199 and Ser202 was unchanged between Thy-Tau22 and T5x mice, several other pathological phospho-epitopes including AT100 (p = 0.0002), AT270 (p = 0.0002), and PHF1 (p = 0.003) exhibited robust 2-4 fold increases in T5x hippocampal fractions versus Thy-Tau22 lysates (B). Whereas total levels of insoluble human tau (HT7) and Ser199 and Ser202 epitopes were unchanged between T5x and Thy-Tau22 mice, T5x mice exhibited dramatic 7-10 fold increases of insoluble AT100, AT270, and PHF1 phosphorylated tau (p<0.0001). Representative immunohistochemical labeling of AT8 (C) and PHF1 (D) immunoreactive tangles in the hippocampus further illustrate the enhancement of tau hyperphosphorylation induced by Aβ pathology in T5x mice. Labeling for the tau conformational epitope MC1 at both low (E) and high (F) power magnification likewise reveals a large increase in MC-1 immunoreactive CA1 neurons (quantification shown in Supp. Figure 2). Data are represented as mean ± SEM, normalized to % of WT group, n ≥ 8 mice/group. *Indicates p<0.05 for both ANOVA and Fisher’s protected least-significant difference (PLSD) post hoc tests. Scale Bar = 100 mm in C-E and 50 mm in F.
Figure 6. Tau hyperphosphorylation within the cortex is also increased by Aβ accumulation. (A) Although levels of soluble unphosphorylated human tau (HT7 60 kDa, p = 0.19), p-S199, and p-S201 are not significantly different between T5x and Thy-Tau22 littermates, phosphorylation of tau at the 65 kDa HT7 band and AT100, AT270, and PHF1 pathological epitopes is greatly increased (p<0.01). (B) Levels of insoluble human tau (HT7), p-S199, and p-S201 within the cortex are also unchanged, but again insoluble accumulation of AT100, AT270, and PHF1 phosphorylated tau is dramatically elevated in T5x mice (p<0.0001). Immunohistochemical labeling of AT8 (C) and PHF1 (D) in the cortex further demonstrate the considerable increase in tau accumulation that occurs in T5x mice. Likewise cortical labeling of MC1 at low (E) and high (F) magnification (quantified in Supp. Figure 2) again reveal a significant increase in this pathological conformational tau epitope. Data are represented as mean ± SEM, normalized to % of WT group, n ≥ 8 mice/group. * Indicates p<0.05 for both ANOVA and Fisher’s protected least-significant difference (PLSD) post hoc tests. Scale Bar = 100 mm in C-E and 50 mm in F.
Figure 7. T5x mice exhibit increased levels of Tau misfolding. Equivalent hippocampal sections were examined from 7-month old T5x and Thy-Tau22 mice and immunohistochemically labeled with the conformational-specific tau antibody; MC-1 (see Figures 2 and 3). Quantification revealed a significant increase in the numbers of MC-1-positive neurons in hippocampus CA1 (p<0.0001) and an even greater increase in MC-1-immunoreactive cells within the perirhinal/entorhinal cortex. Data are represented as mean ± SEM of optical density (O.D.), n ≥ 8 mice/group. * Indicates p<0.05 for both ANOVA and Fisher’s protected least-significant difference (PLSD) post hoc tests.
*T5x mice exhibit differential levels of cytokines in brain.*

Cytokines have been implicated in the modulation of microglia number and function in the presence of beta-amyloid and tau pathologies. To determine the combined effects of tau and amyloid pathology on neuroinflammation, we quantified the protein levels of IL-10, IL-1β, IL-2, IL-5, IL-6, and TNF-α within the cortex (Figure 8). In T5x mice, a surprising three-fold decrease in IL-1β relative to 5xfAD mice was observed (p<0.0001; Figure 8). Interestingly, IL-5, IL-6, and IL-10 levels were also decreased in T5x mice compared to 5xfAD littermates (p<0.05; Figure 8). In contrast, T5x mice showed an approximately 7-fold increase in both IL-2 and TNF-α compared to wild-type and transgenic littermates (p<0.0001). Because IL-2 and TNF-α levels highly correlate with PHF-1 (Figure 9, IL-2: PHF-1 $R^2 = 0.481$, TNF-α: PHF-1 $R^2 = 0.557$), this suggests that increasing tau pathology likely plays a role in the induction of these cytokines. Whereas IL-2 and TNF-α promotes microglial activation and proliferation, IL-10 has been shown to inhibit microglial Aβ phagocytosis [258-261]. Taken together, the cytokine profile of T5x mice suggest that microglial function is differentially affected by varying amounts of tau and amyloid and led us to investigate whether microglia number or activation state were altered in T5x mice.
Figure 8. The combination of Aβ and tau pathology alters the production of cytokines within the brain. MSD multiplex ELISA was utilized to examine several key cytokines. Compared to WT, Tau and 5xfAD littermates, T5x mice showed a significant increase in both TNF-a and IL-2 (p<0.0001). In contrast, T5x mice exhibited a significant decrease in IL-1β in comparison to 5xfAD littermates (p<0.0001) as well as decreases in IL-5, IL-6, and IL-10 (p<0.05). Data are represented as mean ± SEM, n ≥ 8 mice/group. * Indicates p<0.05 for both ANOVA and Fisher’s protected least-significant difference (PLSD) post hoc tests.
Figure 9. Changes in tau pathology correlate closely with alterations in TNF-α and IL-2. (A) Further demonstrating the shift in cytokines that occurs in T5x mice, IL-6 and TNF-α exhibit a bimodal distribution, with high levels of TNF-α but low levels of IL-6 in T5x mice (green) versus low TNF-α and high IL-6 in WT (purple), Thy-Tau22 (blue), and 5xfAD (red) mice. (B) TNFa and IL-2 expression are very closely correlated \( R^2 = 0.925 \) especially in T5x mice (green), illustrating a strong concordance between these two pro-inflammatory cytokines. Both soluble (C) and insoluble (D) measures of cortical PHF-1 tau correlate well with cortical TNFα levels. Likewise, soluble (E) and insoluble (F) measures of PHF-1 tau also correlate closely with IL-2 expression.
**Tau and Aβ synergistically modulate astrocyte and microglia number and morphology**

To determine whether neuroinflammatory changes in T5x mice were associated with astrogliosis, astrocyte morphology was examined in all genotypes (Figure 10A). Astrocyte total volume, as assessed by GFAP immunoreactivity, was increased 2-3 fold in T5x mice compared to 5xfAD and Thy-Tau22 littermates within CA1 and the cortex (Figure 10B, ANOVA and PLSD p<0.05). Western blot analysis of cortical fractions further corroborated these results revealing an approximately two-fold increase in GFAP protein levels in T5x lysates (Figure 10C, ANOVA and PLSD p<0.05). This increase in astrogliosis is suggestive of a synergistic immune response to the combination of tau and beta-amyloid pathology in T5x mice.

Next, we sought to determine the effect of amyloid and tau on microgliosis as microglia are considered the predominant immune cells of the brain, are tasked with the bulk phagocytosis of CNS material including beta-amyloid, and are known to play a prominent role in AD pathogenesis [194, 262-264]. To investigate how microglia are affected by AD pathology in T5x mice, we quantified microglial number and characterized their morphology within the CA1, the dentate gyrus (DG), and the parietal association cortex (PAC) from all four genotypes (Figure 11A, B, C, respectively). Our analysis revealed a more than two-fold increase in the number of microglia (IBA1+) within T5x mice compared to the other genotypes in CA1 (p<0.0001) and this increase was similarly observed within the DG and PAC (Figure 11A, D). Activated pro-inflammatory microglia are typified by short processes and decreased branching [265]. In T5x mice, microglia process length per microglia was significantly decreased compared to all other genotypes, in all three analyzed regions: CA1, DG, and PAC (Figure 11E, ANOVA and PLSD p<0.05).
T5x mice also displayed significant decreases in microglial branching per microglia compared to all genotypes, in each of the same three brain regions (Figure 11F, ANOVA and PLSD, p<0.05). 5xfAD microglia also exhibited significantly shorter processes and decreased branching compared to WT and Thy-Tau22 in PAC and DG, supporting previous studies demonstrating that amyloid accumulation can drive microglia activation [266, 267]. T5x microglia also exhibited significantly shorter processes and less process branching in CA1 relative to their age-matched 5xfAD counterparts—suggesting that tau plays a synergistic role with amyloid in the modulation of microglia activation state and that the dramatic increase in tau pathology in T5x mice can further exacerbate microgliosis.

Additional evidence of tau-mediated microglial activation was observed by examination of the morphology of microglial populations within CA1 of the hippocampus—an area that exhibits abundant tauopathy [149, 268]. T5x brain sections labeled for Beta-III tubulin (B3T) and IBA1 revealed a distinct microglia population uniquely surrounding the dendrites of pyramidal neurons (Figure 11H-K). These microglia are characterized by long rod-like morphology with shortened processes. Rod-shaped microglia of similar morphology have recently been reported to accumulate in areas of acute neuronal injury and axonal degeneration, further supporting the notion that accumulating tau pathology further alters microglial activation in T5x mice [269, 270].
Figure 10. T5x mice exhibit increased astrogliosis. (A, B) Immunohistochemical analysis and IMARIS quantification of GFAP-labeled astrocytes reveal elevated numbers within CA1 of the hippocampus (WT p = 0.13; 5x p = 0.03; Tau p = 0.04) and the parietal association cortex (WT, Tau p<0.0001; 5x p = 0.0002), but no differences within the dentate gyrus. (C) Western blot analysis of GFAP in cortical fractions corroborate these findings by demonstrating a significant increase in GFAP between T5x and both WT and Thy-Tau22 groups (WT p = 0.006; 5x p = 0.10; Tau p = 0.002). Data are represented as mean ± SEM of optical density (O.D.), n ≥ 8 mice/group. *Indicates p<0.05 for both ANOVA and Fisher’s protected least-significant difference (PLSD) post hoc tests. Scale Bar = 100 mm.
Figure 11. Microglial number and morphology is dramatically altered in T5x mice. Microglia were immunohistochemically labeled with IBA1 and then quantified by IMARIS bitplane analysis of confocal Z-stacks to determine the effects of Aβ, and tau pathology on microglial number and morphology. Analysis was performed within the CA1 (A) and dentate gyrus (B) of the hippocampus and the parietal association cortex (C), revealing a significant 2-3 fold increase in microglial number in T5x versus WT mice in each of these regions (p<0.0001). T5x mice exhibited a similar 2-fold increase in microglial number relative to Thy-Tau22 mice in all 3 regions (D). Within CA1, T5x microglial number was also substantial higher than 5xfAD littermates (p<0.0001). However, T5x mice exhibited a more subtle increase in comparison to 5xfAD mice within the dentate gyrus (p=0.02) and cortex (p=0.07). (E) Automated quantification of microglial process length (E) and branching (F) also revealed several significant differences with T5x microglia exhibit significantly shorter process length and decreased microglial branching compared to all other genotypes in CA1 (p<0.01) and to Thy-Tau22 and WT groups within the dentate gyrus and cortex, (p<0.05). (H-K) Examination of microglia in hippocampus CA1 revealed a unique population of rod-like microglia in T5x mice. β3-tubulin immunoreactivity (green, G) were associated with a microglial response, IBA-1 labeled microglia (red, H) were imaged within the stratum radiatum. Interestingly, this examination revealed a very specific pattern of microglial morphology within T5x mice that was not present in any of the other three genotypes. The appearance of elongated, linearly organized microglia is reminiscent of highly activated ‘rod-like’ microglia that are found in association with neurodegenerative changes [270, 271]. Thus, the microglia appear to mount a very specific response to hippocampal dendritic degeneration within T5x mice. Data are represented as mean ± SEM, n ≥ 8 mice/group. * Indicates p<0.05 for both ANOVA and Fisher’s protected least-significant difference (PLSD) post hoc tests. Scale Bar = 100 mm in A-C, 30 mm in G-I, and 10 mm in J.

**Amyloid plaque burden is significantly decreased in T5x mice versus 5xfAD littermates.**

Although the effects of amyloid on tau hyperphosphorylation have been extensively studied, far fewer studies have examined whether tau pathology could potentially influence amyloid burden. While we hypothesized that no differences in Aβ plaque pathology would be detected between T5x and 5xfAD littermates, changes in microgliosis suggested that microglial interactions with Aβ may also be altered. Surprisingly, analysis of Aβ pathology (with Aβ-specific antibody, 82E1) using IMARIS 3-D quantification revealed a ~50% reduction in Aβ plaque volume within multiple brain regions of T5x versus 5xfAD mice (Figure 12A-B, p<0.05). Amyloid plaque deposition was significantly lower in the CA1 of
the hippocampus, the retrosplenial cortex (RC), and parietal association cortex (PAC) of T5x versus 5xFAD littermates (ANOVA and PSLD p<0.05). Within the dentate gyrus of the hippocampus, T5x mice exhibited a non-significant trend towards decreased plaque volume (p = 0.07, **Figure 12B**).

To examine whether the decrease in amyloid burden results from down-regulation of the APP transgene, APP soluble protein levels were quantified by Western blot analysis in both the cortex (**Figure 12C, D**) and hippocampus (data not shown). Our results reveal no changes in APP holoprotein expression between T5x and 5xFAD littermates (p = 0.81). To determine whether alterations in Aβ might be influenced by changes in endogenous amyloidogenic enzymes we examined the mRNA expression of BACE1, BACE2, ADAM10, PSEN1, and PSEN2 and found no significant differences across genotypes (**Figure 13**). These data support the notion that, changes in Aβ load may be mediated by alterations in Aβ clearance mechanisms rather than altered Aβ production.

To further validate the observed effects on Aβ plaque load we used a Mesoscale Discovery (MSD) V-Plex Aβ Peptide ELISA to quantify both soluble and insoluble levels of Aβ38, Aβ40, and Aβ42. MSD analysis of soluble cortical fractions revealed no significant differences between T5x and 5xFAD littermates for Aβ38 Aβ40, or Aβ42, consistent with the notion that production of Aβ is unchanged (**Figure 12E**). In contrast, levels of insoluble Aβ that contribute to fibrillar Aβ plaques exhibited a significant ~2-fold decrease in T5x Aβ38 (p = 0.03), Aβ40 (p = 0.01) and Aβ42 (p = 0.02) versus 5xFAD littermates (**Figure 12F**). Thus, in T5x mice, there is a surprising two-fold decrease in both insoluble Aβ species and total plaque volume when compared to the aggressive 5xFAD model of amyloidosis.
Figure 12. T5x mice exhibit decreased amyloid burden compared to 5xfAD littermates. (A) Amyloid deposition was quantified using immunofluorescent labeling of Aβ (82E1) followed by confocal Z-stack imaging and IMARIS bitplane software analysis within CA1 of the hippocampus (CA1) the dentate gyrus (DG), retrosplenial cortex (RSC), and parietal association cortex (PAC). (B) Amyloid plaque volume was significantly decreased in T5x mice relative to 5xfAD littermates in CA1 (p = 0.03), RSC (p = 0.04), and the PAC (0.03), whereas a nonsignificant reduction was observed in the dentate gyrus (p = 0.07). (C) To determine whether the observed reduction in Aβ pathology resulted from a change in APP transgene expression, human full-length APP (6E10) was examined and quantified by western blot, revealing no differences between T5x and 5xfAD groups (D). Next, levels of soluble and insoluble Aβ38, 40 and 42 were examined using a Mesoscale Devices (MSD) multiplex ELISA. Interestingly, while soluble levels of Aβ where unchanged between T5x and 5xfAD groups (E), levels of insoluble Aβ38, 40 and 42 were significantly decreased in T5x mice relative to 5xfAD littermates (F). Data are represented as mean ± SEM, n ≥ 9 mice/group. * indicates p<0.05 for both ANOVA and Fisher’s protected least-significant difference (PLSD) post hoc tests. Scale Bar = 50 mm.
Figure 13. Nanostring analysis reveals no differences in mRNA expression of APP-processing enzymes. Hippocampal mRNA was isolated from each genotype and examined using a custom Nanostring panel to quantify APP-processing associated genes including murine APP, BACE1, BACE2, ADAM10, PSEN1, and PSEN2. In each case, no differences between genotype were observed.

**T5x microglia exhibit increased Aβ phagocytosis**

While greater microglial numbers alone promote Aβ clearance, the observed changes in T5x microglial morphology and decreased brain IL-10 levels might also indicate increased microglial phagocytic capacity that further contributes to a reduction in Aβ.
Altered brain cytokine profiles indicate changes in microglia activation state, which influence amyloid phagocytosis [272, 273]. For example, decreased IL-10 levels is associated with increased microglial Aβ phagocytosis [163, 256]. To test the hypothesis that reduced Aβ in T5x mice is the result of increased phagocytic capacity of T5x microglia, microglial Aβ internalization was assessed using volumetric IMARIS analysis in matching brain sections of T5x and 5xfAD mice. Microglia were identified by IBA1 immunoreactivity and Aβ phagocytosis was determined by co-localization of Aβ (82E1) and CD68 which is only present in microglial phagolysosomes (Figure 14A). While the total volume of phagolysosome labeling per microglia was not significantly different between 5xfAD and T5x littermates (Figure 14B, p = 0.18), quantification of the proportion of Aβ internalized within CD68+ phagolysosomes was significantly different between T5x and 5xfAD groups. In fact, this analysis revealed a nearly 30% increase in the ability of T5x microglia to internalize Aβ versus 5xfAD littermates (Figure 14C, p = 0.01). Taken together, these data suggest that increases in tau pathology and neuroinflammation can affect microglial Aβ phagocytosis, leading to a profound increase in amyloid clearance and reduction of plaque load in T5x mice.
Figure 14. T5x microglia phagocytose more Aβ than their 5xfAD counterparts. (A) Equivalent 7 month old T5x and 5xfAD sections were labeled for microglia (IBA1, green), Aβ (82E1, red), and microglial phagolysosomes (CD68, blue). IMARIS bitplane software rendering of confocal Z-stacks was then used to calculate the volume of phagolysosomes within microglia and then the volume of internalized Aβ within those CD68+ microglial phagolysosomes, depicted in greyscale in A. Together, this analysis demonstrate that although T5x and 5xfAD microglia exhibit no differences in phagolysosome volume per microglia (B), the amount of Aβ within the phagolysosomes of T5x microglia was significantly increased (C) versus 5xfAD microglia. Data are represented as mean ± SEM, normalized to % of 5xfAD group, n ≥ 9 mice/group. * Indicates p<0.05 for both ANOVA and Fisher’s protected least-significant difference (PLSD) post hoc tests. Scale Bar = 20 mm.

Discussion

Although the notion that amyloid increases tau pathology is well established, much less work has been made on whether there exists a reciprocal relationship between tau and the development of amyloid pathology. Beginning in 2001, two seminal studies demonstrated that Aβ could exacerbate the development of tau pathology in transgenic AD tau models [249, 250]. Subsequently, a similar causal relationship between Aβ and tau pathology was established in AD transgenic mouse models [251, 252, 274, 275]. Even in the absence of the expression of a human tau transgene, many AD transgenic mice exhibit some degree of Aβ-induced tau hyperphosphorylation, a prerequisite for the development of neurofibrillary tangles. Likewise, in humans, triplication of APP, either in some family
pedigrees or in trisomy 21, leads to elevated levels of Aβ and the development of tau pathology [276, 277].

As expected, the accumulation of Aβ in T5x mice leads to a dramatic increase in tau hyperphosphorylation and increased accumulation of neurofibrillary tangle pathology within the hippocampus and neocortex. Yet surprisingly, T5x mice also exhibited a 50% reduction in amyloid plaque burden and insoluble Aβ species versus 5xfAD littermates. Although the expression of both the APP and tau transgenes are driven by the Thy1 promoter, the expression of APP in T5x mice was unchanged and therefore, decreased Aβ levels were not the result of altered transgene expression. Furthermore, levels of soluble Aβ were unaffected; suggesting that the observed decreases in plaques and insoluble Aβ was not due to effects on APP processing.

However, the combination of amyloid and tau pathology did produce a dramatic effect on neuroinflammatory processes, included increased microgliosis and astrogliosis in T5x versus 5xfAD or Thy-Tau22 littermates. Therefore, we examined whether changes in specific cytokines could explain the increase in neuroinflammation. Our data show that the combined presence of amyloid and tau pathology exacerbates the levels of both TNF-α and IL-2 (Figure 5). In contrast, the observed decreases in IL-1β, IL-5, and IL-6 in T5x mice versus 5xfAD littermates may reflect the predominant influence of amyloid on the release and maturation of certain pro-inflammatory cytokines. Although IL-1β has been shown to increase tau phosphorylation, amyloid has also been reported to promote astrocytic and microglial release of IL-1β [256, 278-280]. Thus, while one may expect elevated IL-1β in T5x mice in association with increased tau hyperphosphorylation [177], the influence of reduced amyloid burden on IL1β induction may take precedence over effects caused by
changes in tau. Therefore, it may be the case that IL-1β, IL-5 and IL-6 are less elevated in T5x than 5xfAD mice as a consequence, rather than a cause of reduced amyloid burden.

TNF-α, a pro-inflammatory cytokine that is released by activated microglia was one of the most upregulated cytokines observed in T5x mice [267, 281]. Although TNF-α does not directly increase microglial proliferation, TNF-α promotes astrocyte proliferation and GM-CSF release, which in turn, can stimulate microglial proliferation [282-284]. Taken together, elevated astrocyte numbers and a ~5-fold increase in TNF-α in T5x mice relative to 5xfAD and Thy-Tau22 littermates suggests that the combined stress of amyloid and tau pathology work synergistically to promote both astrocytic and microglial proliferation.

Furthermore, the significant decrease in IL-10 expression in T5x mice suggested that microglial phagocytosis of Aβ may also be altered as recent studies have shown that deletion of IL-10 can enhance microglial Aβ phagocytosis [261]. Our finding that T5x mice exhibit a similar decrease in IL-10 relative to Thy-Tau22 and 5xFAD littermates suggest that decreased IL-10 is a result of the combined stress of tau and amyloid pathology. Subsequent analysis confirmed that T5x microglia indeed have increased Aβ phagocytosis, providing further support that tau pathology contributes to microglial regulation of amyloid.

While cytokines are often determined by the extent of AD pathology, they can also control the progression of the disease through the modulation of microglial response. The influence of microglia in AD has received growing attention with the recent discovery of genetic risk polymorphisms in several microglial-enriched genes [15]. Some of these risk factor genes such as CD33 have themselves been implicated in microglial Aβ phagocytosis [239, 285]. Thus, the role of microglia in limiting Aβ pathology continues to garner new
attention. Microglia have long been implicated in the modulation of tau hyperphosphorylation and misfolding through cytokine-mediated neuroinflammatory activation of tau kinases [177, 286]. More recently, studies have shown that the misfolding of tau can promote microglial activation [287], and tau oligomers and fibrils have been found to interact directly with microglia [176]. In addition, microglia have also recently been implicated in the propagation of tau within the brain [288]. The fact that microglia exhibit the capacity to directly phagocytose the same tau oligomers that can promote their activation, highlights the possibility that tau pathology is not only exacerbated by microglial activation, but likely in turn modulates microglial responses within the brain [289]. We reasoned that changes in cytokine levels observed in T5x mice would affect microgliosis and our current findings suggest that tau and amyloid pathology in T5x mice synergistically recruit a robust microglial response. Significantly increased activated amoeboid microglia in T5x mice versus Thy-Tau22 littermates suggests that amyloid pathology is the strongest determinant of microglial activation. Amyloid holds an advantage over predominately intracellular tau aggregates in its potential influence over microglial activation as evident of amyloid soluble oligomers released from abundant extracellular plaques [267, 290, 291]. However, our findings of increased microglial activation, phagocytosis, and numbers in T5x mice, suggest that tau pathology can also contribute to or further modulate microglia activation state.

The remaining question is exactly how development of tau pathology influences the neuroinflammatory response. One possibility is that amyloid driven tau hyperphosphorylation and subsequent release of intracellular factors and tau oligomers from degenerating neurons drives microglial activation. Previous studies have reported the
activation of microglia by the release of apoptotic factors and intracellular molecules such as ADP or ATP [292-294]. When we examined T5x mice for dendritic degeneration, we observed a reduction in hippocampal Beta-III tubulin staining in T5x mice compared to transgenic and WT littermates (Figure 15). Notably, hippocampal regions displaying the significant reduction in Beta-III tubulin were accompanied by the previously described rod microglia (Figure 5 G-J) [295]. Although not extensively characterized, rod microglia have been found in human AD cases and exhibit abnormally frequent interactions with synaptic clefts [269, 296, 297]. T5x rod-like microglia are similar in morphology to recently described rod-microglia which appear highly activated and respond to diffuse neuronal injury [270]. The association between reduced β3-Tubullin density and rod microglia presence therefore suggests that degenerating dendrites and subsequent release of intracellular factors such as ADP may contribute to the altered microglia activation state in T5x mice. However, further studies are clearly needed to elucidate the exact mechanisms by which tau synergizes with Aβ to modulate microglial activation.
Figure 15. The combination of Aβ and Tau pathology leads to reductions in hippocampal β3-tubulin. (A-D) To determine whether T5x mice begin to exhibit early signs of neurodegeneration, dendritic architecture was examined by β3-tubulin immunolabeling of all four genotypes. (E-G) Quantification of β3-tubulin revealed a significant reduction in T5x mice compared to WT and transgenic littermates within the pyramidal cell layer (E; p<0.05), stratum radiatum (F; p<0.05), and molecular layer (G; WT, Tau p<0.05; 5x p = 0.27) of the hippocampus. Data are represented as mean ± SEM of optical density (O.D.), n ≥ 8 mice/group. *Indicates p<0.05 for both ANOVA and Fisher's protected least-significant difference (PLSD) post hoc tests. Scale Bar = 100 mm in A-D, 30 mm in H-J, and 10 mm in K.

It is important to acknowledge that our findings both corroborate and differ from other recently examined amyloid-tau crosses. We report a significant reduction in amyloid plaque burden and insoluble Aβ in T5x mice relative to 5xfAD littermates that appears to be mediated via increased microglial-mediated Aβ clearance. Using a similar model, one group has shown that 5xfAD crossed with Tg30 tau mice likewise exhibit decreased Aβ pathology, although the potential role of microglia in this finding was not examined [253]. In contrast, two other studies crossed 5xfAD mice with other tau models and reported no change in Aβ plaques by optical densitometry, although only 3 or 4 mice per group were
compared versus group sizes of 9-10 mice for the current Aβ ELISA analysis [298, 299]. Yet, one of these studies nevertheless showed a non-significant 45% reduction in Aβ plaque load at 3 months of age [298]. It is therefore quite possible that the use of 3D plaque volume quantification, ELISA, or a larger sample size would have revealed a similar significant reduction in Aβ to that observed in the current study. In another study, Tg2576 APP mice were crossed with mutant tau VLW mice. Surprisingly, this study reported a significant increase in amyloid deposition in bigenic mice [300]. These varying reports suggest that perhaps the magnitude of tau pathology and/or microgliosis likely influences the effects on Aβ pathology. In support of this notion, we find that T5x mice exhibit a dramatic increase in microglial number, shift in morphology, and change in cytokines that is not apparent in Thy-Tau22 littermates with lower levels of tau pathology (Figures 4 and 5).

Recently, a new pathological diagnosis termed Primary Age-Related Tauopathy (PART) has been described that is apparent in nearly all aged individuals when brains are examined at autopsy [301, 302]. As the name implies, this condition is associated with predominate AD-like tau pathology but minimal Aβ plaques. These subjects also typically exhibit relatively mild or even no cognitive dysfunction. Given our current findings, it is interesting to speculate that perhaps in this subset of patients; robust tau pathology may help to limit Aβ accumulation by a similar neuroinflammatory mechanism. Although clearly a great deal of additional study will be needed to determine if that is indeed the case.

In conclusion, we have established an AD model that provides further insight into the potential reciprocal relationships between amyloid, tau and neuroinflammation. The
combination of these pathologies significantly increases tau hyperphosphorylation and microgliosis, yet decreases amyloid burden. Our data also suggest that tau-induced increases in microglial number, and phagocytic activity may explain the reductions in amyloid burden. The changes observed in amyloid accumulation and microgliosis in the presence of tau pathology suggests that therapies targeting tauopathy could have increased benefit towards treating additional underlying proponents of AD. Therefore, our study adds to the growing understanding of the role of microglia in AD pathogenesis and suggests that differential activation of these cells in response to Aβ and tau pathology can have both detrimental as well as beneficial effects on disease progression.

Competing interest
The authors declare that they have no competing interest.

Author Contributions
W.C., W.W.P., E.M.A., and M.B-J. designed research;
W.C., A.L., and M.B-J. performed research;
W.C., A.L., and M.B-J. analyzed data;
L.B. and D.B provided the Thy-Tau22 mice;

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Chapter One Addendum A: T-Cell Infiltration in T5x mice

Introduction

T-cells are lymphocytes and are considered a major component of the adaptive immune system which responds with a high degree of specificity to infectious agents or foreign cells. T-cells originate from hematopoietic stem cells, migrate to and mature within the thymus, and are characterized by the expression of mature cell surface receptors such as CD3 [303]. A number of different T-cell subtypes exist that serve varying physiological functions and can be distinguished by expression of specific cell surface markers. In general, these different T-cells subsets respond to varying stimuli and antigens to produce differential cytokine responses [304]. Cytotoxic T-Cells for example express CD8+ and are responsible for killing virus infected and abnormal cancerous cells as well as rejecting foreign tissue. CD4+ Helper T-cells activate cytotoxic T-Cells in addition to contributing to the differentiation and maturation of B-cells. Another class of CD4+ T-cells is called T-regulatory cells (T-regs) and achieves the termination of an immune response by suppressing activated cytotoxic T-cells. Disruption of the delicate balance between T-regs and effector T-cells can result in autoimmune disease [305]. Memory T-cells maybe either CD4 or CD8 positive and persist longer than the average T-cell. Their function is to build a ‘memory’ of invading foreign antigens and they are responsible for building up the body's resistance to a former bacterial or viral infection.

Although T-cells and their role in immune response have been implicated in a number of diseases, the purpose of T-cells in the brain has been a debated topic of AD research. While the healthy human brain parenchyma has been considered an immune privileged site and generally lacks T-cells [306], a number of studies have reported finding
CD4+ and CD8+ T-cells within AD brain tissue [307-309]. The contributions of these brain-localized T-cells towards the progression of the disease however, are not yet well understood. While peripheral T-cells proliferate during an immune response to foreign antigens, previous studies have reported that T-cells in the human brain lack proliferative markers—suggesting that invading T-cells are most likely not mediating an antigen response against AD pathology [308]. A recent study of invading T-cells in amyloid transgenic mice further supports the human findings that resident T-cells in the brain exhibit minimal proliferative activity and do no become antigen-producing effector cells [310]. Instead, invading T-cells may influence microglial clearance of Aβ or participate in the process of AD related neurodegeneration. Prior studies have suggested that IFN-γ producing T-cells could act beneficially by promoting macrophage and microglial phagocytosis and subsequent Aβ clearance [310, 311]. On the other hand, invading helper T-cells could also promote neuroinflammation and macrophage-driven neurodegeneration [312]. Furthermore, cytotoxic T-cell levels in the CSF of AD patients have direct correlations with the extent of cognitive impairment and parahippocampal tissue damage [313].

To investigate whether T-cells may play a role in mediating AD pathology, I examined peripheral and cerebral T-cell within T5x, 5xfAD, Tau22, and wild-type littermates. These experiments revealed that T5x mice have elevated levels of CD3+/CD4+ and CD3+/CD8+ T-cells in the spleen and also exhibit increased T-Cell infiltration into several brain regions.
Methods

**Flow Cytometry.** Spleenocytes were isolated from spleens following a standard protocol. Briefly, spleens were mashed and treated with RBC (Red Blood Cell) Lysing Buffer (Sigma-Aldrich) to remove erythrocytes followed by filtration through 70μm nylon mesh. Cells were counted and 1.5-2x10⁶ cells were resuspended in sterile 5ml polystyrene tube labeling with cell surface marker antibodies. Cells were incubated with the following fluorescent-conjugated antibodies (0.5-1µg) against cell surface markers: FITC-CD3, Vioblue-CD4 and APC-CD8 (all antibodies from affimetrix/eBioscience, San Diego). Following antibody incubation cells were washed and resuspended for flow cytometry. Cells were run on a Macs Quant cytometer (Miltenyi Biotec, Germany) and analyzed with MACSQuant Analysis Software (Miltenyi Biotec, Germany). The numbers of mice used to collect splenocyte samples are as follows: WT, n = 6; 5xFAD, n = 5; Tau22, n = 4; T5x, n = 5.

**DAB staining.** T5x, Tau22, 5xFAD, and WT tissue sections were taken for light-level immunohistochemistry staining of T-cells. Anti-CD3ε antibody (Clone 500A2) were applied to sections overnight and subsequently incubated with biotin-conjugated secondary antibody. Staining was then visualized using Vectastain Elite ABC kit (Vector Labs; Burlingame, CA) followed by 3,3’-diaminobenzidine (DAB) with Nickel Peroxidase (HRP) Substrate Kit (Vector Labs). Sections were mounted and coverslipped using DPX (DBH) mounting medium (VWR, Radnor, PA). Sections from one animal of each genotype were used for staining.
**Statistical analysis.** Statistical analysis was performed using StatView software (SAS Institute Inc.). Statistical comparisons between multiple groups were performed using ANOVA followed by Fischer’s PLSD post-hoc tests. Error bars represent the standard error of the mean. Groups were considered statistically significant when *p<0.05 for both ANOVA and posthoc analysis.

**Results**

**T5x mice exhibit trends toward increased numbers of peripheral T-cells**

To examine whether adaptive immune response could be elevated by AD pathology, I examined T5x mice for levels of peripheral T-cells. I collected splenocytes from WT, 5xFAD, Tau22 and T5x mice and sorted for the T-cell surface marker CD3. CD3+ T-cells were then further sorted by CD4+ and CD8+ labeling to differentiate helper and cytotoxic T-cell populations respectively *(Figure 16).* Compared to WT littermates, T5x mice exhibited trends toward an almost two-fold increase in total CD3+ T-cells (p = 0.12), a ~1.5 increase in CD4+ helper T-cells (p = 0.26), and doubling of CD8+ cytotoxic T-cells (p = 0.08), although none of these comparisons reached significance. T5x mice also displayed nearly identical trends toward increased T-cell numbers relative to 5xFAD littermates, with a ~2 fold elevation in CD3+ (p = 0.13) and CD3+/CD8+ (p = 0.10) T-cells and a ~1.5 fold increase in CD3+/CD4+ (p = 0.26) T-cells. The suggestion that T-cell populations may be slightly elevated in T5x and not in 5xFAD or Tau22 mice relative to WT littermates suggests that the combined tau and amyloid pathology is necessary to evoke a peripheral T-cell
response although these changes failed to reach significance with this relatively small samples size (n=4-6).

**Figure 16. T-cell numbers are non-significantly elevated in T5x mice.** Splenocytes collected from all genotypes were gated for CD3+ T-cell staining and then sorted for CD4+ helper and CD8+ cytotoxic T-cells. Measurements of T-cell populations were quantified as the percent of total cells collected. Data are represented as mean ± SEM of optical density (O.D.), n 4-6 mice/group. Statistical analysis was calculated by ANOVA and Fisher's protected least-significant difference (PLSD) post hoc tests.

**T-cells infiltrate the brains of T5x mice**

After establishing that T5x mice exhibit trends toward increased peripheral T-cell numbers, I investigated whether T-cells also could be detected within the brain parenchyma. DAB staining for CD3 revealed a number of infiltrating T-cells in Tau22, 5xFAD, and T5x mice, but not in WT mice (Figure 17). From preliminary staining, it appears that T-cell infiltration may be greater in 5xFAD and T5x mice compared to Tau22 littermates. Furthermore, the pattern of invading T-cells may also be region specific. In Tau22 mice, I observed T-cells only within the amygdala whereas 5xFAD and T5x mice exhibited T-cells within the amygdala, parahippocampus, and cortex. While these
preliminary results are intriguing, further studies with a larger cohort of animals will be needed to conclusively elucidate how T-cell numbers might change in T5x mice and what disease-associated patterns of T-cell infiltration occur in the brains of these mice.

Figure 17. T-cells infiltrate the brains of transgenic mice. DAB staining of T-cell surface marker CD3 reveal the presence of T-cells in 5xFAD, Tau22, and T5x mice, but not in WT mice. A few T-cells infiltrate the amygdala in 5xFAD mice, and a greater number of T-cells infiltrate the amygdala of T5x and Tau22 mice.

Discussion

While the contributions of T-cells to progression of AD are still under debate, changes in T-cell populations are seen in the blood and brain of AD patients. Similar to human AD cases, T5x mice exhibit elevated T-cell counts in the blood and the brain relative to healthy WT mice. Specifically, T5x mice recapitulate a number of previous reports observing increased CD3+ and CD8+ peripheral T-cells in human AD patients [314-316]. The overall, albeit non-significant increase in T-cells within the spleen of T5x mice relative to WT and transgenic littermates, and the lack of significant changes in T-cell populations between WT, 5xFAD and Tau22 mice suggest that tau and amyloid pathology may synergize to elicit a peripheral immune response. Exactly how T5x brain pathology can induce changes in T-cell activation and number in the periphery is yet to be determined, although several hypotheses can be proposed. One possibility is that infiltrating T-cells
enter the brain, are activated, then cross back into the bloodstream and affect resident T-cells. A previous study has reported that Aβ peptides induces a peripheral immune response in patients with AD, suggesting that AD pathology has the capacity to affect T-cell activation if leaked out of the brain [317]. In T5x mice, the combined accumulation of tau and amyloid pathology could contribute to increased leakiness of the blood brain barrier as observed in human AD patients [318, 319]. Previous studies have also shown that Aβ is actively transported across the blood brain barrier [320, 321]. Increased T-cell response to Aβ in the blood could therefore potentially result from increased Aβ transport in T5x mice. Further studies are required to fully understand what mechanisms induce these potential changes in peripheral T-cells in T5x mice.

Following the same pattern of peripheral T-cell counts, the number of infiltrating T-cells into T5x mice brains are analogously elevated. My observation of infiltrating T-cells in the brain are supported by a recent study demonstrating the invasion of CD3+ T-cells into the brains of several amyloidgenic transgenic models [310]. Similarly, both in T5x mice and in the reported study, T-cell infiltration was not directly associated with the geographically specific location of amyloid plaques. The lack of obvious attraction of invading T-cells to amyloid plaques suggest that T-cells are likely drawn into brain by general blood brain barrier leakiness and/or neuroinflammatory signaling rather than by local microhemorrhages caused by amyloid plaques [310]. While further quantification of T-cell counts in the brains of T5x, Tau22 and 5xFAD mice are needed, qualitative assessment of a small cohort of animals suggests that amyloid pathology plays a larger role in T-cell recruitment relative to tau pathology. This assessment is supported by substantially
greater number of studies that show amyloid, and not tau pathology can modulate transport across of the blood brain barrier [322-324].

The observation of T-cells in the brains of T5x mice poses the question whether infiltrating T-cells could contribute to the neuroinflammatory changes exhibited by T5x mice. Past studies have suggested that IL-2 could play a significant role in AD pathology as elevated levels of IL-2 have been detected in patients with moderate-to-severe AD [325]. Additionally, IL-2 knockout mice have decreased number of activated microglia in response to injury [326], whereas we observe a dramatic increase in both microglial number and IL-2 expression. IL-2 is a pro-inflammatory cytokine that can be produced not only by astrocytes and microglia, but also by neurons and T-cells [327-330]. Recent studies have implicated regulatory T-cells in the development of AD pathology and cognitive deficits in transgenic mice [331, 332]. A number of different AD models have also reported T-cell infiltration in areas of amyloid pathology [332, 333]. Similarly, I find that T5x and 5xFAD mice both exhibit significantly more T-cell infiltration relative to their Tau22 and wild-type littermates (Figure 17). The differences in cytokine levels between T5x and 5xFAD mice reported in Chapter 1 (Figure 8) suggest that in addition to an increase in T-cell number, there may also be a shift from a Th2 response to Th1 T-cells in T5x mice. Typically, Th1 type T-cells are associated with pathogens that require internalization whereas Th2 cells are protective toward large extracellular parasites [334]. Some of the key distinguishing characteristics between Th1 and Th2 T-cells include their cytokine expression profile. Th2 cells for example exhibit increased release of IL-2 and TNF-α and decreased production of IL-1β, IL-5, IL-6, and IL-10 in comparison to Th1 cells [335, 336]. Interestingly, this differential expression of Th2 and Th1 cytokines match extremely well with the shift in
cytokines exhibited by T5x mice compared to 5xFAD and Tau22 littermates (Figure 8 Table 1). Previous studies have reported that human T-cells can exhibit both Th1 and Th2 responses to Aβ stimulation [337]. Immunization of Aβ1-42 in wild-type mice has also been shown to reduce Th1 and enhance Th2 T-cell responses [338]. Taking into account the results of previous studies and the current findings in T5x mice, an interesting question arises as to whether concomitant tau pathology is sufficient to shift the T-cell phenotype in the brain from Th1 to a more Th2 response. This is an exciting new question for which additional studies within the Blurton-Jones lab are planned to determine whether amyloid and tau have a synergistic effect on the T-cell response in the AD brain.

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**Chapter Two: Behavior in T5x mice**

**Introduction**

One of the rationales for developing T5x mice by crossing two aggressive transgenic AD lines, 5xFAD and Tau22 mice, was to establish a mouse model that could better recapitulate advanced stages of AD. To investigate whether T5x mice mimicked the aggressive aspects of AD, it was important to not only examine AD pathology, but behavioral changes as well. In that respect, 5xFAD and Tau22 mice were attractive lines to cross since both models have reported cognitive impairments.

5xFAD mice show frontal cortex dependent cognitive deficits by 4 months of age, and anxiety and hippocampal-dependent cognitive deficits beginning at 6 months of age [144, 145]. 5xFAD mice also exhibit significant age dependent decreases in anxiety starting at 6 months of age [146]. Additional amygdala and hippocampal dependent contextual fear condition deficits manifest in 6 month old 5xFAD mice [144]. Furthermore, 6 month old 5xFAD mice have also been reported to exhibit disruption in sleep patterns and behavior [339]. Finally, 5xFAD mice also demonstrate abnormal social behaviors analogous to impairments in social recognition observed in human AD patients [340].

Similar to 5xFAD mice, Tau22 mice also exhibit behavioral deficits. Starting at 6 months of age, Tau22 mice begin to show the first signs of cognitive disruption, exhibiting anxiety deficits, whereas hippocampal dependent cognitive deficits first manifest several months later at about 10 months of age [149]. By this age, medial septal cholinergic neuronal number and NGF uptake from the hippocampus is significantly reduced [151] and by 12 months of age, Tau22 mice have begun to recapitulate other behavioral and psychological symptoms seen in human AD patients including increased depression,
aggressive behavior, and disturbances in nocturnal activity [150]. At 12 months of age, Tau22 mice demonstrate depression, aggressive behavior and nocturnal disturbances [150]. An additional study has also reported disruption in appetitive response [341]. The recapitulation of substantial tau pathology, cognitive deficits without motor impairments, and neuroinflammatory response highlight the strengths of Tau22 mice as a model of AD-associated tau accumulation.

I investigated whether T5x mice recapitulate AD behavioral changes and tested T5x mice and transgenic and WT mice in a battery of behavioral tasks. T5x mice develop anxiety deficits by 3 months of age and cognitive deficits by 6 months. Importantly, T5x mice also do not exhibit motor impairments that could confound behavior results.

Methods

Behavior. Two cohorts of mice, one at 3 months old and one at 6-7 month old, were used for behavioral analyses. The number of mice tested for each genotype at 3 months old was: WT (n = 7), 5x (n = 9), Tau (n = 7), and T5x (n = 6). The number of mice tested for each genotype at 6-7 months old was: WT (n = 8), 5x (n = 11), Tau (n = 14), and T5x (n = 9). A battery of motor and cognitive tasks were performed and analyzed by a blinded observer as follows:

Pole Test. Mice were tested in the Pole Test of motor coordination following previously described protocols [342]. Briefly, a soft wooden pole of 1 cm in diameter and 1 foot in length was based in a Styrofoam block and placed on the floor of a mouse cage. The Styrofoam base was covered with bedding until roughly 1 inch above the top of the block.
Mice were given two consecutive days of training in which the mice were initially placed on the top end of the pole facing the ceiling with all four limbs clasping the pole. For a successful trial, mice were required to re-orientate themselves until they were clasping the pole and facing the floor of the cage before climbing down into the bedding. Each mouse was given four training trials per day and allowed to fully attempt to re-orientate itself on the pole without time restrictions. On the third day, mice were given four test trials and timed for their ability to re-orientate themselves on the pole. Each of these trials was capped at a 30 second time limit and mice that were unable to complete the task were given a re-orientation value of 30 seconds.

**Rotarod.** A standard Rotarod apparatus (Ugo Basile, Italy) was used to test five mice concurrently in each trial. On Day 1 (Training Day), mice were placed atop the rotarod and run at a fixed speed of 24 rpm for 2 minutes for one trial. Mice that fell off the rotarod or were inverted while running were manually assisted back onto the rotarod and placed into the proper orientation. On Day 2 (Fixed Test) mice were given four trials at a fixed speed of 24 rpm for a maximum of 2 minutes per trial. The time mice spent on top of the rotarod was recorded. Mice that clasped onto the rotating beam without running for more than 1 rotation throughout an entire trials was considered a “falling” at the time of the second rotation. To prevent mice from suffering from fatigue, two cohorts of mice were run in alternating trials at a time. The rotarod was cleaned in between trials with 70% ethanol and allowed to dry before the beginning of the next trial. On Day 3 (Accelerating Test), the rotarod was programmed to accelerate at a constant rate from 4 rpm to 40 rpm in a span of 5 minutes. The length of time mice stayed atop the rotarod was recorded.
Elevated Plus Maze. Mice were tested for anxiety using an elevated plus maze (EPM). The EPM was purchased from Stoelting Co. and is shaped in a symmetrical “+” with pairs of opposing closed and open arms. Each arm is 14 inches long and 2 inches wide, and the entire maze is elevated 20 inches above the floor. The closed arms of the EPM are enclosed with removable panels on three sides excluding the entrance towards the center of the maze and are 6 inches in height. Mice were placed in the center of the maze facing a closed arm and allowed to freely explore the maze for 5 minutes in low light conditions (14 Lux) while an overhead camera recorded the trial. In between all trials, the arms and walls of the maze were cleaned with 70% ethanol and dried to prevent odor cues from affecting performance. A more anxious mouse will spend more time exploring the closed arms compared to the open arms [343]. Mouse movement during the EPM trial was analyzed by an observer blinded to genotype using ANY-Maze software and the percentage of total time spent exploring the open arms was calculated as a measure of anxiety.

Spontaneous Y-maze. Next, spontaneous alternations in the Y-maze were assessed. This test does not involve any task learning and repeated testing in the Y-maze was previously shown to not induce any training or habituation effects [344]. The apparatus was a three-arm horizontal maze in which the arms were arranged at 120° angles to each other. All arms (A, B and C) were 15 inches in length, 2 inches in width, and the walls of the maze were 16 inches tall. The maze was made out of white opaque Plexiglas and was attached to a square floor panel. The maze walls and floor were made opaque with 0.5 cm thick poster board. Mice were habituated to the room for 1h prior to the test to reduce anxiety. The
maze was placed in a dimly lit room (14 Lux). Mice were initially placed in the designated start arm (A) with their head facing the center of the maze (toward the point at which all arms meet). The animals were given 7 minutes to explore the maze, while an overhead video camera tracked their behavior. The experimenter could not be seen by the animals during the 7 minutes. The animal was removed from the maze and returned to its home cage. The maze was thoroughly cleaned with a 70% ethanol solution that was allowed to dry before preforming the next trial. An alternation was defined as three consecutive entries into unique arms (i.e., ABC, CAB, or BCA, but not BAB). Alternations were quantified from video files for all of the overlapping sequences of three consecutive entries by an observer who was blind to genotype. The percentage of alternations was calculated as follows: \[
\text{percentage of alternations} = \left( \frac{\text{number of alternations}}{\text{total number of arm visits} - 2} \right) \times 100
\] (modified from Yang et al., 2009).

**Morris Water Maze.** T5x mice and their corresponding 5x, Tau, and WT littermates were tested for spatial learning and memory using Morris Water Maze (MWM). The maze was a large circular tub made out of thick opaque plastic 50 inches diameter and 15 inches high. A circular platform made out of white plastic (4.5 inches in diameter and 11 inches high) was placed inside the maze 10 inches from the center of the maze. The maze was submerged 0.5 inches under white paint colored water at room temperature. On each of the four walls surrounding the MWM a different shape made out of black construction material was placed on a white background to provide spatial cues. Five starting points equidistant apart were drawn in the three quadrants outside the maze quadrant containing the submerged platform. Mice received 6 consecutive days of training followed by probe
trials on the 7th and 14th day. On each training day mice were given four 60 second trials from one of the 5 starting points preselected at random. The starting points were consistent for all mice for each given day, but different on each consecutive training day. Prior to the first training trial on the first day of training, mice were left on top of the platform for 30 seconds to show the existence of the platform. The time taken for the mouse to reach the platform for each trial was recorded via observation through an overhead camera that allowed the experimenter to remain outside the view of the mouse. For the probe tests, the platform was removed from the maze and each mouse was given one 60 second trial starting from the starting point furthest from the platform. The time it took each mouse to cross over the original location of the platform and the number of times the location was crossed was recorded.

Results

*The combination of beta-amyloid and tau pathology accelerates the development of cognitive dysfunction.*

Previous reports have shown that 5xfAD mice begin to develop mild cognitive deficits beginning at 6 months of age [146]. Tau22 mice in comparison exhibit impairments in spatial and working memory that first manifest at 10 months [345]. We therefore hypothesized that the combined burden of Ab and NFT pathologies would accelerate the onset of cognitive dysfunction, promoting mild dysfunction by 3 months of age and leading to robust impairments in multiple cognitive domains by six months of age. Purebred C57Bl6 littermates with Wildtype, 5xfAD, Tau22, and T5x genotypes (n=8-14/genotype, all) were therefore subjected to a battery of behavioral tasks in two
differently aged cohorts: one at 3 months and one at 6 months of age. 3 month old T5x mice exhibit no significant impairments in motor or cognitive tasks (Figure 18).

Prior studies have shown that Tau22 mice exhibit no signs of motor impairment out to at least 18 months of age. In contrast, 5xfAD mice develop progressive motor deficits beginning at 9 months of age [146]. We therefore began by assessing motor function in each of the four genotypes using Pole and Rotarod tests. Mice from each genotype were tested on this task at 6 months of age and all groups performed equivalently to wild-type littermates (Figure 19A). Next, mice were tested on the accelerating Rotarod, a commonly used measure of motor coordination and again all four groups performed equally well in both the fixed (Figure 18B) and accelerating Rotarod tests (Figure. 19B). Thus, T5x mice and littermates exhibit no signs of motor impairment at 6 months of age, thereby allowing thorough examinations of cognitive function to be pursued.

In AD transgenic models anxiety deficits have been shown to accompany cognitive decline. Mice, whom are naturally prey animals, manifest their anxiety deficits in a loss of their normal sense of fear and spend increasing time in open exposed environments. Each genotype was therefore tested in the elevated plus maze (EPM) at 6-months of age and the percent of total time spent in the open versus closed arms was calculated. Interestingly, at this age 5xfAD show no significant impairments in this task, although they exhibit a trend toward impaired anxiety (p = 0.07). Tau22 mice however spend significantly more time in the open arms than wild-type controls (p = 0.01). Furthermore, T5x mice exhibit an even greater deficit, spending more than twice as much time in the open arms than wild-type controls (p = 0.0004) and exhibiting greater impairments than 5xfAD littermates (Figure. 19C, ANOVA p<0.05, Fisher’s PLSD <0.01).
Next, we tested working and short-term memory using the spontaneous Y-maze paradigm. Video recordings were analyzed by a blinded observer to quantify the number of correct alternations, revealing a significant impairment in T5x mice versus wild-type controls (p = 0.001) and 5x (p = 0.03) littermates at 6 months old (Figure 19D). Tau22 mice also exhibited a moderate impairment in Y-maze performance that was significantly different from wild-type littermates (p = 0.02). These results suggest that cortical disease pathology in T5x mice induce impairments in short-term and working memory.

All groups were next assessed for spatial learning and memory deficits using the Morris Water Maze (MWM) at 6 months of age. On the first three days of MWM testing, no significant differences between T5x, 5x, Tau22 or WT littermates were detected in platform latency. However, by the fourth day of training, WT mice began to show substantial improvements in latency, whereas T5x mice continued to have trouble finding the hidden platform and exhibited significant impairments versus WT littermates (p = 0.04). These T5x-specific impairments in performance continued on Days 5 (p = 0.007) and 6 (p = 0.02), and the T5x performance across the acquisition phase was also significantly impaired via repeated-measures ANOVA (p = 0.001). Next, two probe trials were performed 1-day and 7-days after training. Although T5x mice showed no significant impairments in the 1-day probe trial, by 7 days their long-term memory of the platform location was impaired revealing a significant impairments in platform crosses (p = 0.03) and trend towards impaired latency (p = 0.06) versus wild-type controls. 3 month old T5x mice did not have significant MWM impairment during training or probe trials (Figure 18D, E).
Figure 18. T5x mice show no significant motor or cognitive deficits at 3 months of age. T5x mice are not impaired in either the Pole test (A) or rotarod performance (B), consistent with our results in older 6-7 month old mice. At this young age, T5x mice also exhibit no significant impairments in a Spontaneous Y-maze measure of working memory (C) or Morris Water Maze acquisition (D, Day 3; T5x vs. WT P = 0.11). (E) T5x mice also exhibit no significant deficits relative to WT littermates in latency to platform location during the 1 Day (P = 0.07) and 7 Day (P = 0.46) probe trials. T5x mice also were not significantly different from WT littermates for number of platform crosses during the 1 Day (P = 0.23) and 7 Day (P = 0.53) probe trials. Data are represented as mean ± SEM, ANOVA p>0.05.
Figure 19. Aβ and tau pathologies synergize to promote anxiety and cognitive deficits in T5x transgenic mice without impairing motor function. Motor function was examined in 6-7 month old wild type (WT), 5xfAD, Tau22, and T5x littermates using the Pole test (A) and Rotarod Acceleration Test (B). Together these tests revealed no differences between groups and no evidence of motor impairment for any genotype. Mice were next examined in an elevated plus maze (C), which demonstrated that T5x mice development significant anxiety deficits in comparison to both WT (P = 0.0004) and 5xfAD (P = 0.01) groups. While T5x performance was not significantly different from Tau22 littermates (P = 0.18), Tau22 mice were themselves less anxious than WT mice (P = 0.01). (D) T5x mice also exhibited significantly impairments in the spontaneous Y-maze task relative to WT (P = 0.001) and 5xfAD (P = 0.03) littermates and Tau22 mice likewise performed significantly worse than WT mice (P = 0.02). (E) T5x mice also exhibit significant learning deficits during Morris Water Maze acquisition (Repeated Measures ANOVA P = 0.001), taking significantly longer to reach the hidden platform on days 4 (P = 0.04), 5 (P = 0.007), and 6 (P = 0.02) of training versus WT controls. 24 hours later all mice were tested in a probe trial (F, H) which revealed no significant deficits between the four groups. When the probe trial was performed 7 days later, T5x mice again showed no impairments in latency (G) but did demonstrate a significant reduction in the number of platform crosses versus WT controls, suggesting that this group exhibits a subtle impairment in long-term memory (I). Data are represented as mean ± SEM. * indicates p<0.05 for both ANOVA and Fisher’s protected least-significant difference (PLSD) post hoc tests. n ≥ 8 mice/group.
Discussion

Despite a multitude of promising therapeutics that have been developed in AD mice, the failure to successfully translate treatments for AD for human patients highlights a need for more relevant transgenic models. One potential explanation for the deficiency in translation of treatments from mice to humans is that most mouse models recapitulate AD pathology at stages that preclude diagnosis of the disease in human patients [13]. We created T5x mice from two mouse models that exhibited aggressive AD pathology with the aim of accelerating disease progression to better mimic AD as seen in human patients. To that extent, T5x mice exhibit significant anxiety and cognitive deficits as early as 6 months of age. It is important to recognize that because AD is intrinsically a model of aging, cognitive decline should be a progressive process rather a developmental effect. Although T5x mice accumulate disease pathology at an early age, they do no exhibit cognitive deficits at 3 months of age—suggesting that the development of behavioral impairment is indeed progressive rather than developmental. To properly assess cognitive behavior, it was imperative to establish that T5x mice do no exhibit motor deficits that would act as a performance confound. Although, 5xFAD mice have reported motor deficits by 9 months of age, Tau22 mice exhibit no motor impairment—a distinct advantage over other Tau transgenic models that accumulate significant spinal tau pathology [146, 149, 346]. Similar to their Tau22 counterparts, T5x mice crucially exhibit no motor deficits at the onset of cognitive impairments at 6 months of age.

Behavioral differences between T5x mice and Tau22 and 5xFAD littermates suggest that the effects of amyloid and tau on anxiety and cognition are intertwined. In EPM, spontaneous y-maze and MWM tasks, T5x mice have the greatest deficits relative to their
transgenic littermates (Figure 19). In EPM and spontaneous y-maze tasks, both Tau22 and 5xFAD mice have impaired compared to WT littermates, recapitulating results from previous studies [146, 149]. The fact that Tau22 mice exhibit greater impairments in both tasks compared to 5xFAD mice suggest that tau pathology may have a greater impact on cortical and amygdala dependent tasks. Furthermore, the extent of EPM and spontaneous y-maze deficits in T5x mice suggest that effects of tau and amyloid on these tasks are additive. Interestingly, the influence of tau and amyloid on T5x performance in the hippocampal-dependent MWM task appears to synergize rather than be simply additive. During MWM training trials, Tau22 mice are not impaired in contextual memory acquisition relative to WT littermates. In contrast 5xFAD and T5x mice show significant learning deficits. The fact that T5x mice exhibit greater learning deficits compared to 5xFAD mice, however, suggests that tau pathology synergizes with amyloid to exacerbate impairment of hippocampal dependent learning. Thus, differential performance in anxiety and cognitive tasks between T5x mice and transgenic and WT littermates suggests that the synergistic or summative effects of amyloid and tau pathology on behavior can vary depending on the task at hand.

In summary, T5x mice present an aggressive, yet progressive model of AD. T5x mice develop anxiety deficits by 3 months of age and cognitive deficits by 6 months old. The onset of cognitive deficits precludes the appearance of any motor deficits, making T5x mice an ideal model to test potential therapies for AD symptoms. Examination of behavioral performance of T5x mice and transgenic and WT littermates for several tasks suggests that amyloid and tau and combine for both summative and synergistic exacerbation of anxiety and cognitive impairment. Future studies utilizing drugs or other novel therapies to reduce
either tau or amyloid pathology in T5x mice may further elucidate the specific impacts of either pathology on anxiety and cognition.
Chapter Three: Examining the effects of tau and amyloid on α-synuclein pathology

Introduction

Although AD is typically characterized by two hallmark pathologies, amyloid and tau, one can make the argument that α-synuclein can be included as a third major player in AD pathogenesis. Up to 70% of Alzheimer’s patients exhibit α-syn positive Lewy bodies upon autopsy [120]. Yet despite the prevalence of α-syn in human AD cases, there are considerably less studies focused on α-syn in AD and transgenic models of this disease. From the available literature, however, one can find reports of numerous interactions between α-synuclein and other AD pathologies. The role of α-syn in disease progression is relatively unexplored and remains an open area of research that could further elucidate the underlying mechanisms of AD.

Since α-syn was first identified as the non-Aβ component (NAC) of amyloid plaques in human AD patients [91], subsequent studies have further corroborated the occurrence of α-syn deposits within plaques, tangles, and Lewy bodies [92, 93, 95, 347]. More recent human studies have examined whether levels of CSF α-syn correlate with dementia and can differentiate patients with AD from patients with LBD. In general, increasing CSF α-syn levels correlate with increased cognitive impairments [348]. While there are some reports that CSF α-syn levels do not differ between DLB and AD patients [349, 350], several contrasting studies have reported greater levels of α-syn in CSF AD patients than DLB subjects[351-353]. In addition, CSF α-syn levels also correlate with brain atrophy rates in the caudate and brainstem of AD patients and the correlation is significantly stronger for AD than healthy elderly controls [354]. CSF α-syn levels have also been linked to other AD
pathologies with a strong correlation between CSF α-syn and CSF Aβ42 in DLB patients [353].

Correlations between α-syn and Aβ are a reflection of the numerous direct interactions α-synuclein shares with the amyloid and tau components of AD pathology. Under pathological conditions, previous studies suggest that α-syn and Aβ may interact in AD and promote oligomeric aggregation of α-synuclein and amyloid, form nanopores that deleteriously increase intracellular calcium, induce oxidative stress, increase lysosomal leakage, and elevate mitochondrial dysfunction [355-358]. The effects that tau-α-synuclein relationships have on the progression of AD are less clear, but numerous studies have observed the coincidence of tau and α-syn in Lewy bodies as well as their shared interactions in pathological fibrillization and phosphorylation of one another [119, 125, 138, 359]. While previous studies have examined the simultaneous interactions between all three pathologies in animal models using transgenes for amyloid, tau and α-synuclein, I wanted to investigate whether the combination of aggressive tau and β-amyloid pathology could potentially induce α-syn accumulation in the absence of an α-synuclein transgene [119].

Here, I report that T5x mice not only exhibit robust human Aβ and phospho-tau accumulation, but also develop significant α-syn pathology. T5x mice develop both axonal spheroid and neuronal Lewy body-like formations that closely resemble the α-synuclein pathologies observed in human AD cases. Surprisingly, the morphology and distribution of these aggregates in T5x mice mimics that of human Lewy bodies more closely than those observed in an established α-synuclein transgenic mouse. At 7 months of age, T5x mice exhibit significant PS129+ α-syn inclusions in the amygdala and cortex. Compared to WT,
5xFAD and Tau22 littermates, T5x mice also exhibit a general increase in insoluble, but not soluble α-syn protein levels by western blotting.

**Methods**

*Generation of T5x mice.* Thy-Tau22 mice express human 4 repeat tau with two frontotemporal dementia-associated point mutations (G272V and P301S) under control of the neuronal driven promoter Thy1.2 and are maintained on a C57Bl6/J background [149]. The 5xfAD mice used in this study are also maintained on a congenic C57Bl6/J background and were purchased from the Mutant Mouse Resource and Research Center (MMRRC, stock# 034848-JAX). The 5xfAD model co-expresses human amyloid precursor protein (APP695) carrying the Swedish, Florida, and London mutations and a human presenilin-1 (PS1) transgene carrying the M146L and L286V mutations under the Thy-1 promoter. Both APP and PS1 transgenes are co-integrated and thus co-inherited. Heterozygous Thy-Tau22 and 5xfAD mice were crossed to create Thy-Tau22-5xfAD (T5x) mice, as well as Thy-Tau22, 5xfAD, and WT littermates that were genotyped via PCR amplification of human tau, PS1, and APP transgenes. The number of mice used 7 month old cohorts from each sex-balanced genotype was: WT (n = 8), 5x (n = 11-17), Tau (n = 8-14), and T5x (n = 9). All mice were maintained on a purebred C57Bl/6 background and group housed (2-4 mice/cage) on a 12h/12h light/dark cycle with access to food and water ad libitum. All animal procedures were performed in strict accordance to the National Institutes of Health and University of California Institutional Animal Care and Use Committee.
**Tissue preparation and Neuropathological analysis.** Following behavioral testing, all mice were sedated with euthasol and sacrificed by cardiac perfusion with 0.1M phosphate buffered saline (PBS). Brains were removed and one hemisphere was snap frozen on dry ice while the other hemisphere was postfixed in 4% paraformaldehyde for 48 hours then stored in PBS + 0.05% sodium azide. Fixed half-brains were placed in 30% sucrose for at least 48 hours before being cut in the coronal plane (40 µm sections) using a freezing sliding microtome.

**Immunohistological staining.** Brain sections were rinsed in PBS three times and blocked in PBS+0.05% Triton-X with 5% donkey or goat serum for one hour. Primary antibodies used included: PS129 (Abcam; 1:500) against α-syn, 82E1 (ABL America; 1:500) against human Aβ, HT7 (ThermoFisher; 1:1000) against human tau, and SMI32 (Millipore; 1:4000) against neurofilament. Sections were incubated in primary antibodies at 4 degrees Celsius overnight. Sections were then washed three times with PBS and incubated with appropriate Alexa fluor-conjugated secondary antibodies at room temperature for one hour. Sections were then rinsed three additional times, mounted on slides and coverslipped with Fluoromount-G with DAPI.

**Biochemical Analysis.** Cortex was microdissected from frozen brains and processed to collect both soluble and insoluble extracts. Briefly, microdissected tissue was homogenized in TPER (ThermoFisher) and centrifuged at 12,000 RPM for 15 minutes. Supernatant was collected as the soluble fraction and the pellet was treated with 70% formic acid and spun down at 25,000 rpm. The resulting supernatant was collected as the insoluble fraction.
Insoluble protein samples were neutralized for Western blotting and further precipitated with trichloroacetic acid (TCA) when probing for insoluble tau. Protein samples were denatured at 95°C for 15 minutes before being loaded onto 4-20% TGX precast polyacrylamide gels (Bio-rad). Antibodies used for western blotting include: PS129 (Abcam; 1:500) and Syn211 (Invitrogen; 1:1000).

Confocal microscopy and quantification. Equivalent brain sections were picked and immunofluorescent sections were imaged using Olympus FX1200 confocal microscope. α-synuclein inclusions identified by PS129 were visualized through a Z-stack image taken through the entire depth of the section at 1 µm intervals. α-synuclein inclusions were then manual counted by a blind observer and analyzed throughout the Z-stack. Areas of the brain quantified included the rostral cortex (AP: -0.38), caudal cortex (AP: -2.06), basal lateral amygdala (BLA) (AP: -2.06), posterior basal lateral amygdala (BLP) (AP: -2.80), and the CA1, subiculum and dentate gyrus of the parahippocampal area (AP: -2.06).

Statistical analysis. Statistical analysis was performed using StatView software (SAS Institute Inc.). Statistical comparisons between multiple groups were performed using ANOVA followed by Fischer’s PLSD post-hoc tests. Error bars represent the standard error of the mean. Groups were considered statistically significant when *p<0.05 for both ANOVA and posthoc analysis.
Results

*Amyloid, Tau and α-synuclein pathologies co-localize in T5x mice.*

While α-syn can be phosphorylated at a number of different sites under physiological stress, the phosphorylation of Serine-129 (PS129) has been strongly implicated in the development of α-synuclein pathology [360, 361]. Previous studies have shown that PS129 promotes α-syn aggregation, oligomeric formation and neurotoxicity, and is enriched in Lewy body formations [362-365]. I have recently shown that T5x mice exhibit significant Aβ and phospho-tau accumulation throughout the brain by 7 months of age. To determine if this aggressive accumulation of Aβ and tau leads to the appearance of α-syn pathology I performed immunohistological staining of PS129 on T5x brain sections. Punctate, most likely synaptic PS129+ staining was readily detected throughout the brain whereas PS129+ pathological inclusions were mainly present within the cortex and amygdala (Figure 20), with some inclusions also detected within the parahippocampal area. Incidence of PS129+ labeling colocalized around Aβ plaques and areas of significant tau and amyloid pathology. Staining of human tau (HT7) and murine P129+ synuclein in T5x mice closely resemble the distribution of occasional co-localization of human tau and α-synuclein pathology as observed in human AD brains (Figure 21A, B). Cortical PS129+ inclusions in T5x mice also closely resembled the appearance of human α-syn spheroids and Lewy bodies (Figure 21C-F).
Figure 20. **T5x mice exhibit amyloid, tau and α-synuclein pathology.** AD pathology was labeled using immunohistochemistry by antibodies 82E1 (human amyloid), HT7 (human tau) and PS129 (α-syn) in the cortex and amygdala.
Figure 21. α-Synuclein labeling in T5x mice resembles human AD α-synuclein pathology. (A, C, E) Immunohistochemical labeling of tau (HT7; green) and α-syn (PS129; red) was carried out in T5x mice brain sections. The subcellular distribution and morphology of α-syn aggregates appears to be highly similar between T5x mice (A) and human AD patients (B). T5x mice exhibit α-syn positive axonal swellings and inclusions that resemble human patient spheroids (D). T5x mice also develop intraneuronal Lewy-body like inclusions (E) that closely resemblance human Lewy bodies (F). Human spheroid image adapted from Kruer et. al., 2011. Human Lewy body image borrowed from ‘FrontalCortex.com.’
**α-Synuclein pathology is elevated in T5x mice.**

To determine whether α-synuclein pathology was specifically elevated in T5x mice relative to WT, 5xFAD and Tau22 littermates, I carried out quantification of PS129+ inclusion numbers in the cortex, amygdala and hippocampus (Figure 22A, B). No PS129+ inclusions were detected in any brain region of WT mice. In contrast, T5x mice exhibited significantly greater number of PS129+ inclusions in comparison to WT, Tau22, and 5xfAD littermates in the caudal cortex (p<0.05). T5x and Tau22 littermates also exhibited similarly higher numbers of PS129+ inclusions compared to 5xFAD mice in the rostral cortex (p<0.001) and the posterior basolateral nucleus of the amygdala (BLP) (T5x vs. 5xFAD, p = 0.057; Tau22 vs. 5xFAD, p <0.0001). Whereas within the anterior portion of the BLA, only T5x mice exhibited significantly more PS129+ inclusions than Tau22 littermates (p<0.01). Within the subiculum, the dorsal hippocampus, and the dentate gurus both T5x mice and 5xFAD mice displayed significantly more α-synuclein deposits than Tau22 and WT littermates  (p<0.05). Although T5x inclusion numbers were consistently higher than 5xFAD and Tau22 mice in all brain regions examined, differential numbers of PS129+ inclusions in varying brain regions of 5xFAD and Tau22 mice suggest that individual amyloid or tau pathology can influence α-syn pathology to varying degrees.

To further confirm that α-synuclein pathology is indeed elevated in T5x mice and transgenic littermates, I performed western blot analysis of soluble and insoluble cortical brain lysates (Figure 22C). Corroborating the quantification of PS129+ inclusions, insoluble PS129 protein levels were elevated in T5x (p<0.05), 5xFAD (p<0.05) and Tau22 (p = 0.059) littermates compared to WT mice (Figure 22F). Interestingly, PS129 could not be detected in soluble cortical fractions of transgenic mice—suggesting that this
phosphoepitope is predominantly associated with insoluble α-syn aggregates in these mice, consistent with findings in human Lewy body diseases [365]. In contrast, detection of soluble total α-synuclein by western blot revealed no significant differences between WT and transgenic littermates, confirming that endogenous murine monomeric α-synuclein production is not significantly altered, but rather the solubility of this protein is changed in transgenic mice (Figure 22G). Consistent with this hypothesis, larger species of insoluble mouse α-synuclein detected at a 50 kDa were significantly elevated in T5x mice relative to WT littermates (p<0.05) and non-significantly increased compared to 5xFAD (p = 0.13) and Tau22 (p = 0.12) mice (Figure 22D). Furthermore, high molecular weight labeling of mouse α-synuclein was also greatly increased in T5x mice relative to WT and transgenic littermates (p<0.05; Figure 22E). Thus, while T5x mice exhibit no significant increases in soluble α-syn relative to WT and transgenic littermates, phosphorylated and pathological insoluble α-syn species are greatly elevated in T5x cortical fractions.
**Figure 22. α-Synuclein pathology is elevated in T5x mice.** (A) Immunohistochemical labeling of pathological α-syn reveal PS129+ inclusions in 5xFAD, Tau22, and T5x mice. (B) T5x mice exhibit greater numbers of PS129+ inclusions relative to all transgenic and wild type littermates in the caudal cortex (p<0.05), relative to 5xFAD mice in the rostral cortex (p<0.0001), and relative to Tau22 mice in the BLA (p<0.001), subiculum (p<0.01), hippocampus CA1 (p<0.01), and dentate gyrus (p<0.05). (C-G) Analysis of cortical α-syn protein levels by western blot (C) reveal increases in aggregated mouse synuclein in T5x mice that can be observed at 50 kDa (D; vs. WT, p<0.05; vs. 5xFAD p = 0.30; vs. Tau22 p = 0.31) and higher molecular weights (E; p<0.05). (F) Insoluble PS129 levels are also significantly elevated in T5x relative to WT mice (p<0.05), but not significantly different from Tau22 and 5xFAD mice. (G) In contrast, soluble α-syn monomers are not significantly different between WT and transgenic littermates. Data are represented as mean ± SEM, n ≥ 8 mice/group. * groups indicates p<0.05 for both ANOVA and Fisher’s protected least-significant difference (PLSD) post hoc tests.
Discussion

While AD research has focused primarily on the study of amyloid and tau pathologies, the potential role of α-synuclein in this disease has been considerably less explored. Yet, α-syn pathology is readily apparent in the great majority of AD cases [366, 367]. Given this, the development of α-synuclein pathology in an AD transgenic model that lacks an α-synuclein transgene, could potentially contribute invaluable knowledge toward our understanding of the mechanisms that underlie this disease. I reasoned that to induce α-syn pathology in a mouse model without an α-synuclein transgene, the model would need to exhibit extensive pathology that mimicked advanced stages of AD. In that respect, I crossed two aggressive models of AD pathology: amyloidgenic Thy1-5xFAD mice and human mutant tau transgenic Thy1-Tau22 mice. The resulting T5x mice develop robust amyloid and tau pathology by 7 months of age. Furthermore, we find that 7-month old T5x mice also exhibit a significant degree of Lewy body-like α-syn pathology. Amyloid, tau and α-synuclein pathology co-localize within multiple brain regions of T5x mice and appear to synergize to create spheroid and Lewy-body like α-syn inclusions. To our knowledge, T5x mice are the first reported transgenic AD mice to develop spheroid and Lewy body-like α-syn pathology without an α-synuclein transgene. Prior studies have reported that AD pathology can accelerate the accumulation of α-syn in AD transgenic mice that co-express a human α-synuclein transgene with either tau or amyloid transgenes [119]. Although a few studies have also reported α-syn accumulation in highly amyloidgenic models carrying APP [368] or APP/PS1 [369] transgenes, the α-syn labeling in these mice manifest mostly in plaque-associated dystrophic neurites and do not achieve the α-synuclein inclusion morphology exhibited by T5x mice. As the first reported AD transgenic model to exhibit α-
syn inclusions pathology without a α-synuclein transgene, T5x mice appear to closely recapitulate the pathogenesis of human AD with Lewy bodies. By studying how and when PS129+ α-syn inclusions arise in T5x mice, one may therefore be able to determine how amyloid and tau pathologies synergize to induce α-synuclein accumulation without the caveats that come from transgenic overexpression of human α-synuclein. T5x mice display a number of different forms of α-syn labeling in the brain. While PS129+ inclusions are the largest and most obvious form of α-synuclein labeling, T5x mice also exhibit α-syn positive dystrophic neurites surrounding amyloid plaques and synaptic/punctate labeling throughout the brain. These forms of α-synuclein pathology recapitulate observations made in human cases of AD. α-syn normally plays a role in synaptic transmission and synaptic α-syn labeling has also been previously reported in transgenic AD mice [370]. Furthermore, phosphorylated α-syn is highly enriched in synaptic protein fractions in human AD patients [131]. Dystrophic neurites have also long been reported in human cases of AD [92, 371]. Perhaps most strikingly, the occurrence of T5x PS129+ spheroid and Lewy body-like inclusions in the amygdala and cortex mirror the anatomical distribution of Lewy bodies in human AD patients which begins in cortical and limbic region [131, 365], and exhibits the highest occurrence within the amygdala [372, 373]. Additionally, PD patients exhibiting AD pathology also exhibit higher cortical and limbic α-syn burden than PD only patients, further supporting the association between AD and α-syn pathologies in these regions [374, 375]. Thus, it appears that likely due to the aggressive nature of amyloid and tau pathology in T5x mice, the natural progression of α-synuclein pathology in human AD is closely recapitulated in T5x mice.
The induction of PS129+ inclusions in T5x mice allow one to examine how and to what extent amyloid and tau pathology affect α-synuclein accumulation. As previously mentioned, Aβ and phospho-tau have shown the propensity to recruit α-synuclein pathology and induce the formation of α-syn oligomers and fibrils [118, 125, 357]. The presence of PS129+ inclusions in 5xFAD and Tau22 mice confirm that indeed amyloid and tau can independently induce α-synuclein pathology. The question then remains as to which pathology, amyloid or tau, plays a greater role in the induction of α-synuclein pathology and whether their effects on α-syn synergize. The quantification of PS129+ inclusions in T5x mice suggests that either amyloid or tau can have a greater impact on α-syn pathology depending on the anatomical region. For example, 5xFAD mice have more PS129+ inclusions in the parahippocampal area and BLA relative to Tau22 littermates, corroborating prior studies on α-syn pathology in amyloidgenic models. It is surprising however that in the hippocampus, where tau pathology is abundant, but amyloid deposition is minimal compared to cortex and amygdala in T5x mice, that PS129+ inclusions are seen in 5xFAD mice, but not in Tau22 mice. This finding is perhaps not as surprising taking into account that Lewy body formation in AD is observed in fAD cases that have greatly increased amyloidosis [92]. It is worth also noting that despite the fact that T5x mice exhibit reduced insoluble Aβ and plaque burden relative to 5xFAD littermates, T5x and 5xFAD mice exhibit similar numbers of PS129+ inclusions in the hippocampus. These results can perhaps be explained by the relatively low plaque burden seen in the hippocampus and that difference in low amyloid numbers is not sufficient to induce a significant difference in PS129+ inclusions number. On the other hand, the quantification of PS129+ inclusions also suggests that tau may be the major mediator of α-
synuclein pathology. In rostral cortex and BLP, where Aβ and phospho-tau pathology is abundant, I observed greater numbers of PS129+ inclusions in Tau22 mice relative to 5xFAD littermates. A prior study in human PD patients has reported that in the CSF, total and phospho-tau levels correlate significantly better than Aβ42 to α-syn levels [376]. Furthermore, previous examination of AD pathology in the olfactory bulb, an area of the brain that directly connects to the amygdala, found that α-syn localized with tau, but not amyloid pathology in AD brains [377]. Thus, in certain areas of high tau pathology, phospho- and oligomeric tau may play a greater role in formation of PS129+ α-syn inclusions. Lastly, in the caudal cortex T5x mice exhibit significantly greater number of PS129+ inclusions compared to both 5xFAD and Tau22 littermates—suggesting that pathological effects of amyloid and tau on α-synuclein can likely also synergize with one another.

While looking at the individual effects of amyloid or tau on α-synuclein pathology is valuable for elucidating specific mechanisms of AD, the reality is that in the AD brain all three pathologies are continuously interacting with one another. The examination of all pathologies in a single context in T5x mice may therefore provide a new approach to examine these important interactions and their combined influence on neuronal function and cognition in vivo. In fact, one study has reported in Parkinson’s Disease Dementia (PDD) patients, the combined correlation of Aβ, tau, and α-syn pathology was a greater predictor of dementia than any single pathology by itself [378]. Prior in vitro studies [125, 379] and experiments in transgenic mice [380, 381] also demonstrate synergistic interactions between amyloid, tau, and α-syn pathologies and lend support to the hypothesis that these disorders may involve prion-like seeding and propagation of
Further studies in T5x mice on whether tau and Aβ oligomer conformations precede and subsequently recruit formation of PS129+ inclusions and α-syn oligomers could be pursued to elucidate whether such cross-seeding of these pathologies occurs. Another potential explanation for how amyloid and tau pathology may induce accumulation of α-syn involves the underlying mechanisms for tau and α-synuclein subcellular transport. One study has shown that α-syn and tau axonal transport are both mediated via the fast transport-motor kinesin-1 [386]. This same motor has also been implicated in APP transport [387]. In T5x mice, it is therefore possible that Aβ-mediated exacerbation of tau phosphorylation can destabilize transport machinery leading to blockage of axonal transport and subsequent accumulation of α-syn. In T5x mice, we observe build-up of α-syn pathology in axonal spheroids and intraneuronal Lewy body-like inclusions (Figure 23). Thus, the induction of α-syn pathology by phospho-tau and Aβ in T5x mice is most likely a combination of several processes that may include, but are not limited to prion-like propagation and axonal transport dysfunction.

In summary, I have shown that T5x mice accumulate murine α-syn pathology coinciding with robust expression of human Aβ and tau. T5x α-syn pathology co-localizes anatomically with amyloid and tau pathology in the cortex and amygdala. T5x mice also exhibit elevated levels of PS129+ inclusions and insoluble α-syn protein levels. To our knowledge, T5x mice are the first AD transgenic mice to exhibit significant PS129+ spheroid and Lewy body-like α-syn inclusions without the expression of a α-synuclein transgene. Thus, T5x mice are able to recapitulate α-syn, amyloid and tau aspects of AD and serve as an attractive model to examine the interactions between these three pathological proteins and to test future therapies that target them.
Figure 23. T5x mice exhibit various forms of α-syn accumulation. (A) Labeling of neurofilament (SMI32; green), α-syn (PS129; red) and nuclei (DAPI; blue) reveals synaptic and axonal aggregation of α-syn. (B) High magnification confocal images, stained for Aβ (82E1; blue), α-syn (PS129; red) and nuclei (DAPI; green), also reveal intraneuronal accumulation of Lewy-body like α-syn inclusions in the cell body of a neuron.
Chapter Four: Examining the role of adaptive immunity in tau pathology

Introduction

While researchers have extensively studied the role of innate immunity in AD pathogenesis, the potential influence of the adaptive immune system in AD remains largely unknown. The adaptive immune system, as the name implies, adapts to and forms a memory of invading pathogens and is responsible for building up long-term immune resistance against viruses and microbes. In addition, the adaptive immune system can recognize and destroy cancerous growths, differentiate between foreign antigens and endogenous cells, and is responsible for immune rejection of grafted or transplanted tissue. The cells that comprise the adaptive immune system are divided into two main types of lymphocytes: B-cells and T-cells. While the role of T- and B-cells in AD are still under debate, recent studies have begun to implicate the adaptive immune system in disease pathogenesis.

T-cells are involved a wide range of functions including defense against foreign antigens and endogenous tumors, release of pro- and anti-inflammatory cytokines, differentiation and maturation of B-cells, and self-regulation of T-cell response. While the detection of T-cells in the AD brain has been previously reported [388, 389], whether their role in AD is beneficial or harmful remains unclear. It is however recognized, that T-cell’s have the capacity to react to AD pathology. For example, a study examining T-cell response to Aβ41-42 vaccination in patients showed increased Aβ-specific T-cell reactivity in older and AD subjects relative to younger and healthy controls [337]. Regulatory T-cells (Tregs), which normally function to suppress immune response, may also play important roles in progression of AD. Tregs infiltrating the brain through the choroid plexus have been
implicated in CNS surveillance and repair [390]. In AD, however, the infiltration of Tregs through the choroid plexus may be impaired. In a recent study, examination of the choroid plexus in 5xFAD mice revealed significant reduction of adhesion molecules and local cytokines [332]. In the same study, Tregs were transiently depleted and then allowed to repopulate after 3 weeks. 5xFAD mice with repopulated T-cells exhibiting greater number of brain-infiltrating Tregs, reduced amyloid burden and ameliorated cognitive deficits—suggesting that infiltrating Tregs protect against AD [332]. Other studies have also supported the neuroprotective role of Tregs in AD. One study reported that early transient depletion of Tregs exacerbated cognitive deficits in APP/PS1 mice without affect Aβ pathology [391], while a follow-up study showed that amplification of Treg response by IL-2 dosing rescued cognitive deficits [392]. Additionally, our lab has also previously reported that amyloidgenic Rag-5xFAD mice lacking T-cells have increased Aβ pathology [163]. While these studies suggest that some T-cells may play a beneficial part in AD, a few studies suggest otherwise. In contrast to our lab’s findings, one study found that PSAPP mice also lacking functional T- and B-cells had decreased levels of Aβ pathology [393]. Furthermore, while there is evidence that Tregs promote microglia colocalization to and clearance of Aβ plaques [392], other studies have reported that T-cells can restrain microglia responses to Aβ via secretion of IL-10 [260, 261, 394]. Lastly, a previous study has also reported that microglia can exacerbated nitric-oxide mediated neurotoxicity of Th1 T-cells in the presence of Aβ [395]. In summary, whether T-cells ultimately restrain or exacerbate AD remains unclear suggesting that further studies are needed to elucidate their potential role in pathogenesis.
The second major component of adaptive immunity; B-cells generate antibodies that recognize, bind, and target foreign antigens for removal. Antibodies, also known as immunoglobulins (Ig), occur in five different forms in humans and mice: IgA, IgD, IgE, IgG and IgM. IgGs are the most common form of antibody, making up to 75% of all serum immunoglobulins [396] and have been thought to play a role in AD. Plasma IgG levels have been correlated with development of AD, specifically post-translational glycosylation of IgG and subsequent cleavage of IgG-Fc fragments correlate with mild cognitive impairment and intensity of the disease [397]. The production of non-specific IgG has also been thought to be a potentially neuroprotective mechanism against the accumulation of Aβ. Prior studies have shown that IgG given in vitro to culture human primary neurons can protect against oxidative stress [398]. Intracranial injection of mouse IgG has also been shown to reduce Aβ deposition in APP/PS1 mice [399]. Our lab have corroborated this finding and also show that removal of IgG’s by ablation of the adaptive immune system in immune deficient Rag-5xFAD result in decreased microglial clearance of Aβ [163]. Additionally, our lab reported that replenishing of IgG-producing B-cells by bone marrow adoptive transfer in Rag-5xFAD mice decreased Aβ burden [163]. It should be noted that while intravenous injection of human polyclonal IgG solutions in late-stage AD patients has also decreased plasma and CSF Aβ42 in a dose-dependent manner, unfortunately no significant cognitive benefits were detected [400].

While nonspecific IgG’s have been suggested to promote Aβ clearance, there has also been much research done on the potency of Aβ-specific IgG’s/antibodies in AD. Numerous studies have previously reported that passive or active immunization against Aβ in transgenic AD mice can reduce Aβ fibril formation, plaque burden and ameliorate cognitive
deficits [401-403]. In AD patients, B-cells also produce naturally occurring autoantibodies against Aβ [404-406]. Aβ-autoantibodies administered in human neuronal cell cultures, transgenic APP mice and humans AD patients have been reported to promote Aβ clearance and inhibit Aβ fibrillization [407-410]. Interestingly, Aβ-specific autoantibodies are lower in AD patients relative to healthy controls—suggesting that B-cell dysregulation or dysfunction in AD patients may lead to decreased Aβ clearance and increased plaque deposition [411, 412]. Furthermore, human Aβ-specific autoantibodies have been shown to specifically bind Aβ oligomers, but not Aβ monomers and fibrils [413]. The fact that Aβ-specific autoantibodies bind Aβ oligomers, the Aβ conformation thought to be the initial step in the disease [234, 414-416], suggests that Aβ-autoantibodies act as an initial neuroprotective mechanism against accumulation of pathological Aβ but may lose their efficacy once AD has sufficiently progressed and Aβ fibrillization occurs.

Although past research has examined the effects of the adaptive immune system on Aβ, very little is known about the role of the adaptive immune system in tau pathology. To investigate the interactions between the adaptive immune and tau pathology I therefore developed RagTau mice, a model of tau pathology with genetic deletion of the adaptive immune system. At 9 months of age, RagTau exhibit robust tau hyperphosphorylation and anxiety and cognitive deficits, yet tau pathology is not significantly different between RagTau and Tau22 mice. To further confirm that the adaptive immune system does not have a significant impact on tau pathology, I performed adoptive transfer of bone marrow (BM) cells in 3-month old RagTau mice. Despite successful engraftment of BM cells and the replenishment of T- and B-cells, RagTau mice did not exhibit increased tau pathology
compared to vehicle controls. Thus, my results suggest that the adaptive immune system does not play a substantial role in the regulation of tau pathology.

**Methods**

*Generation of RagTau mice.* Thy-Tau22 (Tau22) mice express human 4 repeat tau with two frontotemporal dementia-associated point mutations (G272V and P301S) under control of the neuronal driven promoter Thy1.2 and are maintained on a C57Bl6/J background [149]. Rag2−/−/Il2rγ−/− (Taconic) mice are maintained on a C57/BL6/Bl10 background and exhibit constitutive knockout of recombinase activating gene 2 (Rag2) and interleukin 2 (IL2) receptor gamma, resulting in complete ablation of B-, T- and natural killer (NK) Cells [154-157]. Heterozygous Thy-Tau22 and homozygous Rag2−/−/Il2rγ−/− mice were crossed and then backcrossed over multiple generations to create Tau22-Rag2−/−/Il2rγ−/− (RagTau) mice, as well as WT-Rag2−/−/Il2rγ−/− (RagWT) littermates. All mice were group housed (2-4 mice/cage) on a 12h/12h light/dark cycle with access to food and water ad libitum. All animal procedures were performed in strict accordance to the National Institutes of Health and University of California Institutional Animal Care and Use Committee.

*Tissue preparation and Neuropathological analysis.* Following behavioral testing, all mice were sedated with euthasol and sacrificed by cardiac perfusion with 0.1M phosphate buffered saline (PBS). Brains were removed and one hemisphere was snap frozen on dry ice while the other hemisphere was postfixed in 4% paraformaldehyde for 48 hours then
stored in PBS + 0.05% sodium azide. Fixed half-brains were placed in 30% sucrose for at least 48 hours before being cut in the coronal plane (40 µm sections) using a freezing sliding microtome.

**Immunohistological staining.** Brain sections were rinsed in PBS three times and blocked in PBS+0.05% Triton-X with 5% donkey or goat serum for one hour. Primary antibodies used included: CD4 (1:250; BioRad) against T-cells. Sections were incubated in primary antibodies at 4 degrees Celsius overnight. Sections were then washed three times with PBS and incubated with appropriate Alexa fluor-conjugated secondary antibodies at room temperature for one hour. Sections were then rinsed three additional times, mounted on slides and coverslipped with Fluoromount-G with DAPI.

**Biochemical Analysis.** Cortex was microdissected from frozen brains and processed to collect both soluble and insoluble extracts. Briefly, microdissected tissue was homogenized in TPER (ThermoFisher) and centrifuged at 12,000 RPM for 15 minutes. Supernatant was collected as the soluble fraction and the pellet was treated with 70% formic acid and spun down at 25,000 rpm. The resulting supernatant was collected as the insoluble fraction. Insoluble protein samples were neutralized for Western blotting and further precipitated with trichloroacetic acid (TCA) when probing for insoluble tau. Protein samples were denatured at 95°C for 15 minutes before being loaded onto 4-20% TGX precast polyacrylamide gels (Bio-rad). Antibodies used for western blotting include: total human tau antibody VH2-VL2 and IC9 (1:1000; provided by the Dr. Agadjanyan lab), and phospho-tau antibodies PS199 (Abcam; 1:1000), PS202 (Abcam; 1:1000), AT8 (1:1000;
ThermoFisher), AT100 (ThermoFisher; 1:1000), PHF-1 (1:1000; provided by the Dr. Cotman lab), PS396 (Abcam; 1:1000), and PS404 (Abcam; 1:1000).

**Bone marrow adoptive transfer.** GFP+ bone marrow cells were isolated from 3 month old C57/BL6 CAG-GFP mice for adoptive transfer in 3 month old RagWT and RagTau mice. Donor GFP-mice were euthanized by CO₂ asphyxiation and dissected quickly for femurs on ice. PBS was then injected into femur cavities with a syringe and whole bone marrow was collected. BM cells were then mixed with ammonium chloride–potassium buffer to lyse red blood cells, filtered through a 70-μm nylon mesh, and counted by a hemocytometer. RagWT or RagTau mice were then anesthetized using isoflurane and given retroorbital injections of 500,000 live cells in 100 μL or equivalent volume of. The numbers of mice from each genotype and treatment group are as follow: RagWT Vehicle (n = 5), RagTau Vehicle (n = 10), RagWT BM (n = 6), and RagTau BM: (n = 10). To confirm engraftment, blood was collected 1, 2 and 3 months after BM injection and analyzed for B, T, and NK cells by flow cytometry.

**Confocal microscopy and quantification.** Equivalent brain sections were picked and immunofluorescent sections were imaged using Olympus FX1200 confocal microscope. Synuclein inclusions identified by PS129 were visualized through a Z-stack image taken through the entire depth of the section at 1 μm intervals. GFP+ bone marrow cells were visualized using confocal microscopy and then manual counted for equivalent sections. Areas of the brain quantified included the amygdala and hippocampus dentate gyrus at the following rostral-caudal coordinates: AP -1.58, -2.06, -2.70, and -3.00. Microglia
quantification by confocal microscopy was also analyzed using IMARIS. Microglia number was quantified using IMARIS spot function and process morphology was measured using filament length and branching functions.

**Behavior.** 9 month old WT, Tau22, RagWT and RagTau mice were used for behavioral analyses. The number of mice tested for each genotype was: WT (n = 11), Tau22 (n = 10), RagWT (n = 12), and RagTau22 (n = 11). A battery of motor and cognitive tasks were performed and analyzed by a blinded observer as follows:

**Pole Test.** Mice were tested in the Pole Test of motor coordination following previously described protocols [342]. Briefly, a soft wooden pole of 1 cm in diameter and 1 foot in length was based in a Styrofoam block and placed on the floor of a mouse cage. The Styrofoam base was covered with bedding until roughly 1 inch above the top of the block. Mice were given two consecutive days of training in which the mice were initially placed on the top end of the pole facing the ceiling with all four limbs claspings the pole. For a successful trial, mice were required to re-orientate themselves until they were claspings the pole and facing the floor of the cage before climbing down into the bedding. Each mouse was given four training trials per day and allowed to fully attempt to re-orientate itself on the pole without time restrictions. On the third day, mice were given four test trials and timed for their ability to re-orientate themselves on the pole. Each of these trials was capped at a 30 second time limit and mice that were unable to complete the task were given a re-orientation value of 30 seconds.
**Rotarod.** A standard Rotarod apparatus (Ugo Basile, Italy) was used to test five mice concurrently in each trial. On Day 1 (Training Day), mice were placed atop the rotarod and run at a fixed speed of 24 rpm for 2 minutes for one trial. Mice that fell off the rotarod or were inverted while running were manually assisted back onto the rotarod and placed into the proper orientation. On Day 2 (Fixed Test) mice were given four trials at a fixed speed of 24 rpm for a maximum of 2 minutes per trial. The time mice spent on top of the rotarod was recorded. Mice that clasped onto the rotating beam without running for more than 1 rotation throughout an entire trials was considered a “falling” at the time of the second rotation. To prevent mice from suffering from fatigue, two cohorts of mice were run in alternating trials at a time. The rotarod was cleaned in between trials with 70% ethanol and allowed to dry before the beginning of the next trial. On Day 3 (Accelerating Test), the rotarod was programmed to accelerate at a constant rate from 4 rpm to 40 rpm in a span of 5 minutes. The length of time mice stayed atop the rotarod was recorded.

**Elevated Plus Maze.** Mice were tested for anxiety using an elevated plus maze (EPM). The EPM was purchased from Stoelting Co. and is shaped in a symmetrical “+” with pairs of opposing closed and open arms. Each arm is 14 inches long and 2 inches wide, and the entire maze is elevated 20 inches above the floor. The closed arms of the EPM are enclosed with removable panels on three sides excluding the entrance towards the center of the maze and are 6 inches in height. Mice were placed in the center of the maze facing a closed arm and allowed to freely explore the maze for 5 minutes in low light conditions (14 Lux) while an overhead camera recorded the trial. In between all trials, the arms and walls of the maze were cleaned with 70% ethanol and dried to prevent odor cues from affecting
performance. A more anxious mouse will spend more time exploring the closed arms compared to the open arms [343]. Mouse movement during the EPM trial was analyzed by an observer blinded to genotype using ANY-Maze software and the percentage of total time spent exploring the open arms was calculated as a measure of anxiety.

**Novel Arm Y-maze.** Next, hippocampal dependent memory was tested in the Novel Arm Y-maze (NAY-maze) were assessed. The apparatus was a three-arm horizontal maze in which the arms were arranged at 120° angles to each other. All arms (A, B and C) were 15 inches in length, 2 inches in width, and the walls of the maze were 16 inches tall. The maze was made out of white opaque Plexiglas and was attached to a square floor panel. The maze walls and floor were made opaque with 0.5 cm thick poster board. Each arm was given a unique background (spots, stripes, and crosses) by layer of black tape. Mice were habituated to the room for 1h prior to the test to reduce anxiety. The maze was placed in a dimly lit room (14 Lux). Before training, one of two exploratory arms (either arm B or C) was pre-chosen and blocked off by an insert the same height as the maze walls. For the training trial, mice were placed in the start arm (arm A) and allowed to explore the maze for 7 minutes. Mice were then removed from the maze and placed into a holding cage for 2 minutes while the maze is cleaned with ethanol and dried. For the test trial, the insert blocking the pre-chosen arm was then removed and mice were placed back into the maze for 7 minutes. During the test trial an overhead camera records the mice’s movements. Video files of the training trial were then loaded into ANY-Maze software and percentage of total time spent in the novel (blocked off arm during training trial) arm was quantified.
Statistical analysis. Statistical analysis was performed using StatView software (SAS Institute Inc.). Statistical comparisons between multiple groups were performed using ANOVA followed by Fischer’s PLSD post-hoc tests. Error bars represent the standard error of the mean. Groups were considered statistically significant when *p<0.05 for both ANOVA and posthoc analysis.

Results

RagTau mice have constitutive knockout of the adaptive immune system.

To examine the role of the adaptive immune system on tau pathology, I created an immune deficient transgenic tau model to study how tau pathology would progress in the absence of the adaptive immune system. Heterozygous Thy1-Tau22 mice were backcrossed with Rag2−/−/Il2rγ−/− mice over multiple generations to achieve homozygous constitutive knockout of both Rag2 and Il2rγ genes. Resulting ‘RagTau’ mice lack lymphocytes including B- and T-cells (Figure 24) and NK-cells (data not shown). Importantly, RagTau mice breed normally and do not exhibit signs of early morbidity, living up to 14 months of age.
Figure 24. RagTau mice lack their adaptive immune system. Blood was extracted through the tail veins of 3-6 month old RagTau mice and sorted for CD45+ hematopoietic cells. CD45-low expressing lymphocyte cell population was not detected, confirming knockout of the adaptive immune system.

Ragtau mice exhibit anxiety and cognitive deficits without motor impairment.

Tau22 mice have been previously reported to exhibit cognitive impairment without motor deficits by 9 months of age [149]. To examine whether knocking out the adaptive immune system in Tau22 mice would have an effect AD symptoms, 9-month old RagTau, RagWT, Tau22 and WT mice were tested for behavior. Unlike numerous other tau models of AD, Tau22 mice lack motor impairments due to the relatively low presence of tau pathology in their spinal cords [149]. Encouragingly, RagTau mice also exhibit no
significant motor deficits in the fixed and acceleration trails of the rotarod test (Figure 25A, B) and in the reorientation time of the pole test (Figure 25C).

RagTau mice were also examined for anxiety behavior, a symptom that precludes cognitive deficits in human AD patients and many mouse models of AD [417]. Mice were tested for exploratory behavior in the elevated plus maze which measures decreasing anxiety as more time spent in the open arms compared to the closed arms of the maze (Figure 25D). Recapitulating results from prior studies [149], Tau22 mice exhibit decreased anxiety relative to WT mice (p<0.001). RagTau mice exhibited even greater anxiety deficits, spending significantly more time in the open arms of the EPM relative to WT, Tau22, and RagWT (p<0.01) mice. Interestingly, RagWT mice exhibited similar anxiety deficits compared to WT mice (p<0.05) as Tau22 mice did, also spending almost ~1.5 as much time exploring the open arms of the EPM compared to WT mice. These results suggest that the adaptive immune system may contribute to modulating anxiety behavior and that knocking out the adaptive immune system may in itself induce anxiety deficits. However, the finding that RagTau mice spent ~2.5 times the amount of time exploring EPM open arms relative to WT mice, suggests that the effects of tau pathology and knockout of the adaptive immune system combine to exacerbate anxiety dysfunction in RagTau mice.

In AD, tau pathology accumulates in the hippocampus and contributes to cognitive impairment. Thus, I also tested RagTau mice for cognitive deficits in the hippocampal-dependent novel arm y-maze task (NAY-maze) (Figure 25E). In the NAY-maze, mice are given an exploratory training trial with one arm of the maze blocked off and then after a brief respite, given a subsequent exploratory test trial with all maze arms available. Mice that are cognitively intact will preferentially spend more time in the novel, previously
blocked-off arm of the NAY-maze. Compared to WT mice, Tau (p = 0.87) and RagWT (p = 0.47) mice perform no worse and spend similar amounts of time exploring the novel arm. Surprisingly, however, RagTau mice spend significantly less time exploring the novel arm compared to WT, Tau22, and RagWT (p<0.001) mice. The findings that RagTau mice exhibit cognitive deficits relative to WT mice in NAY-maze while Tau22 and RagWT do not, suggests that tau pathology and deletion of the adaptive immune system synergize to induce NAY-maze deficits.

Figure 25. RagTau mice exhibit impaired anxiety and cognition without motor dysfunction. (A, B) 10-month old WT, Tau22, RagWT and RagTau mice motor performance was tested using a rotarod apparatus. Mice were recorded for time spent on the rotarod without falling during trials of fixed (A) or accelerating (B) speed. (C) Mice were also test for motor performance in the pole test, where the time needed for each mouse to reorientate was recorded. (D) Ragtau exhibit significantly less anxiety in the elevated plus maze relative to transgenic and WT mice (p<0.05). (E) RagTau mice also exhibit significant cognitive deficits in the hippocampal-dependent novel arm y-maze in comparison to WT, Tau22, and RagWT groups. Data are represented as mean ± SEM. *indicates significance versus all groups, whereas ‘WT, RagTau’ indicates significant differences versus WT and RagTau groups with p<0.05 for both ANOVA and Fisher’s protected least-significant difference (PLSD) post hoc tests. n ≥ 10 mice/group.
Deletion of the adaptive immune system does not significantly affect tau pathology in RagTau mice.

After observing that RagTau mice exhibit behavioral impairments, I investigated whether tau pathology was also accordingly elevated in RagTau mice. I performed analysis of tau protein levels from 10-month WT, Tau22, RagWT and RagTau mice by western blot (Figure 26A, 27A). First, I measured total human tau levels in hippocampal soluble protein fractions of Tau22 and RagTau mice and observed no differences (Figure 26B, C)—confirming that transgene production of human tau was not altered by Rag2/IL2rγ knockout. Contrary to my expectations, however, RagTau mice did not exhibit significant elevation of phospho-tau epitopes compared to Tau22 mice (Figure 27D-G). While phospho-tau levels were expectedly elevated in Tau22 and RagTau mice compared to WT and RagWT mice (p<0.0001), tau hyperphosphorylation was only minimally and non-significantly increased in RagTau mice relative to Tau22 mice. Similarly, insoluble phospho-tau species was also not significantly different between Tau22 and RagTau mice (Figure 27). While the levels of phospho-tau in RagTau mice were uniformly increased relative to Tau22 mice, these difference failed to reach significance—suggesting that knockout of the adaptive immune system may only minimally effects tau pathology.
Figure 26. Hippocampal soluble tau phosphorylation is unaltered in RagTau mice. 

(A) Soluble protein fractions homogenized from micro-dissected hippocampi from 10-month old WT and transgenic mice were analyzed for total and phospho-tau levels by western blot. (B, C) Total human tau levels probed by antibodies IC9 (B, p = 0.60) and VH2-VL2 (C, p = 0.32) are equivalent between Tau22 and RagTau22 mice. (D-G) Phospho-tau levels probed by antibodies AT8 (E, p = 0.57), AT100 (E, p = 0.18), PS396 (F, p = 0.16) and PS404 (G, p = 0.21), also do not significantly differ between Tau22 and RagTau mice. Data are represented as mean ± SEM. Statistical analysis was carried out by ANOVA and Fisher’s protected least-significant difference (PLSD) post hoc tests. n ≥ 10 mice/group.
Figure 27. Hippocampal insoluble tau phosphorylation is not significantly altered in 
RagTau mice. (A) Hippocampal insoluble fractions from 10-month old mice were analyzed 
by western blot. (B-F) No significant differences were detected in insoluble tau species 
probed by PS199 (B, p = 0.87), PS202 (C, p = 0.71), AT100 (D, p = 0.69), PS396 (E, p = 0.95) 
and PS404 (F, p = 0.60). Data are represented as mean ± SEM. Statistical analysis was 
carried out by ANOVA and Fisher’s protected least-significant difference (PLSD) post hoc 
tests. n ≥ 10 mice/group.

RagTau exhibit increased microglial activation, but not astrogliosis.

Given that RagTau exhibit behavioral deficits without significant increases in tau 
pathology, I investigated whether changes in the innate immune system could potentially 
account for the cognitive deficits observed in RagTau mice. Recent evidence suggests that 
increased microglial activation correlates with reduced cognitive function and 
neurotransmitter metabolism in human AD patients [418]. Thus, I examined microglial 
activation in RagTau mice by confocal microscopy and IMARIS 3D-analysis. Quantification 
of microglial morphology by Iba-1 immunohistochemistry labeling revealed evidence of 
increased microglial activation in RagTau mice (Figure 28). RagTau microglia exhibited 
shorter process length compared to WT, Tau22 and RagWT microglia in hippocampus CA3.
(WT, Tau22, p<0.001; RagWT, p = 0.10), CA1 (p<0.05) and DG (WT, p<0.001; Tau22, p<0.05; RagWT, p = 0.35) (Figure 28A). RagTau microglia were also characterized by lower process branching numbers in CA3 (WT, Tau22, p<0.001; RagWT, p = 0.10), CA1 (p<0.05) and DG (WT, p<0.01; Tau22, p<0.05; RagWT, p = 0.21) (Figure 28B). Interestingly, RagWT microglia are also activated to a lesser extent compared to WT microglia (p<0.05), supporting evidence that the adaptive immune system can suppress microglial activation [394]. Additionally, while microglial activation is clearly increased in RagTau mice, microglial number is not as significantly elevated in hippocampus CA3 (WT, p<0.05; Tau22, p = 0.25; RagWT, p = 0.72), CA1 (WT, p = 0.11; Tau22, p = 0.67; RagWT, p = 0.33) or DG (WT, p = 0.41; Tau22, p = 0.53; RagWT, p = 0.32) (Figure 28C)—suggesting that the adaptive immune system plays a larger role in modulating microglial morphology and activation state rather than proliferation.

After observing that RagTau mice exhibit increased microglial activation, I examined whether astrogliosis was also elevated. I performed immunohistochemistry labeling of reactive astrocyte marker GFAP and IMARIS quantification of GFAP volume in hippocampal Z-stack images captured by confocal microscopy. Quantification of RagTau GFAP volume revealed in no significant differences between mice in hippocampus CA1 (WT, p = 0.31; Tau22, p = 0.62; RagWT, p = 0.30) (Figure 29). These findings therefore suggest that tau pathology and the adaptive immune system do not have a significant part in modulating astrogliosis in Tau22 and RagTau mice. It should be noted that while these results corroborate GFAP quantification of Tau22 microglia in my previous study of T5x mice (Figure 9), increased astrogliosis in T5x suggests that higher levels of tau pathology may still be able to influence astrocyte reactivity.
Figure 28. RagTau mice exhibit increased microglial activation. (A) Immunohistochemical labeling of microglia by Iba-1 was quantified using confocal microscopy and IMARIS software analysis. (B) RagTau microglia are significantly activated relative to WT and transgenic mice, displaying shorter process length and less process branching number in hippocampus CA3, CA1, and DG. (C) Microglia number is significantly elevated in RagWT mice, but not in RagTau mice. Data are represented as mean ± SEM. * indicates p<0.05 for both ANOVA and Fisher’s protected least-significant difference (PLSD) post hoc tests. n ≥ 5 mice/group.
Figure 29. RagTau mice exhibit no significant differences in astrogliosis. IMARIS quantification of GFAP labeling hippocampus CA1 revealed no significant changes in astrogliosis across WT, Tau22, RagWT and RagTau22 mice. Data are represented as mean ± SEM. Statistical analysis was calculated by ANOVA and Fisher’s protected least-significant difference (PLSD) post hoc tests. n ≥ 5 mice/group.

Adoptive transfer of bone marrow cells into RagTau mice restores adaptive immunity.

Given the non-significant trend toward increased tau pathology in RagTau mice we speculated that these subtle changes might occur because of small differences in the underlying background strain of these mice. Tau22 mice are maintained on C57/BL6 background, whereas the RagTau mice were generated by crossing purebred Tau22 mice with Rag2/il2rg knockouts that are maintained on a Bl6/B10 background. To address this concern, we restored the adaptive immune system in RagWT and RagTau mice and examined whether any changes in tau pathology could be detected. To achieve this, I performed adoptive transfer of bone marrow (BM) cells isolated from age-matched GFP+ mice into ~3-month old RagWT and RagTau mice. I chose to perform adoptive transfer on younger mice such that the influence of the adaptive immune system on the development of tau pathology could take effect before significant phospho-tau accumulation had begun. RagTau mice received retro-orbital injections of either GFP+ BM-cells or PBS-vehicle and
were bled 1-, 2- and 3-months after injection to confirm engraftment. Sorting of GFP+ cells from collected blood samples revealed that RagWT and RagTau mice receiving adoptive transfer exhibited successful engraftment of BM-cells that persisted until the 3-month experimental endpoint (Figure 30). Additionally, quantification of lymphocyte populations showed no significant differences in engraftment efficiency in RagTau mice compared to RagWT mice that received adoptive transfers (Figure 31).

**Figure 30.** Adoptive transfer of GFP+ bone marrow cells successfully engraft in RagTau mice. Flow cytometry sorting GFP+ cells from the blood of RagTau mice reveal successful engraftment of BM-cells 3-months after injection.
Engraftment of bone marrow cells is unaltered in RagTau mice compared to RagWT mice. Engraftment efficiency was measured by % of total CD45+ hematopoietic cells that were lymphocytes in the blood. Quantification at 1- (p = 0.98), 2- (p = 0.49), and 3 months (p = 0.26) after adoptive transfer revealed no significant differences in total lymphocyte numbers in RagTau mice relative to RagWT mice. Data are represented as mean ± SEM. Statistical analysis was calculated by student T-test. n ≥ 6 mice/group.

Bone marrow-derived cells infiltrate the brains of RagWT and RagTau mice.

3-months after adoptive transfer, RagWT and RagTau mice were perfused and their brains collected for subsequent examination. Initial analysis of coronal brain tissue sections revealed infiltrating GFP+ BM-cells predominantly within the amygdala and to a lesser extend the dentate gyrus of the hippocampus (Figure 32A). Next, we used immunohistochemistry to determine the phenotype of these infiltrating GFP cells. As I had previously shown that endogenous T-cells infiltrate the brains of Tau22 mice (Figure 17), I began by examining the pan T-cell marker CD3. Interestingly, immunohistochemistry staining confirmed that all infiltrating GFP+ cells were positive for CD3 (Figure 32B). Furthermore, labeling with CD4 revealed that a significant subset of these are CD4+ T-helper cells, (Figure 32C). These results corroborate my previous observations of
endogenous T-cell infiltration in the amygdala of Tau22 mice and support the hypothesis that tau pathology locally recruits T-cells into the brain.

Next, we examined whether tau pathology might influence the degree of GFP+ T-cell infiltration by quantifying the number of GFP+ cells within the dentate gyrus and amygdala (Figure 32D). GFP+ cell counts were relatively equivalent between RagWT and RagTau mice in hippocampus DG at rostral-caudal coordinates -1.58 (p = 0.33), -2.06 (p = 0.67), -2.70 (p = 0.86), and -3.00 (p = 0.36). In contrast, GFP+ cell counts were non-significantly greater in the amygdala of RagTau mice relative to RagWT mice at rostral-caudal coordinates -1.58 (p = 0.19), -2.06 (p = 0.10), -2.70 (p = 0.11), and -3.00 (p = 0.45). Although not significant, the trend toward increased GFP+ T-cell infiltration in the amygdala of RagTau mice relative to RagWT counterparts, combined with equivalent BM engraftment efficiency between both groups (Figure 31), suggests that tau pathology may promote the infiltration of adaptive immune cells into the brain.
Figure 32. Analysis of infiltrating bone marrow cells in the brains of RagWT and RagTau mice. (A) Post-mortem examination of brain tissue sections from RagWT and RagTau mice 3-months after adoptive transfer show GFP+ BM cells in the amygdala and hippocampus DG. (B) All GFP+ cells in the brains of RagWT and RagTau mice are positive for T-cell marker CD3+ by immunofluorescent staining. (C) Immunohistochemistry staining of CD4 reveal both GFP+/CD4- cells (white arrows) and GFP+/CD4+ (yellow arrows) cells, suggesting that some infiltrating BM-cells are helper T-cells. (D) Quantification of GFP+ cells from brain sections of several rostral-caudal coordinates (-1.58, -2.06, -2.70, -3.00) reveal minimal and similar GFP+ cell counts in hippocampus DG between RagWT and RagTau mice, and a greater, but non-significant increase in GFP+ cell numbers in the amygdala (Am) of RagTau mice relative to RagWT mice.

Restoring the adaptive immune system in RagTau mice does not significantly alter tau pathology.

After establishing that T-cells infiltrate the brain of RagWT and RagTau mice following adoptive transfer, I investigated whether these cells may influence tau pathology.
I examined soluble and insoluble amygdala and hippocampal protein fractions, the two areas that exhibit the greatest degree of T-cell infiltration, from RagTau mice that received either BM-cells or vehicle (Figure 33, 34). Analysis of soluble hippocampal fractions revealed no significant differences between RagTau Veh and RagTau BM groups when probed with phospho-tau antibodies PS199 (p = 0.68), PS202 (p = 0.29), AT100 (p = 0.27), PS396 (p = 0.48) and PS404 (p = 0.92) (Figure 33A). Similarly, insoluble hippocampal fractions also exhibited no significant differences between RagTau Veh and RagTau BM groups when probed with PS199 (p = 0.77), PS202 (p = 0.80), AT100 (p = 0.42), PS396 (p = 0.86) and PS404 (p = 0.83) (Figure 33B). I then examined soluble amygdala fractions and again observed no significant changes in PS199 (p = 0.24), PS202 (p = 0.28), AT100 (p = 0.35), PS396 (p = 0.64) and PS404 (p = 0.91) between RagTau Vehicle and RagTau BM groups (Figure 34A). Finally, I analyzed RagTau BM and RagTau vehicle insoluble amygdala fractions and again detected no significant differences in PS199 (p = 0.36), PS202 (p = 0.54), AT100 (p = 0.93), PS396 (p = 0.39) and PS404 (p = 0.48) (Figure 34B). In summary, adoptive transfer of BM-cells into RagTau mice does not significantly alter tau hyperphosphorylation, suggesting that the adaptive immune system does not have a major impact on tau pathology and further supporting the conclusions of our prior comparison between RagTau and Tau22 mice.
Figure 33. Restoring the adaptive immune system in RagTau mice has no effect on hippocampal phospho-tau. Western blot analysis was performed on hippocampal soluble (A) and insoluble (C) protein fractions. Although quantification of soluble (B) and insoluble (D) phospho-tau species confirmed an expected increase in tau phosphorylation between RagTau versus RagWT groups, there were no significant differences between RagTau mice that received BM-cells or vehicle. Data are represented as mean ± SEM. P>0.05 for ANOVA and Fisher’s protected least-significant difference (PLSD) post hoc tests. n ≥ 5 mice/group.
Figure 34. Restoring the adaptive immune system in RagTau mice does not significantly alter phospho-tau levels within the amygdala. Western blot analysis was performed on amygdala soluble (A) and insoluble (C) protein fractions. While quantification of soluble (B) phospho-tau species did not show significant changes in tau hyperphosphorylation, analysis of insoluble (D) amygdala fractions suggest a non-significant decrease in insoluble phospho-tau species in RagTau mice that received BM-cells relative to RagTau mice that received PBS vehicle. Data are represented as mean ± SEM. P>0.05 for ANOVA and Fisher’s protected least-significant difference (PLSD) post hoc tests. n ≥ 5 mice/group.
Microglial morphology is not significantly different between RagTau mice that received BM adoptive transfer versus vehicle.

Considering that RagTau that did not undergo adoptive transfer exhibit increased microglial activation compared to Tau22 mice, I wondered whether the lack of T-cells could be responsible for the changes observed in of RagTau microglia. I therefore performed immunofluorescent staining of Iba-1+ microglia and analyzed microglial morphology in the amygdala, the region of the brain exhibiting highest T-cell infiltration. Microglia in the amygdala of RagTau mice that received BM-cells appeared to potentially exhibit longer process lengths and increased branching than vehicle-injected RagTau mice. However, quantification of microglial process demonstrated that these measures were highly variable and non-significant (Figure 35A,B; p = 0.42 and 0.61). RagTau BM and vehicle mice also showed no difference in microglia number within the amygdala compared to RagTau mice that received vehicle (Figure 35C, p = 0.38).

![Figure 35](image_url)

**Figure 35.** Amygdala microglial activation in RagTau mice is not significantly altered by adoptive transfer of bone marrow cells. Immunofluorescent labeling of Iba-1 positive microglia and subsequent analysis and quantification by IMARIS software reveal no significant differences in microglial process length (A), branching (B), and microglia number (C) between RagTau mice that received BM-cells versus RagTau mice that received PBS vehicle. Data are represented as mean ± SEM. Statistical analysis was done by student T-test. n ≥ 8 mice/group.
Discussion

While there is emerging evidence that the adaptive immune system modulates Aβ in the context of AD, little is known about the role of the adaptive immune system in tau pathology. To study the effects of adaptive immunity on the pathological progression of tau, I created an immune deficient tau model by crossing Thy1-Tau22 mice with Rag2−/−/Il2rγ−/− mice. The resulting RagTau mice express mutant human tau and lack B-, T- and NK- cells. Interestingly, 9-month old RagTau mice exhibit increased anxiety and cognitive deficits relative to immune-intact Tau22 mice yet no significant changes in tau pathology. RagTau mice also exhibit elevated microglia activation without increased astrogliosis compared to WT, Tau22, and RagTau mice. To further investigate whether the adaptive immune system can modulate tau pathology I restored adaptive immunity in RagTau mice by adoptive transfer of GFP+ bone marrow cells. Immunohistological staining revealed that all infiltrating BM-cells were CD3+ T-cells and a subset were CD4+ helper T-cells. Although there was a trend toward increased GFP cell infiltration in RagTau versus RagWT mice, these differences failed to reach significance. Likewise, BM adoptive transfer did not significantly alter tau pathology—suggesting that the adaptive immune system does not play a significant role in modulating tau pathology.

The biggest remaining question to come out of this study is why RagTau mice exhibit increased anxiety and cognitive deficits despite a lack of changes in tau pathology. In respect to anxiety deficits, increased infiltration of T-cells into the amygdala of RagTau mice may provide a potential hint. There is for example emerging evidence that CD3+/CD4+ helper T-cells play a neuroprotective role in AD and contribute to the clearance of Aβ [391, 392]. Helper T-cells infiltrating the amygdala of Tau22 mice might
also attenuate tau-induced anxiety deficits and the lack of T-cells in RagTau mice may be the source of exacerbated RagTau anxiety deficits. To my knowledge, there has no previous evidence showing that T-cells can directly impact tau pathology, and my own findings in RagTau mice further support this. While the changes in tau pathology were negligible in RagTau mice with restored adaptive immunity, it is interesting that a non-significant trend toward reduced insoluble tau PS396/404 was observed in the amygdala (Figure 34D)—coincidentally the brain region with the greatest helper T-cell infiltration. Despite not significantly affecting tau pathology, infiltrating helper T-cells may still play a role in maintaining neural homeostasis in amygdala and normal anxiety behavior in the presence of tau pathology. Infiltrating T-cells have been previously shown to attenuate microglia activation via cytokine release [394]. The finding that RagTau mice, lacking T-cells, have increased hippocampal microgliosis relative to Tau22 mice and that RagTau mice receiving BM adoptive transfer exhibit infiltrating T-cells and a trend toward decreased microglial activation in the amygdala (Figure 35), suggests that T-cells may play a role in suppressing or modulating microglial activation. Thus, increased microglial activation in RagTau mice may contribute to neuronal dysfunction in the amygdala and explain the development of anxiety deficits in RagTau mice. It should also be noted that repeating the study with larger cohorts of mice would likely help to reduce the variability and uncover potentially significant findings regarding the potential interactions between T-cells, microglia, and anxiety in Tau22 mice. The hippocampal-dependent cognitive deficits that are exhibited by RagTau mice are less readily explained. Like amygdala tau pathology, hippocampal tau pathology is not significantly altered in RagTau mice relative to Tau22 mice. Thus the major differences observed between the hippocampi of RagTau and Tau22, increased
microglial activation, is also a probable contributor to hippocampal cognitive dysfunction. A recent study has shown that increased microglia activation correlates with decrease cognitive performance in human AD patients [419]. The remaining question is then why are RagTau hippocampal microglia increasingly activated? It may be tempting to suggest that the lack of infiltrating T-cells in RagTau mice (that did not receive adoptive BM transfer) is responsible for increased microglial activation, as supported by the trend toward decreases microglial activation in the amygdala of RagTau mice that received bone marrow transfer. However, comparatively minimal number of infiltrating T-cells in the hippocampi of RagTau mice that received BM transfer suggests that local microglial modulation by infiltrating T-cells is a less likely mechanism in the hippocampus. Instead, microglial activation in the hippocampi may be modulated more by peripheral T-cells rather than T-cells infiltrating into the hippocampus. Peripheral T-cells have been previously shown to release a number of different cytokines depending on the specific T-cell type [304, 420, 421]. Some cytokines released by T-cells can then cross the blood brain barrier and potentially modulate microglial activation [422-424]. Thus, to answer why hippocampal microglia exhibit increased activation despite the minimal amount of infiltrating T-cells into the hippocampus, future studies can examine peripheral and brain levels of T-cell produced cytokines in RagTau mice with and without adoptive transfer of BM-cells.

Another remaining question that arises from my findings is why T-cell infiltration is predominantly restricted to the amygdala of RagTau (Figure 32) and Tau22 (Figure 16) mice. One idea is that areas of dense tau pathology produce signals that attract and draw in T-cells into the brain. While this idea fits the amygdala, it does not explain why T-cell
infiltration is low in the hippocampus—another region of the brain that displays significant tau pathology. Alternatively, T-cells may be drawn into the brain not by tau, but by synuclein pathology. In Tau22 mice, α-syn pathology and inclusions are greatest in the amygdala and cortex (Figure 21), similar to human AD patients [131, 372]. It may be that accumulated α-syn pathology attracts T-cell infiltration, and the reason T-cells are seen in the amygdala and not the cortex of RagTau mice is because 1) amygdala α-syn pathology is much more dense while cortical α-syn is spread out, and 2) the amygdala lies proximal to the lateral ventricle where T-cells circulating in the CSF can enter through the choroid plexus [425]. Future studies can investigate whether α-syn pathology has an effect on T-cell infiltration by analyzing whether endogenous T-cell infiltration numbers in the amygdala of 5xFAD, Tau22 and T5x mice correlate with the extent of α-syn pathology in the amygdala of each genotype.

While my findings in RagTau mice suggest that T-cells are the major component of the adaptive immune system that interacts with AD, B-cells may also play a role in modulating tau pathology. A number of Tau active and passive vaccination studies in transgenic mice have previously been reported to effectively reduce tau pathology [426]. Thus, there is an argument that endogenous tau-specific antibodies could play a role in modulating AD pathology. While this possibility could hold some truth in human AD patients that exhibit elevated levels of phospho-tau circulating in the CSF [427-429], there is no evidence that B-cells produce tau-specific antibodies in our tau transgenic mouse models. B-cells isolated from the spleen of Tau and T5x mice exhibit no greater number of recombinant human tau-reactive B-cells relative to WT littermates (Figure 36). Although it is unlikely that B-cells may directly modulate tau pathology via production of tau-specific
antibodies, B-cells may indirectly influence the accumulation of tau via the production of unspecific IgG’s. As I previously discussed, our lab and others have shown that IgG’s can increased general microglia phagocytic activity and lead to increased microglial Aβ clearance [163, 430]. Thus, B-cell production of unspecific IgG’s may slow the progression of tau pathology either through the initial reduction of Aβ or through increased microglial phagocytosis and potential clearance of tau oligomers. In RagTau mice receiving BM adoptive transfer, this phenomenon is unlikely to be observed due to the fact that RagTau mice lack the amyloid pathology that contributes to IgG’s infiltration into the brain. Our lab and others have shown that amyloidgenic transgenic mice have significantly elevated levels of IgG in the brain, most likely due to amyloid-induced choroid plexus dysregulation [163, 332]. Thus, it would be useful to develop immune-deficient RagT5x expressing both amyloid and tau pathology to study whether unspecific IgG’s play a role in regulating tau pathology.

In summary, my findings in RagTau mice suggest that the adaptive immune system can modulate microglial activation, but does not play a significant role in tau pathology. Further studies are needed to elucidate whether modulation of microglia by the adaptive immune system in the presence of tau pathology can lead to cognitive dysfunction. Additionally, while the adaptive immune system does not alter tau pathology in RagTau mice, yet undiscovered roles for the adaptive immune system in tau pathology may be observed in transgenic models that include both amyloid and tau pathology. As the importance of the adaptive immune system in AD comes to light, new transgenic models revealing the mechanisms that drive immune response to AD pathology will prove to be invaluable.
Figure 36. Detection of B cells producing Aβ- and tau-specific antibodies by ELISPOT. 
(A) Sorting of CD19+/B220+ splenocytes reveal no significant differences in B-cell numbers between WT, 5xFAD, Tau22, and T5x mice. (B) Antibody-forming B cells (AFC) specific to Aβ42, Aβ40, full length human tau and Tau2-18 protein were detected in splenocytes by ELISPOT (Mabtech Inc, Cincinnati, OH) as described previously [163]. Splenocytes were incubated for 24 hours in 96-well plates coated with tau protein. After incubation the assay was performed as recommended by the manufacturer (Mabtech Inc, Cincinnati, OH). The number of mice used to isolate splenocytes from each genotype was as follows: WT (n = 2), 5xFAD (n = 2), Tau (n = 1), and T5x (n = 1).
Chapter Five: Reprogramming of induced pluripotent stem cells and transplantation of neural stem cells into immune deficient mice

Introduction

In addition to studying the effects of the adaptive immune system on tau pathology, RagTau mice also serve as an attractive model for studying the use of stem cell transplantation to treat AD. Our lab has previously established the use of mouse neural stem cells (mNSC) transplantation to ameliorate BDNF and cognitive deficits in 3xTgAD mice [218]. The study of human neural stem cell transplantation (hNSC) in transgenic mouse models, however, is greatly hindered by the complications that are involved with immunosuppression and xenotransplantation. For example, calcineurin inhibitors such as cyclosporine or FK506 can influence the hyperphosphorylated and accumulation of tau, inhibit tau-mediated tubulin polymerization, and increases phospho- and total tau levels in vitro [431-434].

To overcome these obstacles, I developed immune deficient RagTau mice that do not require immunosuppressants to achieve robust xenotransplantation and stem cell engraftment. I also reprogrammed induced pluripotent stem cells (iPSCs) from AD patient fibroblasts, differentiated them into NSCs, and transplanted these iPSC-derived NSCs (iPSC-NSC) into RagTau mice. By combining iPSC technology with the RagTau model, I was able to investigate the differentiation of human cells in an AD mouse model and determine whether tau pathology can propagate or spread to engrafted stem cells.

Methods

Human Patient Fibroblasts. Skin biopsy samples were taken from patient volunteers recruited through UC Irvine’s Alzheimer’s Disease Research Center longitudinal cohort.
Patients are clinically diagnosed as unaffected, qualitative cognitive impairment (QCI), mild cognitive impairment (MCI), or probable AD at the time of sample collection. Skin biopsy samples are about 0.25 to 0.5 cm in diameter and are immediately placed on ice in fibroblast media (DMEM+ 1% glutamax + 1% anti anti media). Skin biopsy samples are transported from the collection center to the hood and transport equipment is sterilized to minimize chance of contamination. 1 hour prior to skin biopsy collection, one 6-well plate per patient sample is coated with 0.1% gelatin and left in a 37 °C incubator. Skin biopsy samples are then dissected in the hood to about 10-12 pieces and plated in the 6-well plate at one to two pieces per well. After biopsy pieces are plated, 500 μL of fibroblast media is very slowly added to each well. 24 hours after plating, an additional 1 mL of media is added every 48 hours for one week. During the first 48-72 hours after plating karyocytes will begin to grow out from attached biopsy pieces. After 72 hours, fibroblasts will then expand out from the karyocyte later and begin to grow throughout the well. Once each well is confluent, fibroblasts were passaged using 0.05% trypsin and replated in T75 flasks for further expansion.

**iPSC reprogramming using Cytotune 2.0 Sendai Reprogramming Kit.**

**iPSC reprogramming of patient fibroblasts.** The following protocol is adapted from the Cytotune 2.0 Sendai Reprogramming Guide from Life Technologies. One day before transduction human fibroblasts are plated onto two wells in a 6-well plate at 3 x 10^5 cells/well. One well should be trypsinized on the day of transduction and used to check for proper cell density. Using the formula bellow and the virus titer given with the Certificate of Analysis for the viral lot#, calculate how much virus to add to the appropriate amount of
fibroblast media. One the day of transduction, warm 1ml of fibroblast media in the dry bath and remove the viral vectors form the -80 degree C freezer. Viral vectors should be thawed at room temperature and kept on ice after being thawed.

\[
\text{Volume of virus (µL)} = \frac{\text{MOI (CIU/cell)} \times \text{number of cells}}{\text{titer of virus (CIU/mL)} \times 10^{-3} (µL/mL)}
\]

Sendai 2.0 has three viral vectors: hKlf4, hc-Myc, and KOS (vector with Sox2 and Oct4 combined). Transduce each viral vector at the following MOIs: KOS=5; hc-Myc=5; hKlf4=3. The MOI’s of each viral vector can be reduced by half and still generate an ample amount of iPSC colonies. Remove one set of CytoTuneR 2.0 Sendai tubes from the −80°C storage. Aspirate out the media from the wells designated for transduction and add 1mL of fresh fibroblast media containing the viral vectors to each well. 24 hours after transduction replace the media with fresh Fibroblast media. Change media every day with fresh fibroblast media for the next 6 days.

**Feeder Dependent reprogramming.** Six days after transduction, plate irradiated mouse embryonic fibroblasts (MEFs) onto 0.1% gelatin coated 6-well plates at a density of 2.5 x 10⁴ cells/cm². On the seventh day after transduction remove reprogrammed cells from 6-well plates with 0.05% trypsin and plate onto MEFs at a cell density of ~1 x 10⁵ cell/well in fresh fibroblast media. 24 hours later change media with fresh iPSC media. Change media every day for the next three to four weeks and observe regularly for formation of potential iPSC colonies to pick. Depending on the quality of MEFs, the MEFs may begin to die off.
before three to four weeks of reprogramming has occurred. In this scenario, supplement iPSC media with MEF conditioned media at a 1:1 ratio to keep colonies at a higher viability.

**Feeder Independent reprogramming.** Six days after transduction, coat 6-well plates with Vitronectin or Geltrex. On the seventh day after transduction remove reprogrammed cells from 6-well plates with 0.05% trypsin and plate onto coated plates at a cell density of ~1 x 10^5 cell/well in fresh fibroblast media. 24 hours later change media with fresh Essential 8 media. Change media every day for the next three to four weeks and observe regularly for formation of potential iPSC colonies to pick.

**Live Staining of iPSC colonies.** For identification and confirmation of pluripotent iPSC colonies to pick for expansion live staining is performed using mouse Anti-Tra1-60 antibody. Before starting, make sure antibodies are sterile and filtered to prevent contamination. Wash iPSC colonies with KO DMEM/F12. Add Tra1-60 antibody at a 1:1000 dilution and incubate at 37 degrees for 60 minutes. Aspirate primary antibody solution and wash 3x with KO DMEM/F12. Add anti-mouse Alexa555 secondary antibody solution to the cells at a 1:500 dilution. Incubate cells in secondary antibody for 60 minutes then wash cells 3x with KO DMEM/F12. Visualize cells under a fluorescent microscope to check for positively stained cells. If live staining is used for picking colonies circle colony locations with a pen on the bottom of the plate.

**iPSC expansion, characterization, and storage.** After picking iPSC colonies, transfer colonies on matrigel coated plates and continue to feed with Essential 8 media. Continue to
monitor colonies without maintenance for the first five passages. Once colonies have grown large enough to start touching and fusing together, passage colonies using collagenase or accutase and expand colonies into additional plates. Some colonies will begin to die off or spontaneous differentiate so be sure to start with at least 20 colonies when initially expanding. Once surviving colonies have reached passage 6, choose five iPSC clones that have the best iPSC morphology and continue expanding while removing spontaneous differentiation. After additional passages, choose three final clones from the five previously selected and characterize the iPSCs for pluripotency and karyotype. Pluripotency characterization was done through Oct4/Sox2 immunocytochemistry staining, and RT-qPCR pluripotency Scorecard assay (ThermoFisher). Briefly, undifferentiated iPSCs or iPSC embryo bodies (EB) were pelleted, RNA was isolated, and cDNA synthesized before loading onto Taqman hPSC Scorecard Panels. Once loaded, Scorecards were then run on a ViiA7 Real-Time qPCR system (Applied Biosystems). After the RT-qPCR run was completed, qPCR run data files were loaded onto the Scorecard website and analyzed for pluripotency. iPSC lines are frozen in Bambanker freezing media and stored at -150 degrees for long-term storage.

**NSC Differentiation.**

**Neural induction.** NSC differentiation was achieved using PSC Neural Induction Medium (Gibco). iPSCs colonies are first plated in feeder-free matrigel coated 6-well plates at a cell concentration of 3 x 10^5 cells per well or at a 15-25% confluence. 24 hours after plating iPSC colonies, switch media to neural induction media to begin neural induction. Add neural induction media every 48 hours for 6 days. On day 4, cells should be 70-80%
confluent. Before cells reach 100% confluency, remove any iPSC colonies that remain undifferentiated by day 4. By the end of day 6, the well should be 100% confluent. On day 7 after neural induction passage cells onto geltrex coated 6-well plates using accutase and switch to neural expansion media with rock inhibitor. 24 hours after passaging switch to neural expansion media without rock inhibitor. At this point cells should begin to grow showing mixed or partially reprogrammed morphology and multiple expansions are necessary to achieve homogenous NSC morphology. Once cells reach near 100% confluence, passage cells onto poly-ornithine/laminin coated plates and continue feeding with either neural expansion media or NSC media.

**Terminal neural differentiation.** Plate NSCs in poly-ornithine/laminin coated plates at a 70-80% confluence. For neuronal and astrocyte differentiation switch to neuronal and astrocyte differentiation media respectively (media detailed on page 12). The first week of differentiation will result in a significant amount of cell death so switch media daily to remove cell debris. After the first week of differentiation change the media every other day for 2-3 weeks while observing change in morphology. Cells can then be fixed in paraformaldehyde (PFA) and stained for NSC markers such as Sox2 and Nestin.

**Immunocytochemistry staining.** Cell cultures were first washed 3x with PBS before being fixed in 4% PFA for 15 minutes at room temperature. Following fixation, cells are rinsed 3x with PBS for 5 minutes per wash. Cells are blocked in PBS + 0.1% Triton-X plus 5% donkey or goat serum for one hour. Cells were then kept in primary antibody (at a stand 1:1000 dilution) overnight at 4 degrees C. The following day, cells are washed 3x with PBS for 5
min per wash and then placed in secondary antibody (diluted 1:500 in PBS) for 1 hour. After three more 5 min washes with PBS cells are then visualized under a fluorescent microscope. Primary antibodies used included: Sox2 (Millipore; 1:1000) and Nestin (1:500; Millipore) as a neural stem cell markers.

**Stereotactic Surgery.** 9 month old RagTau mice were transplanted with IPSC-NSCs. Cells are resuspended in PBS + FGF/EGF (20 μg/mL), counted using BioRad Automated Cell Counter and kept on ice for the duration of surgery. 100,000 cells per site will be transplanted bilaterally into the hippocampus (Bregma AP: -2.06; ML: +/- 1.75; DV: -1.95). Mice are anesthetized under isoflurane and placed on the stereotax. Cells are injected at a rate of 0.5 μL/min with a 5 μL Hamilton mmicrosyringe (33-gauge).

**Generation of RagTau mice.** Thy-Tau22 (Tau22) mice express human 4 repeat tau with two frontotemporal dementia-associated point mutations (G272V and P301S) under control of the neuronal driven promoter Thy1.2 and are maintained on a C57Bl6/J background [149]. Rag2−/−/Il2rγ−/− mice are maintained on a C57/BL6/Bl10 background and exhibit constitutive knockout of recombinase activating gene 2 (Rag2) and interleukin 2 (IL2) receptor gamma, resulting in complete ablation of B-, T- and natural killer (NK) Cells [154-157]. Heterozygous Thy-Tau22 and homozygous Rag2−/−/Il2rγ−/− mice were crossed and then backcrossed over multiple generations to create Tau22-Rag2−/−/Il2rγ−/− (RagTau) mice, as well as WT-Rag2−/−/Il2rγ−/− (RagWT) littermates. All mice were maintained on a purebred C57Bl/6 background and group housed (2-4 mice/cage) on a 12h/12h light/dark cycle with access to food and water ad libitum. All animal procedures
were performed in strict accordance to the National Institutes of Health and University of California Institutional Animal Care and Use Committee.

**Tissue preparation and Neuropathological analysis.** Following behavioral testing, all mice were sedated with euthasol and sacrificed by cardiac perfusion with 0.1M phosphate buffered saline (PBS). Brains were removed and one hemisphere was snap frozen on dry ice while the other hemisphere was postfixed in 4% paraformaldehyde for 48 hours then stored in PBS + 0.05% sodium azide. Fixed half-brains were placed in 30% sucrose for at least 48 hours before being cut in the coronal plane (40 µm sections) using a freezing sliding microtome.

**Immunohistological staining.** Brain sections were rinsed in PBS three times and blocked in PBS+0.05% Triton-X with 5% donkey or goat serum for one hour. Primary antibodies used included: DCX (1:500; Abcam) a immature neurons, Nestin (1:500; Millipore) a neural stem cells, NeuN (1:500; Abcam) a nuclear neuronal marker, MC-1 (1:1000; Cotman Lab) a misfolded/pathological tau, and human cell markers SC121 (1:500; Stem Cells Inc) and Ku80 (1:500; Abcam). Sections were incubated in primary antibodies at 4 degrees Celsius overnight. Sections were then washed three times with PBS and incubated with appropriate Alexa fluor-conjugated secondary antibodies at room temperature for one hour. Sections were then rinsed three additional times, mounted on slides and coverslipped with Fluoromount-G with DAPI.
Results

**AD patient fibroblasts can be reprogrammed into induced pluripotent stem cells.**

Human patient skin biopsies were collected from probable AD patients recruited by UC Irvine’s Alzheimer’s Disease Research Center (ADRC). Biopsies were then plated down in fibroblast media, expanded, and fibroblasts were passaged multiple times to achieve a homogenous population. After several passages, fibroblasts were then either replated with irradiated, non-proliferating mouse embryonic stem cells (MEFs) into fibronectin-coated 6-well plates or replated in feeder-free conditions and grown to ~90% confluence. To initiate iPSC reprogramming, we applied Cytotune 2.0 sendai viruses expressing Oct4, Klf4, Sox2, and c-Myc (Day 0). One day after adding Sendai viruses, cells began to exhibit initial signs of reprogramming and become more punctate, characterized by shrinking cell bodies (Figure 37A). The first iPSC colonies begin to form by Day 8 (Figure 37B), and expand and round out in shape by Day 20 (Figure 37C). As iPSC colonies continued to grow, non-reprogrammed human fibroblasts began to die off and detach from the bottom of the plate. In non-feeder free condition, if MEFs began to prematurely die reprogramming MEF media was supplemented with feeder-free Tesr-8 media (StemCells Technologies). By Day 21, iPSC colonies could be live-stained in culture for pluripotency marker Tra1-60 (Figure 37D). Pluripotent colonies identified by Tra1-60 staining were then picked, transferred to new cell culture vessels, and adapted to feeder-free conditions. During expansion and passaging of newly reprogrammed iPSC lines, differentiating iPSCs were manually removed to prevent spontaneous differentiation. After several passages, stable iPSC lines exhibited reduced spontaneous differentiation and iPSC colonies were smoothly rounded around the parameter (Figure 37E).
Stable iPSC lines were characterized and confirmed for pluripotency. First, iPSCs underwent immunocytochemistry labeling and stained positive for proliferative marker Sox2 and pluripotency marker Oct4 (Figure 37F). Next, iPSC lines were analyzed using the human pluripotent stem cell (hPSC) Scorecard panel. RNA was first isolated from either undifferentiated iPSCs or embryo bodies (EB) formed from iPSC lines. cDNA was then synthesized from RNA samples and loaded onto Scorecard panels for RT-qPCR. Data files from each RT-qPCR run were subsequently uploaded online and analyzed by Scorecard analysis software. Analysis of undifferentiated iPSCs (Figure 38A, B, D) and respective EB’s (Figure 38C) confirm pluripotency in patient iPSC lines.

**Patient iPSCs are differentiated into multipotent neural stem cells.**

To differentiate iPSCs into NSCs, iPSCs feeder-free adapted iPSCs were seeded onto Matrigel coated plates at low density (Figure 39A). Thereafter, iPSCs were switched into GIBCO Neural Induction Medium to initiate neural differentiation. By Day 5 following addition of neural induction media, iPSCs begin to exhibit signs of differentiation and exhibit heterozygous cell populations (Figure 38B). By 7 days after initial differentiation, iPSCs grew to 100% confluence and were replated into Geltrex-coated vessels (Figure 39C). Upon growing to 100% confluence again, cells were then transferred onto Poly-Ornithine/Laminin (PO/Lam). Cells initially plated on PO/Lam retain some undifferentiated morphology (Figure 39D), but after several passages NSC populations become homogenous and lose undifferentiated characteristics (Figure 39E). To accelerate purification of NSC cultures, NSCs were maintained in alternating passages either as a monolayer on PO/Lam or as free-floating neurospheres. Following multiple passages,
neural differentiation of NSCs was confirmed by immunofluorescent staining of NSC markers Sox2 and Nestin (Figure 37G). Finally, multipotency of NSCs was examined by further neuronal (Figure 37H) and astrocyte (Figure 37I) differentiation.

*iPSC-derived NSCs intracranially injected into RagTau mice survive for at least 3 months after transplantation.*

To test whether immune deficient RagTau mice could support long-term transplantation of human brain cells, I transplanted iPSC-derived NSCS (iPSC-NSC) into the hippocampi of RagTau mice. After stereotaxic injection of iPSC-NSCs, I waited 3 months before perfusing mice and extracting brains for analysis. I performed immunohistochemistry labeling of RagTau brain sections using SC121, a human cytoplasmic marker, to identify transplanted iPSC-NSCs (Figure 40A). I observed significant masses of iPSC-NSCs located proximal to initial injections sites, suggesting that transplanted iPSC-NSCs have no issues surviving in RagTau mice. Additionally, while iPSC-NSCs seem to grow and extend out multiple processes, they exhibit very little migration. Furthermore, positive staining of iPSC-NSCs by neuronal nuclear marker NeuN and immature neuronal marker DCX suggest that iPSC-NSCs lean towards a neuronal lineage, but undergo minimal differentiation (Figure 40A).

I was also curious whether tau pathology in RagTau mice could spread into transplanted iPSC-NSCs. Previous studies have suggested that tau pathology accumulates and propagates via prion-like mechanisms in AD [90, 435, 436]. To examine whether tau pathology propagated into iPSC-NCSs residing in RagTau mice, I performed immunohistological labeling of iPSC-NSCs and tau pathology (Figure 40B). iPSC-NSCs
identified by human nuclear marker Ku80 and neuronal marker NeuN did not exhibit any colocalized labeling of pathological tau marker MC-1 despite significant tau pathology in the hippocampus. Thus, it does not appear that tau pathology propagates into transplanted iPSC-NSCs nor inhibit iPSC-NSC survival.
Figure 37. AD patient fibroblasts are reprogrammed in iPSCs and then differentiated into multipotent NSCs. Cytotune 2.0 iPSC reprogramming sendai viruses were added to AD patient fibroblast in feeder free conditions (Day 0). (A) One day after addition of reprogramming factors (Day 1), cells begin to exhibit signs of reprogramming and shrink in size. (B) On Day 8, the first iPSC colonies begin to form over the underlying fibroblast monolayer. (C) By Day 20, iPSC colonies have expanded and rounded out. Non-reprogrammed fibroblasts have also died off by this time. (D) iPSC colonies that have formed 3 weeks after initial reprogramming stain positive for extracellular pluripotency marker Tra1-60. (E) Tra1-60+ iPSC colonies are then picked and expanded over multiple passages. (F) Immunofluorescent labeling of stable iPSC lines stain positive for pluripotency markers Oct4 and Sox2. (G) iPSCs differentiated into NSCs express NSC markers Sox2 and Nestin. (H, I) iPSC-derived NSCs are multipotent and can differentiate into neurons (H) and astrocytes (I).
**Figure 38. hPSC Scorecard panels confirm pluripotency in reprogrammed iPSC lines.** After RNA was isolated and cDNA synthesized from either human undifferentiated iPSCs or iPSC EB’s, cDNA samples were run on hPSC Scorecard panels and analyzed using ThermoFisher’s Scorecard webtool. Algorithms analyzing system genetics determined whether samples tested positive for self-renewal and ectoderm, mesoderm and endoderm differentiation. Reprogrammed undifferentiated iPSC samples (A, B, D) tested positive for self-renewal without significant signs of differentiation. In contrast, EB’s formed for reprogrammed iPSC lines (C) tested positive for ectodermal, mesodermal, and endodermal differentiation, confirming iPSC pluripotency.
Figure 39. Neural differentiation of iPSCs. (A) Feeder-free adapted iPSCs were plated at low density on Matrigel and switched into GIBCO Neural Induction Medium (Day 0). (B) After 5 days in neural induction media, iPSCs exhibit signs of differentiation into dermal lineages. (C) By Day 7, cells reach 100% confluence and exhibit robust differentiation. (D) Cells replated onto Poly-Ornithine/Laminin on Day 8 exhibit both NSC and undifferentiated morphologies. (E) After multiple passages, NSCs lose undifferentiated characteristics and achieve a homogenous cell population. Scale bars represented 1000 microns (A-C) and 5000 microns (D,E).
Figure 40. iPSC-derived NSCs transplanted into the hippocampi of RagTau persist three months after transplantation without immunosuppression. (A) Human iPSC-NSCs are transplanted into RagTau hippocampi and survive 3-months after transplantation. iPSCs labeled by human cytoplasmic marker SC121 (Blue) exhibit limited neuronal differentiation and express neuronal markers DCX (Red) and NeuN (Green). (B) iPSC-NSCs persist in the presence of tau pathology and do not exhibit evidence of propagation of tau pathology into human cells. iPSC-NSCs were identified with human nuclear cell marker Ku80 (Green) and neuronal marker NeuN (purple), and misfolded, pathological tau was labeled by MC1 (Red).

Discussion

When our lab first decided to pursue the research of iPSC reprogramming in the context of AD, iPSC technology was still in its infancy. Through multiple efforts of trial-and-error, I was able to optimize and establish an iPSC protocol for the reprogramming of human AD patient fibroblasts. To fully utilize AD iPSCs for AD research, I also optimized a protocol for differentiating iPSCs into NSCs. I was then able to transplant iPSC-NSCs into RagTau mice and establish that RagTau mice are a viable model for the study of long-term NSC transplantation in AD.

The development of AD iPSC lines opens up a number of unique opportunities to study and model AD in vitro. Previous studies have proven that AD iPSCs and iPSC-differentiated
neurons can recapitulate multiple facets of AD pathology [211-213, 437, 438]. By exhibiting AD-related phenotypes, AD iPSC-derived cells can also serve as an attractive and viable cell culture model for the testing of therapeutic drugs for AD [213]. Although iPSC reprogramming of fAD patient cells can recapitulate AD pathology and serve as a cell culture version of AD transgenic mouse carrying fAD mutations [437, 438], AD iPSCs also have the unique advantage of modeling and studying the more complex genetic differences involved in sAD [211, 213, 439, 440]. The inherited genetic backgrounds and signatures in AD iPSCs allows for future studies of how relevant AD mutations may affect functional and pathological aspects of different cell types. For example, the study of iPSC-differentiated microglia from patients exhibiting mutations in AD-associated microglial gene TREM2 [441]; or the study of iPSC-differentiated astrocytes from patients carrying the APOE4/4 alleles, the greatest known risk factor for AD [205], both projects now being pursued in our lab. In summary, AD iPSCs and iPSC-differentiated cell types may pave the way for exciting new discoveries in AD research.

Our lab has previously shown that transplantation of mouse neural stem cells in 3xTgAD mice can rescue cognitive deficits [218]. The next logical step for translating NSC transplantation as a therapy for AD is the study of human NSC transplantation in transgenic AD mice. The development of immune deficient RagTau mice now presents a unique opportunity to study the effects of xenotransplantation on tau pathology without the mitigating off targets effects of immunosuppression. Additionally, my findings also highlight the importance of selecting the appropriate neural cell type for transplantation. In different models of neurodegenerative disease, the rescue of cognitive deficits has coincided with the migration and terminal differentiation of transplanted mouse NSCs.
In my study, however, transplanted human iPSC-NSCs fail to migrate and exhibit minimal signs of terminal differentiation (Figure 40). It may be that while mouse NSCs respond and differentiate in response to extrinsic signals within the mouse brain, human NSCs respond less favorably in RagTau mice and lack the appropriate signaling for migration and terminal differentiation. Thus, for the study of human neural cell transplantation in the treatment of AD, it may be more beneficial to choose further differentiated neuronal or glial progenitor cells for transplantation. Lastly, RagTau mice may also be utilized to study the effects of transplantation of other human cells types, such as microglia or macrophages, on tau pathology.

In conclusion, I have established protocols for AD iPSC reprogramming and NSC differentiation that allow for future modeling and studies of AD in cell culture. The successful long-term transplantation of iPSC-NSCs in RagTau also suggest that RagTau mice can be used in future studies to test whether human cell transplantation can target tau pathology and ameliorate AD symptoms. I hope that by establishing novel cell culture and animal models of AD, we can better elucidate the underlying mechanisms that drive AD.
Chapter Six: DNA immunization with tau epitope vaccine induced a strong immune responses and reduced tau pathology in THY-Tau22 mice

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Abstract

Background: By the time clinical symptoms of Alzheimer’s disease (AD) manifest in patients there is already substantial tau pathology in the brain. There is evidence that Tau pathology may also become self-propagating. We therefore propose using tau immunotherapy as a potential treatment for AD. Here we tested the immunological and therapeutic potency of the first DNA anti-tau vaccine, AV-1980D based on the highly immunogenic and universal MultiTEP platform technology. Methods: Beginning at three months of age, THY-Tau22 mice were injected intramuscularly with AV-1980D vaccine targeting tau epitope spanning aa2-18. To enhance the transduction of muscle cells with the DNA vaccine we used an AgilPulse™ electroporation system. Humoral and cellular immune responses in vaccinated animals were then analyzed by ELISA and ELISpot, respectively. Six months later, neuropathological changes in the brains of experimental and control mice were examined using biochemical (WB) and immunohistochemical (IHC) methods. Results: Electroporation-mediated AV-1980D vaccinations of THY-Tau22 did not induce potentially harmful autoreactive Th cells responses specific to endogenous tau species. Instead, vaccination strongly activated Th cells specific to the MultiTEP vaccine platform and triggered a robust humoral immunity response specific to human tau. The maximum titers of anti-tau antibodies were reached after two immunizations and remained slightly lower, but steady during five subsequent monthly immunizations. DNA vaccinations significantly reduced total tau levels in the brains extracts of vaccinated mice and reduce to some extent levels of phosphorylated tau detected in soluble and insoluble fractions, as well as in brain sections. The binding of these antibodies to tau in human AD brain sections suggests that this approach could be translated toward potential clinical
testing. **Conclusions:** Immunological data generated in THY-Tau22 mice and other tau/Tg animals (PS19 and Tg4510) demonstrated that MultiTEP-based epitope vaccine is highly immunogenic in several mouse models of tauopathy. Anti-tau antibodies generated with this approach are therapeutically potent and can reduce tau deposition *in vivo.*

1. Introduction

Alzheimer’s disease (AD) is a devastating neurodegenerative disease and the most common form of dementia[443], AD symptoms manifest in cognitive, memory, and functional impairments[444]. Neuropathological features of AD include deposition of the amyloid-β (Aβ) fragment of amyloid precursor protein (APP) in senile plaques, accumulation of neurofibrillary tangles (NFT) composed of tau protein, and death of neurons[445-449]. Although Aβ may be the primary initiator of AD pathogenesis, it is clear that pathological tau also plays a critical role in AD[450]. Importantly, by the time clinical signs of AD appear there is already substantial tau pathology in the brain[451, 452], which may become self-propagating[88, 453-455]. Although tau immunotherapy may not be as advanced as Aβ-targeted therapy, preclinical data supports its development toward clinical trials. In several mouse models, tau immunotherapy has effectively targeted various neurotoxic tau species such as misfolded tau, tau oligomers and phosphoepitopes[456, 457]. In these previous studies, various antibodies have been examined using passive immunotherapy and a reduction in tau pathology in several mouse models has been observed including: MC1 (conformation specific) and PHF1 (pS396/S404) antibodies in JNPL3 and P301S mice[458-461], DA31 (pan-tau) antibody in JNPL3 mice[461], range of anti-tau monoclonal antibodies in P301S mice[454, 462], tau oligomer-specific monoclonal
antibody (TOMA) in JNPL3 mice[463], AT8 (pS202/T205) antibody in 3xTg-AD mice [464], Ta1505 (pS413) antibody in tau609 and tau784 mice [465]. While vaccination with full-length tau caused encephalitis[466], several tau short peptides were also evaluated in tau transgenic mice using active immunization approaches, and showed efficacy by preventing pathology in the following mice: Tau379-408[pS396/S404] peptide in JNPL3 mice[467] and htau/PS1 M146L mice[468], Tau395-406[pS396/S404] peptide in P301L pR5 mice[469], Tau195-213[pS202/T205], Tau207-220[pT212/S214] and Tau221-231[pT231] peptides in K257T/P301S mice[470], Y10A[pS422] peptide in THY-Tau22 mice[471], pBri peptide in 3xTg-AD mice[472].

To develop a DNA vaccine targeting pathological tau we decided to use our immunogenic MultiTEP platform incorporating the tau2-18 epitope, AV-1980D. We chose this epitope because it was previously shown that tau2-18 is normally hidden in microtubule bound tau conformation but becomes highly exposed during tau aggregation[473, 474] and that truncation of the N-terminal region of tau may remove a toxic region and have a neuroprotective role[475]. To improve the in vivo uptake and expression of our DNA vaccine and to induce a robust immune response, AgilePulse™ electroporation device was used. In this report, THY-Tau22 mice were immunized with AV-1980D epitope vaccine in order to evaluate tau active immunotherapy.

2. Materials and methods

2.1. Mice

In this study we have used heterozygous female THY-Tau22 mice[476]. Mice were anesthetized with 4% isoflurane (Vedco, Inc., St. Joseph, MO) and maintained in 3-3.5%
isoflurane during all injections. All animals were housed in a temperature and light-cycle controlled facility, and their care was under the guidelines of the National Institutes of Health and an approved IACUC protocol at University of California, Irvine.

2.2. Antigen

The AV-1980D vaccine, composed of small B-cell epitopes of tau protein (tau2-18) linked to the MultiTEP platform, consisting of a string of twelve foreign Th epitopes was generated as described below. A polynucleotide encoding three copies of tau2-18 epitope separated by GS linkers was synthesized by GenScript (Piscataway, NJ) and subcloned with MultiTEP minigen (from AV-1959D construct[477]) using a restriction sites. The resulting gene was cloned into the pVAX1 vector (Invitrogen, Carlsbad, CA)[478], that was designed to be coherent with current Food and Drug Administration (FDA) guidelines, using Nhel/XhoI restriction sites. DNA sequencing was performed to confirm that the generated plasmids contained correct sequences. Plasmid was designated as AV-1980D. Map of AV-1980D is presented in Fig. 1A. Plasmids were prepared and purified by Aldevron (Fargo, ND). The expression of the plasmid was detected in transfected CHO cells by western blot (WB) using anti-tau 1C9 monoclonal antibody (generated at The Institute for Molecular Medicine, Huntington Beach, CA, Fig. 41B).

2.3. Immunizations

Female, three months old THY-Tau22 mice (n=6) were injected into both tibialis anterior muscles with 40µg (20µg per leg) of AV-1980D vaccine. The EP pulses were applied using the AgilPulse™ device from BTX Harvard Apparatus (Holliston, MA) as
described previously[479]. Control mice (n=7) were injected with pMultiTEP plasmid. Two vaccinations were performed with a 2-week interval and continued with monthly immunizations (total 7). On day 12 after each immunization (starting from second one) blood was collected for analysis of anti-tau antibody. Mice were terminated at age 9 months old for further neuropathological analysis. More detailed experimental protocols are provided in Fig. 1C.

2.4. Detection of tau-specific antibodies and isotyping

The concentrations of anti-tau antibodies in mouse sera were determined by ELISA as we described previously[480]. Anti-tau antibody concentrations were calculated using a calibration curve generated with 1C9 mAb. HRP-conjugated anti-IgG1, IgG2a, IgG2b and IgM specific antibodies (Bethyl Laboratories, Inc., Montgomery, TX) were used to characterize the isotype profiles of anti-tau antibodies. The isotypes of antibodies were detected in individual sera of mice at dilution 1:800.

2.5. Detection of IFN-γ producing splenocytes

Analysis of IFN-γ producing T helper (Th) cells was performed in splenocyte cultures from immunized mice by ELISpot assay (BD Biosciences, San Jose, CA), as previously described[477, 481-483]. Cultures of splenocytes were re-stimulated in vitro with 10µg/ml of individual peptides representing epitopes from the MultiTEP platform, tau2-18 or irrelevant peptides (GenScript, Piscataway, NJ) for 20 hours. The numbers of SFC per 10^6 splenocytes stimulated with Th peptides or tau2-18, were then counted.
2.6. Detection of tau tangles in human brain tissues by IHC

Sera from mice immunized with AV-1980D and pMultiTEP were screened for the ability to bind to tau tangles using 50 μm brain sections of formalin-fixed cortical tissues from severe AD case (received from Brain Bank and Tissue Repository, MIND, UC Irvine) using immunohistochemistry as described previously[477, 482, 483]. A digital camera (Olympus, Tokyo, Japan) was used to capture images of the plaques at 60x magnification.

2.7. Biochemical Analyses

Right hemispheres, previously frozen on dry ice and stored at −80 °C, were crushed on dry ice using mortar and pestle, then homogenized in solution of T-PER (Thermo Scientific, Waltham, MA) and phosphatase and protease inhibitor mixtures (Thermo Scientific, Waltham, MA and Roche, San Francisco, CA) and processed as previously described[484, 485]. Soluble and insoluble SDS-page Western blot was performed following standard protocols as previously described [485]. Primary antibodies used for Western blot analysis included the following: 1C9 (IMM, Huntington Beach, CA), AT180 (pT231), anti-tau (pS404), anti-tau (pS199), anti-tau (pS396) (all from Abcam, Cambridge, United Kingdom), HT7, AT-270 (pT181), AT8 (pS202/T205), anti-tau (pS214), AT100 (pT212/S214), anti-tau (pT212), anti-tau (pS396/S404) (all from ThermoFisher Scientific, Waltham, MA), anti-tau (pS422; WuXi AppTec, San Diego, CA). All blot membranes with soluble samples were also labeled with anti-GAPDH antibodies (ThermoFisher Scientific, Waltham, MA) as loading control.
2.8. Immunohistochemistry, Confocal Microscopy and Quantitative Analysis

Following perfusion, one hemisphere from each mouse was postfixied in 4% paraformaldehyde for 48 hours then stored in PBS + 0.05% sodium azide. Fixed half-brains were placed in 30% sucrose for at least 48 hours before being cut in the coronal plane (40 µm sections) using a freezing sliding microtome. Brain sections were rinsed in PBS before blocking in PBS+0.05% Triton-X with 5% donkey or goat serum for one hour. The following primary antibodies were used: 1C9 (IMM, Hungtington Beach, CA; 1:500) and HT7 (ThermoFisher; 1:1000) against human Tau, total Tau (human+mouse; Dako; 1:1000), conformational tau epitope MC-1 (generously provided by Peter Davies; 1:1000), and phospho-tau (PS199, Abcam, 1:1000). Sections were then incubated in primary antibodies at 4 degrees Celsius overnight. The next day, sections were washed three times with PBS and placed in appropriate Alexa fluor-conjugated secondary antibody solutions at room temperature for one hour. Sections were rinsed three additional times, mounted onto slides and coverslipped using Fluoromount-G with DAPI. For confocal microscopy, immunofluorescent staining was performed on equivalent brain sections and imaged on the Olympus FX1200 confocal microscope. Tau protein was visualized through a Z-stack image taken through the entire depth of the section at 1 µm intervals. Z-stacks were compressed into a single slice image and quantified. For quantification of 1C9, HT7 and PS199 staining, four randomly selected square sub-areas were selected in hippocampus CA1 and quantified by optical density using ImageJ. Quantification of MC-1 labeling was carried out by manual counting of MC-1 positive pyramidal neurons in hippocampus CA1.
2.9. Statistical analysis

Statistical parameters (mean, standard deviation (SD), standard errors (SE), significant difference, etc.) were calculated using the Prism 6 software (GraphPad Software, Inc., La Jolla, CA). Statistically significant differences were examined using a two-tailed t-test (a P value of less than 0.05 was considered significant).

3. Results

3.1. Immunogenecity of AV-1980D vaccine in THY-Tau22 mice

We have previously reported that the MultiTEP platform-based anti-Aβ vaccine induced strong immune responses in mice, rabbits and monkeys[477-479, 486]. Using the same MultiTEP platform, we generated an anti-tau DNA vaccine, AV-1980D, using three copies of N-terminal B cell epitope from tau protein (aa2-18) fused to a string of T helper cell epitopes (Fig. 41A). Immunizations of THY-Tau22 mice with AV-1980D followed by EP induced T cell immune responses (INF-γ secreting Th cells) specific to three different Th epitopes (PADRE, P30 and P17) incorporated into the MultiTEP platform of the AV-1980D vaccine (Fig. 42A), but not to the B cell epitope of tau. Control mice immunized with pMultiTEP plasmid generated cellular immune responses specific to the same three epitopes, PADRE, P30 and P17 (Fig. 42A).

THY-Tau22 mice generated strong humoral immune responses after two immunizations with AV-1980D (246.5 ± 119.8 µg/ml), then maintained the antibody response during five subsequent monthly immunizations (Fig. 42B). We measured the production of IgG1, IgG2a,b, IgG2b, and IgM isotypes of anti-tau antibodies to characterize
the type of humoral immune responses. The levels of IgG1, IgG2\text{ab}, and IgG2\text{b} immune responses were robust and stable, whereas the level of IgM was low (Fig. 42C). More specifically, EP-mediated i.m. delivery of AV-1980D induced equal amounts of IgG1 and IgG2\text{ab}, and more IgG2\text{b} antibodies specific to tau (Fig. 42C).

To show the therapeutic potency of anti-tau antibodies generated in THY-Tau22 mice after AV-1980D vaccination, we analyzed the binding of these antibodies to pathological forms of tau in AD case. As we had expected, antibodies generated by AV-1980D, but not pMultiTEP plasmid, bound neuritic threads and NFTs in human brain tissue (Fig. 42D).
Figure 41. Schematic representations of DNA vaccine construct; analyses of its expression and experimental design in THY-Tau22 transgenic mice. (A) Strategy for cloning the gene encoding 3Tau2.18-MultiTEP (AV-1980D) into the pVAX1 vector and schematic representation of AV-1980 construct encoding 3 copies of Tau2.18 fused to one universal synthetic Th epitope, PADRE and eleven foreign promiscuous Th epitopes from infectious agents. (B) Intracellular and secreted AV-1980D from transfected CHO cells demonstrated by WB, visualized by staining with anti-tau Mab, 1C9. (C) Design of experimental protocol in Tg mice vaccinated with AV-1980D and pMultiTEP (control group).
Figure 42. Cellular and humoral immune responses in THY-Tau22 mice vaccinated with AV-1980D. (A) Numbers of IFN-γ producing T-cells were calculated by ELISPOT in splenocyte cultures obtained from vaccinated animals. Bars represent average ± SD (n=6-7 per group). (B) Concentration of anti-tau antibodies was detected in sera by ELISA. Lines indicate the mean values of antibody concentration (n=6). (C) Isotypes of anti-tau antibodies were analyzed by ELISA. Sera collected after two immunizations were used at dilution 1:800.
3.2. Changes of tau pathology in THY-Tau22 mice after immunization with AV-1980D

As a preclinical test of the efficacy of AV-1980D vaccination, we studied neurological changes in brains of nine month old THY-Tau22 mice vaccinated with AV-1980D. First, we investigated the impact of immunization on tau pathology in transgenic mice by performing immunohistochemistry (Fig. 43). Staining of brain tissue showed some reduction in total tau (1C9, \(p=0.8344\) and HT7, \(p=0.0562\); Fig. 3A-D), as well as phosphorylated tau (pS199, \(p=0.1852\): Fig. 43G, H) following vaccination with AV-1980D. Similarly, we also observed a decrease in MC1+ cells in brain sections from AV-1980D vaccinated mice compared with pMultiTEP injected mice (\(p=0.3294\), Fig. 43E, F).

To further investigate tau aggregation, biochemical analyses were performed with soluble and insoluble fractions. Western blot analysis was carried out using antibodies raised against total and/or phosphorylated forms of tau. We observed that vaccination significantly reduced the levels of total tau in soluble fractions of brain extracts from THY-Tau22 mice (1C9, \(p=0.0436\) and HT7, \(p=0.0297\); Fig. 43A-C). There were no obvious differences in the level of soluble phospho-tylated tau epitopes AT100 (\(p=0.7387\)), pS396 (\(p=0.6848\)), pS404 (\(p=0.7463\)), pS422 (\(p=0.7836\)), and pT212 (\(p=0.8086\)) in vaccinated mice compared with control mice (Fig. 44D and F, G, H, I, L, respectively). We did, however, observe a trend of reduction in soluble phosphorylated tau species in AV-1980D vaccinated mice, examining tau epitopes AT8 (\(p=0.0704\)), AT180 (\(p=0.3328\)), and AT270 (\(p=0.0746\)) (Fig. 44D and E, J, K, respectively). Although, levels of soluble phosphorylated tau decreased in brains of vaccinated animals compared with that of control mice, these
changes were not significant mainly due to a large variability of tau pathology in individual animals.

Next, we compared the level of total and phosphorylated tau in insoluble extracts from experimental and control brain homogenates by WB. We observed significant reduction of the level of total tau after staining with 1C9 mAbs ($p=0.0196$; Fig. 45A, B), but not with HT7 mAbs ($p=0.4936$; Fig. 45A, C) in AV-1980D immunized mice vs. control mice. We did not detect any changes in levels of phosphorylated insoluble tau after staining with αPHF-1 ($p=0.5663$), AT180 ($p=0.3841$), AT270 ($p=0.7552$) and pS199 ($p=0.8667$) antibodies (Fig. 45D and E, G, H, I, respectively). Trend of reduction of the level of insoluble phosphorylated tau in AV-1980D vaccinated mice was detected at the AT100 epitope ($p=0.3224$; Fig 45D, F).

![Figure 43. Immunohistochemical changes measured in mice after DNA vaccination. Level of total tau protein (A-D) and several phosphorylated tau species (E-G) in brain sections were analyzed by Confocal analysis. Representative images of hippocampal CA1 region from vaccinated and non-vaccinated mice are presented (A, C, E, G, I).]
Figure 44. Effect of DNA vaccination on tau proteins in THY-Tau22 mice. Level of total tau protein (A-C) and several phosphorylated tau species (D-L) in brain soluble extractions were analyzed by WB.
Figure 45. Effect of DNA vaccination on tau proteins in THY-Tau22 mice. Level of total tau protein (A-C) and several phosphorylated tau species (D-I) in brain insoluble extractions were analyzed by WB.

4. Discussion

Immunotherapeutic approaches to treat tauopathies are promising strategy for AD. Prior studies have shown that both active and passive tau-based immunotherapies clear some pathological forms of tau[487]. There is, however, a high possibility of inducing encephalitis after active immunization with full-length tau, which was observed in clinical trials with Aβ vaccination[488]. Previously, Rosenmann et al. reported that immunization of C57BL/6 mice with full-length recombinant tau produced neurological deficits, NFT-like changes, gliosis, and inflammatory infiltration[466]. To reduce the risk of an adverse T cell-
mediated immune response to tau immunotherapy, the design of a vaccine that will target
the immunogenic B cell epitope of tau is critical for safe clearance of tau pathology. Last
decade several anti-tau vaccines were generated and evaluated in preclinical studies[487].
Subsequently, the same group showed that harmful effects associated with vaccinations
using phosphorylated tau as an epitope may still persist. In one study where E257T/P301S-
tau Tg mice and wild-type mice were repetitively immunized with a mixture of three
phospho-tau peptides (Tau195–213[p202/205], Tau207–220[p212/214], Tau224–
238[p231]), mice developed a paralytic disease accompanied by significant neurological
disability[489]. Similar effects with other phosphor-tau epitopes are not shown.

In this study, we suggested targeting PAD, the non-phospho-epitope located in N-
terminal region of tau spanning aa2-18 (tau2-18). This region normally is folded and hidden
in native protein, but becomes exposed due to different biochemical modifications of
tau[473, 490]. PAD plays an important role in the activation of a signaling cascade that
leads to FAT inhibition, resulting in early synaptic dysfunction characteristic of AD and
tauopathies [475]. It was reported that phosphorylation of Y18 in tau can prevent the
inhibitory effect of tau filaments on anterograde FAT, suggesting that physical blocking or
binding of PAD can provide protection against the neurotoxic effects of pathogenic
tau[475]. Also, it is shown that deletion of tau2-18 decreased the polymerization of tau
molecule, suggesting that this region is a potential therapeutic target [491]. We generated
the MultiTEP platform[477, 478, 486] based anti-tau DNA vaccine (AV-1980D; Fig. 41A)
and analyzed the potency of this vaccine in transgenic mice. We hypothesized that this
MultiTEP-based vaccine can stimulate immune responses in a broader population of
vaccinated subjects with high MHC class II genes polymorphisms.
It's well known that DNA vaccination provides a unique alternative method of immunization and the rapid accumulation of pre-clinical data demonstrating the potency of DNA vaccines in animal species ranging from mice to non-human primates has led to numerous early-stage human clinical trials[492, 493]. For the last two decades, there have been numerous approaches used to improve the *in vivo* uptake and expression of plasmid DNA for the induction of a more robust immune response[479]. In this study we tested the efficacy of AV-1980D in THY-Tau22 mice delivered *in vivo* by AgilePulse™ electroporation device from BTX Harvard Apparatus. THY-Tau22 mice are characterized by the over expression of human 4-repeat tau mutated at sites G272V and P301S under the control of Thy1.2 promoter and display a hippocampal astrogliosis progressing along with the development of hippocampal tau pathology[476]. THY-Tau22 mice have many advantages compared with other transgenic models since they do not display motor deficits and they have late neuropathology[471]. Repeated immunization of young THY-Tau22 mice with AV-1980D induced strong T cell responses specific to three epitopes from MultiTEP (Fig. 42A), but not to tau<sub>2-18</sub> (data not shown). Here we eliminated the risk of an adverse T cell-mediated immune response to AV-1980D vaccination. Previously, we reported a positive correlation between the concentration of anti-Aβ antibody and a reduction of insoluble, cerebral Aβ plaques without any adverse events, such as CNS T cell or macrophage infiltration or microhemorrhages[482]. We believed that it was very important to generate potent concentrations of anti-Aβ and anti-tau antibodies that were able to clear AD-like pathology and improve cognitive functions. THY-Tau22 mice immunized with our vaccine generated strong IgG-type anti-tau antibodies (Fig. 42B, C). We also observed that MultiTEP AV-1980D vaccination demonstrated therapeutic efficacy towards the targeting
of non-phosphorylated tau. It was shown that monoclonal antibodies specific to tau$_{25-35}$ that are capable of inhibiting tau propagation from cell to cell, may reduce tau pathology and improve cognitive functions in PS19 mice after intracerebroventricular (ICV) infusions[454]. Another group tested the Mab specific to oligomeric tau conformational epitope in JNPL3 mouse models of tauopathy and showed a single intravenous (IV) injection of Mab cleared oligomeric tau, rescued the locomotor phenotype, and reversed the memory deficits associated with oligomeric tau pathology[463].

The goal of our study was to examine whether we could generate an effective DNA vaccine that reduces tau in transgenic AD mice. In that respect, immunization with AV-1980D lead to significant decreases in the levels of total tau in soluble (Fig. 44A-C) and insoluble (Fig. 45A-C) fractions of brain extracts from vaccinated mice. DNA vaccination showed some trends of reduction in levels of phosphorylated tau detected in both soluble (Fig. 4) and insoluble (Fig. 5) fractions, as well as by immunohistochemistry (Fig. 43). These results are consistent with findings from the other groups[467, 468] reporting that antibodies generated by peptide vaccination were found to cross the BBB, bind phosphorylated tau, and reduce pathology without any adverse effect. Our data provides further support that active immunization can be an effective therapy for the targeted reduction of tau-related pathology.

In summary, the present study showed that MultiTEP based anti-tau DNA vaccine, AV-1980D, induced strong immune responses in THY-Tau22 mice and generated anti-tau antibodies that reduced tau pathology in nine months old animals.
Acknowledgments

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Conflict of interest

MGA is co-founder of Neuroimmune that licensed MultiTEP vaccine platform technology from the Institute for Molecular Medicine. The remaining author(s) declare no financial and commercial conflict of interests.
Concluding Remarks

Alzheimer disease is the leading cause of dementia [494] and its cost on society extends far beyond simply the affected individuals [495]. Yet despite AD’s substantial effects on the world’s population, no effective cure has yet to be discovered. The development of AD therapies is often handcuffed by the limited success of research translation from AD models into human patients. To improve the efficacy of transgenic animal models, the AD research field should strive to either develop more relevant AD models that better represent the disease or utilize models to study specific mechanisms that contribute to AD. In those respects, I have generated two novel transgenic mouse models: T5x and RagTau mice. As much as AD is a disease of aging, it is also a disease of inflammation [173]. Thus, I pursued a better understanding of AD by examining the interactions between the immune system and AD pathology in my mice.

The development of AD is often accepted to follow the amyloid cascade hypothesis, a theory describing the initial accumulation of Aβ in amyloid plaques leading to subsequent hyperphosphorylation of tau and formation of neurofibrillary tau tangles [14]. While the study of AD pathology has predominantly focused on the unidirectional effects of Aβ on tau phosphorylation, I have shown in T5x mice that tau pathology can also affect the accumulation of amyloid. T5x mice recapitulate several facets of AD. Aged T5x mice exhibit robust amyloid and tau pathology throughout the brain and mimic the pathology present similar to that of late-stage human AD patients. Increases in tau hyperphosphorylation in T5x mice contribute to elevated levels of neuroinflammation and release of pro-inflammatory cytokines. Elevated levels of inflammatory cytokines coincide with exacerbated astro- and microgliosis. Surprisingly, T5x mice also exhibited decreased
amyloid plaque burden and insoluble Aβ. Upon further examination, lower amyloid levels could be attributed to increased microglial phagocytosis of Aβ in T5x mice. Thus, my findings in T5x mice suggest that while Aβ induces pathological hyperphosphorylation of tau, tau pathology can also retroactively mediate amyloid load via inflammatory mechanisms.

As I previously discussed, the vast majority of research on AD pathology is limited to the study of amyloid and tau. In over 70% of human AD cases, however, post-mortem analysis of patient brains reveal significant α-syn pathology [92, 93]. In the study of DLB, AD with LB, and PD+AD, emerging evidence strongly suggests that interactions between amyloid, tau and α-syn synergize to recruit pathological conformations of the proteins, increase neuroinflammation and induce neuronal dysfunction [120, 385, 496]. I show that in addition to amyloid and tau pathology, T5x mice also exhibit significant α-syn without a synuclein transgene. To my knowledge, T5x mice are the first reported PSAPP/Tau mouse model to exhibit significant accumulation of α-syn positive spheroid and Lewy body-like inclusions in the brain. Furthermore, T5x mice exhibit specific accumulation of α-syn pathology in the amygdala and cortex—the two main brain regions of α-syn pathology observed in human AD patients [93]. My results suggest that both Aβ and phospho-tau contribute to the induction of α-syn pathology, although further studies are needed to elucidate the exact mechanisms by which amyloid and tau pathology engage α-syn in T5x mice.

In addition AD pathology, I also examined T5x mice for behavioral deficits. The clinical diagnosis of human AD cases is characterized by changes in anxiety and decline of cognitive faculties [417]. T5x mice exhibit anxiety deficits at 3 months at age without
significant cognitive deficits. By 6 months of age, however, T5x mice develop significant impairment in cortical and hippocampal based learning and memory tasks. Importantly, T5x mice also exhibit no motor deficits up to at least 7 months of age. In contrast to numerous other tau AD mice that report motor deficits [146, 149, 346], the testing of T5x anxiety and cognitive behavior is not complicated by performance confounds. Thus, I showed that T5x mice are an effective mouse model for studying the changes in anxiety and cognition that are observed in AD.

I generated RagTau mice, expressing tau pathology in addition to lacking B-, T-, and NK- cells, to examine the effects of the adaptive immune system on tauopathy. Similar to T5x mice, RagTau mice also develop anxiety and cognitive deficits without motor impairment. Compared to Tau22 mice, RagTau mice exhibit no significant differences in tau pathology. RagTau mice do, however, display increased microglial activation. To further examine the role of the peripheral immune system in AD, I restored the adaptive immune system in RagTau mice by adoptive transfer of bone marrow cells. BM-cells retrorbitally injected into RagTau mice successfully engrafted and persisted 3-months after adoptive transfer. Examination of tau pathology in RagTau mice receiving adoptive transfer revealed no significant changes in tau hyperphosphorylation—further suggesting that the adaptive immune system does not play a major role in modulating the development of tauopathy. Interestingly, I also observed that a significant number of BM-cells infiltrated the amygdala of RagTau mice while a few BM-cells were also observed in the hippocampus DG. I found that all infiltrating BM-cells were T-cells, and a subset of infiltrating cells were Tregs. Furthermore, RagTau mice receiving BM-cells potentially displayed trends towards decreased microglial activation in the amygdala—supporting previous findings showing
increased microglia activation in RagTau mice relative to Tau22 mice. Thus, these findings suggest that T-cells and the adaptive immune system may play a role in suppressing microglial activation.

The lack of the adaptive immune system also allows for xenotransplantation of human cells into RagTau mice. Our lab and others have previously shown that the transplant of mouse neural stem cells can ameliorate deficits in BDNF and cognition in AD transgenic mice [218, 497]. To test the transplantation of human neural stem cells in a model of AD, I first reprogrammed iSPCs from AD patient fibroblasts. I then differentiated neural stem cells from AD iPSC and confirmed that NSCs could further differentiate into astrocytes and neurons. Finally, I transplanted iPSC-derived NSCs into the hippocampus of RagTau mice. Transplanted iPSC-NSCs survived 3-months after transplantation and exhibited minimal signs of differentiation or migration. iPSC-NSCs also did not exhibit an evidence of developing tau pathology via the propagation of endogenous pathological tau in RagTau mice. Thus, I showed that RagTau mice serve as a viable model for the study of xenotransplantation of human cells and their effects on tau pathology and mouse behavior.

Finally, we also tested the first DNA anti-tau vaccine, AV-1980D, in Tau22 mice. AV-1980 targets the pathological Tau2-18 epitope and is developed on the MultiTEP vaccine platform. Tau22 mice received intramuscular injection of AV-1980D and AgilePulse™ electroporation every two weeks for 7 months. Immunization with the MultiTEP platform induced a strong, but safe T-cell response. AV-1980D also induced a tau-specific humoral immune response that subsisted during the course of immunization with only minimal decreases in tau antibody levels over time. Immunohistochemistry and western analysis revealed significant reduction of unphosphorylated human tau and some trends in
decrease of phospho-tau species. In summary, we have shown the first effective application of a DNA Tau-specific vaccine for the targeted reduction of human tau in transgenic mice.

My research has shown the significance of the immune system in AD. In both T5x and RagTau mice, increased microglial activation is observed alongside development of anxiety and cognitive impairment. I also showed that T-cells infiltrate the brain of RagTau and T5x mice. The significant increases in AD pathology coinciding with T-cell infiltration in T5x mice, but not in RagTau mice suggests that the peripheral immune system does not play a significant role in modulating tau pathology alone, but that amyloid pathology may be a critical component in the modulation of tauopathy by the adaptive immune system. I also showed that the adaptive immune system could be recruited to target the reduction of tau via DNA vaccination. My findings highlight the diverse roles the adaptive and innate immune system can play in the progression, suppression and targeted treatment of Alzheimer’s disease and emphasize the importance of studying immunity for the future development of therapies for AD.
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