UNIVERSITY OF CALIFORNIA,
IRVINE

Investigating the role of T cells in Alzheimer's Disease Pathogenesis

DISSERTATION

Submitted in partial satisfaction of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

In Biological Sciences

by

Jessica Ramirez Sanchez

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DEDICATION

To my family, friends, and colleagues who have been supportive and proud of my academic career.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

AD	Alzheimer's Disease
ACK	ammonium-chloride-potassium
ANOVA	analysis of Variance
APC	antigen presenting cell
APP	amyloid precursor protein
Aβ	amyloid-beta
BBB	blood brain barrier
BDNF	brain derived neurotrophic factor
BMT	bone marrow transplant
CD	cluster of differentiation
CFSE	carboxyfluorescein succinimidyl ester
cLN	cervical lymph nodes
CNS	central nervous system
CSF	cerebrospinal fluid
DAM	disease associated microglia
DC	dendritic cell
DG	dentate gyrus
EAE	experimental autoimmune encephalomyelitis
EBV	Epstein-Barr virus
FACS	Florescent Activated Cell Sorting
fAD	familial Alzheimer's Disease
GFP	green fluorescent protein
GWAS	genome wide associative studies
HPC	hematopoietic progenitor cell
HSC	hematopoietic stem cell
Iba1	ionophore calcium-binding adapter molecule 1
iPSC	induced pluripotent stem cell
Ig	immunoglobulin
INFγ	interferon gamma
IL	interleukin
KO	knockout
MHC	major histocompatibility complex
PBS	phosphate buffered saline
PSEN1	presenilin-1
RAG	recombination activating gene
ROS	reactive oxygen species
sAD	sporadic Alzheimer's disease
scRNA seq	single-cell RNA sequencing

TCR	T cell receptor
TGFβ	transforming growth factor beta
TNFα	tumor necrosis factor alpha
Treg	regulatory T cell
WT	wildtype
YFP	yellow fluorescent protein

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- Claes C, England WE, Danhash EP, Kiani Shabestari S, Jairaman A, Chadarevian JP, Hasselmann J, Tsai AP, Coburn MA, **Sanchez J**, Lim TE, Hidalgo JLS, Tu C, Cahalan MD, Lamb BT, Landreth GE, Spitale RC, Blurton-Jones M, Davtyan H. The P522R protective variant of PLCG2 promotes the expression of antigen presentation genes by human microglia in an Alzheimer's disease mouse model. Alzheimers Dement. 2022 Feb 9. doi: 10.1002/alz.12577. Epub ahead of print. PMID: 35142046.
- Gallagher, K. L., Alfonso-Garcia, A., **Sanchez, J**., Potma, E. O., and Santos, G.M. (2015) Plant growth conditions alter phytolith carbon. Front Plant Sci. 6:753. doi: 10.3389/fpls.2015.00753

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- Sanchez, J.**,** Marsh, S., McIntyre, L.L., Kiani, S., Silva, J., Davtyan, H., Walsh, C.M., Blurton-Jones, M. *CD8+ T cells Infiltrate the Brain and Reduce Alzheimer's Disease Pathogenesis*. **Institute for Immunology 17th Annual Immunology Symposium,** Irvine, CA. December 2019.
- Sanchez, J., Marsh, S., McIntyre, L.L., Kiani, S., Davtyan, H., Walsh, C.M., Blurton-Jones, M. *Adaptive Immune Cells Infiltrate the Brain and Reduce Alzheimer's Disease Pathogenesis.* **Southern California Flow Summit**, Irvine, CA. May 2019.
- Sanchez, J., Marsh, S., McIntyre, L.L., Kiani, S., Davtyan, H., Walsh, C.M., Blurton-Jones, M. *Adaptive Immune Cells Infiltrate the Brain and Reduce Alzheimer's Disease Pathogenesis.* **Institute for Immunology 16th Annual Immunology Symposium**, Irvine, CA. December 2018.
- Sanchez, J., Marsh, S., Davtyan, H., Blurton-Jones, M. *Transplantation of C1q KO Microglia May Affect Synapse Loss in Alzheimer's Disease.* UCI MSP IMSD Summer Symposium, Irvine CA. August 2017.

Conference Abstracts

- Sanchez, J.**,** Marsh, S., McIntyre, L.L., Kiani, S., Silva, J., Davtyan, H., Walsh, C.M., Blurton-Jones, M. (2020) *Investigating the role of Cytotoxic T cells in*

Alzheimer's Disease Pathogenesis. **La Jolla Immunology Conference.** Zoom. December 2020.

- Sanchez, J.**,** Marsh, S., McIntyre, L.L., Kiani, S., Silva, J., Davtyan, H., Walsh, C.M., Blurton-Jones, M. (2020) *Investigating the role of Cytotoxic T cells in Alzheimer's Disease Pathogenesis*. **StemCell SoCal Virtual Poster Session.** Zoom. November 2020.
- Sanchez, J.**,** Marsh, S., McIntyre, L.L., Kiani, S., Silva, J., Davtyan, H., Walsh, C.M., Blurton-Jones, M. (2019) *CD8+ T cells Infiltrate the Brain and Reduce Alzheimer's Disease Pathogenesis*. **La Jolla Immunology Conference.** La Jolla, CA. October 2019.
- Sanchez, J.**,** Marsh, S., McIntyre, L.L., Kiani, S., Silva, J., Davtyan, H., Walsh, C.M., Blurton-Jones, M. (2019) *Adaptive Immune Cells Infiltrate the Brain and Reduce Alzheimer's Disease Pathogenesis.* **Keystone Symposia: Neuroinflammation.** Keystone, CO. June 2019.
- Sanchez, J.**,** Marsh, S., McIntyre, L.L., Kiani, S., Davtyan, H., Walsh, C.M., Blurton-Jones, M. (2019) *Adaptive Immune Cells Infiltrate the Brain and Reduce Alzheimer's Disease Pathogenesis*. **UCI AGS Symposium.** Irvine, CA. April 2019.
- Sanchez, J., Marsh, S., McIntyre, L.L., Kiani, S., Davtyan, H., Walsh, C.M., Blurton-Jones, M. *Adaptive Immune Cells Infiltrate the Brain and Reduce Alzheimer's Disease Pathogenesis.* **Alzheimer's Disease/Parkinson's Disease Congress.** Lisbon, Portugal. March 2019. Funded by NIH-IMSD GM055246.
- Sanchez, J., Marsh, S., McIntyre, L.L., Kiani, S., Davtyan, H., Walsh, C.M., Blurton-Jones, M. *Adaptive Immune Cells Infiltrate the Brain and Reduce Alzheimer's Disease Pathogenesis.* **UCI REMIND Symposium.** Irvine, CA. February 2019. Funded by NIH-IMSD GM055246.
- Sanchez, J., Marsh, S., McIntyre, L.L., Kiani, S., Davtyan, H., Walsh, C.M., Blurton-Jones, M. *Adaptive Immune Cells Infiltrate the Brain and Reduce Alzheimer's Disease Pathogenesis.* **La Jolla Immunology Conference.** La Jolla, CA. October 2018. Funded by NIH-IMSD GM055246.

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ABSTRACT OF THE DISSERTATION

Investigating the role of T cells in Alzheimer's Disease Pathogenesis

By
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Doctor of Philosophy in Biological Sciences

University of California, Irvine, 2022

Professor Mathew Blurton-Jones, Chair

Alzheimer's Disease (AD) is the most common cause of age-related dementia and currently the sixth leading cause of deathin the United States. AD neuropathology is primarily characterized by the accumulation of extracellular amyloid plaques and intraneuronal neurofibrillary tangles that are composed of insoluble proteinaceous aggregates of beta-amyloid (Aβ) and tau. Although these hallmark AD pathologies have been well established and described in numerous studies, many other important pathological changes occur in the brains of AD patients including synaptic and neuronal loss and neuroinflammation. Most recently, genome-wide association studies (GWAS) have provided substantial new evidence that immune dysfunction may contribute to the development and progression of AD. To date over 60 GWAS loci have been identified and almost two thirds of these genes are highly expressed in microglia, the resident innate immune cell of the brain. These genetic discoveries have in turn inspired many new studies of microglial biology and the role of these cells in AD. In contrast, the role of the

adaptive immunity in AD remains understudied, despite evidence that many of AD risk genes are also highly expressed in T and B cells. To investigate the role of T cell infiltration in AD, my thesis research utilized both bone marrow transplanted immune-deficient and immune intact transgenic mouse models of AD coupled with flow cytometry and single cell RNA sequencing. Taken together my experiments demonstrate that multiple T cell subtypes infiltrate the AD brain and effector memory CD8 T cells are specifically enriched in response to Aβ pathology or a combination of Aβ and Tau pathologies. Single-cell sequencing analysis of T-cell receptor (TCR) repertoires further reveal clonality dependent on amyloid; and high expression of several AD risk genes. Ultimately, this study further demonstrates the crucial importance of investigating the role of T cells in AD and provides a guide for future experiments to continue to enhance our understanding of AD immunology.

INTRODUCTION

Alzheimer's Disease Pathogenesis

Alzheimer's Disease (AD) is the most common form of dementia and the leading cause of age-related neurodegeneration and currently affecting over 6 million people in the United States alone (Alzheimer's Association, 2022). AD is a progressive and debilitating disease that gradually impairs learning and memory and overall cognitive function. Eventually AD robs patients of the ability to perform basic daily functions (Alzheimer's Association, 2019). Currently, there is no cure or disease modifying treatment for AD. Drugs that reduce Aβ have all failed in Phase 3 clinical trials and the only 6 FDA- approved drugs provide at best 6-12 months of relief from symptoms and only in mild-moderate AD (Russ & Morling, 2012; Tan et. al., 2014). Most recently the FDA-approved the use of Aducanumab, an anti-Aβ monoclonal antibody infusion, for the treatment of AD. However, this approval has been highly controversial as one phase 3 trial revealed no benefit and a second trial revealed only subtle slowing of progression in a small subgroup analysis. Interestingly, the European Medicines Agency has rejected Aducanumab and Medicare has refused to cover the costs of this controversial drug. Thus, there continues to be a desperate need to develop and test novel therapeutic approaches for AD. It is also clear that additional efforts need to be

made to explore alternative hypotheses and to better understand the role of immune dysfunction in AD.

AD pathology is primarily characterized by two hallmark protein aggregates, amyloid plaques and neurofibrillary tangles (Alzheimer et al., 1995). Extracellular plaques are composed of aggregated amyloid-beta (Aβ) peptide, whereas the intraneuronal tangles are composed of hyperphosphorylated insoluble forms of the microtubule-binding protein tau (Selkoe, 2001). AD is also characterized by significant neuronal and synaptic loss, which correlates with cognitive decline, and is thought to be driven at least in part by these two key pathologies (Davies & Maloney, 1976; Dekosky & Scheff, 1990; Terry et al., 1991; Masliah et al., 2001). Over the past three decades the predominant hypothesis regarding AD pathogenesis has been the 'amyloid cascade hypothesis' which states that amyloid accumulation serves as the initial trigger that begins a pathogenic cascade leading to inflammation, tau phosphorylation, and neuronal and synaptic loss (Hardy & Allsop, 1991; Selkoe, 1991; Hardy & Higgins, 1992; Hardy & Selkoe, 2002; Hardy, 2009; Hardy & Selkoe, 2016). Interestingly, amyloid deposition begins as many as 20-25 years before the onset of clinical symptoms. Aβ deposition is then thought to activate other aspects of the pathological cascade including tau hyperphosphorylation and inflammation. Together, these pathologies combine to induce synaptic and neuronal loss which can only typically be detected by longitudinal MRI imaging and a loss of cortical thickness just prior to the onset of

cognitive symptoms (Jack et al., 2013). Additional data has shown that synaptic loss correlates more strongly with cognitive decline than levels of either Aβ or tau (Terry et al., 1991; Masliah et al., 2001). The exact role that amyloid plays in the subsequent neurodegeneration and cognitive decline observed in AD is still unclear, but evidence from genetically inherited familial AD (fAD), Down's syndrome, as well as genetic risk factors with direct links to Aβ generation implicate amyloid in the initiation of this disease (Tanzi, 2012; Jack et al., 2013).

The production of the Aβ peptide occurs by cleavage of a larger transmembrane protein, amyloid precursor protein (APP). The sequential proteolytic cleavage of APP can be divided into pathogenic and non-pathogenic pathways (Selkoe, 2001; LaFerla et al., 2007). Cleavage by α-secretases (ADAM9, ADAM10, or ADAM17) cuts APP within the Aβ sequence, precluding the generation of Aβ. On the pathogenic side, APP is cleaved first by a β-secretase (BACE1) to create two fragments; sAPPβ and the C-terminal fragment C99, which contains the Aβ sequence. C99 is then subsequently cleaved by γ-secretase, which results in the production of Aβ. When γ-secretase cleaves C99 it can cut at several different sites leading to Aβ peptides of various lengths. The most common are 40 or 42 amino acid in length, known as Aβ40 and Aβ42. Substantial evidence suggests that Aβ42 is the more pathogenic species (Selkoe, 2001; LaFerla et al., 2007). The two cleavage pathways of APP are in an equilibrium, where increased pathogenic processing results in a decrease of non-pathogenic processing and vice

versa. The disturbance of this equilibrium can lead to increased production of Aβ which can slowly begin to aggregate and accumulate, which is in turn thought to drive the pathogenic 'amyloid cascade hypothesis ‘of AD.

There are several factors that can play a role in increasing the production of either APP or Aβ. Rare patients with dominantly inherited familial forms of AD (~2% of total AD patients) can carry mutations in the APP gene near to the α- or β-cleavage sites that influence the production of Aβ (Selkoe & Podlisny, 2002; Wilcock, 2010). Other familial AD patients can exhibit triplication of the APP gene either alone or as a consequence of Downs’ Syndrome (trisomy 21) which also leads to a life-long overproduction of Aβ. Familial mutations in either prensenilin-1 or 2 (PS1/PS2) genes, the proteolytic component of the γ-secretase complex, promote the cleavage of C99 toward the more pathogenic Aβ42 species (Selkoe & Podlisny, 2002; Tanzi 2012). These mutations, although rare, have been harnessed to generate many different transgenic animal models of AD, which are commonly used as preclinical models for this disease (LaFerla & Green, 2012). While fAD patients exhibit overproduction of Aβ, that does not seem to occur in most sporadic Alzheimer’s disease (sAD) subjects. Instead, growing evidence indicates that the majority of Aβ accumulation that occurs in sAD patients most likely results from impaired clearance of the peptide (Wildsmith et al., 2013). This insufficient clearance of Aβ in sAD patients is thought to be due to several factors, including a loss of phagocytic clearance by the immune system, specifically

microglia. A number of studies have shown that the chronic inflammatory state present in these innate immune cells may impair their ability to phagocytose and clear Aβ (Meyer-Luehmann & Prinz, 2015; Mhatre et al., 2015). However, more recent studies suggest that this relationship may be far more complex and that microglia may play a more important role in regulating the compaction of Aβ plaques and the subsequent induction of neuritic dystrophy and tau pathology (Linker et al., 2020; Yuan et al., 2016).

Although AD pathology has been well established and described in numerous studies, the role of immune function in this disease was largely ignored for many years (Heneka et al., 2015). However, in the past decade significant advances have been made in our understanding of the role of inflammation in the pathogenesis of Alzheimer's disease. Specifically, many groups have employed new tools and genetic models to investigate the interactions between the brain's endogenous innate immune cells, microglia and Aβ and tau pathology (Bradshaw et al., 2013; Griciuc et al., 2013; Guerreiro et al., 2013; Heneka et al., 2015; Jay et al., 2015; Wang et al., 2015). There have also been several highly influential genome-wide association studies (GWAS) which have strongly implicated polymorphisms in multiple immune-related genes that are associated with altered risk of developing AD (Lambert et al., 2013). Follow up studies on several of these GWAS genes have further confirmed the importance of microglia in AD (McQuade et al., 2020; Kiani et al., 2020; Claes et al., 2022). Taken together this data has led

to the hypothesis that alterations in inflammation are not just a byproduct or downstream consequence of amyloid and tau pathologies, but rather are important contributing factors in the development and pathogenesis of AD (Meyer-Luehmann & Prinz, 2015). Many of these studies have specifically advanced our understanding of the innate immune system and its potential role in AD. However, the other branch of the immune system, adaptive immunity, has remained highly understudied in the field of AD. Given that innate and adaptive immunity rarely work in isolation, we propose that a logical next step in understanding the pathogenesis of AD should involve the examination of adaptive immunity and the potentially important interactions that may occur between T cells and microglia.

Role of Innate Immunity in Alzheimer's Disease

For many years, the brain has been considered "immune privileged" because of the blood brain barrier (BBB) and the lack of a true lymphatic system. It was argued that the brain did not require signals from the peripheral immune system and that immune activity in the brain was minimal (reviewed in: Schwartz & Kipnis, 2011; Louveau et al., 2015). However, recent research has shown that even though the central nervous system (CNS) is typically less immunologically active than peripheral tissues, it is not as truly isolated from the rest of the body as once believed. There are many cell types in the brain and periphery that contribute to the inflammatory processes in AD, however in this proposal I will focus on the two

cell types that are the most relevant to my overall hypothesis; T cells and microglia. The adaptive immune system, which is primarily composed of B and T cells, has remained understudied in the context of AD. Yet recent studies have begun to demonstrate the potential importance of adaptive immunity in AD (Gate at al., 2019; Marsh et al., 2016). However, the main CNS resident macrophage in the brain, microglia, have also been heavily implicated in the pathogenesis of AD and are part of the innate immune system (Akiyama et al., 2000; Wyss-Coray & Rogers, 2012). To better understand how T cells and microglia may interact in AD, one needs to first review our current understanding of each of these cell types.

Microglia

Microglia, the primary immune cells of the brain, are derived from primitive hematopoietic progenitors within the yolk-sac that migrate into the brain during embryonic development and become restricted to the CNS due to the formation of the BBB (Ginhoux et al., 2010; Yona et al., 2013; Meyer-Luehmann & Prinz 2015). Microglia are maintained throughout life via proliferation (Ajami et al., 2007; Mildner et al., 2007; Elmore et al., 2014) and in the normal healthy brain play an important role in tissue surveillance and removal of dead cells, myelin debris, and pathogens via phagocytosis (Nimmerjahn et al., 2005). Microglia have also been shown to play critical roles in synaptic pruning, neurogenesis, myelin turnover, and neuronal homeostasis (Hanisch & Kettenmann, 2007; Ransohoff &

Perry, 2009; Hong et al., 2016). In several neurological diseases microglia have been found to shift from a homeostatic state to a highly 'activated' phenotype. With the advent of single cell sequencing studies the field has further begun to understand that microglia can adopt a complex array of 'activation states', each of which can exhibit varying cell surface receptor expression, cytokine secretion, morphology, and functional activity (Town, 2010; Wyss-Coray & Rogers, 2012; Murray et al., 2014; Karen-Shaul et al., 2017; Hasselmann et al., 2019; Sala et al., 2019).

In the brains of both human Alzheimer's patients and transgenic AD mice models, microglia consistently surround amyloid plaques (Rogers et al., 1988; Haga et al., 1989; Itagaki et al., 1989; Frautschy et al., 1998; Stalder et al., 1999). These plaque-associated microglia exhibit an altered more ameboid morphology and increased expression and secretion of numerous inflammatory cytokines and chemokines (Lue et al., 2001; Nimmerjahn et al., 2005; Mandrekar- Colucci & Landreth, 2010). As a response to Aβ, microglia are known to release many important pro-inflammatory and potentially detrimental factors, including: reactive oxygen species (ROS), IL-1β, IL-6, TNF-α, interferon γ (IFNγ), MIP-1α, MIP-1β, MCP-1, and complement components C1q, C3, C4, C9 (Lue et al., 2001; Coraci et al., 2002; Walker & Lue, 2005; Wyss-Coray & Rogers, 2012). Microglia also exhibit chemotaxis that drives them towards amyloid plaques via expression of a variety of cell surface receptors which have been shown to detect Aβ including the

AD risk gene Triggering Receptor Expressed on Myeloid Cells 2 (TREM2). Microglia can also be drawn toward other inflammatory signals including cytokines and chemokines that are associated with plaques and secreted by other cell types adjacent to the plaques including astrocytes (Fassbender et al., 2004; Liu et al., 2012; Wyss-Coray & Rogers, 2012).., 2004; Liu et al., 2012; Wyss-Coray & Rogers, 2012).

Early studies suggested that plaque-associated microglia may play an important protective role by actively phagocytosing Aβ. This notion was formulated because of their close association with amyloid plaques, their known role as phagocytic cells, and the occasional observation of beta-amyloid material within some microglia in AD brains. Functional studies using acute slice culture models and microglial monocultures further demonstrated that microglia are indeed capable of phagocytosing amyloid *in vitro*. However, *in vivo* studies showed that most plaque-associated microglia do not actively phagocytose amyloid (Hickman et al., 2008; Lee & Landreth, 2010; Krabbe et al., 2013; Meyer-Luehmann & Prinz, 2015). Nevertheless, GWAS studies have identified a number of genes that are highly expressed by microglia in associated with altered risk of developing AD (Lambert et al., 2016; Bellenguez et al., 2022). So how else might these microglial genes alter function to impact AD risk? Many ongoing studies include several from our lab are exploring this question. To date one of the more compelling and consistent findings is that AD risk genes influence the ability for

microglia to sense and respond to amyloid plaques and to compact more diffuse amyloid pathology (Kiani et al., 2022; McQuade et al., 2020; Claes et al., 2022). A potentially important beneficial outcome of this activity is that secondary plaque-associated neuronal damage is reduced. In contrast, microglia carrying AD risk mutations exhibit impaired plaque compaction and increased neuritic dystrophy (McQuade et al., 2020).

For many years clinical trials have tested active and passive immunotherapies that target Aβ, with the goal of utilizing endogenous immune defense mechanisms to combat the potentially toxic buildup of amyloid. Several studies in AD mouse models have shown the use of passive Aβ immunotherapy, including antibodies that target various forms of Aβ (Bard et al., 2000; Wilcock et al., 2004; Jordão et al., 2010). Other studies have demonstrated that one of the primary mechanisms by which anti-Aβ antibodies impact amyloid load is through recruitment of microglia and increased microglial phagocytosis of antibody-opsonized Aβ (Das et al., 2003; Wilcock et al., 2003; Wilcock et al., 2004; Koenigsknecht-Talboo et al., 2008). Recent studies have also demonstrated that anti-Aβ antibodies can reduce amyloid burden in human patients (Liu et al., 2015). However, anti-Aβ antibody approaches continue to fail in the clinic. In fact, one of the last hopes for this approach, a primary prevention trial in fAD patients carrying a PSEN1 mutation (Tariot et al., 2018), recently reported no benefits, despite initiation of treatments up to 8 years prior to symptom onset. Thus, the failure of

these antibody trials are likely not a result of the inability of human microglia to migrate to and phagocytose amyloid following immunotherapy treatment. Rather, these studies suggest that perhaps amyloid plaques are not the key pathological component that underlies cognitive dysfunction.

Instead, GWAS studies that have begun to define AD risk genes and the response of microglia to AD pathology, strongly implicates the innate immune system in Alzheimer's pathogenesis. While there are of course well established differences between the innate and adaptive immune systems, these systems frequently work together when the body encounters a disease pathology, a local injury, or a pathogenic infection (Town et al., 2005; Ransohoff & Engelhardt, 2012; Engelhardt & Ransohoff, 2012). In the context of AD, it is clear that this important crosstalk between these two arms of the immune system needs to be further explored. However, I will first discuss the current state of knowledge regarding the potential role of adaptive immunity in AD.

Role of Adaptive Immunity in Alzheimer's Disease

The idea that the CNS was "immune privileged" has likely contributed to the relatively sparse investigation of adaptive immunity in AD and many other CNS disorders (reviewed in: Schwartz & Kipnis, 2011). Studies have recently provided major advances that have begun to challenge this overly simplistic idea by exploring how the BBB functions, identifying and examining brain lymphatic and

glymphatic systems, examining the immune cells that circulate through the CSF, seeking to better understand the blood CSF barrier (BCSFB), and looking more closely into the role of the adaptive immune cells that survey both the healthy and diseased CNS. Most peripheral immune cells observed within the heathy brain likely play a role in immune surveillance. On the other hand, in the diseased brain adaptive immune cells can perform a variety of functions that can either promote or in some cases, protect against the development and progression of disease pathology.

T cells

The most widely investigated adaptive immune cell population in neurodegenerative disorders are T cells. In particular, research on multiple sclerosis (MS) has shown that infiltrating T cells play a significant role in disease pathogenesis (McIntyre et al, 2020). In contrast, our understanding of the potential role of T cells in AD remains in its infancy. T cells were first reported in the AD brain though immunohistological studies over three decades ago (Itagaki et al., 1988; Rogers et al., 1988; McGeer et al., 1989). However, the relatively small number of T cells observed in AD brains, especially in comparison to MS lesions, led researchers to conclude that they likely played little to no role in AD. Since then, there have been conflicting reports on the presence or absence of T cells in AD versus control brains including discussions as to whether T cell infiltration was

simply associated with other comorbidities such as stroke (Togo et al., 2002; Parachikova et al., 2007; Hartwig, 1995). Several of these studies also reported that infiltrating T cells did not closely associate with either amyloid plaque or tau tangle pathologies. As the understanding of T cells in other neurological disease such as MS has grown, it has become clear that a more thorough examination of T cell response in AD is desperately needed. T cells can of course differentiate into various subtypes include CD8+ cytotoxic cells, CD4+ T helper subtypes (Th1, Th2, Th17), and regulatory T cells (Treg) (Oestreich & Weinmann, 2012; Walsh et al., 2014; Hemmer et al., 2015). Each of these different T cell subtypes can provide very different functions that could be either beneficial or detrimental in AD by interacting with microglia or astrocytes or secreting specific cytokines, chemokines or other proteins that mediate their effector functions (Aloisi et al., 2000).

The first prominent studies on T cell influence in AD focused on the Treg subtype, which play a primary role in modulating autoimmunity and suppression of effector immune responses (Fontenot et al., 2003; Sakaguchi et al., 2008; Baruch et al., 2015). A study that utilized the 5xfAD transgenic AD mouse model (Oakley et al., 2006) in combination with a FoxP3-diptheria toxin receptor (FoxP3-DTR) mouse (Mayer et al., 2014), showed that depletion of the Treg population during AD pathogenesis was surprisingly beneficial to disease progression (Baruch et al., 2015). This group found that transient depletion of FoxP3+ Tregs using either the genetic model method or a pharmacological approach lead to a significant decrease

in the amyloid plaque load, and a corresponding improvement in cognition. The researchers argued that the significant decrease in plaque load was due to changes in the choroid plexus following Treg depletion which led to an increased ability of both monocyte-derived macrophages and repopulating Tregs to infiltrate the AD brain. They also made the conclusion that AD inflammation in the brain can result in Tregs responding in a detrimental manner that blocks the ability of other peripheral immune cells to respond to and infiltrate the AD brain. They further argued that temporarily removing Tregs could restore balance to the immune system allowing for a beneficial response from other peripheral immune cells, which could in turn decrease pathology and inflammation. They also suggested that Tregs that repopulate after depletion were now helpful because they have not been exposed to the inflammatory environment like the Tregs prior to depletion.

However, a second study that examined Tregs in AD contradicted these prior results. This study found that transient depletion of Tregs using instead an anti-CD25 antibody actually increased cognitive deficits without altering Aβ pathology. They also found that increasing Treg numbers with IL-2 rescued cognitive function (Dansokho et al., 2016). However there are problems with the methods used in each of these experiments that may explain these distinct conclusions. Anti-CD25 antibody is arguably not an optimal method to deplete Tregs since it does not lead to depletion of FoxP3+/CD25- Tregs (Fontenot et al., 2003; Fontenot et al., 2005), but does deplete CD4+/CD25+/FoxP3- effector T cells (Couper et al., 2009).

Likewise, diphtheria toxin-induced Treg depletion would likely produce many pleotropic effects including altered BBB function. The use of human IL-2 to activate mouse Tregs by Fontenot et. al., is also problematic as human IL-2 can effect CD4+ effector T cells and CD8+ memory T cells (Boyman & Sprent, 2012). There have been many other studies conducted that show the complicated influence that Tregs have in CNS diseases. In other studies, depleting Tregs worsened the neurodegeneration observed in both Parkinson's disease (PD) and stroke animal models, while increases in Tregs were beneficial (Reynolds et al., 2007; Liesz et al., 2009). On the other hand, another study found that depletion of T cells was beneficial in a stroke model (Kleinschnitz et al., 2013). Given these conflicting reports, it is clear that additional research is needed to fully understand how Tregs respond to and influence the progression of AD (Zhou et al., 2009; Komatsu et al., 2009; Walsh et al., 2014).

T cells could provide either beneficial or problematic effects in AD through many other potential mechanisms, including secretion of BDNF. Increases in brain BDNF levels have been shown to rescue cognitive, motor, and synaptic deficits in models of AD and PD (Blurton-Jones et al., 2009; Goldberg et al., 2015). Other studies have examined mice that have been modified to lack BDNF in both their T cells and myeloid cells; leading to worsened outcome in an EAE model. The over expression of BDNF in T cells transferred into this model decreased the disease compared to GFP-overexpressing T cells (Linker et al., 2010). Another potential T

cell mechanism that could influence AD is via modulation of astrocyte funciton. The clearance of extra synaptic glutamate is an important role performed by astrocytes in the brain since excess glutamate can lead to excitotoxicity. One study used primary neurons that were subjected to oxidative stress and incubated them with conditioned media from astrocytes co-cultured with T cells. Results demonstrated that there was a decrease in neuronal apoptosis compared to naïve astrocyte conditioned media. The study also showed that the cytokines produced by T cells were alone able to restore the fundamental role of the astrocytes in clearing extracellular glutamate (Garg et al., 2008).

Another important mechanism by which T cells may influence the pathogenesis of neurodegenerative disorders, is by interacting directly with antigen presenting cells. The interactions between the adaptive and innate immune systems are important for each system to function properly. The adaptive immune system requires antigen presentation by innate immune cells in order to properly respond to an inflammatory state in neurodegenerative diseases. Earlier studies have argued that peripherally-derived dendritic cells (DCs) infiltrate the brain in disease conditions and serve as the primary antigen presenting cells (APCs). Yet in the healthy CNS only a small number of DCs can be detected (Ransohoff & Engelhardt, 2012). Studies have shown that innate immune cells such as microglia and bone marrow derived macrophages can also serve as antigen presenting cells. Therefore, it is important to fully understand and examine the role of microglia in

AD as antigen presenting cells and how this may influence their interactions with infiltrating T cells.

Antigen presentation to T cells involves the process of forming an immunological synapse between the antigen presenting cell and the T cell. This interaction occurs between major histocompatibility complex class I or class II (MHC I or MHC II) molecules expressed on the APC and on the T cell receptor (TCR) expressed by CD8+ or CD4+ T cells (Dustin et al., 1998; Comrie et al., 2015; Markey et al., 2015). There also needs to be binding between Lymphocyte Function-associated Antigen 1 (LFA-1) and Intracellular Adhesion Molecule 1 (ICAM-1; aka CD54) and one or more costimulatory factor for a successful antigen presentation to occur in these cells (Dustin et al., 1998; Comrie et al., 2015; Markey et al., 2015; Ransohoff & Engelhardt, 2012). Dendritic cells in the periphery express these APC markers but also express CD11c (Bulloch et al., 2008; Prodinger et al., 2011). In a healthy CNS, dendritic cells are the only immune cells expressing most of these antigen presenting markers. However, using CD11c green fluorescent protein (GFP) and yellow fluorescent protein (YFP) reporter mice researchers first identified a small population of microglia that also express CD11c (Hanisch & Kettenmann, 2007; Bulloch et al., 2008; Prodinger et al., 2011). Plaque-associated microglial have also recently been shown to upregulate CD11c and many other APC genes (Deczkowska et al., 2018' Hasselmann & Coburn et al., 2019).

Microglia usually have low levels of APC-associated markers; however they have the ability to upregulate these markers and perform antigen presentation in the brain (Ransohoff & Engelhardt, 2012; Bennett et al., 2016). When T cells were first reported to infiltrate the brain parenchyma of AD patients, concurrent reports demonstrated the presence of MHC II positive microglia in the AD brain as well (McGeer et al., 1987; McGeer et al., 1988; Styren et al., 1990). In response to neurodegeneration and inflammation in the brain, microglia have been shown to upregulate several co-stimulatory molecules including CD40, ICAM-1, CD80 (B7.1), CD86 (B7.2) (Aloisi et al., 2000; Town et al., 2005; Almolda et al., 2015; Rice et al., 2015). After APCs detect antigens through pattern recognition receptors and Toll-like receptor family (TLRs), they are induced to upregulate those co-stimulatory molecules (Town et al., 2005). Aβ is a ligand for both TLR2 and TLR4 and thus could provide a potential mechanism in which Aβ can induce increase microglial expression of APC molecules (Fassbender et al., 2004; Liu et al., 2005; Liu et al., 2012). Despite these studies, it remains unclear the extent to which microglial upregulation of APC molecules leads to or promotes antigen presentation to infiltrating T cells in the AD brain. However, my studies have shown that there may be an important role for microglia in presenting antigen to T cells which can likely in turn influence the development and progression of AD pathology.

In the field of multiple sclerosis, microglia have previously been shown to serve as efficient antigen presenting cells. Studies have compared the antigen-presenting function of microglia to infiltrating CD11c+ cells and found that during EAE microglia upregulate many of the same stimulatory molecules and can induce the proliferation of CD4+ helper T cells. Research has also found that microglia produce lower levels of Th1 and Th17 cytokines and do not produce Th2 cytokines compared to peripheral APCs that infiltrate the CNS (Wlodarczyk et al., 2014). This shows that microglia may affect the T cell response in a differential manner from that of infiltrating DCs. Although studies have begun to examine the role of infiltrating T cells in AD pathogenesis, additional work is needed to better understand the influence of both infiltrating and local APCs such as microglia.

Recent Studies on CD8+ T cells in Alzheimer's Disease

Immunohistochemical studies in advanced AD patient brains have shown an increased number of CD3+ CD8+ T cells in the hippocampus that significantly correlated with tau pathology compared to nondemented controls (Merlini et al., 2018). In mice models of AD, infiltrating hippocampal T cells correlate with tau pathology (Laurent et al., 2017) and amyloid pathology (Ferretti et al., 2016). Most recently, studies of AD and control patient CSF revealed an enrichment of effector memory CD8+ T cells in AD subjects that were negatively associated with cognition. Furthermore, CD8+ T cells were found adjacent to cerebral blood

vessels and TCR repertoire analysis revealed AD-associated increases in clonality (Gate et al., 2019). Further exploration of CD8+ T cells in AD brains, both in humans and mouse models, is clearly needed. My thesis research aimed to examine the impact of the adaptive immune system in AD mouse models with a specific focus on the infiltration of cytotoxic T cell into the brain.

To further investigate the role of adaptive immune cell infiltration in AD, my thesis studies utilized both immune-deficient and immune intact transgenic mouse models of AD coupled with bone marrow transplantation, flow cytometry, and single cell RNA sequencing. Together these experiments reveal a broad array of T cell subtypes that infiltrate the brain and identify specific subsets of CD8 T cells that are enriched in response to Aβ pathology or a combination of Aβ and Tau pathologies. Examination of T cell receptor (TCR) repertoires further demonstrate an amyloid-responsive induction of TCR clonality. Taken together these studies provide an important new resource that can be used to guide future investigations and further inform our collective understanding of AD immunology.

CHAPTER ONE

CYTOTOXIC CD8 T CELLS INFILTRATE THE BRAINS OF IMMUNE DEFICIENT RAG-5XFAD MICE FOLLOWING BONE MARROW TRANSPLANTATION

INTRODUCTION

The innate immune system, particularly microglia, have been strongly implicated in Alzheimer's disease (AD) pathogenesis. In contrast the role of adaptive immunity in AD remains poorly understood still. To investigate the impact of adaptive immunity on AD pathogenesis, our lab generated two complementary transgenic models of AD. First, to determine whether genetic deletion of adaptive immunity alters AD pathogenesis, we backcrossed the well-established 5xfAD model onto a Rag2/il2rγ double knockout background. Hereafter referred to as Rag-5XFAD mice, this model lacks B cells, T cells and NK cells and overexpresses mutant forms of APP and PS1, leading to the robust development of Aβ plaques. Interestingly, genetic deletion of adaptive immune cells in these mice led to a dramatic increase in amyloid beta plaque load, an activated microglial neuroinflammatory state, and a corresponding decrease in the ability of microglia to phagocytose beta-amyloid. Furthermore, restoration of the adaptive immune system via bone marrow transplantation (BMT) led to a corresponding decrease in amyloid plaques, indicating a fundamental role for the adaptive immune system in AD pathology (Marsh et al., 2016). To further investigate the specific role of T cells in these findings, we performed adoptive transfer experiments including bone marrow transplantation into Rag-5XFAD and wild-type littermate controls; Rag-WT mice. Immunohistochemistry (IHC) and

flow cytometry revealed an increase in CD8+ T cells within the Rag-5XFAD brain parenchyma versus Rag-WT controls, suggesting that beta-amyloid plaque pathology promotes the specific recruitment of CD8 T cells to the brain.

MATERIALS AND METHODS

Animals

All animal procedures were performed in strict accordance with National Institutes of Health and University of California guidelines. The Rag-5XFAD immune-deficient AD mouse model was created by backcrossing 5XFAD transgenic mice onto a Rag2/il2rγ double knockout background as previously described (Marsh et al., 2016). Briefly, 5XFAD mice (MMRRC Strain: 034848-JAX), express two cointegrated and co-inherited transgenes (APP and PS-1). The APP transgene includes three familial AD mutations (Swedish, Florida, London) and the PS-1 transgene includes two mutations (M146L & L286V) (Oakley et al., 2006). Purebred C57Bl6 5XFAD mice were crossed with Rag2/il2rγ double knockout mice (Taconic #4111) (Cao et al., 1995), followed by repeated littermate crosses from each generation, to create mice that are heterozygous for the APP/ PS-1 transgenes and lack both copies of the Rag2 and Il2rγ genes (Il2rγ -/y in males). We also simultaneously generated strain matched immune-deficient mice wild-type for the AD transgenes hereafter referred to as Rag-WT. For bone marrow adoptive transfer experiments, strain-matched WT-5XFAD mice were crossed with

CAG-eGFP mice (JAX Stock#: 006567), which express “enhanced” GFP under the control of chicken beta-actin promoter and cytomegalovirus enhancer, hereafter referred to as GFP-5XFAD.

Tissue Processing

Immune deficient (Rag-5XFAD and Rag-WT) mice were sacrificed by carbon dioxide asphyxiation and intracardial perfusion was performed with 0.01M phosphate buffered saline (PBS). Immediately following perfusion, brains were isolated and cerebellum and olfactory bulbs removed and then hemispheres separated along the mid-sagittal plane. Half brains were then dissociated using a glass homogenizer and a 23% percoll density gradient centrifugation was used to remove myelin and debris prior to flow cytometry. The other half of each brain was drop fixed in 4% paraformaldehyde for 48 hours at 4°C and then transferred to 0.01M PBS and 0.02% NaN3 and stored at 4°C. Prior to sectioning, fixed hemibrains were cryoprotected in 30% sucrose for three days and then sectioned in the coronal plane on a freezing microtome (Leica) at 40μm thickness.

Flow Cytometry

Single cell suspensions isolated from the brain, spleen, deep cervical lymph nodes, and blood were immunophenotyped with fluorescent antibodies for the following cell surface markers for pan T cells: PE-conjugated CD3e (500A2; BD

Biosciences; San Jose, CA), PerCP-eFluor710-conjugated CD3e (17A2, eBioscience). CD4 and CD8 T cells: PE-conjugated CD4 (GK1.5, BD Biosciences), Pacific Blue-conjugated CD4 (RM4-5, BioLegend; San Diego, CA) APC-conjugated CD8 (Ly-2, BD Biosciences), PE/Cy7- conjugated CD8 (53-6.7, eBioscience). NK cells: APC-conjugated CD49b (DX5; BD Biosciences), PE-conjugated NK1.1 (PK136, BioLegend). B cells: FITC-conjugated CD45R/B220 (RA3-6B2; BD Biosciences), Alexa Fluor 710-conjugated CD19 (ebio1D3, eBioscience). Memory markers: BV421-conjugated CD44 (Biolegend), APC-conjugated CD62L (Biolegend). Hematopoietic lineage and monocyte markers: BUV496-conjugated CD45 (Biolegend), Alexa 700-conjugated CD11b (Biolegend). All cells examined by flow cytometry were first FC blocked with anti-CD16/32 (1:200; BD Biosciences). Cells were examined on a Fortessa flow cytometer (BD Biosciences) or BD FACSAria II (BD Biosciences) and analyzed with FlowJo v10.7 software (FlowJo; Ashland, OR).

Immunohistochemistry

Fluorescent immunohistochemical analysis followed previously established protocols (Hasselmann et al., 2019). Primary antibodies used for immunohistochemical analysis included: Anti-GFP Alexa 488 conjugated (Life Technologies); Anti-GFP (Millipore; Temecula, CA); Iba1 (Wako; Richmond, VA), CD3e (Clone 500A2; BD Biosciences), CD4 (Clone RM4-5; AbD Serotec &

BioLegend), CD8 (Clone YTS105.18; AbD Serotec; Raleigh, NC). Sections were incubated in primary antibodies overnight followed by detection with either appropriate Alexa Fluor® conjugated secondary antibody (Life Technologies) or biotinylated secondary antibodies followed by Alexa Fluor conjugated streptavidin and coverslipped using Fluoromount-G with or without DAPI (Southern Biotech; Birmingham, AL). Fibrillar amyloid was visualized using Amylo-Glo (Biosensis; Temecula, CA) diluted 1:100 in 0.01M PBS.

Confocal microscopy and quantification

Immunofluorescent sections were imaged using an Olympus FX1200 confocal microscope. To avoid non-specific bleed-through, each laser line was excited and detected independently. All images shown represent either single confocal z-slices or z-stacks. All image analyses were conducted by a blind observer using coded images.

Bone Marrow Adoptive Transfer

Adoptive transfer experiments were performed according to previously published protocols (Marsh et al., 2016). Briefly, age- and sex-matched immune-intact GFP-5XFAD mice served as donors for bone marrow adoptive transfer. Donor mice were euthanized by CO2 asphyxiation, femurs and tibias were removed, and whole bone marrow harvested by flushing the marrow contents with

PBS. Marrow was then treated with Ammonium-Chloride-Potassium (ACK) buffer to lyse red blood cells, filtered through a 40μm nylon mesh, and cell numbers counted by hemocytometer and automated cell counter. Recipient mice were Rag-5XFAD and Rag-WT mice. A total of 13 Rag-5XFAD (5F & 8M) and 9 Rag-WT (6F & 3M) mice were injected with BM. Each recipient was anesthetized with isoflurane prior to receiving 550,000 live cells in 100μl or equivalent volume of PBS via retro-orbital injection. To confirm engraftment, mice were bled 3 weeks post transplantation and analyzed for the presence of GFP+ B, T, and NK cells by flow cytometry.

Statistical Analysis

Statistical analysis was performed using Graphpad Prism 6 software and R. Comparisons involving more than two groups utilized one way analysis of variance (ANOVA). Comparisons of two groups utilized two-tailed Students t-test. All differences were considered significantly different when $p<0.05$.

RESULTS

T cells infiltrate the brain parenchyma of immune deficient AD mice

In previous studies we generated immunodeficient AD mice by backcrossing 5XFAD mice onto a Rag2 and il2rγ double knockout background (RAG2$^{-/-}$,IL2rɣ$^{-/-}$,5XFAD$^{+/0}$). The resulting mice, hereafter referred to as Rag-5XFAD, lack T, B, and NK cells and remarkably exhibited a greater than two-fold increase in beta-amyloid plaque pathology (Marsh et al., 2016). Conversely, bone marrow transplantation (BMT) into Rag-5XFAD mice partially reversed this effect. In the current study we sought to further determine whether these changes in amyloid load might be accompanied by infiltration of specific peripheral immune cells into the brain parenchyma. We therefore transplanted GFP-expressing bone marrow hematopoietic stem cells (HSCs) into 2-month old Rag-5XFAD mice and wild-type 'Rag-WT' immunodeficient controls (RAG2$^{-/-}$,IL2rɣ$^{-/-}$,WT)(RAG2-/-, ɣc-/-, WT) controls (Figure1A). Because Rag-5XFAD and Rag-WT mice lack an adaptive immune system, successful engraftment of donor-derived hematopoietic cells can be achieved without the need for preconditioning irradiation or chemoablation (Marsh et al., 2016). Four months after BMT, mice were sacrificed and brains examined by iImmunohistochemistrycal (IHC), revealing an increase in GFP+ donor cell infiltration within 5XFAD brains in comparison to Rag-WT mice (Figure 1B,C). Thus, amyloid accumulation or other neuropathological changes induced by plaque pathology, promote the recruitment of donor derived immune

cells into the brain. Interestingly, GFP+ cells were observed throughout the brain of Rag-5XFAD mice including the hippocampus, cortex, and meninges (Figure 1D,E), but also in greater numbers adjacent to the lateral ventricles (Figure 1F). To determine whether GFP+ cells might simply be localized within cerebral blood vessels, sections were counterstained with the blood vessel marker GLUT1, demonstrating that virtually all GFP+ cells were located within the parenchyma (Figure 1G).

As BMT into immune-deficient recipients repopulates various blood lineages, we began by examining CD45, a marker that labels all hematopoietic cells. As expected, all GFP+ cells within either Rag-5XFAD or RAG-WT recipient brains co-expressed CD45. Interestingly, CD45/GFP double-positive cells exhibited small circular morphologies that contrasted with a subset of CD45+/ GFP- brain resident cells that based on their morphology likely represent microglia (Figure 1H). Further staining with the microglial/monocyte marker IBA-1 revealed no examples of GFP-expressing IBA-1 cells (Figure 1I). Thus, donor-derived cells did not give rise to brain-infiltrating monocytes or macrophages in this chemoablation- and irradiation-free BMT paradigm. Given the ameboid morphology of GFP-expressing cells and prior reports demonstrating T cell infiltration into AD mouse models (Unger et al., 2020; Ferretti et al., 2016), we next examined the pan T-cell marker CD3. IHC staining revealed that virtually all GFP+ cells detected in the AD brain co-expressed CD3+ (Figure 1J-L).

Interestingly, T cells were sometimes observed adjacent to IBA-1+ microglia (Figure 1M-O) and Aβ plaques (Figure 1P) within Rag-5XFAD brains, suggesting that activated microglia may play a role in T cell recruitment and/or antigen presentation in the AD brain environment.

To further understand whether specific subtypes of T cells are recruited to the brains of Rag-5XFAD mice, we next performed flow cytometry (gating strategy shown in Figure 6A). This analysis revealed a significant increase in GFP/ CD3/CD8+ T cells (P=0.0390) and a non-significant trend **(**P=0.4363**)** toward increased GFP+/CD3+/CD4+ T cells in bone marrow transplanted Rag-5XFAD mice in comparison to Rag-WT BMT recipients (Figure Q,R). As bone marrow transplantation into immune-deficient recipients can induce homeostatic expansion it is possible that T cell infiltration might be amplified in this paradigm. Our findings in Rag-WT mice suggest this is unlikely to be the case. Nevertheless, we sought to either corroborate or refute our initial Rag-5XFAD findings by examining immune-intact 5XFAD mice. Both male and female 6-month old 5XFAD and wildtype littermates (n=6/genotype) were sacrificed via intracardiac perfusion of PBS and then brains processed and stained for flow cytometry. This analysis revealed similar results to our Rag-5XFAD studies, demonstrating a significant increase in CD8+ T cells in 5XFAD brains versus wildtype littermates, but no significant changes in CD4+ cells (Figure 1S).

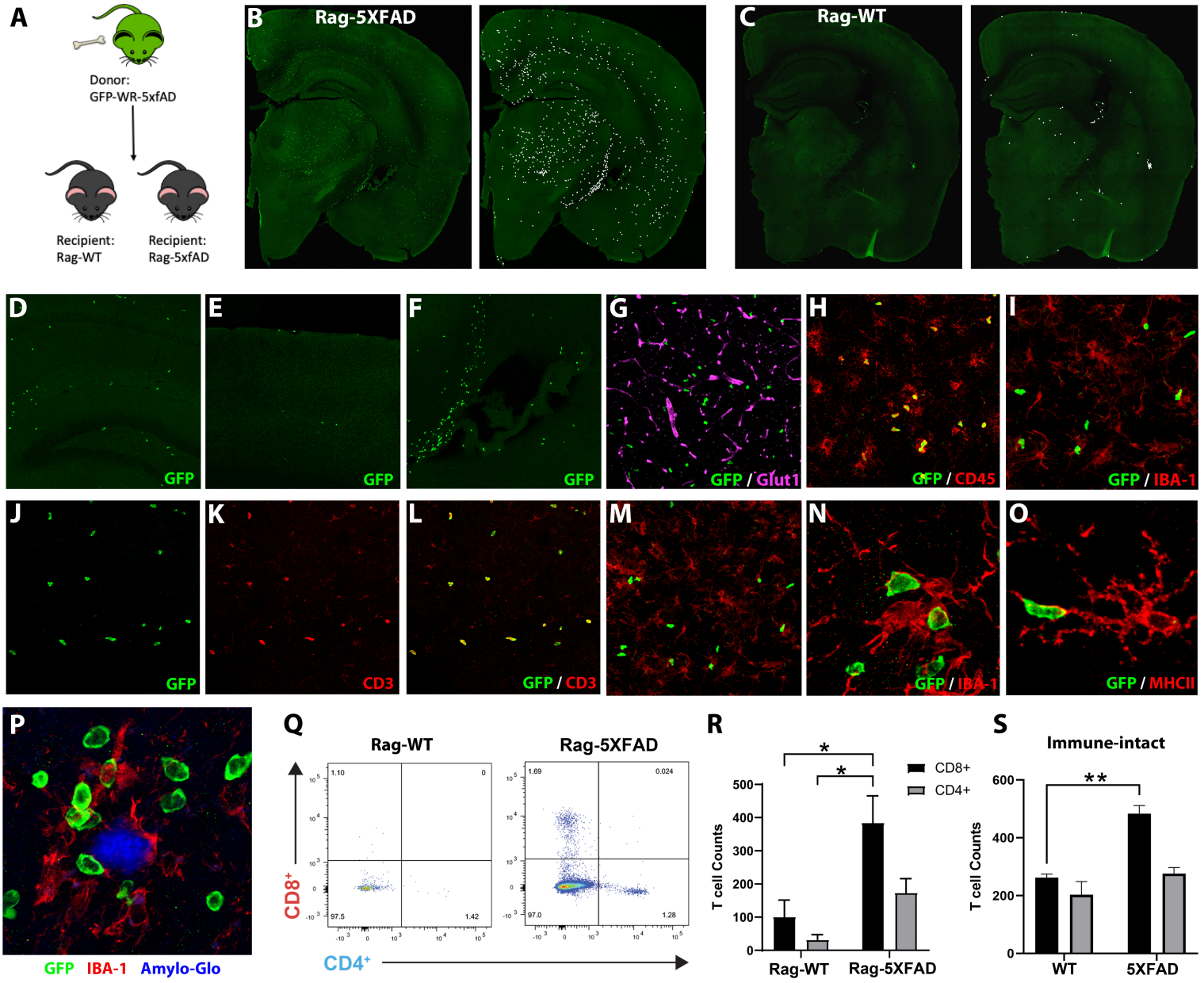


Figure 1. **Cytotoxic T cells infiltrate Rag-5XFAD mice brains.** (A) Adoptive transfers were conducted using GFP+ bone marrow cells into 2 month-old Rag-WT and Rag-5XFAD recipients via retro-orbital injections. (B) Increased numbers of GFP+ cells infiltrate the brain parenchyma in AD mice (B) but not WT mice (C), GFP cells highlighted with white spots. (D,E) IHC images of GFP+ cells in hippocampus, cortex, and meninges. (F) IHC image showing GFP+ cells adjacent to the lateral ventricles. (G) IHC staining of GLUT1 blood vessel marker (purple) demonstrates that GFP+ cells are located within the brain parenchyma. (H) IHC staining of GFP+/CD45+ cells and GFP-/CD45+ microglia. (I) IHC staining of microglial/monocyte marker IBA-1 and GFP cells shows a lack of GFP+ monocyte infiltration. IHC staining of GFP+ cells (J), CD3 T cell marker (K) and colocalization of GFP+/CD3+ cells (L). IHC staining of GFP+ cells, IBA-1+ (M) and MHCII+ (N) microglia, and Amylo-glo+ plaques (P). (Q,R) Flow cytometry analysis of CD8+ and CD4+ T cells in Rag-5xfAD and Rag-WT mice brains. (S) Flow cytometry analysis of CD8+ and CD4+ T cells in immune intact 5xfAD and WT mice brains. Statistical significance was determined if $p < 0.05$.

CHAPTER TWO

EFFECTOR MEMORY CD8 T CELLS INFILTRATE THE BRAINS OF IMMUNE INTACT PS-5X MICE ACROSS MULTIPLE AGES

INTRODUCTION

In the past decade significant advances have been made in our understanding of the role of innate immunity in the pathogenesis of Alzheimer's disease. Many studies have employed new tools and genetic models to investigate the interactions between the brain's endogenous innate immune cells, microglia and Aβ and tau pathology (Heneka et al., 2015; Jay et al., 2015; Amit et al., 2018; Wes et al., 2016; Shi et al., 2018).). Genome-wide association studies (GWAS) also continue to implicate microglial-enriched genes in the risk of developing AD (Lambert et al., 2013; Bassett et al., 2021). Collectively, these studies have greatly advanced our understanding of the role of innate immunity in AD. In contrast, only recently have a handful of researchers begun to examine the potential role of adaptive immunity in this disease.

Given the robust infiltration of CD8+ T cells observed in bone marrow transplanted Rag-5XFAD mice, we sought to determine whether a similar effect can be observed within immune intact AD mice. Using a bigenic model of AD and flow cytometry, I have indeed confirmed that CD8 T cell infiltration is dramatically increased in immune intact AD mice. It is crucial to investigate the infiltration of T cells in both immune-deficient and immune-intact mice to establish that cytotoxic T cells have a role in AD pathology. The immune-deficient mice served as preliminary experiments to facilitate the identification of which adaptive immune cells infiltrate the AD brain. Studies in immune-intact AD mice have allowed us to

further explore the impact of T cells in bigenic Tau-5x mouse brains and to compare the varying response of T cells to amyloid, tau, or combined neuropathologies.

MATERIALS AND METHODS

Animals

All animal procedures were performed in strict accordance with National Institutes of Health and University of California guidelines. PS19 transgenic mice (Strain #:008169; B6C3F1/J) and 5XFAD mice (Strain #034848, B6 congenic) were purchased from the Jackson Laboratories. PS19 mice express mutant human microtubule-associated protein tau, MAPT, driven by the mouse prion protein (Prnp) promoter. Hemizygous PS19 and hemizygous 5XFAD mice were crossed to create a colony of mice that carried one of four genotypes; WT/WT, bigenic $PS19^{+/0}$/$5XFAD^{+/0}$, $PS19^{+/0}$/WT, or WT/$5XFAD^{+/0}$. The colony was subsequently expanded via additional crosses of PS19 and 5XFAD hemizygous F1 progeny.

Sex- and age-matched littermates were used for all studies except single cell sequencing analysis which focused specifically on female mice from all four genotypes. All animals were group housed on a 12h/12h light/dark cycle with access to food and water ad libitum.

Tissue Processing

For immune intact mouse studies, WT, 5XFAD, PS19, and PS-5X mice were sacrificed at 6, 9, and 12 months of age by carbon dioxide asphyxiation and spleen, deep cervical lymph nodes, and blood were gently removed prior to intracardial perfusion with 0.01M phosphate buffered saline (PBS). Hemibrains were processed and one side drop-fixed, cryoprotected, and sectioned as described above. The other hemibrain was immediately placed in complete RPMI medium, cut into fine pieces and placed in dissociation tubes along with Collagenase IV+ DNase1 in complete RPMI. Tissue was then placed into a gentleMACS Dissociator for 30 mins on the ABDK setting at 37°C and then filtered through 70μm strainers. Further removal of myelin and debris was conducted using 23% Percoll density gradient centrifugation. After isolation, cells were processed for flow cytometry as described below.

Flow Cytometry

Single cell suspensions isolated from the brain, spleen, deep cervical lymph nodes, and blood were immunophenotyped with fluorescent antibodies for the following cell surface markers for pan T cells: PE-conjugated CD3e (500A2; BD Biosciences; San Jose, CA), PerCP-eFluor710-conjugated CD3e (17A2, eBioscience). CD4 and CD8 T cells: PE-conjugated CD4 (GK1.5, BD Biosciences), Pacific Blue-conjugated CD4 (RM4-5, BioLegend; San Diego, CA)

APC-conjugated CD8 (Ly-2, BD Biosciences), PE/Cy7- conjugated CD8 (53-6.7, eBioscience). NK cells: APC-conjugated CD49b (DX5; BD Biosciences), PE-conjugated NK1.1 (PK136, BioLegend). B cells: FITC-conjugated CD45R/B220 (RA3-6B2; BD Biosciences), Alexa Fluor 710-conjugated CD19 (ebio1D3, eBioscience). Memory markers: BV421-conjugated CD44 (Biolegend), APC-conjugated CD62L (Biolegend). Hematopoietic lineage and monocyte markers: BUV496-conjugated CD45 (Biolegend), Alexa 700-conjugated CD11b (Biolegend). All cells examined by flow cytometry were first FC blocked with anti-CD16/32 (1:200; BD Biosciences). Cells were examined on a Fortessa flow cytometer (BD Biosciences) or BD FACSAria II (BD Biosciences) and analyzed with FlowJo v10.7 software (FlowJo; Ashland, OR).

In vitro T cell stimulation

T cells were isolated from deep cervical lymph nodes of 6-month-old female mice using a negative magnetic bead selection and co-cultured with whole brain homogenates at a 1:1 ratio. 100,000 brain T cells from either PS-5X mice or WT mice were cultured with 100,000 brain cells from either female PS-5X mice or WT mice. One mouse per group was used and the same set of cells were run in replicate wells. T cells from both genotypes were also cultured alone as a negative control. 96-well plates were coated with IgG and 1ug/mL anti-CD28 and T cells were activated by adding 1ug/mL anti-CD3 when cultured. T cells were stained

with CFSE proliferation dye. Cells were co-cultured for five days and then analyzed using a Fortessa Flow Cytometer and FlowJo software v10.7 (FlowJo; Ashland, OR).

Statistical Analysis

Statistical analysis was performed using Graphpad Prism 6 software and R. Comparisons involving more than two groups utilized one way analysis of variance (ANOVA). Comparisons of two groups utilized two-tailed Students t-test. All differences were considered significantly different when $p<0.05$.

RESULTS

CD8+ T cells infiltrate the brains of immune intact PS-5X mice across multiple ages

Given the robust infiltration of CD8 T cells observed in bone marrow transplanted Rag-5XFAD mice and corroborating evidence of infiltration in immune intact 5XFAD mice, we next sought to determine whether accumulation of another hallmark AD pathology, neurofibrillary tangles, could similarly induce T cell infiltration. As human AD patients exhibit both amyloid plaques and tau-laden neurofibrillary tangles, we also examined the impact of combined amyloid and tau pathologies by crossing 5XFAD mice with the PS19 model of tau pathology to generate bigenic AD mice that develop both plaques and tangles. PS19 mice express a human MAPT transgene carrying the frontotemporal dementia (FTD)-linked P301S mutation and develop progressive tau hyperphosphorylation and tangle formation {Yoshiyama, 2007 #11869}. While the P301S tau mutation leads to FTD, not AD, these mice develop neurofibrillary tangle pathology that closely mimics that of AD patients and are therefore widely used to examine FTD- and AD-related aspects of tau pathology {Gratuze, 2020 #11648;Iba, 2015 #11649;Sayed, 2018 #11650;Yoshiyama, 2007 #11794}.

As amyloid and plaque pathologies can exhibit substantial sex-dependent differences and also continue to accumulate as mice age, we examined T cell infiltration by flow cytometry in both male and female mice, across three different

ages: 6-, 9-, and 12-months of age (gating strategy shown in Figure 6B). At 6-months of age, sex-specific analysis of female or male 5XFAD mice reveals a non-significant trend towards increased CD8 T cell infiltration in female mice (p=0.0559), but no differences in male 5XFAD mice (Figure 2A). In contrast, tau pathology alone has no effect on CD8 recruitment in female mice but leads to a significant increase in male PS19 mice (P=0.0007). These sex-dependent differences in CD8 T cell recruitment are consistent with prior reports that female 5XFAD mice exhibit increased plaque pathology at early ages, whereas PS19 male mice exhibit increased Tau pathology versus females (Sil et al., 2022, Li et al., 2016). When amyloid and tau pathologies are combined in 6-month-old bigenic PS-5X mice, flow cytometry reveals a greater than 4-fold increase in CD8 T cell recruitment to the brain of both female and male PS-5X mice versus wild-type littermates (Figure 2A; female P=0.0005, male P=<0.0001). Representative FACS plot of 6-month old wild-type and PS-5X female mice shown in Figure 2A. Interestingly, the effects of amyloid, tau, and combined pathology appear to be restricted to CD8 T cells, as no differences in CD4 T cell recruitment are detected across any genotype.

By 9-months of age, increasing amyloid pathology in female mice is associated with a more than 3-fold increase in CD8 T cells recruitment to the brain (P=0.0023), but no significant difference in male 5XFAD mice **(**P=0.9694**)**. Combined amyloid and tau pathology also continues to recruit significant numbers

of CD8 T cells into the brains of both male and female 9-month old PS-5X mice (Figure 2B; female P=<0.0001, male P=<0.0001), representative FACS plot shown in Figure 2B. In contrast, CD4 T cell recruitment continues to show no significant differences. Similar results are observed at 12-months of age, except that the magnitude of CD8 T cell recruitment continues to be further increased in bigenic PS-5X mice with both sexes exhibiting a more than 4-fold increase in CD8 T cells in the brain in comparison to wild-type littermates (Figure 2C; female P=0.0496**,** male P=0.0057), representative FACS plot shown in Figure 2C. To further understand whether T cell recruitment is dependent on age, flow cytometry data was plotted across each of the three timepoints. Analysis by repeated-measures ANOVA revealed a significant effect of age on CD8 T cell recruitment in both female and male mice (Figure 2D). In contrast, no significant main effect of age was detected in CD4 T cell recruitment. To determine whether CD8 T cells can also be observed within the brain of PS-5X mice via immunohistochemical approaches we examined representative fixed coronal brain sections by confocal microscopy. This analysis revealed evidence of scattered CD8+ immunoreactive cells (red) adjacent to both amyloid plaques (blue) and tau immunoreactive neurons (white) within the hippocampus (Figure 2E**)** and multiple other brain regions. Taken together, our experiments confirm that either amyloid or tau pathology can induce the recruitment of CD8 T cells to the brain, although the effects appear to be sex-dependent. Furthermore, we find that combined amyloid

and tau pathologies lead to a substantial increase in CD8 T cell recruitment to the brain in PS-5X mice that is well beyond that observed in 5XFAD or PS19 mice alone, demonstrating that AD pathologies produce a synergistic effect on CD8 T cell recruitment to the brain.

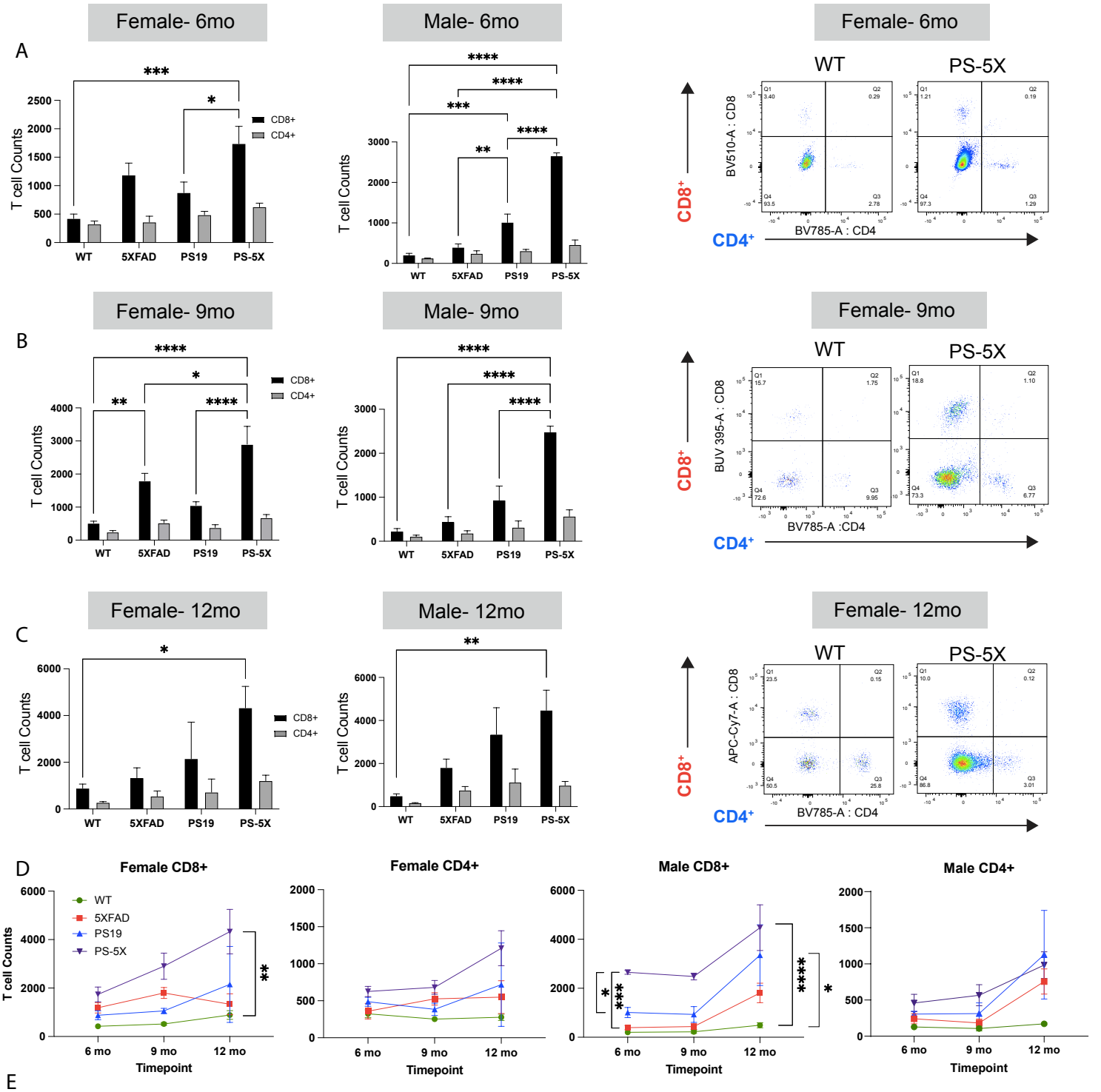

A
Female- 6mo
Male- 6mo
Female- 6mo
T cell Counts
CD8+
CD4+
WT
5XFAD
PS19
PS-5X
BV510-A : CD8
BV785-A : CD4
B
Female- 9mo
Male- 9mo
Female- 9mo
BUV 395-A : CD8
BV785-A :CD4
C
Female- 12mo
Male- 12mo
Female- 12mo
APC-Cy7-A : CD8
BV785-A : CD4
D
Female CD8+
Female CD4+
Male CD8+
Male CD4+
Timepoint
6 mo
9 mo
12 mo
E
Female -6mo - PS-5X
AmyloGlo
Merged
CD8
Tau

Figure 2. **CD8+ cytotoxic T cells infiltrate the bigenic PS-5X mice model at 6, 9, and 12 months old.** (A) Flow cytometry of live PI- CD45+ CD8+ and CD4+ T cell counts in female and male WT (no pathology), 5xFAD (plaque pathology), PS19 (tangle pathology), and PS-5X (tangle & plaque pathology) 6mo mice. Representative flow graphs of CD8+ and CD4+ T cells infiltrating 6mo female WT and PS-5X mice brains. (B) Flow cytometry of live PI- CD45+ CD8+ and CD4+ T cell counts in female and male WT, 5xFAD), PS19, and PS-5X 9mo mice. Representative flow graphs of CD8+ and CD4+ T cells infiltrating 9mo female WT and PS-5X mice brains. (C) Flow cytometry of live PI- CD45+ CD8+ and CD4+ T cell counts in female and male WT, 5xFAD, PS19, and PS-5X 12mo mice. Representative flow graphs of CD8+ and CD4+ T cells infiltrating 12mo female WT and PS-5X mice brains. (D) Flow cytometry analysis across the three timepoints for T cells counts of female CD8+ T cells, female CD4+ T cells, male CD8+ T cells, and male CD4+ T cells for each genotype. Statistical significance was determined if p value is <0.05.

Enrichment of Effector Memory CD8 T cells within the brains of AD transgenic mice

Recent reports have shown that effector memory CD8 T cells are enriched within the cerebral spinal fluid of AD patients and exhibit evidence of T cell receptor clonality (Gate et al., 2019). We therefore hypothesized that increased CD8 T cells observed within AD transgenic mice might also exhibit an effector memory phenotype. Whereas human effector memory cells (TEMRA) can be examined via co-expression of CCR7 and CD45RA, these markers do not accurately distinguish murine effector memory cells. However, murine effector memory cells (TEM) can be measured via a combination of high expression of the effector marker CD44 and low expression of the central memory marker CD62L (Bachmann et al., 2005; Reiser et al., 2016). Flow cytometry analysis using a panel of antibodies against CD45, CD3, CD8, CD4, CD44, and CD62L was performed to distinguish between naive (CD44-CD62+), effector (CD44-/CD62-), central memory (CD44+/CD62+), and effector memory (CD44+/CD62-) T cell subsets (gating strategy shown in Figure 6C). These experiments revealed a significant enrichment of effector memory CD8 T cells in male PS-5X mice across all three ages and a similar increase in female PS-5X mice at both 9- and 12-months of age (Figure 3 A,B,C). Interestingly, both male and female 5XFAD mice and PS19 mice also exhibited significant increases in TEM cells versus wild-type littermates at 9-months of age (Figure 3B). Thus, it appears that either amyloid pathology, tau

pathology, or combined pathologies can induce a significant increase in effector memory CD8 T cell recruitment to the brain. Whereas effector and central memory T cell subsets show no significant differences across any age or genotype, the proportion of naive CD8 T cells is significantly reduced in male PS-5X mice at 6- and 9-months age and in female PS-5X mice at 12-months of age (Figure 3 A,B,C). Results show that almost 80% of CD8 cells are effector memory T cells in 9mo female mice and almost 70% in 9mo male mice (Figure 3B). Effector memory cells make up almost 50% of CD8 T cells in 6mo female mice, and almost 60% in 6mo male mice brains (Figure 3A)**.** Lastly, effector memory cells make up almost 80% of CD8 T cells in 12mo female mice and almost 70% in 12mo male (Figure 3C). This demonstrates that the majority of infiltrating CD8 T cells are antigen-experienced and were likely recruited to brain tissue, rather than circulating randomly.

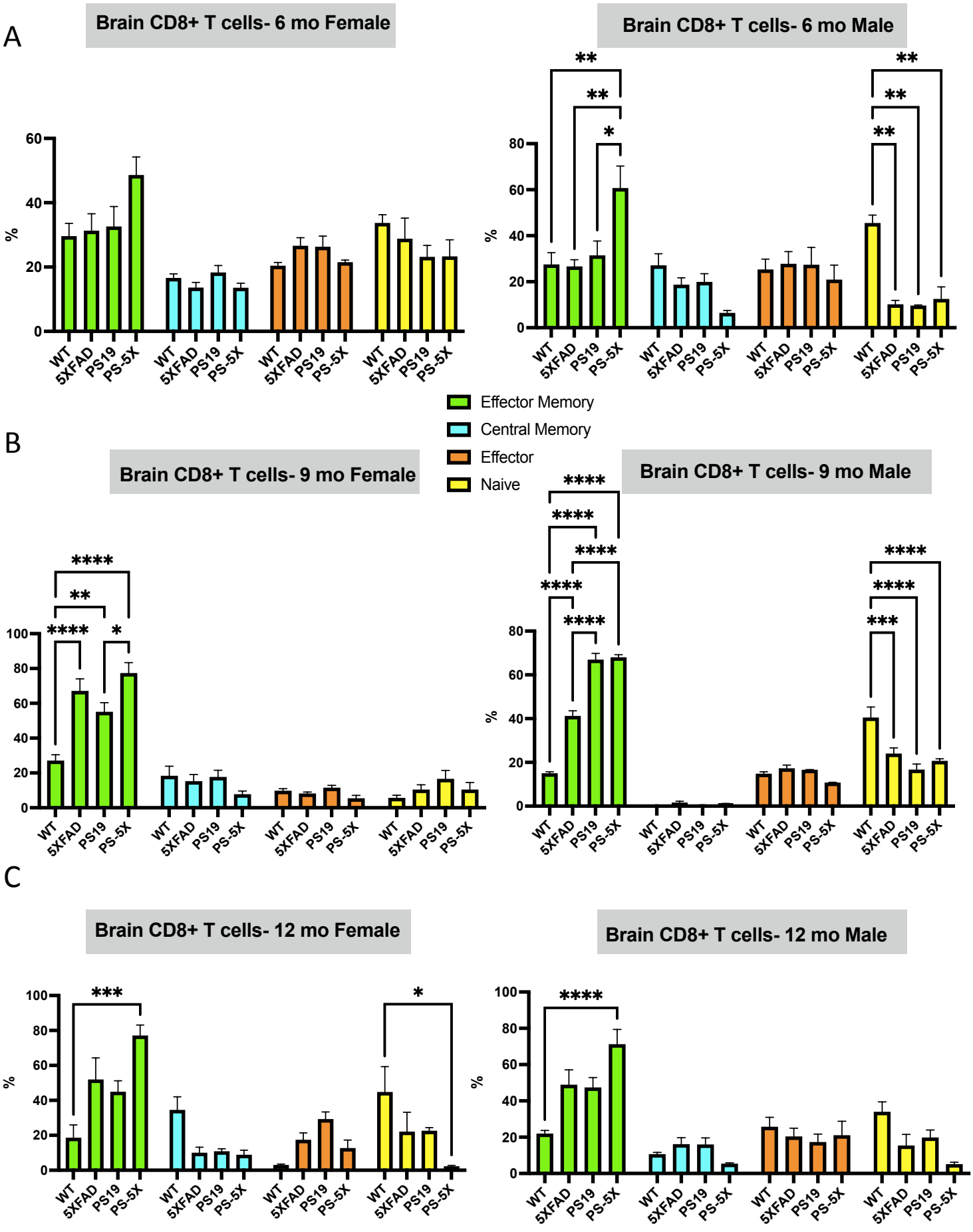
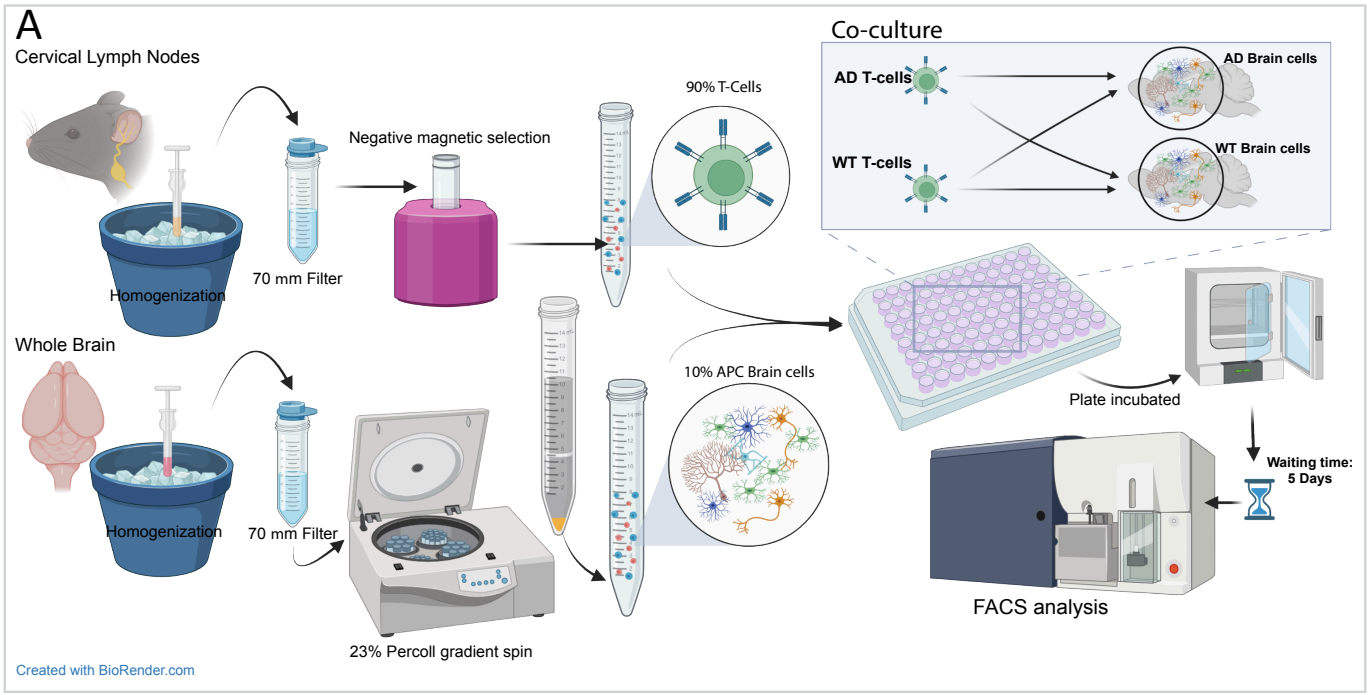

A
Brain CD8+ T cells- 6 mo Female
Brain CD8+ T cells- 6 mo Male
B
Brain CD8+ T cells- 9 mo Female
Brain CD8+ T cells- 9 mo Male
C
Brain CD8+ T cells- 12 mo Female
Brain CD8+ T cells- 12 mo Male
Effector Memory
Central Memory
Effector
Naive
%
WT
5XFAD
PS19
PS-5X

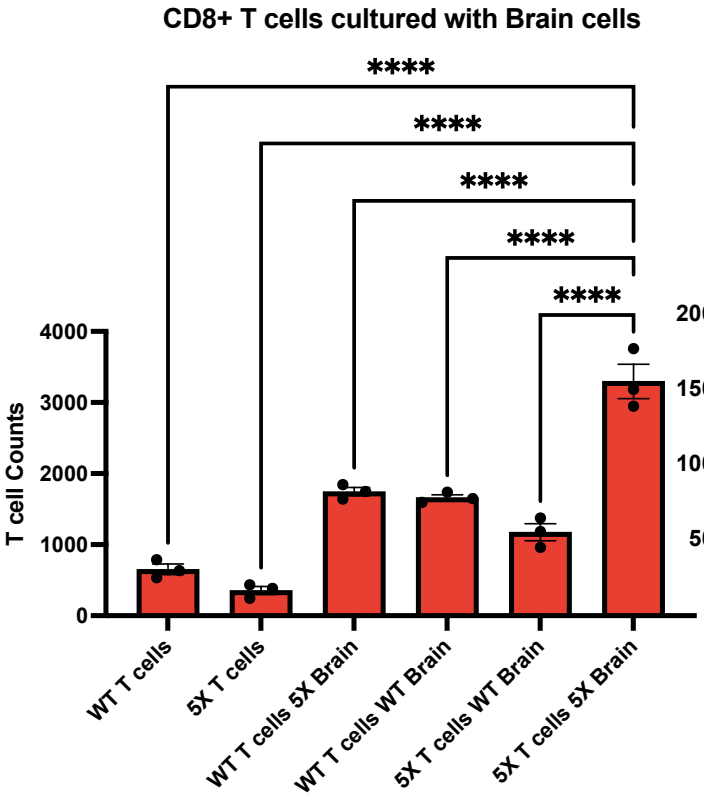
Figure 3. **Effector memory CD8+ T cells infiltrate the brains of bigenic PS-5X mice.** (A) 6mo female and male PS-5X, PS19, 5XFAD, and WT CD8+ T cells were analyzed via flow with memory markers CD44 and CD62L. Memory T cells were gated on as follows: central memory (CD44+,CD62L+), effector memory (CD44+, CD62L-), effector (CD44-, CD62L-), or naive (CD44-, CD62L+). (B) 9mo female and male PS-5X, PS19, 5XFAD, and WT CD8+ T cells were analyzed via flow with memory markers CD44 and CD62L. Memory T cells were gated on as follows: central memory (CD44+,CD62L+), effector memory (CD44+, CD62L-), effector (CD44-, CD62L-), or naive (CD44-, CD62L+). (C) 12mo female and male PS-5X, PS19, 5XFAD, and WT CD8+ T cells were analyzed via flow with memory markers CD44 and CD62L. Memory T cells were gated on as follows: central memory (CD44+,CD62L+), effector memory (CD44+, CD62L-), effector (CD44-, CD62L-), or naive (CD44-, CD62L+). Statistical significance was determined if $p < 0.05$.

Cytotoxic T cells respond and proliferate in an AD environment *in vitro*

To determine whether brain cells from AD mice can elicit T cell proliferation, indicative of AD- specific memory T cell activation, we conducted *in vitro* co-culture studies (Figure 4A). T cells were isolated from deep cervical lymph nodes via negative magnetic bead sorting, then co-cultured with whole brain homogenates. T cells from either PS-5X or WT mice were cultured with brain cells from either PS-5X or WT mice. After 5 days of co-culture, the cells were analyzed by flow cytometry. Results showed that the T cells responded robustly to an AD brain environment. CD8+ T cells from AD mice proliferated significantly when co-cultured with AD brain cells and had a 2-fold increase compared to CD4+ T cells (Figure 4B), suggesting an AD-related antigen-specific response. These T cells come from an AD environment where they have been exposed to an AD antigen, when they are cultured with brain cells that also come from an AD environment, they respond and proliferate. In contrast, very little proliferation was observed when T cells isolated from wild type mice were cultured with AD homogenates or when T cells from AD mice were cultured with homogenates derived from wild type mice.


A
Cervical Lymph Nodes
Homogenization
70 mm Filter
Negative magnetic selection
90% T-Cells
Co-culture
AD T-cells
WT T-cells
AD Brain cells
WT Brain cells
Whole Brain
Homogenization
70 mm Filter
23% Percoll gradient spin
10% APC Brain cells
Plate incubated
Waiting time:
5 Days
FACS analysis
Created with BioRender.com

B


CD8+ T cells cultured with Brain cells
T cell Counts
4000
3000
2000
1000
0
****
****
****
****
****
WT T cells
5X T cells
WT T cells 5X Brain
WT T cells WT Brain
5X T cells WT Brain
5X T cells 5X Brain

C

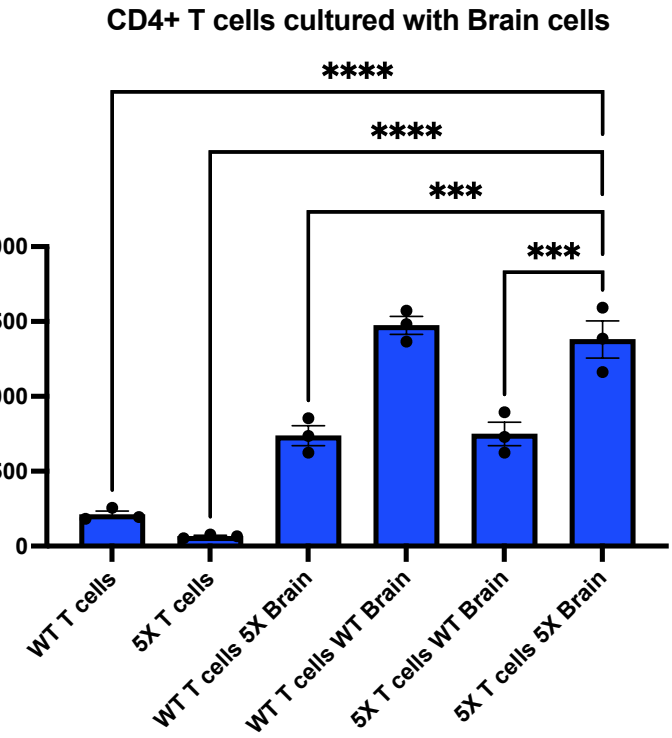

CD4+ T cells cultured with Brain cells
2000
1500
1000
500
0
****
****
***
***
WT T cells
5X T cells
WT T cells 5X Brain
WT T cells WT Brain
5X T cells WT Brain
5X T cells 5X Brain

Figure 4. **AD cytotoxic T cells proliferate significantly when co-cultured with AD brain cells in vitro.** (A) Deep cervical lymph nodes were extracted from mice, homogenized, filtered, and then T cells were isolated using a negative magnetic bead selection. Whole brains were extracted from mice, homogenized, filtered, and then brain cells were isolated as a pellet using 23% percoll gradient. Isolated T cells and brain cells were co-cultured at a 1:1 ratio (100,000 cells each). T cells from PS-5X (amyloid plaque & tau tangle pathology) and WT (no pathology) 6mo mice were co-cultured with brain cells from PS-5X (amyloid plaque & tau tangle pathology) and WT (no pathology) 6mo mice. (B) Flow cytometry analysis of CD8+ and CD4+ T cells in different co-culture conditions. Statistical significance was determined if $p < 0.05$.

CHAPTER THREE

SINGLE CELL SEQUENCING REVEALS A DIVERSE ARRAY OF T CELL SUBTYPES THAT INFILTRATE THE BRAIN, SHOW A HIGH EXPRESSION OF MULTIPLE AD RISK GENES, AND DEMONSTRATE SIGNIFICANT CLONAL EXPANSION IN AD MICE

INTRODUCTION

We utilized a novel version of 10x single-cell sequencing, that allows one to simultaneously examine both the transcriptome of T cells and the specific T cell receptor (TCR) sequences that each cell processes. In doing so this approach allows one to not only examine the many different subtypes of T cells that may infiltrate the AD brain but also to determine whether infiltrate T cells exhibit evidence of clonality that would indicate a highly specific response to restricted antigens. Importantly, this new version of the 10X single cell sequencing platform enables concurrent sequencing of the T cell receptors (both alpha and beta chains) from each cell, thus future experiments can be designed to identify the specific antigens that drive any observed clonality.

MATERIALS AND METHODS

Animals

All animal procedures were performed in strict accordance with National Institutes of Health and University of California guidelines. PS19 transgenic mice (Strain #:008169; B6C3F1/J) and 5XFAD mice (Strain #034848, B6 congenic) were purchased from the Jackson Laboratories. PS19 mice express mutant human microtubule-associated protein tau, MAPT, driven by the mouse prion protein (Prnp) promoter. Hemizygous PS19 and hemizygous 5XFAD mice were crossed to create a colony of mice that carried one of four genotypes; WT/WT, bigenic PS19$^{+/}$

^{0}/5XFAD$^{+/0}$, PS19$^{+/0}$/WT, or WT/5XFAD$^{+/0}$. The colony was subsequently expanded via additional crosses of PS19 and 5XFAD hemizygous F1 progeny. Sex- and age-matched littermates were used for all studies except single cell sequencing analysis which focused specifically on female mice from all four genotypes. All animals were group housed on a 12h/12h light/dark cycle with access to food and water ad libitum.

Tissue Processing

For immune intact mouse studies, WT, 5XFAD, PS19, and PS-5X mice were sacrificed at 6, 9, and 12 months of age by carbon dioxide asphyxiation and spleen, deep cervical lymph nodes, and blood were gently removed prior to intracardial perfusion with 0.01M phosphate buffered saline (PBS). Hemibrains were processed and one side drop-fixed, cryoprotected, and sectioned as described above. The other hemibrain was immediately placed in complete RPMI medium, cut into fine pieces and placed in dissociation tubes along with Collagenase IV+ DNase1 in complete RPMI. Tissue was then placed into a gentleMACS Dissociator for 30 mins on the ABDK setting at 37°C and then filtered through 70μm strainers. Further removal of myelin and debris was conducted using 23% Percoll density gradient centrifugation. After isolation, cells were processed for flow cytometry as described below.

Flow Cytometry

Single cell suspensions isolated from the brain, spleen, deep cervical lymph nodes, and blood were immunophenotyped with fluorescent antibodies for the following cell surface markers for pan T cells: PE-conjugated CD3e (500A2; BD Biosciences; San Jose, CA), PerCP-eFluor710-conjugated CD3e (17A2, eBioscience). CD4 and CD8 T cells: PE-conjugated CD4 (GK1.5, BD Biosciences), Pacific Blue-conjugated CD4 (RM4-5, BioLegend; San Diego, CA) APC-conjugated CD8 (Ly-2, BD Biosciences), PE/Cy7- conjugated CD8 (53-6.7, eBioscience). NK cells: APC-conjugated CD49b (DX5; BD Biosciences), PE-conjugated NK1.1 (PK136, BioLegend). B cells: FITC-conjugated CD45R/B220 (RA3-6B2; BD Biosciences), Alexa Fluor 710-conjugated CD19 (ebio1D3, eBioscience). Memory markers: BV421-conjugated CD44 (Biolegend), APC-conjugated CD62L (Biolegend). Hematopoietic lineage and monocyte markers: BUV496-conjugated CD45 (Biolegend), Alexa 700-conjugated CD11b (Biolegend). All cells examined by flow cytometry were first FC blocked with anti-CD16/32 (1:200; BD Biosciences). Cells were examined on a Fortessa flow cytometer (BD Biosciences) or BD FACSAria II (BD Biosciences) and analyzed with FlowJo v10.7 software (FlowJo; Ashland, OR).

10x scSeq Gene Expression and TCR Profiling

A second cohort of 9 month old female mice from each of the four genotypes was used for 10X single cell sequencing. Mouse brains were processed for cell isolation using the gentleMACS Dissociator as described above and cells were then stained with the following antibodies: APC-CD8, APC-Cy7-CD45, FITC-CD4, PE-TCRg/d, BV421-CD11b, and PI (all with 1:200 dilution). T cells were then sorted on a BD FACSAria II and cells from multiple mice were pooled for each genotype into 2 samples each for WT and 5XFAD and 1 sample each of PS19 and PS-5x: n=10 WT-1, n=8 WT-2, n=4 5XFAD-1, n=3 5XFAD-2, n=4 PS19, n=4 PS-5X. A total of six 10X libraries were then generated from these samples.; 2 each for WT and 5XFAD and 1 each for PS19 and PS-5X. Single cell 5' gene expression libraries and single cell V(D)J enriched libraries were prepared according to the 10x Genomics Chromium Next GEM Single Cell V(D)J Reagent Kits v1.1 user guide (CG000207 Rev F). The Chromium Single Cell Mouse T Cell Enrichment Kit only enriches for αβ TCRs, so 5' gene expression was obtained for all T cells but clonotype data was limited to αβ T cells. The 10x Genomics workflow was then followed according to the manufacturer protocol and libraries were pooled at equimolar concentrations for sequencing on an Illumina NovaSeq 6000, targeting ~50,000 reads per cell. Gene expression and V(D)J FASTQ files from each sample were aligned to the GRCm38 (mm10) mouse transcriptome with the 10x Genomics Cell Ranger 6.0.1 multi command, using references provided by

10X Genomics ("refdata-gex-mm10-2020-A" for gene expression and "refdata-cellranger-vdj-GRCm38-alts-ensembl-5.0.0" for V(D)J alignment). For cellranger multi, "expect-cells" was set to 10,000 and all other arguments were set to default. Results from all samples were combined using cellranger aggr, with "normalize" set to none.

Quality control and filtering of single cell gene expression

Analysis of single-cell count and clonotype data was conducted in R using the Seurat package (version 4.1.0). A Seurat object was created from the filtered count matrix, as output by *cellranger aggr*, using min_cells = 10 and min_features = 200. Cells that contained multiple distinct TCR-β chains, as identified by TCR sequencing, were considered doublets and removed from the data set. Cells which did not have a recognized TCR *and* did not express at least one count of Cd3d, Cd3e, or Cd3g were also removed from the data set. Remaining cells were further filtered to only examine cells that had more than 800 genes expressed, less than 5% mitochondrial gene expression, and between 10% and 55% of ribosomal gene expression. These parameters were determined by visual inspection of population distribution along these variables. To preserve the cluster of proliferating cells but remove possible doublets missed by TCR filtering, cells were given a "complexity" score, defined as the total count of RNA in the cell divided by the number of genes. Cells with a complexity score greater than 4.75 or with more than 20,000 RNA

counts were discarded as doublets. After filtering, the data set contained a total of 16,135 T cells distributed across samples as follows: WT-1 (680 cells), WT-2 (3335 cells), 5XFAD-1 (2326 cells), 5XFAD-2 (1940 cells), PS19 (2186 cells), and PS5X (5668 cells).

Assigning a "stress score" to cells

During preliminary analysis, we discovered a batch effect between the first sequencing run of samples (WT-1, 5XFAD-1, PS19, and PS-5X) and the second run (WT-2 and 5XFAD-2). Differential expression between batches showed many genes known to be upregulated in response to heat dissociation of immune cells during preparation for RNA sequencing, including heat shock proteins (Hspa8, Hspa1a) and other stress-related chaperones (Dnajb1, Fkbp4). Differential genes were also enriched for the Reactome pathway "cellular response to heat stress". This effect was most likely attributable to differences in preparation time between the two batches. To account for these differences in stress pathways, we created a "stress score" for each cell based on expression of 16 genes (Atm, Bag3, Dnajb1, Hsp90aa1, Hsp90ab1, Hspa1a, Hspa1b, Hspa4l, Hspa5, Hspa8, Hsph1, Mapkapk2, Nup205, Nup98, Rpa3, Sirt1). These genes were selected by starting with the list of 83 mouse genes in the Reactome "cellular response to heat stress" pathway, and filtering it down to genes that were expressed in at least 5% of cells in the data set and which were in the top 4000 most variable genes (as determined by the Seurat

function *FindVariableFeatures*). The Seurat *AddModuleScore* function was used to calculate the stress score for each cell. This score was used as a latent variable in downstream analysis.

Clustering and subpopulation analysis

For clustering analysis, the filtered data set was scaled using the Seurat integration pipeline. Briefly, the Seurat object was split into multiple objects, one per sample. Each sample was scaled separately using *SCTransform*, regressing out total RNA count and stress score. Samples were combined back into one Seurat object with *IntegrateData,* using 4,000 integration features. The integrated data was clustered using *FindNeighbors* and *FindClusters* using PCA dimensions 1-40 and a resolution of 0.1. We were also interested in examining CD8+, CD4+, and γδ cells separately, so subpopulation analyses were performed as described below:

Although αβ T cells can express TCRγ genes at the RNA level, for both CD8+ and CD4+ analysis, we chose to remove all cells that expressed *any* markers of γδ (Trdc, Tcrg-V1, Tcrg-V3, Tcrg-V4, Tcrg-V5, Tcrg-V6, Tcrg-V7, Tcrg-C1, Tcrg-C3, Tcrg-C4, Trdv1, Trdv2-1, Trdv2-2, Trdv3, Trdv4, Trdv5, Sox13, and Blk), regardless of if they seemed likely to be αβ T cells. We noted, however, that Trgv2 and Tcrg-C2 were expressed in an unusually large number of cells that was inconsistent with our own flow cytometry data and with the literature, so we did not use those two genes as a γδ marker.

For CD8+ analysis, we filtered the data set to retain only cells that expressed at least one count of Cd8a or Cd8b1, and did not express Cd4 or any γδ markers described above. This left 9,127 CD8+ αβ cells. These cells were re-integrated using *SCTransform* and *IntegrateData* as above. Data was clustered using PCA dimensions 1-40 and a resolution of 0.5.

For CD4+ analysis, we filtered the original data set to retain cells that expressed at least one count of Cd4 and did not express either Cd8a or Cd8b1, or any γδ markers described above. This left 2,441 CD8+ αβ cells. There were too few cells to use the integration pipeline, so *SCTransform* was run on all cells together, using 4000 variable features and regressing out total RNA count and stress score. The SCT assay was used to calculate PCA, and data was clustered using PCA dimensions 1-25 and a resolution of 0.3.

For γδ analysis, we used a more stringent list of markers to retain only cells that were highly likely to be true γδ cells. We filtered the original data set to contain only cells that expressed at least 1 count of any Tcrδ gene (as alpha rearrangement removes the delta locus) or γδ-specific transcription factor (Trdc, Trdv1, Trdv2-1, Trdv2-2, Trdv3, Trdv4, Trdv5, Sox13, or Blk). This left 1,828 γδ cells. There were too few cells to use the integration pipeline, so SCTransform was run on all cells together, using 4000 variable features and regressing out total RNA count and stress score. The SCT assay was used to calculate PCA, and data was clustered using PCA dimensions 1-30 and a resolution of 0.3.

Differential expression analysis

For all differential gene testing, Seurat's *FindAllMarkers* or *FindMarkers* functions were called on log-normalized counts using MAST to test for significance and regressing out total RNA count and stress score as latent variables. All p-values were adjusted using Bonferroni correction. Genes were considered significantly different if adjusted p <= 0.01, log fold-change >= 0.25, and at least 10% of the cells in the cluster (or genotype) expressed the gene.

Differential composition analysis.

To assess potential differences in population proportion between genotypes, given only one to two samples per genotype, we used a modified version of scDC (version 0.1.0). Briefly, the scDC_NoClustering method was used to resample cells from each sample 10,000 times. This method then counted the number of cells with each cluster identity in each resample. To account for the large differences in total cell count between samples, re-sampled population counts were normalized to be between 0 and the minimum total cell count among the six samples. We then fit a generalized linear model to the distribution of counts using the formula glm(cellCount ~ cellType*genotype, family = poisson(link=log)) where cellCount is the number of cells identified as a certain cell type (cluster) for a certain genotype. Statistics from each GLM were pooled into a single statistic as in Cao et al., 2019 using the R package mice's pool function. Significance of the pooled

estimates was determined by the univariate Wald test. We found significant interactions between multiple clusters and genotypes, so we also conducted post-hoc pairwise comparisons. For each GLM, we ran the multcomp function glht(fit, linfct=emm(pairwise~genotype|cellTypes, adjust="tukey")), which generated pairwise comparisons between genotypes within each cluster. Estimates from each GLM for each comparison were pooled using mice's pool.scalar function, using the Benjamini-Hochberg correction for multiple comparisons. Population differences were considered significant if $p <= 0.05$.

Code Availability

The code written to process 10X scSeq data is publicly available on GitHub: https://github.com/jaclynrbeck/TCellsAD2022

RESULTS

Single cell sequencing reveals a diverse array of T cell subtypes that infiltrate the brains of AD mice

To further investigate the role of T cells in the AD brain environment, we profiled T cell gene expression and T cell receptor (TCR) clonality at the single-cell level using 10x Genomics' single cell immune profiling. We sequenced CD8+, CD4+, and γδ T cells from 9 month old female mice from four different genotypes (WT, 5XFAD, PS-19, and PS-5X) (Figure 5A). Each sample contained a pool of T cells from multiple mice of the same genotype. Clustering of all samples based on gene expression revealed 7 distinct clusters, including cytotoxic cells, naive cells, and a large population of γδ-17 cells (Figure 5B). Consistent with our flow cytometry data, CD8+ T cells made up the majority of the population of infiltrating cells. Additionally, there was a significant increase in the proportion of cytotoxic CD8+ T cells in the amyloid-model mice (5XFAD and PS-5X) compared to WT or PS19 (Figure 5D,E). This corresponded with a significant increase in the proportion of naive-like T cells in the WT and PS19 samples. This suggests that amyloid pathology, but not tau pathology, promotes recruitment of antigen-experienced cytotoxic T cells into the brain.

Differential gene expression between genotypes further supports this. Both the 5XFAD and PS-5X cells show significantly increased expression of cytotoxic and effector genes including Gzmk, Xcl1, Ccl4, Ctsw, and Cd69, while down-

regulating genes associated with naive T cells, including Sell, Ccr7, Tcf7, and Lef1 (Figure 5F). The amyloid models also show increased expression of genes associated with tissue residency (Cxcr6, Cxcr3, Itgae, Litaf, and Cd69) and proliferative exhaustion (Pdcd1, Ctla4, Lag3, Tigit), revealing a higher level of T cell recruitment and clonal expansion in the 5XFAD and PS-5X brain. Interestingly, 5XFAD and PS-5X cells also show a significant up-regulation of interferon-stimulated genes (ISGs) including Ifit3, Ifitm3, Irf7, and Bst2, with PS-5X cells having the largest increase in expression.

We note a disproportionate increase of "activated" CD8+ cells in one of our WT samples which is not reflected in the other WT sample or in samples from the other genotypes. Cells in the "activated" cluster are the only cells in the data set that express high levels of Gzma, Cx3cr1, Zeb2, Klrg1, and S1pr5, and they express higher levels of Ifng, Gzmb, Prf1, Klrk1, Ccl5, and Nkg7 than other clusters. This suggests that these are highly activated effector cells responding to an acute insult. Additionally, of the top 10 clonally-expanded TCRs in this cluster, 8 belong to this one WT sample. Given the gene expression profile and the amount of clonal expansion in these WT cells, we believe the cells are likely to be from the same mouse and reflect an anomalous acute immune response not present in the other mice. The effect of these cells is visible in population distribution statistics (Figure 5D,E) and differential gene expression between genotypes (Figure 5F), so

we are cautious about making claims about this cluster or its genetic profile in relation to genotype differences.

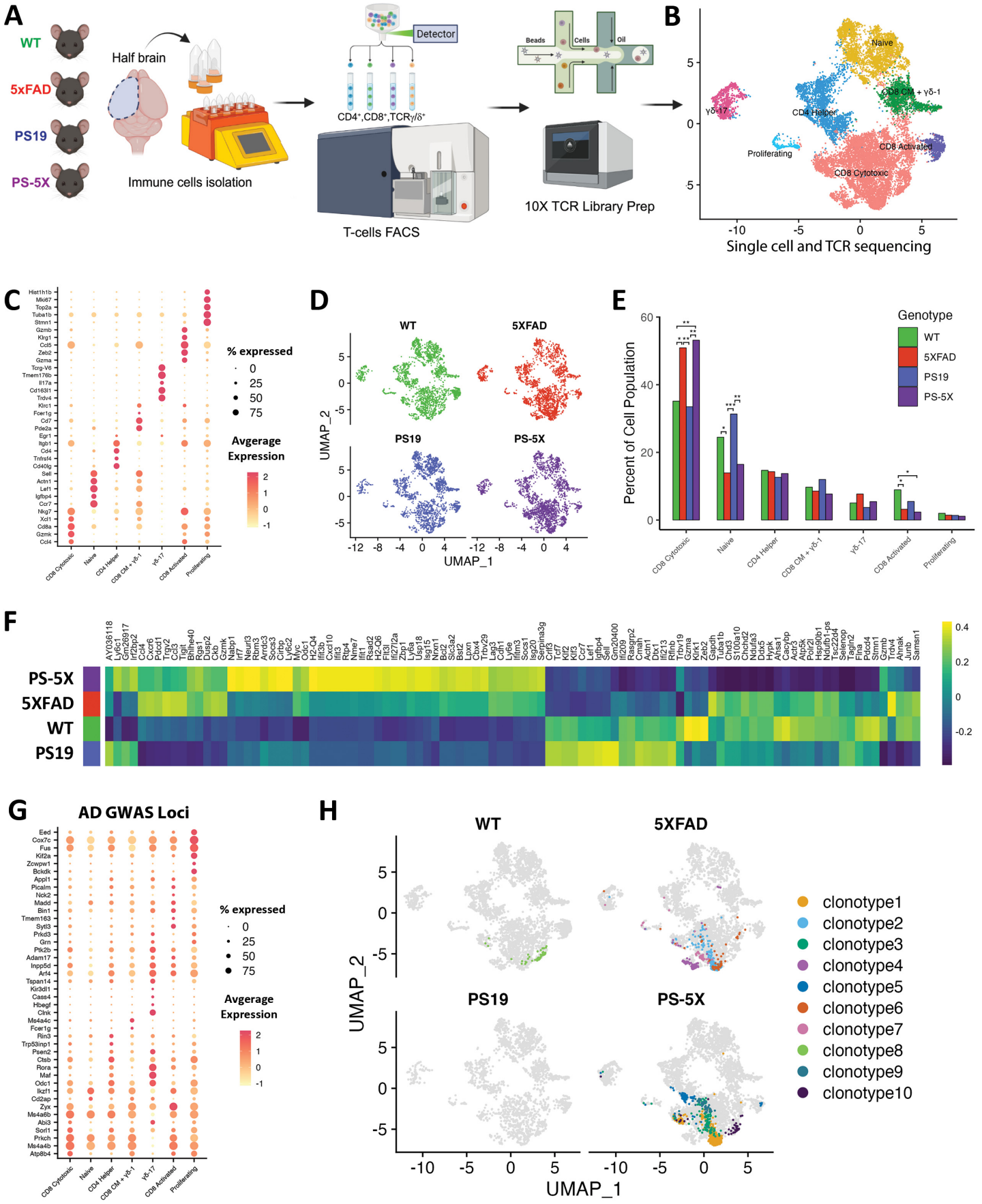
T cells show high expression of multiple AD risk genes

Recent genome-wide association studies have implicated many immune-related genes in AD risk. As microglia are the primary and most abundant immune cell within the brain many groups, including our own, have sought to understand the impact of AD risk genes on microglia function. However, some of these AD risk genes have also been shown to be expressed in adaptive immune cells. To better understand whether AD risk genes might influence the infiltration or function of T cells, we have examined the expression levels of mouse homologues of AD risk genes identified from GWAS genes in our single cell sequencing data. Remarkably, we find that many of these AD risk genes are highly expressed in multiple subtypes of T cells. 42 AD risk genes were significantly upregulated in at least one cluster, including 16 upregulated in the γδ-17 cluster alone (Figure 5G). Notably, the risk genes Clnk and Hbegf are almost exclusively expressed in γδ cells. Furthermore, these genes are significantly upregulated in T cells and gamma delta cells in the bigenic model that develops both hallmark AD pathologies (Figure 5). While ongoing studies are further exploring the implications of these findings, these data suggest that mutations in AD risk genes may not only alter

microglia function but also influence the function and/or infiltration of T cells. These findings also show that the role of γδ cells in AD bears further scrutiny.

TCR clonality in AD mice

Lastly, single-cell TCR sequencing reveals consistent evidence of significant clonal expansion of T cells in AD mice compared to wild-type controls. Clonally expanded TCRs were found to be enriched specifically in the cytotoxic CD8 T cells, with 9 of the top 10 expanded clonotypes in the data set belonging to cells from amyloid-model mice (5XFAD and PS-5X clonotypes), primarily from the CD8 cytotoxic cluster. Interestingly both of the amyloid genotypes (5X and PS-5X) elicited more TCR response compared to Tau (Figure 5H). Notably, the top PS-5X clonotype makes up 7.2% of all PS-5X cells in our data set, and the top 5XFAD clonotype makes up 4.2% of 5XFAD cells. In contrast, the top clonotypes for WT and PS19 cells represent only 1.3% and 1.2% of those groups, respectively.


A
WT
5xFAD
PS19
PS-5X
Half brain
Immune cells isolation
Detector
CD4+,CD8+,TCRγ/δ+
T-cells FACS
Beads
Cells
Oil
10X TCR Library Prep
B
Naive
CD8 CM + γδ-1
γδ-17
CD4 Helper
Proliferating
CD8 Activated
CD8 Cytotoxic
Single cell and TCR sequencing
C
% expressed
0
25
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CD8 Cytotoxic
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CD4 Helper
CD8 CM + γδ-1
γδ-17
CD8 Activated
Proliferating
D
WT
5XFAD
PS19
PS-5X
UMAP_2
UMAP_1
E
Genotype
WT
5XFAD
PS19
PS-5X
Percent of Cell Population
F
PS-5X
5XFAD
WT
PS19
G
AD GWAS Loci
% expressed
0
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H
WT
5XFAD
PS19
PS-5X
UMAP_2
UMAP_1
clonotype1
clonotype2
clonotype3
clonotype4
clonotype5
clonotype6
clonotype7
clonotype8
clonotype9
clonotype10

Figure 5. **All Cells single-cell sequencing.** (A) Schematic of workflow to obtain single-cell sequencing data. T cells were isolated from half-brains, sorted based on the presence of CD8, CD4, or γδ TCR, and then sequenced for both general RNA and TCR-specific RNA using the 10X Genomics workflow. (B) Clustering of all samples together revealed grouping by common T cell phenotypes. The seven clusters were identified by characteristic markers as Naive (Sell, Ccr7, Cd44-), Central Memory (Sell, Ccr7, Cd44) + γδ type 1 (Trdc, Nkg7, Il4-, Il17a-), Activated (Ccl5++, Cx3cr1, Klrk1), Cytotoxic (Gzmb, Gzmk, Nkg7), CD4 Helper (Cd4, Cd40lg, Cd44), γδ type 17 (Trdc, Blk, Il17a), and Proliferating (Mki67, Top2a). (C) Dot plot of the top 5 most up-regulated genes in each cluster. Dot color represents average expression value in the cluster, and dot size represents the proportion of cells in the cluster that express each gene. (D) UMAP of cell clusters split by genotype. (E) Population analysis revealed significant differences in cluster proportions between genotypes. Significance markers: * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$. (F) Heatmap of the top 100 most up- or down-regulated genes when comparing each genotype to WT cells. Mitochondrial and ribosomal genes, as well as genes related to heat stress, have been removed from the list. (G) List of GWAS-identified AD risk genes which are significantly changed in at least one of the 7 clusters, ignoring genotype. (H) UMAP of the location of the top 10 most clonally-expanded clonotypes, split by genotype.

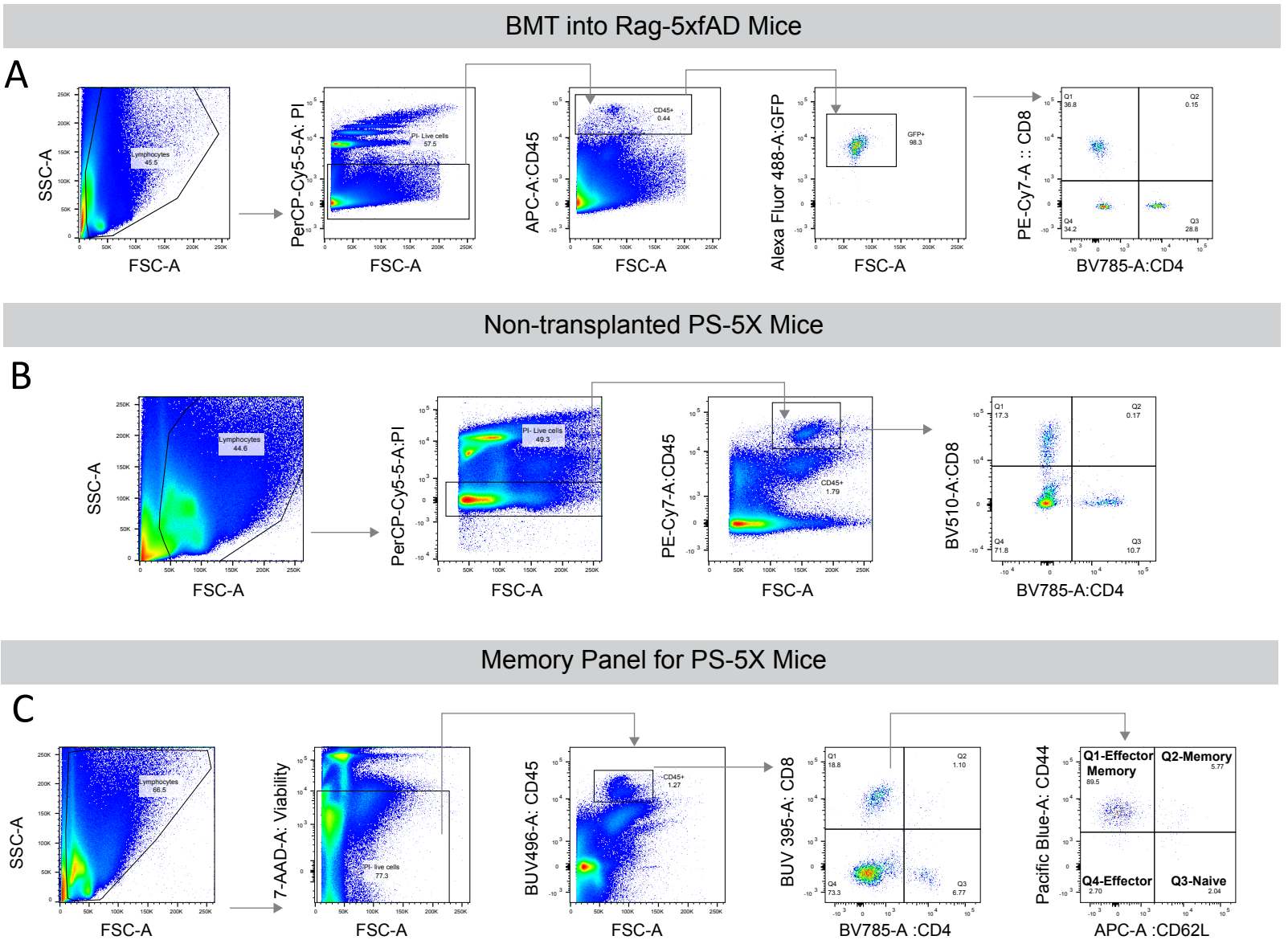

Figure 6. **Flow cytometry gating strategy for different T cells panels.** (A) GFP+ T cells in the BMT immune-deficient Rag-5xfAD mice were gated on as lymphocytes that are singlet, live, CD45hi, GFP+, CD4+ or CD8+ T cells. (B) Non-transplanted T cells from immune-intact PS-5X mice were gated on as lymphocytes that are singlet, live, CD45hi, CD4+ or CD8+ T cells. (C) PS-5X mice were analyzed with memory markers CD44 and CD62L.. These cells are lymphocytes, singlet, live, CD45hi, CD8+ that are either central memory (CD44+,CD62L+), effector memory (CD44+, CD62L-), effector (CD44-, CD62L-), or naive (CD44-, CD62L+) T cells.

DISCUSSION

Despite many years of research and considerable progress, Alzheimer's disease continues to represent a major emotional and financial burden to patients, caregivers, and society as a whole. Current therapies are largely palliative and once promising approaches heralded as "disease-modifying" continue to fail in late stage clinical trials. It therefore remains critically important to continue to improve our understanding and to expand our thinking and inquiry regarding the cell types and mechanisms that may underlie this devastating disease. Within the last decade a great deal of progress has been made in our collective understanding regarding the role of innate immunity in AD. Yet innate and adaptive immunity rarely work in isolation. Instead complex signaling between innate brain-resident microglia and T cells that routinely survey the CNS are likely important in AD and many other neurodegenerative disorders.

To further expand and advance our understanding of the potential roles of adaptive immunity in AD, my thesis coupled traditional immunological methods such as flow cytometry and bone marrow transplantation paradigms with modern approaches such as single cell RNA sequencing and TCR repertoire analysis. Using these complimentary methods we discovered that memory CD8+ T cells infiltrate the brains of AD mice *in vivo* and can further respond by proliferating in response to an AD-like environment *in vitro*. Using the Rag-5X FAD immune-deficient AD mouse model we began by exploring the role of the adaptive immune

system in AD via flow cytometry and IHC. Collectively we have found that a significant amount of memory cytotoxic T cells respond to AD brain-derived signals and infiltrate the brain. We have also learned that infiltrating cytotoxic T cells can be detected adjacent to microglia, and sometimes surrounding plaque-associated microglia within AD mouse brains. We also found that unlike T cells in transgenic AD brains, WT mouse T cells do not respond equivalently to transgenic AD brain lysates *in vitro* and do not infiltrate the brain parenchyma of WT mice *in vivo*.

To understand whether tau pathology or combined amyloid and tau pathology in bigenic PS-5X mice can further enhance the recruitment of T cells to the brain, I utilized three different immune-intact AD mouse models; 5XFAD mice, PS19 Tau mice, and PS-5X biogenic mice that develop both hallmark AD pathologies. By examining mice across multiple ages, my studies demonstrated that that, memory CD8+ T cells are significantly elevated in both male and female bigenic PS-5X mouse brains at 6 months and 9 months of age. Using these immune-intact bigenic mice also we conducted co-culture experiments, demonstrating that PS-5X brain tissue samples elicit robust proliferation of T cells derived from PS-5X deep cervical lymph nodes. In contrast, co-culture of PS-5X T cells with WT brain tissue samples or WT T cells with wither WT or PS-5X tissue samples has little effect on T cell proliferation. These experiments therefore further confirm that amyloid and tau pathology induce pathology specific memory CD8

which can be activated in response to AD mouse brain samples. While these *in vitro* findings are exciting they also point to the need to pursue additional future co-culture experiments to determine whether cytotoxic T cells responses are triggered specifically by amyloid pathology or tau pathology alone, or via some other downstream pathological component such as neuronal apoptosis.

Our single-cell RNA analysis of infiltrating T cells revealed a significant increase in interferon-stimulated genes (ISGs), including Irf7, Ifit3, and Bst2, in cytotoxic CD8 T cells from both 5XFAD and PS-5X mice. This suggests that amyloid induces pro-inflammatory interferon-responsive signaling between cytotoxic T cells and other cell types. However, it is unclear whether this heightened INF-responsive signaling arises from other T cells versus degenerating neurons, activated microglia, or other brain resident cell types. A prior study suggested that type I interferon responses within plaque-associated microglia enhances inflammation and drives synaptic loss in AD mice (Roy et al., 2020). However, the role and impact of interferon-responsive T cells remains unclear. Future studies will likely be needed to determine whether INF-responsive T cells similarly drive synaptic degeneration and/or enhance the microglia proinflammatory response.

Our single-cell analysis additionally revealed 42 GWAS-identified AD risk genes that were significantly upregulated in at least one cluster of T cells, demonstrating that microglia are not the only immune cell population with the

brain of AD mice that may be affected by these risk alleles. Surprisingly, the γδ-17 cluster alone accounted for 16 of these genes, including genes like Clnk that are almost exclusively expressed in this cluster. Both neurons and glia directly respond to IL-17 (Moynes et al., 2014), and IL-17-secreting γδ cells in the brain have been shown to negatively affect cognition in AD (Brigas et al., 2021) and to have harmful effects in other CNS diseases (Wo et al., 2020; Chen et al., 2020). Unlike αβ TCRs, γδ TCRs are not restricted to MHC I or II binding for antigen recognition, and antigen targets of γδ-17 cells are mostly unknown (Chien et al., 2014; Constantinides et al., 2021). Our data shows that this population of cells warrants further investigation, including exploration of potential AD-related antigens that might be recognized by γδ cells and examination of interactions between these cells and neurons and glia in the AD brain.

Our IHC data has shown that T cells are sometimes observed adjacent and even surrounded by microglial processes, suggesting that direct interaction and perhaps even antigen presentation by microglia occurs within the brain of AD mice. If this is indeed the case, it will be important to determine what specific antigen(s) these CD8 T cell receptors (TCRs) are responding to. The TCRs of cytotoxic T cells are very unique and can respond to a specific antigen that is being presented to them by an APC. The 10X single cell sequencing platform used in this study enabled concurrent sequencing of T cell receptors (both alpha and beta chains) and RNA from each cell, allowing us to correlate clonal expansion with T

cell function. We identified several massively clonally-expanded TCRs on CD8 cytotoxic cells from 5XFAD and PS-5X mice, so further experiments should be conducted to identify the specific antigen that these clonotypes recognize within the AD brain, and to determine whether this antigen is related to AD pathology or AD-related cellular dysfunction. Future experiments can be designed to identify the specific antigens that drive this observed clonality.

Cytotoxic T cells are commonly referred to as killer T cells because their effector function is to release inflammatory cytokines and enzymes that kill target cells. Release of IFNγ, TNFα, granzyme, and perforin mediate these cytotoxic effects of CD8+ T cells. Cytotoxic T cells release the protein perforin to destroy target cells by creating pores in their membranes that allow granzymes to enter the cell and induce apoptosis. Given this important role of perforin in CD8 T cell induced cell death, future studies should focus on whether perforin expression is necessary to induce the loss of neurons that occurs at later ages in the PS-5X bigenic mice or whether CD8 T cells can directly influence the survival and turnover of microglia in AD. Likewise, future studies should determine whether neuronal loss correlates with a specific T cell subpopulation or T cell receptor sequences. Taken together, we have demonstrated that CD8 T cells infiltrate the brain of AD transgenic mice and predominantly exhibit an effector memory phenotype. These infiltrating CD8 T cells also exhibit TCR clonality in association with beta-amyloid and combined amyloid and tau pathologies, interact with brain-

resident microglia, and express several AD risk genes. While the direct impact of T cells on synaptic and neuronal degeneration in AD mice remains unclear, the current studies provide an important model, resource, and clues that will can inform future studies that seek to further unravel this intriguing and complex biology.

REFERENCES

Arancio, O, Chao, MV (2007) Neurotrophins, synaptic plasticity and dementia. Curr Opin Neurobiol, 17:325–330.

Armulik, A, Genové, G, Mäe, M, Nisancioglu, MH, Wallgard, E, Niaudet, C, He, L, Norlin, J, Lindblom, P, Strittmatter, K, Johansson, BR, Betsholtz, C (2010) Pericytes regulate the blood-brain barrier. Nature, 468:557–561.120

Auld, DS, Kornecook, TJ, Bastianetto, S, Quirion, R (2002) Alzheimer's disease and the basal forebrain cholinergic system: relations to beta-amyloid peptides, cognition, and treatment strategies. Prog Neurobiol, 68:209–245.

Bachmann MF, Wolint P, Schwarz K, Jäger P, Oxenius A. Functional properties and lineage relationship of CD8+ T cell subsets identified by expression of IL-7 receptor alpha and CD62L. J Immunol. 2005 Oct 1;175(7):4686-96. doi: 10.4049/jimmunol.175.7.4686. PMID: 16177116.

Baglietto-Vargas, D, Moreno-Gonzalez, I, Sanchez-Varo, R, Jimenez, S, Trujillo-Estrada, L, Sanchez-Mejias, E, Torres, M, Romero-Acebal, M, Ruano, D, Vizuete, M, Vitorica, J, Gutierrez, A (2010) Calretinin interneurons are early targets of extracellular amyloid-beta pathology in PS1/AbetaPP Alzheimer mice hippocampus. J Alzheimers Dis, 21:119–132.

Bard, F, Cannon, C, Barbour, R, Burke, RL, Games, D, Grajeda, H, Guido, T, Hu, K, Huang, J, Johnson-Wood, K, Khan, K, Kholodenko, D, Lee, M, Lieberburg, I, Motter, R, Nguyen, M, Soriano, F, Vasquez, N, Weiss, K, Welch, B, Seubert, P, Schenk, D, Yednock, T (2000) Peripherally administered antibodies against amyloid beta-peptide enter the central nervous system and reduce pathology in a mouse model of Alzheimer disease. Nat Med, 6:916–919.

Barkalow, FJ, Goodman, MJ, Gerritsen, ME, Mayadas, TN (1996) Brain endothelium lack one of two pathways of P-selectin-mediated neutrophil adhesion. Blood, 88:4585–4593.

Barker, RA, Widner, H (2004) Immune problems in central nervous system cell therapy. NeuroRx, 1:472–481.

Baruch, K, Deczkowska, A, David, E, Castellano, JM, Miller, O, Kertser, A, Berkutzki, T, Barnett-Itzhaki, Z, Bezalel, D, Wyss-Coray, T, Amit, I, Schwartz, M (2014) Aging. Aging induced type I interferon response at the choroid plexus negatively affects brain function. Science, 346:89–93.

Baruch, K, Deczkowska, A, Rosenzweig, N, Tsitsou-Kampeli, A, Sharif, AM, Matcovitch-Natan, O, Kertser, A, David, E, Amit, I, Schwartz, M (2016) PD-1 immune checkpoint blockade reduces pathology and improves memory in mouse models of Alzheimer's disease. Nat Med, 22:135–137.

Bellenguez C, Küçükali F, Jansen IE, Kleineidam L, Moreno-Grau S, Amin N, Naj AC, Campos-Martin R, Grenier-Boley B, Andrade V, Holmans PA, Boland A, Damotte V, van der Lee SJ, Costa MR, Kuulasmaa T, Yang Q, de Rojas I, Bis JC, Yaqub A, Prokic I, Chapuis J, Ahmad S, Giedraitis V, Aarsland D, Garcia-Gonzalez P, Abdelnour C, Alarcón-Martín E, Alcolea D, Alegret M, Alvarez I, Álvarez V, Armstrong NJ, Tsolaki A, Antúnez C, Appollonio I, Arcaro M, Archetti S, Pastor AA, Arosio B, Athanasiu L, Bailly H, Banaj N, Baquero M, Barral S, Beiser A, Pastor AB, Below JE, Benchek P, Benussi L, Berr C, Besse C, Bessi V, Binetti G, Bizarro A, Blesa R, Boada M, Boerwinkle E, Borroni B, Boschi S, Bossù P, Bråthen G, Bressler J, Bresner C, Brodaty H, Brookes KJ, Brusco LI, Buiza-Rueda D, Bûrger K, Burholt V, Bush WS, Calero M, Cantwell LB, Chene G, Chung J, Cuccaro ML, Carracedo Á, Cecchetti R, Cervera-Carles L, Charbonnier C, Chen HH, Chillotti C, Ciccone S, Claassen JAHR, Clark C, Conti E, Corma-Gómez A, Costantini E, Custodero C, Daian D, Dalmasso MC, Daniele A, Dardiotis E, Dartigues JF, de Deyn PP, de Paiva Lopes K, de Witte LD, Debette S, Deckert J, Del Ser T, Denning N, DeStefano A, Dichgans M, Diehl-Schmid J, Diez-Fairen M, Rossi PD, Djurovic S, Duron E, Düzel E, Dufouil C, Eiriksdottir G, Engelborghs S, Escott-Price V, Espinosa A, Ewers M, Faber KM, Fabrizio T, Nielsen SF, Fardo DW, Farotti L, Fenoglio C, Fernández-Fuertes M, Ferrari R, Ferreira CB, Ferri E, Fin B, Fischer P, Fladby T, Fließbach K, Fongang B, Fornage M, Fortea J, Foroud TM, Fostinelli S, Fox NC, Franco-Macías E, Bullido MJ, Frank-García A, Froelich L, Fulton-Howard B, Galimberti D, García-Alberca JM, García-González P, Garcia-Madrona S, Garcia-Ribas G, Ghidoni R, Giegling I, Giorgio G, Goate AM, Goldhardt O, Gomez-Fonseca D, González-Pérez A, Graff C, Grande G, Green E, Grimmer T, Grünblatt E, Grunin M, Gudnason V, Guetta-Baranes T, Haapasalo A, Hadjigeorgiou G, Haines JL, Hamilton-Nelson KL, Hampel H, Hanon O, Hardy J, Hartmann AM, Hausner L, Harwood J, Heilmann-Heimbach S, Helisalmi S, Heneka MT, Hernández I, Herrmann MJ, Hoffmann P, Holmes C, Holstege H, Vilas RH, Hulsman M, Humphrey J, Biessels GJ, Jian X, Johansson C, Jun GR, Kastumata Y, Kauwe J, Kehoe PG, Kilander L, Ståhlbom AK, Kivipelto M, Koivisto A, Kornhuber J, Kosmidis MH, Kukull WA, Kuksa PP, Kunkle BW, Kuzma AB, Lage C, Laukka EJ, Launer L, Lauria A, Lee CY, Lehtisalo J, Lerch O, Lleó A, Longstreth W Jr, Lopez O, de Munain AL, Love S, Löwemark M, Luckcuck L, Lunetta KL, Ma Y, Macías J, MacLeod CA, Maier W, Mangialasche F, Spallazzi M, Marquié M, Marshall R, Martin ER, Montes AM, Rodríguez CM, Masullo C, Mayeux R, Mead S, Mecocci P, Medina M, Meggy A, Mehrabian S, Mendoza S, Menéndez-González M, Mir P, Moebus S, Mol M, Molina-Porcel L,

Montrreal L, Morelli L, Moreno F, Morgan K, Mosley T, Nöthen MM, Muchnik C, Mukherjee S, Nacmias B, Ngandu T, Nicolas G, Nordestgaard BG, Olaso R, Orellana A, Orsini M, Ortega G, Padovani A, Paolo C, Papenberg G, Parnetti L, Pasquier F, Pastor P, Peloso G, Pérez-Cordón A, Pérez-Tur J, Pericard P, Peters O, Pijnenburg YAL, Pineda JA, Piñol-Ripoll G, Pisanu C, Polak T, Popp J, Posthuma D, Priller J, Puerta R, Quenez O, Quintela I, Thomassen JQ, Rábano A, Rainero I, Rajabli F, Ramakers I, Real LM, Reinders MJT, Reitz C, Reyes-Dumeyer D, Ridge P, Riedel-Heller S, Riederer P, Roberto N, Rodriguez-Rodriguez E, Rongve A, Allende IR, Rosende-Roca M, Royo JL, Rubino E, Rujescu D, Sáez ME, Sakka P, Saltvedt I, Sanabria Á, Sánchez-Arjona MB, Sanchez-Garcia F, Juan PS, Sánchez-Valle R, Sando SB, Sarnowski C, Satizabal CL, Scamosci M, Scarmeas N, Scarpini E, Scheltens P, Scherbaum N, Scherer M, Schmid M, Schneider A, Schott JM, Selbæk G, Seripa D, Serrano M, Sha J, Shadrin AA, Skrobot O, Slifer S, Snijders GJL, Soininen H, Solfrizzi V, Solomon A, Song Y, Sorbi S, Sotolongo-Grau O, Spalletta G, Spottke A, Squassina A, Stordal E, Tartan JP, Tárraga L, Tesí N, Thalamuthu A, Thomas T, Tosto G, Traykov L, Tremolizzo L, Tybjærg-Hansen A, Uitterlinden A, Ullgren A, Ulstein I, Valero S, Valladares O, Broeckhoven CV, Vance J, Vardarajan BN, van der Lugt A, Dongen JV, van Rooij J, van Swieten J, Vandenberghe R, Verhey F, Vidal JS, Vogelgsang J, Vyhnalek M, Wagner M, Wallon D, Wang LS, Wang R, Weinhold L, Wiltfang J, Windle G, Woods B, Yannakoulia M, Zare H, Zhao Y, Zhang X, Zhu C, Zulaica M; EADB; GR@ACE; DEGESCO; EADI; GERAD; Demgene; FinnGen; ADGC; CHARGE, Farrer LA, Psaty BM, Ghanbari M, Raj T, Sachdev P, Mather K, Jessen F, Ikram MA, de Mendonça A, Hort J, Tsolaki M, Pericak-Vance MA, Amouyel P, Williams J, Frikke-Schmidt R, Clarimon J, Deleuze JF, Rossi G, Seshadri S, Andreassen OA, Ingelsson M, Hiltunen M, Sleegers K, Schellenberg GD, van Duijn CM, Sims R, van der Flier WM, Ruiz A, Ramirez A, Lambert JC. New insights into the genetic etiology of Alzheimer's disease and related dementias. Nat Genet. 2022 Apr;54(4):412-436. doi: 10.1038/s41588-022-01024-z. Epub 2022 Apr 4. PMID: 35379992; PMCID: PMC9005347.

Blurton-Jones, M, Spencer, B, Michel, S, Castello, NA, Agazaryan, AA, Davis, JL, Müller, F-J, Loring, JF, Masliah, E, LaFerla, FM (2014) Neural stem cells genetically-modified to express neprilysin reduce pathology in Alzheimer transgenic models. Stem Cells Research & Therapy,

Boddeke, E, Dinarello, CA, Breitner, JC, Cole, GM, Golenbock, DT, Kummer, MP (2015) Neuroinflammation in Alzheimer’s disease. Lancet Neurol, 14:388–405.

Bosma, MJ, Carroll, AM (1991) The SCID mouse mutant: definition, characterization, and potential uses. Annu Rev Immunol, 9:323–350.
Boyman, O, Sprent, J (2012) The role of interleukin-2 during homeostasis and activation of the immune system. Nat Rev Immunol, 12:180–190.
Brew, BJ, Pemberton, L, Blennow, K, Wallin, A, Hagberg, L (2005) CSF amyloid beta42 and tau levels correlate with AIDS dementia complex. Neurology, 65:1490–1492.
Britschgi, M, Olin, CE, Johns, HT, Takeda-Uchimura, Y, LeMieux, MC, Rufibach, K, Rajadas, J, Zhang, H, Tomooka, B, Robinson, WH, Clark, CM, Fagan, AM, Galasko, DR, Holtzman, DM, Jutel, M, Kaye, JA, Lemere, CA, Leszek, J, Li, G, Peskind, ER, Quinn, JF, Yesavage, JA, Ghiso, JA, Wyss-Coray, T (2009) Neuroprotective natural antibodies to assemblies of amyloidogenic peptides decrease with normal aging and advancing Alzheimer's disease. Proc Natl Acad Sci U S A, 106:12145–12150.
Brochard, V, Combadière, B, Prigent, A, Laouar, Y, Perrin, A, Beray-Berthat, V, Bonduelle, O, Alvarez-Fischer, D, Callebert, J, Launay, JM, Duyckaerts, C, Flavell, RA, Hirsch, EC Hunot, S (2009) Infiltration of CD4+ lymphocytes into the brain contributes to neurodegeneration in a mouse model of Parkinson disease. J Clin Invest, 119:182–192.
Bulloch, K, Miller, MM, Gal-Toth, J, Milner, TA, Gottfried-Blackmore, A, Waters, EM, Kaunzner, UW, Liu, K, Lindquist, R, Nussenzweig, MC, Steinman, RM, McEwen, BS (2008) CD11c/EYFP transgene illuminates a discrete network of dendritic cells within the embryonic, neonatal, adult, and injured mouse brain. J Comp Neurol, 508:687–710.
Burgess, A, Dubey, S, Yeung, S, Hough, O, Eterman, N, Aubert, I, Hynynen, K (2014) Alzheimer disease in a mouse model: MR imaging-guided focused ultrasound targeted to the hippocampus opens the blood-brain barrier and improves pathologic abnormalities and behavior. Radiology, 273:736–745.122
Caccamo, A, Oddo, S, Sugarman, MC, Akbari, Y, LaFerla, FM (2005) Age- and regiondependent alterations in Abeta-degrading enzymes: implications for Abeta-induced disorders. Neurobiol Aging, 26:645–654.
Cao, X, Shores, EW, Hu-Li, J, Anver, MR, Kelsall, BL, Russell, SM, Drago, J, Noguchi, M, Grinberg, A, Bloom, ET, et, A (1995) Defective lymphoid development in mice lacking expression of the common cytokine receptor gamma chain. Immunity, 2:223–238.
Capotondo, A, Milazzo, R, Politi, LS, Quattrini, A, Palini, A, Plati, T, Merella, S, Nonis, A, di Serio, C, Montini, E, Naldini, L, Biffi, A (2012) Brain conditioning is instrumental for successful microglia reconstitution

following hematopoietic stem cell transplantation. Proc Natl Acad Sci U S A, 109:15018–15023.
Carrillo, MC, Brashear, HR, Logovinsky, V, Ryan, JM, Feldman, HH, Siemers, ER, Abushakra, S, Hartley, DM, Petersen, RC, Khachaturian, AS, Sperling, RA (2013) Can we prevent Alzheimer's disease? Secondary "prevention" trials in Alzheimer's disease. Alzheimers Dement, 9:123–131.e1.
Cavallucci, V, Berretta, N, Nobili, A, Nistico, R, Mercuri, NB, D'Amelio, M (2013) Calcineurin inhibition rescues early synaptic plasticity deficits in a mouse model of Alzheimer's disease. Neuromolecular Med, 15:541–548.
Chakrabarty, P, Li, A, Ceballos-Diaz, C, Eddy, JA, Funk, CC, Moore, B, DiNunno, N, Rosario, AM, Cruz, PE, Verbeeck, C, Sacino, A, Nix, S, Janus, C, Price, ND, Das, P, Golde, TE (2015) IL-10 alters immunoproteostasis in APP mice, increasing plaque burden and worsening cognitive behavior. Neuron, 85:519–533.
Chen, WW, Blurton-Jones, M (2012) Concise review: Can stem cells be used to treat or model Alzheimer's disease. Stem Cells, 30:2612–2618.
Chu, Y, Kordower, JH (2010) Lewy body pathology in fetal grafts. Ann N Y Acad Sci, 1184:55–67.
Chun, JJ, Schatz, DG, Oettinger, MA, Jaenisch, R, Baltimore, D (1991) The recombination activating gene-1 (RAG-1) transcript is present in the murine central nervous system. Cell, 64:189–200.
Cicchetti, F, Lacroix, S, Cisbani, G, Vallières, N, Saint-Pierre, M, St-Amour, I, Tolouei, R, Skepper, JN, Hauser, RA, Mantovani, D, Barker, RA, Freeman, TB (2014) Mutant huntingtin is present in neuronal grafts in Huntington disease patients. Ann Neurol, 76:31–42.
Claes C, England WE, Danhash EP, Kiani Shabestari S, Jairaman A, Chadarevian JP, Hasselmann J, Tsai AP, Coburn MA, Sanchez J, Lim TE, Hidalgo JLS, Tu C, Cahalan MD, Lamb BT, Landreth GE, Spitale RC, Blurton-Jones M, Davtyan H. The P522R protective variant of PLCG2 promotes the expression of antigen presentation genes by human microglia in an Alzheimer's disease mouse model. Alzheimers Dement. 2022 Feb 9. doi: 10.1002/alz.12577. Epub ahead of print. PMID: 35142046.
Cochran, JN, Rush, T, Buckingham, SC, Roberson, ED (2015) The Alzheimer's disease risk factor CD2AP maintains blood-brain barrier integrity. Hum Mol Genet, 24:6667–6674.
Daouk, J, Bouzerar, R, Chaarani, B, Zmudka, J, Meyer, ME, Balédent, O (2016) Use of dynamic (18)F-fluorodeoxyglucose positron emission tomography to investigate choroid plexus function in Alzheimer's disease. Exp Gerontol,

Das, P, Howard, V, Loosbrock, N, Dickson, D, Murphy, MP, Golde, TE (2003) Amyloid-beta immunization effectively reduces amyloid deposition in FcRgamma-/- knock-out mice. J Neurosci, 23:8532–8538.
Davies, P, Maloney, AJ (1976) Selective loss of central cholinergic neurons in Alzheimer's disease. Lancet, 2:1403.
Davtyan, H, Mkrtichyan, M, Movsesyan, N, Petrushina, I, Mamikonyan, G, Cribbs, DH, Agadjanyan, MG, Ghochikyan, A (2010) DNA prime-protein boost increased the titer, avidity and persistence of anti-Abeta antibodies in wild-type mice. Gene Ther, 17:261–271.
Davtyan, H, Petrushina, I, Ghochikyan, A (2014) Immunotherapy for Alzheimer's disease: DNA- and protein-based epitope vaccines. Methods Mol Biol, 1143:259–281.
Deane, R, Sagare, A, Hamm, K, Parisi, M, Lane, S, Finn, MB, Holtzman, DM, Zlokovic, BV (2008) apoE isoform-specific disruption of amyloid beta peptide clearance from mouse brain. J Clin Invest, 118:4002–4013.
Deane, R, Sagare, A, Hamm, K, Parisi, M, LaRue, B, Guo, H, Wu, Z, Holtzman, DM, Zlokovic, BV (2005) IgG-assisted age-dependent clearance of Alzheimer's amyloid beta peptide by the blood-brain barrier neonatal Fc receptor. J Neurosci, 25:11495–11503.
Deczkowska A, Keren-Shaul H, Weiner A, Colonna M, Schwartz M, Amit I. Disease-associated microglia: a universal immune sensor of neurodegeneration (2018). Cell 173(5):1073-1081
DeKosky, ST, Scheff, SW (1990) Synapse loss in frontal cortex biopsies in Alzheimer's disease: correlation with cognitive severity. Ann Neurol, 27:457–464. 124
Del Bo, R, Angeretti, N, Lucca, E, De Simoni, MG, Forloni, G (1995) Reciprocal control of inflammatory cytokines, IL-1 and IL-6, and beta-amyloid production in cultures. Neurosci Lett, 188:70–74.
Derecki, NC, Cardani, AN, Yang, CH, Quinnies, KM, Crihfield, A, Lynch, KR, Kipnis, J (2010) Regulation of learning and memory by meningeal immunity: a key role for IL-4. J Exp Med, 207:1067–1080.
Devi, L, Ohno, M (2012) 7,8-dihydroxyflavone, a small-molecule TrkB agonist, reverses memory deficits and BACE1 elevation in a mouse model of Alzheimer's disease. Neuropsychopharmacology, 37:434–444.
Gustavson, A, Sayre, J, Sofroni, E, Suarez, T, Chiappelli, F, Bernard, G (2005) Ineffective phagocytosis of amyloid-beta by macrophages of Alzheimer's disease patients. J Alzheimers Dis, 7:221–32; discussion 255.
Ferretti MT, Merlini M, Späni C, Gericke C, Schweizer N, Enzmann G, Engelhardt B, Kulic L, Suter T, Nitsch RM. T-cell brain infiltration and immature antigen-presenting cells in transgenic models of Alzheimer's disease-like

cerebral amyloidosis. Brain Behav Immun. 2016 May;54:211-225. doi: 10.1016/j.bbi. 2016.02.009. Epub 2016 Feb 9. PMID: 26872418.
Fitzner, D, Schnaars, M, van Rossum, D, Krishnamoorthy, G, Dibaj, P, Bakhti, M, Regen, T, Hanisch, UK, Simons, M (2011) Selective transfer of exosomes from oligodendrocytes to microglia by macropinocytosis. J Cell Sci, 124:447–458. 125
Fontenot, JD, Gavin, MA, Rudensky, AY (2003) Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. Nat Immunol, 4:330–336.
Fontenot, JD, Rasmussen, JP, Williams, LM, Dooley, JL, Farr, AG, Rudensky, AY (2005) Regulatory T cell lineage specification by the forkhead transcription factor foxp3. Immunity, 22:329–341.
Frautschy, SA, Yang, F, Irrizarry, M, Hyman, B, Saido, TC, Hsiao, K, Cole, GM (1998) Microglial response to amyloid plaques in APPsw transgenic mice. Am J Pathol, 152:307–317.
Fuller, JP, Stavenhagen, JB, Teeling, JL (2014) New roles for Fc receptors in neurodegenerationthe impact on Immunotherapy for Alzheimer's Disease. Front Neurosci, 8:235.
Gage, FH, Temple, S (2013) Neural stem cells: generating and regenerating the brain. Neuron, 80:588–601.
Galea, I, Bernardes-Silva, M, Forse, PA, van Rooijen, N, Liblau, RS, Perry, VH (2007) An antigen-specific pathway for CD8 T cells across the blood-brain barrier. J Exp Med, 204:2023–2030.
Galli, R, Binda, E, Orfanelli, U, Cipelletti, B, Gritti, A, De Vitis, S, Fiocco, R, Foroni, C, Dimeco, F, Vescovi, A (2004) Isolation and characterization of tumorigenic, stem- like neural precursors from human glioblastoma. Cancer Res, 64:7011–7021.
García-García, E, Rosales, C (2002) Signal transduction during Fc receptor-mediated phagocytosis. J Leukoc Biol, 72:1092–1108.
Garg, SK, Banerjee, R, Kipnis, J (2008) Neuroprotective Immunity: T Cell-Derived Glutamate Endows Astrocytes with a Neuroprotective Phenotype. The Journal of Immunology, 180:3866–3873.
Gasque, P, Singhrao, SK, Neal, JW, Götze, O, Morgan, BP (1997) Expression of the receptor for complement C5a (CD88) is up-regulated on reactive astrocytes, microglia, and endothelial cells in the inflamed human central nervous system. Am J Pathol, 150:31–41.
Hasselmann J, Coburn MA, England W, Figueroa Velez DX, Kiani Shabestari S, Tu CH, McQuade A, Kolahdouzan M, Echeverria K, Claes C, Nakayama T, Azevedo R, Coufal NG, Han CZ, Cummings BJ, Davtyan H, Glass CK, Healy LM, Gandhi SP, Spitale RC, Blurton-Jones M. Development of a Chimeric Model to Study and Manipulate Human Microglia In Vivo.

Neuron. 2019 Sep 25;103(6):1016-1033.e10. doi: 10.1016/j.neuron.2019.07.002. Epub 2019 Jul 30. PMID: 31375314; PMCID: PMC7138101.

Hemmati, HD, Nakano, I, Lazareff, JA, Masterman-Smith, M, Geschwind, DH, Bronner-Fraser, M, Kornblum, HI (2003) Cancerous stem cells can arise from pediatric brain tumors. Proc Natl Acad Sci U S A, 100:15178–15183.

Hemmer, B, Kerschensteiner, M, Korn, T (2015) Role of the innate and adaptive immune responses in the course of multiple sclerosis. Lancet Neurol, 14:406-419.

Heneka, MT, Carson, MJ, Khoury, JE, Landreth, GE, Brosseron, F, Feinstein, DL, Jacobs, AH, Wyss-Coray, T, Vitorica, J, Ransohoff, RM, Herrup, K, Frautschy, SA, Finsen, B, Brown, GC, Verkhratsky, A, Yamanaka, K, Koistinaho, J, Latz, E, Halle, A, Petzold, GC, Town, T, Morgan, D, Shinohara, ML, Perry, VH, Holmes, C, Bazan, NG, Brooks, DJ, Hunot, S, Joseph, B, Deigendesch, N, Garaschuk, O, Hickman, SE, Allison, EK, El Khoury, J (2008) Microglial dysfunction and defective betaamyloid clearance pathways in aging Alzheimer's disease mice. J Neurosci, 28:8354–8360.

Hong, HS, Hwang, JY, Son, SM, Kim, YH, Moon, M, Inhee, MJ (2010) FK506 reduces amyloid plaque burden and induces MMP-9 in AbetaPP/PS1 double transgenic mice. J Alzheimers Dis, 22:97–105.

Hong, S, Dissing-Olesen, L, Stevens, B (2016) New insights on the role of microglia in synaptic pruning in health and disease. Curr Opin Neurobiol, 36:128–134.

Hongpaisan, J, Sun, MK, Alkon, DL (2011) PKC ε activation prevents synaptic loss, Aβ elevation, and cognitive deficits in Alzheimer's disease transgenic mice. J Neurosci, 31:630–643.

Huang, Y, Weisgraber, KH, Mucke, L, Mahley, RW (2004) Apolipoprotein E: diversity of cellular origins, structural and biophysical properties, and effects in Alzheimer's disease. J Mol Neurosci, 23:189–204.

Huh, GS, Boulanger, LM, Du, H, Riquelme, PA, Brotz, TM, Shatz, CJ (2000) Functional requirement for class I MHC in CNS development and plasticity. Science, 290:2155–2159.

Ifergan, I, Kébir, H, Bernard, M, Wosik, K, Dodelet-Devillers, A, Cayrol, R, Arbour, N, Prat, A (2008) The blood-brain barrier induces differentiation of migrating monocytes into Th17- polarizing dendritic cells. Brain, 131:785–799.

Keren-Shaul H, Spinrad A, Weiner A, Matcovitch-Natan O, Dvir-Szternfeld R, Ulland TK, David E, Baruch K, Lara-Astaiso D, Toth B, Itzkovitz S, Colonna

M, Schwartz M, Amit I. A Unique Microglia Type Associated with Restricting Development of Alzheimer's Disease. Cell. 2017 Jun 15;169(7):1276-1290.e17. doi: 10.1016/j.cell.2017.05.018. Epub 2017 Jun 8. PMID: 28602351.

Kiani Shabestari S, Morabito S, Danhash EP, McQuade A, Sanchez JR, Miyoshi E, Chadarevian JP, Claes C, Coburn MA, Hasselmann J, Hidalgo J, Tran KN, Martini AC, Chang Rothermich W, Pascual J, Head E, Hume DA, Pridans C, Davtyan H, Swarup V, Blurton-Jones M. Absence of microglia promotes diverse pathologies and early lethality in Alzheimer's disease mice. Cell Rep. 2022 Jun 14;39(11):110961. doi: 10.1016/j.celrep.2022.110961. PMID: 35705056.

Lambert, J. C, et al. Meta-analysis of 74,046 individual identifies 11 new susceptibility loco for Alzheimer's disease (2013). Nat. Genet. 45, 1452-1458.

Lehtinen, MK, Bjornsson, CS, Dymecki, SM, Gilbertson, RJ, Holtzman, DM, Monuki, ES (2013) The choroid plexus and cerebrospinal fluid: emerging roles in development, disease, and therapy. J Neurosci, 33:17553–17559. 130

Leinenga, G, Götz, J (2015) Scanning ultrasound removes amyloid-β and restores memory in an Alzheimer's disease mouse model. Sci Transl Med, 7:278ra33.

Li X, Feng Y, Wu W, Zhao J, Fu C, Li Y, Ding Y, Wu B, Gong Y, Yang G, Zhou X. Sex differences between APPswePS1dE9 mice in A-beta accumulation and pancreatic islet function during the development of Alzheimer's disease. Lab Anim. 2016 Aug;50(4):275-85. doi: 10.1177/0023677215615269. Epub 2015 Oct 30. PMID: 26519428.

Liesz, A, Suri-Payer, E, Veltkamp, C, Doerr, H, Sommer, C, Rivest, S, Giese, T, Veltkamp, R (2009) Regulatory T cells are key cerebroprotective immunomodulators in acute experimental stroke. Nat Med, 15:192–199.

Lindahl, P, Johansson, BR, Levéen, P, Betsholtz, C (1997) Pericyte loss and microaneurysm formation in PDGF-B-deficient mice. Science, 277:242–245.

Ling, EA, Kaur, C, Lu, J (1998) Origin, nature, and some functional considerations of intraventricular macrophages, with special reference to the epiplexus cells. Microsc Res Tech, 41:43–56.

Linker KE, Elabd MG, Tawadrous P, Cano M, Green KN. Microglial activation increases cocaine self-administration following adolescent nicotine exposure (2020). Nature Communications 11, Article number: 306 (2020)

Linker, RA, Lee, DH, Demir, S, Wiese, S, Kruse, N, Siglienti, I, Gerhardt, E, Neumann, H, Sendtner, M, Lühder, F, Gold, R (2010) Functional role of brain-derived neurotrophic factor in neuroprotective autoimmunity:

therapeutic implications in a model of multiple sclerosis. Brain, 133:2248–2263.
Liu, E, Schmidt, ME, Margolin, R, Sperling, R, Koeppe, R, Mason, NS, Klunk, WE, Mathis, CA, Salloway, S, Fox, NC, Hill, DL, Les, AS, Collins, P, Gregg, KM, Di, J, Lu, Y, Tudor, IC, Wyman, BT, Booth, K, Broome, S, Yuen, E, Grundman, M, Brashear, HR, Bapineuzumab, ACTI (2015) Amyloid-β 11C-PiB-PET imaging results from 2 randomized bapineuzumab phase 3 AD trials. Neurology, 85:692–700.
Liu, S, Liu, Y, Hao, W, Wolf, L, Kiliaan, AJ, Penke, B, Rübe, CE, Walter, J, Heneka, MT, Hartmann, T, Menger, MD, Fassbender, K (2012) TLR2 is a primary receptor for Alzheimer's amyloid β peptide to trigger neuroinflammatory activation. J Immunol, 188:1098–1107.
Liu, Y, Walter, S, Stagi, M, Cherny, D, Letiembre, M, Schulz-Schaeffer, W, Heine, H, Penke, B, Neumann, H, Fassbender, K (2005) LPS receptor (CD14): a receptor for phagocytosis of Alzheimer's amyloid peptide. Brain, 128:1778–1789.
Louveau, A, Harris, TH, Kipnis, J (2015) Revisiting the Mechanisms of CNS Immune Privilege. Trends Immunol, 36:569–577.
Lu, P, Jones, LL, Snyder, EY, Tuszynski, MH (2003) Neural stem cells constitutively secrete neurotrophic factors and promote extensive host axonal growth after spinal cord injury.
Marsh SE, Abud EM, Lakatos A, Karimzadeh A, Yeung ST, Davtyan H, Fote GM, Lau L, Weinger JG, Lane TE, Inlay MA, Poon WW, Blurton-Jones M. The adaptive immune system restrains alzheimer's disease pathogenesis by modulating microglial function (2016). PNAS 113(9):E1316-25.
McIntyre LL, Greilach SA, Othy S, Sears-Kraxberger I, Wi B, Ayala-Angulo J, Vu E, Pham Q, Silva J, Dang K, Rezk F, Steward O, Cahalan MD, Lane TE, Walsh CM. Regulatory T cells promote remyelination in the murine experimental autoimmune encephalomyelitis model of multiple sclerosis following human neural stem cell transplant. Neurobiol Dis. 2020 Jul;140:104868. doi: 10.1016/j.nbd.2020.104868. Epub 2020 Apr 8. PMID: 32276110; PMCID: PMC7253332.
McQuade A, Kang YJ, Hasselmann J, Jairaman A, Sotelo A, Coburn M, Shabestari SK, Chadarevian JP, Fote G, Tu CH, Danhash E, Silva J, Martinez E, Cotman C, Prieto GA, Thompson LM, Steffan JS, Smith I, Davtyan H, Cahalan M, Cho H, Blurton-Jones M. Gene expression and functional deficits underlie TREM2-knockout microglia responses in human models of Alzheimer's disease. Nat Commun. 2020 Oct 23;11(1):5370. doi: 10.1038/s41467-020-19227-5. PMID: 33097708; PMCID: PMC7584603.

Oddo, S, Caccamo, A, Shepherd, JD, Murphy, MP, Golde, TE, Kayed, R, Metherate, R, Mattson, MP, Akbari, Y, LaFerla, FM (2003) Triple-transgenic model of Alzheimer's disease with plaques and tangles: intracellular Abeta and synaptic dysfunction. Neuron, 39:409–421.133

Oestreich, KJ, Weinmann, AS (2012) Master regulators or lineage-specifying? Changing views on CD4+ T cell transcription factors. Nat Rev Immunol, 12:799–804.

Parachikova, A, Agadjanyan, MG, Cribbs, DH, Blurton-Jones, M, Perreau, V, Rogers, J, Beach, TG, Cotman, CW (2007) Inflammatory changes parallel the early stages of Alzheimer disease. Neurobiol Aging, 28:1821–1833.

Pearl, JI, Lee, AS, Leveson-Gower, DB, Sun, N, Ghosh, Z, Lan, F, Ransohoff, J, Negrin, RS, Davis, MM, Wu, JC (2011) Short-term immunosuppression promotes engraftment of embryonic and induced pluripotent stem cells. Cell Stem Cell, 8:309–317.

Pellicanò, M, Larbi, A, Goldeck, D, Colonna-Romano, G, Buffa, S, Bulati, M, Rubino, G, Iemolo, F, Candore, G, Caruso, C, Derhovanessian, E, Pawelec, G (2012) Immune profiling of Alzheimer patients. J Neuroimmunol, 242:52–59.

Picard, D, Miller, S, Hawkins, CE, Bouffet, E, Rogers, HA, Chan, TSY, Kim, S-K, Ra, Y-S, Fangusaro, J, Korshunov, A, Toledano, H, Nakamura, H, Hayden, JT, Chan, J, Lafay-Cousin, L, Hu, P, Fan, X, Muraszko, KM, Pomeroy, SL, Lau, CC, Ng, H-K, Jones, C, Van Meter, T, Clifford, SC, Eberhart, C, Gajjar, A, Pfister, SM, Grundy, RG, Huang, A (2012) Markers of survival and metastatic potential in childhood CNS primitive neuro-ectodermal brain tumours: an integrative genomic analysis. The Lancet Oncology, 13:838–848.

Poduslo, JF, Curran, GL, Berg, CT (1994) Macromolecular permeability across the blood-nerve and blood-brain barriers. Proc Natl Acad Sci U S A, 91:5705–5709.

Poon, WW, Blurton-Jones, M, Tu, CH, Feinberg, LM, Chabrier, MA, Harris, JW, Jeon, NL, Cotman, CW (2011) beta-Amyloid impairs axonal BDNF retrograde trafficking. Neurobiol Aging, 32:821–833.

Reiser J, Banerjee A. Effector, Memory, and Dysfunctional CD8(+) T Cell Fates in the Antitumor Immune Response. J Immunol Res. 2016;2016:8941260. doi: 10.1155/2016/8941260. Epub 2016 May 22. PMID: 27314056; PMCID: PMC4893440.

Roy ER, Wang B, Wan YW, Chiu G, Cole A, Yin Z, Propson NE, Xu Y, Jankowsky JL, Liu Z, Lee VM, Trojanowski JQ, Ginsberg SD, Butovsky O, Zheng H, Cao W. Type I interferon response drives neuroinflammation and synapse loss

in Alzheimer disease. J Clin Invest. 2020 Apr 1;130(4):1912-1930. doi: 10.1172/JCI133737. PMID: 31917687; PMCID: PMC7108898.
Sala Frigerio C, Wolfs L, Fattorelli N, Thrupp N, Voytyuk I, Schmidt I, Mancuso R, Chen WT, Woodbury ME, Srivastava G, Möller T, Hudry E, Das S, Saido T, Karran E, Hyman B, Perry VH, Fiers M, De Strooper B. The Major Risk Factors for Alzheimer's Disease: Age, Sex, and Genes Modulate the Microglia Response to Aβ Plaques. Cell Rep. 2019 Apr 23;27(4):1293-1306.e6. doi: 10.1016/j.celrep.2019.03.099. PMID: 31018141; PMCID: PMC7340153.
Shi Y, Holtzman DM. Interplay between innate immunity and Alzheimer disease: APOE and TREM2 in the spotlight. Nat Rev Immunol. 2018 Dec;18(12):759-772. doi: 10.1038/s41577-018-0051-1. PMID: 30140051; PMCID: PMC6425488.
Sil A, Erfani A, Lamb N, Copland R, Riedel G, Platt B. Sex Differences in Behavior and Molecular Pathology in the 5XFAD Model. J Alzheimers Dis. 2022;85(2):755-778. doi: 10.3233/JAD-210523. PMID: 34864660.
Soontornniyomkij, V, Moore, DJ, Gouaux, B, Soontornniyomkij, B, Tatro, ET, Umlauf, A, Masliah, E, Levine, AJ, Singer, EJ, Vinters, HV, Gelman, BB, Morgello, S, Cherner, M, Grant, I, Achim, CL (2012) Cerebral β-amyloid deposition predicts HIV-associated neurocognitive disorders in APOE ε4 carriers. AIDS, 26:2327–2335.
Spangenberg, EE, Lee, RJ, Najafi, AR, Rice, RA, Elmore, MR, Blurton-Jones, M, West, BL, Green, KN (2016) Eliminating microglia in Alzheimer’s mice prevents neuronal loss without modulating amyloid-β pathology. Brain
Stalder, M, Phinney, A, Probst, A, Sommer, B, Staufenbiel, M, Jucker, M (1999) Association of microglia with amyloid plaques in brains of APP23 transgenic mice. Am J Pathol, 154:1673–1684. 136
Stüve, O, Marra, CM, Bar-Or, A, Niino, M, Cravens, PD, Cepok, S, Frohman, EM, Phillips, JT, Arendt, G, Jerome, KR, Cook, L, Grand’Maison, F, Hemmer, B, Monson, NL, Racke, MK (2006) Altered CD4+/CD8+ T-cell ratios in cerebrospinal fluid of natalizumab-treated patients with multiple sclerosis. Arch Neurol, 63:1383–1387.
Styren, SD, Civin, WH, Rogers, J (1990) Molecular, cellular, and pathologic characterization of HLA-DR immunoreactivity in normal elderly and Alzheimer’s disease brain. Exp Neurol, 110:93–104.
Sudduth, TL, Greenstein, A, Wilcock, DM (2013) Intracranial injection of Gammagard, a human IVIg, modulates the inflammatory response of the brain and lowers Aβ in APP/PS1 mice along a different time course than anti-Aβ antibodies. J Neurosci, 33:9684–9692.

Suzuki, M, McHugh, J, Tork, C, Shelley, B, Klein, SM, Aebischer, P, Svendsen, CN (2007) GDNF secreting human neural progenitor cells protect dying motor neurons, but not their projection to muscle, in a rat model of familial ALS. PLoS One, 2:e689.

Tariot PN, Lopera F, Langbaum JB, Thomas RG, Hendrix S, Schneider LS, Rios-Romenets S, Giraldo M, Acosta N, Tobon C, Ramos C, Espinosa A, Cho W, Ward M, Clayton D, Friesenhahn M, Mackey H, Honigberg L, Sanabria Bohorquez S, Chen K, Walsh T, Langlois C, Reiman EM; Alzheimer's Prevention Initiative. The Alzheimer's Prevention Initiative Autosomal-Dominant Alzheimer's Disease Trial: A study of crenezumab versus placebo in preclinical *PSEN1* E280A mutation carriers to evaluate efficacy and safety in the treatment of autosomal-dominant Alzheimer's disease, including a placebo-treated noncarrier cohort. Alzheimers Dement (N Y). 2018 Mar 8;4:150-160. doi: 10.1016/j.trci.2018.02.002. PMID: 29955659; PMCID: PMC6021543.

Taglialatela, G, Hogan, D, Zhang, WR, Dineley, KT (2009) Intermediate- and long-term recognition memory deficits in Tg2576 mice are reversed with acute calcineurin inhibition. Behav Brain Res, 200:95–99.

Tamaki, SJ, Jacobs, Y, Dohse, M, Capela, A, Cooper, JD, Reitsma, M, He, D, Tushinski, R, Belichenko, PV, Salehi, A, Mobley, W, Gage, FH, Huhn, S, Tsukamoto, AS, Weissman, IL, Uchida, N (2009) Neuroprotection of host cells by human central nervous system stem cells in a mouse model of infantile neuronal ceroid lipofuscinosis. Cell Stem Cell, 5:310–10:151–167.

Uchida, N, Buck, DW, He, D, Reitsma, MJ, Masek, M, Phan, TV, Tsukamoto, AS, Gage, FH, Weissman, IL (2000) Direct isolation of human central nervous system stem cells. Proc

Unger MS, Li E, Scharnagl L, Poupardin R, Altendorfer B, Mrowetz H, Hutter-Paier B, Weiger TM, Heneka MT, Attems J, Aigner L. CD8+ T-cells infiltrate Alzheimer's disease brains and regulate neuronal- and synapse-related gene expression in APP-PS1 transgenic mice. Brain Behav Immun. 2020 Oct;89:67-86. doi:10.1016/j.bbi.2020.05.070. Epub 2020 May 29. PMID: 32479993.

Wes PD, Sayed FA, Bard F, Gan L. Targeting microglia for the treatment of Alzheimer's Disease. Glia. 2016 Oct;64(10):1710-32. doi: 10.1002/glia.22988. Epub 2016 Apr 21. PMID: 27100611.

Xia, MQ, Qin, SX, Wu, LJ, Mackay, CR, Hyman, BT (1998) Immunohistochemical study of the beta-chemokine receptors CCR3 and CCR5 and their ligands in normal and Alzheimer's disease brains. Am J Pathol, 153:31–37.

Xu, W, Banchereau, J (2014) The antigen presenting cells instruct plasma cell differentiation. Front Immunol, 4:504.
Yamasaki, TR, Blurton-Jones, M, Morrissette, DA, Kitazawa, M, Oddo, S, LaFerla, FM (2007) Neural Stem Cells Improve Memory in an Inducible Mouse Model of Neuronal Loss. Journal of Neuroscience, 27:11925–11933.
Yoles, E, Hauben, E, Palgi, O, Agranov, E, Gothilf, A, Cohen, A, Kuchroo, V, Cohen, IR, Weiner, H, Schwartz, M (2001) Protective autoimmunity is a physiological response to CNS trauma. J Neurosci, 21:3740–3748. 139
Yona, S, Kim, KW, Wolf, Y, Mildner, A, Varol, D, Breker, M, Strauss-Ayali, D, Viukov, S, Guilliams, M, Misharin, A, Hume, DA, Perlman, H, Malissen, B, Zelzer, E, Jung, S (2013) Fate mapping reveals origins and dynamics of monocytes and tissue macrophages under homeostasis. Immunity, 38:79–91.
Yoshiyama, Y, Higuchi, M, Zhang, B, Huang, SM, Iwata, N, Saido, TC, Maeda, J, Suhara, T, Trojanowski, JQ, Lee, VM (2007) Synapse loss and microglial activation precede tangles in a P301S tauopathy mouse model. Neuron, 53:337–351.
Yu, DY, Luo, J, Bu, F, Song, GJ, Zhang, LQ, Wei, Q (2006) Inhibition of calcineurin by infusion of CsA causes hyperphosphorylation of tau and is accompanied by abnormal behavior in mice. Biol Chem, 387:977–983.
Yuan P, Condello C, Keene CD, Wang Y, Bird TD, Paul SM, Luo W, Colonna M, Baddeley D, Grutzendler J. TREM2 Haplodeficiency in Mice and Humans Impairs the Microglia Barrier Function Leading to Decreased Amyloid Compaction and Severe Axonal Dystrophy. Neuron. 2016 May18;90(4):724-39. doi: 10.1016/j.neuron.2016.05.003. PMID: 27196974; PMCID: PMC4898967.
Zenaro, E, Pietronigro, E, Bianca, VD, Piacentino, G, Marongiu, L, Budui, S, Turano, E, Rossi, B, Angiari, S, Dusi, S, Montresor, A, Carlucci, T, Nanì, S, Tosadori, G, Calciano, L, Catalucci, D, Berton, G, Bonetti, B, Constantin, G (2015) Neutrophils promote Alzheimer's disease-like pathology and cognitive decline via LFA-1integrin. Nat Med, 21:880–886.
Zhang, Y, Chen, K, Sloan, SA, Bennett, ML, Scholze, AR, O'Keeffe, S, Phatnani, HP, Guarnieri, P, Caneda, C, Ruderisch, N, Deng, S, Liddelow, SA, Zhang, C, Daneman, R, Maniatis, T, Barres, BA, Wu, JQ (2014) An RNA-Sequencing Transcriptome and Splicing Database of Glia, Neurons, and Vascular Cells of the Cerebral Cortex. J Neurosci, 34:11929–11947.
Zhao, C, Deng, W, Gage, FH (2008) Mechanisms and functional implications of adult neurogenesis. Cell, 132:645–660.
Zhao, Z, Nelson, AR, Betsholtz, C, Zlokovic, BV (2015) Establishment and Dysfunction of the Blood-Brain Barrier. Cell, 163:1064–1078.

Zhou, X, Bailey-Bucktrout, SL, Jeker, LT, Penaranda, C, Martínez-Llordella, M, Ashby, M, Nakayama, M, Rosenthal, W, Bluestone, JA (2009) Instability of the transcription factor Foxp3 leads to the generation of pathogenic memory T cells in vivo. Nat Immunol, 10:1000–1007.

Ziv, Y, Ron, N, Butovsky, O, Landa, G, Sudai, E, Greenberg, N, Cohen, H, Kipnis, J, Schwartz, M (2006) Immune cells contribute to the maintenance of neurogenesis and spatial learning abilities in adulthood. Nat Neurosci, 9:268–275.

Zlokovic, BV (2013) Cerebrovascular effects of apolipoprotein E: implications for Alzheimer disease. JAMA Neurol, 70:440–444.