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The Role of Apolipoprotein E in Regulating Tau Pathogenesis and Neurodegeneration in a Tauopathy Mouse Model

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by
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Yang Shi

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Dedicated to my family.

ABSTRACT OF THE DISSERTATION

The Role of Apolipoprotein E in Regulating Tau Pathogenesis and Neurodegeneration in a Tauopathy Mouse Model

by

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Professor David M. Holtzman, Chair

APOE4 is the strongest genetic risk factor for late-onset Alzheimer's disease (AD). APOE4 increases brain amyloid-β (Aβ) pathology relative to other APOE isoforms. However, whether APOE independently influences tau pathology, the other pathological hallmark of AD and other tauopathies, or tau-mediated neurodegeneration, is not clear. By generating P301S tau transgenic mice on either a human APOE knock in (KI) or APOE knockout (KO) background, we show that the presence of human APOE, regardless of APOE isoforms, leads to various degrees of brain atrophy in 9-month old P301S mice, whereas APOE ablation strongly protects against neurodegeneration. In particular, P301S/E4 mice develop significantly more brain atrophy compared with P301S/E2 and P301S/E3 mice. Concomitantly, APOE-sufficient P301S mice develop ptau staining patterns distinct from P301S/EKO mice, with P301S/E4 mice displaying a yet another different pattern from P301S/E2 and P301S/E3 mice that indicates severe neurodegeneration. In diseased P301S mouse brain, APOE specifically binds to a high-molecular-weight tau species and may affect tau pathogenesis through direct APOE-tau

interaction. APOE level strongly correlates with ptau levels, and is associated with less soluble tau and more insoluble tau. In addition to affecting tau pathology and neurodegeneration, APOE has potent immunomodulatory functions. In vitro, E4-expressing microglia produce more proinflammatory cytokines following LPS treatment compared with E2- and E3-expressing microglia. In vivo, P301S/E4 mice show markedly enhanced neuroinflammation compared to P301S/E2 and P301S/E3 mice, whereas P301S/EKO mice are largely protected from the change. Co-culturing P301S tau-expressing neurons with mixed glia (80-90% astrocytes + 10-20% microglia) derived from human APOE KI and APOE KO mice results in a marked neuronal loss in the neuron/E4 co-culture with a concomitant elevation of TNFα level in the medium compared to neuron/E2 and neuron/E3 co-cultures, whereas neurons co-cultured with EKO glia exhibit the greatest viability with the lowest level of secreted TNFα. In contrast, treatment of P301S neurons with recombinant APOE (E2, E3, E4) in the absence of glial cells only leads to a mild neuronal impairment compared to the absence of APOE, indicating that APOE regulates neurodegeneration in part through glia-mediated neuroinflammation. The effect of APOE on glia-mediated neuronal loss requires direct contact between neurons and glia cells, and APOE may serve as an opsonin bridging stressed neurons that present ‘eat-me’ signals on cell surfaces with microglia by binding to triggering receptor expressed on myeloid cells 2 (TREM2) to promote neuronal phagoptosis by microglia. APOE is also required for microglial polarization into a neurodegenerative phenotype characterized by a proinflammatory profile and enhanced phagocytotic activity. Depleting microglia from mouse brain using the colony-stimulating factor 1 receptor (CSF1R) inhibitor - PLX3397, rescues neurodegeneration in TE4 mice, but shows no impact on TEKO mice, indicating critical roles of microglia in neurodegeneration regulated by APOE. Reducing APOE levels in P301S mice by overexpressing an APOE receptor, the low-

density lipoprotein receptor (LDLR), significantly attenuates neurodegeneration and shifts the ptau staining towards an early-disease pattern. In addition, LDLR overexpression leads to a significant reduction of ptau levels, similar to that observed in P301S /EKO mice.

Chapter 1: Introduction

This chapter is adapted from the following manuscript

Shi Y, Holtzman DM. Interplay between innate immunity and Alzheimer's disease: APOE and TREM2 in the spotlight. *Nat Rev Immunol.* (Accepted)

1.1 Alzheimer's Disease

Alzheimer's disease (AD) is the most common cause of dementia accounting for 60%-80% dementia cases, and currently affects over 30 million people worldwide. It is a devastating neurological disorder characterized by severe neurodegeneration and cognitive disabilities that eventually lead to death. Two pathological hallmarks stand out in the AD brain: extracellular deposition of amyloid plaques consisting predominantly of amyloid-β (Aβ) peptides, and intraneuronal accumulation of neurofibrillary tangles (NFTs) comprising aggregated, hyperphosphorylated tau protein. AD pathology begins at least 10-20 years prior to clinical symptom onset, with Aβ aggregation being the earliest pathological event[1]. At a later stage, tau pathology begins to build up extending from the limbic system to the neocortex, accompanied by increased synaptic/neuronal dysfunction and inflammation. These pathological lesions eventually lead to brain atrophy and dementia (Fig. 1).

The amyloid hypothesis poses that the initial Aβ accumulation likely initiates downstream pathological events[2]. Aβ peptides are produced via sequential cleavage of the amyloid precursor protein (APP), a transmembrane protein highly expressed in the CNS, particularly in neurons. The cleavage is carried out by two enzymes, β-secretase (BACE) and γ-secretase. BACE cleaves APP outside its transmembrane domain whereas presenilin, the enzymatic component of the γ-secretase complex, cleaves APP at multiple sites within the membrane to produce Aβ peptides of different lengths such as Aβ38, Aβ40, and Aβ42, among which Aβ42 is the most fibrillogenic species[1]. Upon production, Aβ is secreted out of cell and under pathological conditions, Aβ aggregates to form large amyloid deposits in the extracellular space of brain parenchyma. These amyloid plaques contain multiple Aβ species ranging from Aβ 38-43, where Aβ42 constitutes the major plaque component. Some plaques contain insoluble Aβ fibrils adopting a bona fide β-sheet

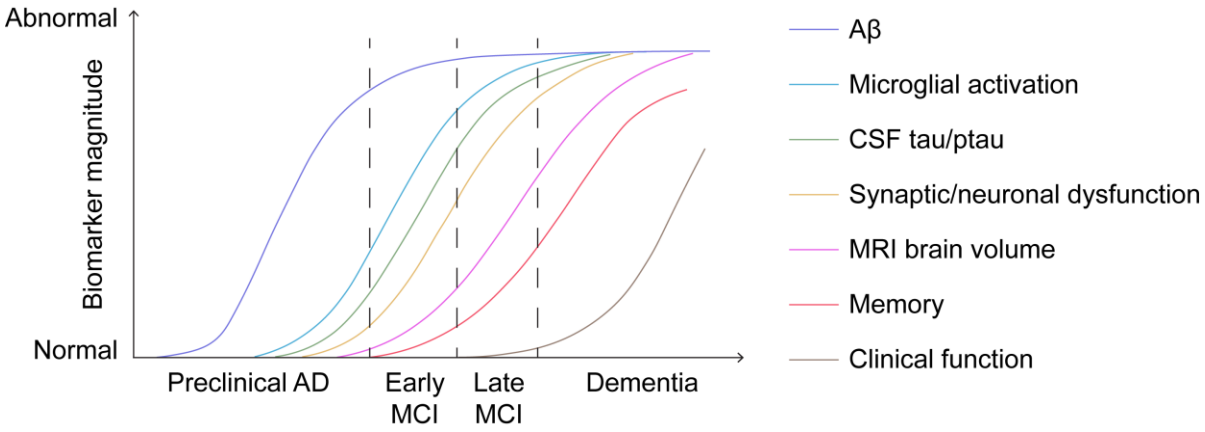

Figure 1 | Time course of biomarker changes in AD in relation to clinical stages. The first pathological event of AD occurs at least 10-20 years prior to the onset of clinical symptoms in cognitively normal people with deposition of amyloid plaques in the brain. At ~5 years before clear-cut cognitive decline, tau pathology begins to accumulate in the neocortex, roughly around the same time, inflammation increases and neuronal and synaptic loss begins to occur, followed by initiation of brain atrophy. This period when AD pathologies build up while a person is still cognitively normal is termed preclinical AD. Once sufficient neuronal damage and neuronal loss are accumulated, early mild cognitive impairment (MCI) becomes clinically detectable. At this time, amyloid deposition has almost reached plateau. As dementia progresses to late-stage MCI, NFT formation increases, accompanied with exacerbated neuronal and synaptic dysfunction, inflammation, and brain atrophy. When the disease progresses to dementia, cognitive deficits becomes severe and deterioration of clinical functions occurs.

structure (fibrillar plaques) that can be recognized by Congo red and thioflavin-S staining, whereas other plaques contain more diffuse Aβ deposits with a non-β sheet conformation (non-fibrillar or diffuse plaques) that can only be recognized by Aβ antibody staining[1]. The amyloid plaques are often surrounded by swollen, degenerating neurites (dystrophic neurites) [1], but otherwise are not associated with notable neurodegeneration.

Tau tangles, the other pathological hallmark of AD, develop at a later disease stage and highly correlates with neuronal dysfunction. In the CNS, tau is produced by neurons, but is also present at low levels in glia and in the extracellular space[3]. As a microtubule-binidng protein, tau normally locates in the axons of neurons and serves to stablize microtubules. In AD, tau becomes hyperphosphorylated, dissociates from microtubules, and transloates from axons to neuronal cell bodies and dendrites[1]. Hyperphosphorylated tau has a tendency to aggregate, forming NFTs in

neuroanl cell bodies and dystrophic neurites. Strong evidence show that tau pathology contributes to clinical progression of AD.

Alzheimer's disease can be divided into two major categories[1]. A small percentage (<1%) of Alzheimer's disease is inherited within families in an autosomal-dominant fashion that manifests with early dementia onset, typically between age 30 and 60. This form of familial Alzheimer's disease (FAD) is typically caused by mutations in genes affecting Aβ production, Aβ42/Aβ40 ratio, or Aβ structure, such as *APP*, presenilin1 *(PSEN1)* and presenilin2 (*PSEN2).* Over 99% of Alzheimer's disease cases occur later in age, typically in people over 65-years old, and are referred to as 'late-onset' Alzheimer's disease (LOAD). While there is no identified 'cause' for LOAD, the two most important risk factors are age and genetics. Among the many AD risk loci identified to date, *APOE* is the first identified AD risk gene and remains the strongest genetic risk factor for LOAD.

1.2 *APOE* as a genetic risk factor for Alzheimer's disease

Apolipoprotein E (APOE) is a lipid-binding protein forming lipoprotein particles with lipids and cholesterol to mediate their transport in body fluids. Human APOE consists of 299 amino acids and contains a mid-region receptor-binding domain (aa 136–150) and a C-terminal lipid-binding domain (aa 244–272)[4]. It binds to lipids and cholesterols and delivers them to cells via APOE receptor-mediated endocytosis of lipoproteins. Upon uptake, intracellular APOE exhibits a significantly longer half-life than the lipids/cholesterols it carries and may either slowly go through degradation or be recycled back to the cell surface[5]. APOE also mediates cellular cholesterol efflux in a process called reverse cholesterol transport, where cellular cholesterol is transferred to the lipoprotein via ATP-binding cassette transporter (ABCA1)[6].

APOE has the highest expression in the liver and the CNS. In the brain, APOE is the most abundantly produced apolipoprotein, and is synthesized primarily by astrocytes and to a lesser extent by microglia[7,8]. Under stressed conditions, neurons can also produce APOE[9]. Three common *APOE* alleles (ε2, ε3, and ε4) are present in human populations, with ε3 being most prevalent (~77%) and ε2 being the rarest (~8%)[4]. Each APOE isoform differs from each other by a single amino acid at position 112 or 158 (E2-Cys112, Cys158; E3-Cys112, Arg158; E4-Arg112, Arg158). These differences affect APOE structures, which subsequently affect APOE property and functions such as APOE receptor-binding capacity and lipid-binding capability. For example, APOE3 and APOE4 bind to low density lipoprotein receptor (LDLR) with >50 folds greater affinity than APOE2[10]. Consequently, APOE2 transports lipids with less efficiency and is associated with type III hyperlipoproteinaemia. The lipid components of lipoprotein particles also differ between APOE isoforms. APOE4 preferentially binds to large, triglyceride-rich very low density lipoprotein (VLDL) particles in the plasma and is associated with a modestly increased risk for cardiovascular disease[4], whereas APOE2 and APOE3 have a preference for small, cholesterol- and phospholipid-enriched high density lipoprotein (HDL)[11]. In the brain where only HDL is present, the sizes of APOE particles in the cerebrospinal fluid (CSF) follow the pattern of APOE 2/3>APOE3/3>APOE3/4>APOE 4/4 in middle-aged nondemented subjects[11]. Similarly, in older adults, the sizes of CSF APOE lipoproteins follow the pattern of APOE4-negative>APOE4-positive[11]. The preference of APOE4 to bind larger lipoprotein particles is believed to be attributable to the presence of Arg at position 112, which affects the conformation of the side chain of Arg61, resulting in domain interaction between Arg61 in the N-terminal domain and Glu255 in the C-terminal lipid-binding domain, therefore affecting APOE lipid-binding capacity[12]. In addition, APOE levels are also isoform-dependent. APOE

level in human in plasma follows the pattern of ε2/ε2 > ε3/ε3 > ε4/ε4 due to the faster turnover rate of plasma APOE4 and lower binding affinity between APOE2 and lipoprotein receptors[13]. APOE levels in human CSF and brain parenchyma in relation to APOE isoforms are less clear[13]. Overall, the different properties of APOE isoforms determine their functional differences in disease.

APOE as an AD risk gene was first reported in 1993[14]. The ε4 allele is a major AD risk factor whereas ε2 is protective relative to the ε3 allele. One copy of ε4 increases AD risk by ~3-fold and two copies by ~12-fold[1]. The ε4 allele is significantly enriched in AD patients (~15% in the general population and ~40% in patients with AD), and ε4-carrying patients show earlier disease onset[15], heavier brain amyloid-β plaque load[16], greater brain atrophy[17], and accelerated disease progression[18] compared to non-carriers. The initial discovery of APOE as an AD risk factor was unexpected. As a lipid-carrier regulating lipid homeostasis, APOE was considered to be primarily involved in influencing atherosclerosis. In the early 1990s, APOE was found to be a constituent of amyloid plaques[19,20], yet its significance was not clear until *APOE* was identified as an Alzheimer's disease risk factor shortly afterwards. The discovery was inspired by two converging lines of evidence, the first showing an Alzheimer's disease linkage at chromosome 19q13[21] where *APOE* is located, and the second indicating a physical interaction of APOE with amyloid-β[14]. The close link between APOE and amyloid-β in the initial discovery studies has largely driven APOE research in the Alzheimer's disease field along an amyloid-β track.

1.3 APOE and Aβ pathology

To date, substantial evidence from animal models has demonstrated APOE affects Aβ deposition in a dosage and isoform-specific fashion[22-26]. In APP transgenic (Tg) mice expressing

human APOE in replacement of the endogenous murine APOE, APOE4 strongly exacerbates amyloid plaque deposition whereas APOE2 reduces plaque load compared to APOE3[24,26]. Genetic ablation of murine or human APOE in Aβ-depositing mice dose-dependently reduces fibrillary amyloid plaques[22,23]. Mechanistically, studies assessing APOE's effect on Aβ fibrilization in vitro have reported contradictory results, which was likely due to inconsistent experimental conditions resulting in different lipid components of APOE[27]. In vivo evidence showed that alteration of APOE lipidation status in the brain has a major impact on Aβ deposition. ABCA1-deficient Aβ-depositing mice that have reduced APOE lipidation demonstrated a marked increase of amyloid deposition[28,29], whereas overexpression of ABCA1 enhanced APOE lipidation and reduced plaque load[30]. In addition to affecting Aβ aggregation, substantial evidence shows that APOE also regulates Aβ clearance. For example, APOE-deficient Aβ-depositing mice showed a faster Aβ clearance rate from the ISF compared to their APOE-sufficient counterpart[31]. In Aβ-depositing mice expressing human APOE isoforms, APOE4-expressing mice exhibited a higher level of ISF Aβ and a lower ISF Aβ clearance rate, whereas APOE2-expressing mice showed lower levels of ISF Aβ and a faster Aβ clearance rate compared to APOE3-expressing mice[26]. This isoform-dependent effect of APOE on ISF Aβ clearance was observed at an early age well before plaque accumulation occurs, indicating an inherent capacity of APOE isoforms to regulate ISF Aβ clearance.

While the role of APOE in regulating Aβ pathogenesis has been well documented, accumulating evidence shows that Aβ pathology poorly correlates with clinical symptoms or tissue loss[32,33]. Extensive amyloid plaque deposition can build up in normal aged brains without causing overt neurodegeneration or dementia. Therefore, whether APOE influences AD solely

by affecting Aβ pathogenesis is questionable. There may be Aβ-independent mechanisms through which APOE impacts AD.

1.4 APOE and tau pathology

Contrary to Aβ, accumulation of pathological tau in the AD brain and in primary tauopathies strongly correlates with clinical signs and neurodegeneration[3-5]. The concurrence of tau pathology and neuronal injury in the timeline of AD disease progression, and substantial evidence from clinical and animal studies strongly suggest tau pathology contributes to neuronal dysfunction.

Interestingly, Recent GWAS studies show a strong and significant association of *APOE* with CSF tau and p-tau after correcting for the effect of *APOE* on Aβ42 levels[34,35]. In frontotemporal dementia (FTD) patients, a large percentage of whom have tauopathy, *ε4* allele frequency was reported to be significantly elevated[36,37], and *ε4* carriers have greater atrophy in affected brain regions[17] as well as exacerbated behavioral deficits[38]. These data suggest that APOE may directly influence tau pathology and tau-mediated neurodegeneration. Early studies showed that APOE interacts with human tau in an isoform-specific manner in vitro[39,40], However, the role of physiologically-expressed APOE in regulating tau pathogenesis and associated neurodegeneration independent of Aβ has never been clearly assessed, although abnormal overexpression of human APOE4 in neurons, but not astrocytes, has been reported to increase tau phosphorylation in mouse brains compared to APOE3 overexpression[41,42]. In the following chapters, we will address the above question in a tauopathy mouse model and investigate underlying mechanisms.

Chapter 2: APOE modulates neurodegeneration in the setting of tauopathy

This chapter is adapted from the following manuscript

Shi, Y. et al. APOE4 markedly exacerbates tau-mediated neurodegeneration in a mouse model of tauopathy. *Nature* 549, 523-527, doi:10.1038/nature24016 (2017).

2.1 Introduction

An appropriate mouse model recapitulating tau pathology and tau-mediated neurodegeneration is critical for the study. Rodents don't naturally develop Alzheimer's disease, nor do they accumulate any AD pathologies. However, mouse models developing amyloid plaques or tau tangles have been generated by overexpressing mutated human transgenes encoding APP/presenilin or tau. Human tau is encoded by the microtubule-associated protein tau gene *(MAPT)*. Six major tau isoforms generated by alternative splicing are present in the adult human brain. These isoforms differ by the number of an N-terminal insert consisting of 29 amino acids. Isoforms containing 0, 1 or 2 inserts are defined as 0N, 1N and 2N respectively. Isoforms are also categorized based on the presence of three or four C-terminal repeat domains (3R or 4R respectively). In the adult human brain, the level of 3R and 4R tau are roughly equal, whereas 1N tau is more abundant relative to 0N and 2N tau[43]. Compared to 3R tau, 4R tau is shown to have higher susceptibility to phosphorylation and aggregation[44]. In this thesis work, we utilized a tau transgenic (Tg) mouse model overexpressing 1N4R human tau carrying the P301S mutation found in FTD patients. These mice develop tau pathology around 4-5 months of age, but are free of Aβ pathology. Unlike Aβ-depositing mouse models that don't show overt neurodegeneration, P301S tau Tg mice demonstrate visibly distinct severe brain atrophy at 9-12 months of age, therefore serving as a good model to assess tau pathology and tau-mediated neurodegeneration.

To study the isoform-dependent effects of human APOE on tau and neurodegeneration, human APOE knock in (KI) mice are utilized. These mice express human APOE under the endogenous murine APOE promoter, therefore representing the physiological pattern of APOE expression regarding APOE levels and expressing cell types. This is crucial as overexpressing APOE can induce significant metabolic changes that affect experimental outcome, and non-

physiological APOE expression by specific cell types other than glia, such as neurons, can cause proteolytic processing of APOE with generation of cytotoxic fragments[23], which would confound the results. To assess the APOE dosage effect, APOE knockout (KO) mice are also utilized. Through a series of genetic crossing, the endogenous murine APOE of P301S mice are either replaced by human APOE isoforms (E2, E3, E4) or deleted. Neurodegeneration was first analyzed in these mice and compared across different APOE genotypes.

Extending the analysis from mouse models to human, we also assessed neurodegeneration affected by APOE genotypes in patients with primary tauopathies. Primary tauopathy is a class of neurodegenerative diseases characterized by accumulation of pathological tau in the brain, including Pick's disease (PiD), progressive supranuclear palsy (PSP), corticobasal degeneration (CBD), argyrophilic grain disease, primary age-related tauopathy, and a recently characterized entity called globular glial tauopathy[45]. The nomenclature of primary tauopathies overlaps significantly with the modern classification of frontotemporal lobar degeneration (FTLD), also frequently referred to as frontotemporal dementia (FTD). Approximately 40% of FTD patients manifest tau pathology, whereas the rest are characterized by other pathological deposits including TAR DNA-binding protein 43 (TDP-43), fused in sarcoma (FUS), and other unknown ubiquitinated proteins[46]. In addition to these predominant pathologies, some patients also harbor certain degrees of co-pathologies such as Aβ and α-synuclein pathologies. Although a previous study demonstrates exacerbated neurodegeneration in affected brain regions in ε4-carrying FTD patients compared to non-carriers, it doesn't differentiate between patients developing tau pathology versus other pathologies, nor do they take into account co-pathologies. We analyzed neurodegeneration in a cohort of PSP, PiD and CBD patients, the three most common sporadic forms of FTLD-tau. All samples are confirmed by histological staining to possess tau pathology,

and the effect of APOE genotype on neurodegeneration is normalized to the co-pathologies in each sample. As a proof of concept, we also assessed APOE genotype effect on clinical disease progression rate in human AD subjects. Alzheimer's disease is deemed as a secondary tauopathy, and the disease progression is believed to be driven by tau-mediated neurodegeneration.

2.2 Experimental procedures

Animals. Human APOE2, APOE3, and APOE4 knock-in mice (C57BL/6) were provided by Dr. Patrick M. Sullivan (Duke University) and APOE knockout mice (C57BL/6) were purchased from The Jackson Laboratory (#002052). P301S tau transgenic mice (The Jackson Laboratory, #008169) on a B6/C3 background expressing human P301S 1N4R tau driven by PrP promoter were crossed to human APOE KI mice to generate P301S hE/hE mice for all three APOE isoforms, which were then crossed to APOE KO mice to generate P301S hE/- mice. Separately, APOE KI mice were crossed to APOE KO mice to generate hE/- mice. P301S hE/- mice were then crossed with hE/- mice to generate P301S hE/hE, P301S hE/- and P301S -/- littermates for all three APOE isoforms. Only P301S hE/hE and P301S -/- male mice were used for analysis. All tau transgenic mice for final analysis were kept at the same generation and had ~90% C57BL/6 background by congenic test. P301S APOE KO mice derived from three APOE isoforms showed no difference in analysis and were combined as one single group (TEKO). WT mice were purchased from Charles River Laboratories (#027) on a C57BL/6 background and were bred separately from the P301S/APOE mice. Due to a slightly different background of the WT mice, we did not perform statistical analysis comparing WT to other mice. All animal procedures and experiments were performed under guidelines approved by the animal studies

committee at Washington University School of Medicine. Sample sizes were chosen based on estimates to provide 80% power to see significant differences of 20% or greater.

Volumetric analysis. Every sixth coronal brain section (300 um between sections) starting rostrally at bregma +2.1mm to the dorsal end of the hippocampus at bregma -3.9 mm were mounted for each mouse. All mounted sections were stained with 0.1% Sudan black in 70% ethanol at RT for 20 min, then washed in 70% ethanol for 1 min, 3 times. The sections were finally washed in Milli-Q water for 3 times and coverslipped with Fluoromount. The stained slices were imaged with the NanoZoomer and areas of interest were traced and measured in each slice using the NDP viewer. The volume was calculated using the following formula: volume = (sum of area) * 0.3 mm. For hippocampus and posterior lateral ventricle, quantification started from bregma -1.1 and ended at bregma -3.9. For piriform/entorhinal cortex, quantification started at bregma -2.3 and ended at bregma -3.9.

Neuronal layer thickness measurement. Three sections (bregma -1.4, -1.7, and -2.0 mm) from each mouse were mounted and stained in cresyl violet for 5 min at RT. The slices were then sequentially dehydrated in 50%, 70%, 95% (3 times) and 100% ethanol (twice) for 1min, then cleared in xylene for 4min (twice), and coverslipped in cytoseal60 (Thermo Fisher Scientific, 8310-16). The thickness of the CA1 pyramidal cell layer and dentate gyrus granular cell layer were measured by drawing a scale perpendicular to the cell layer at two spots in all three slices and taking the average value for each mouse.

Study of neurodegeneration in human primary tauopathies. We searched the University of California, San Francisco Neurodegenerative Disease Brain Bank database for patients with APOE genotyping and a pathological diagnosis of CBD, Pick's disease, or PSP, the three most common sporadic forms of FTLD-tau. Authorization for autopsy was provided by patient's next-

of-kin, and procedures were approved by the UCSF Committee on Human Research. We constrained our search to patients autopsied since 2007, when systematic, prospective recording of neuropathological findings began. Such data were available from 29 patients with CBD, 17 with Pick's disease, and 33 with PSP. Demographics and relevant variables are shown in Table 2.1.

Postmortem brains were processed as previously described[47]. Fixed tissue slabs were dissected into blocks representing dementia-relevant brain regions, embedded in paraffin wax, and cut into 8 microns-thick sections. Regional neurodegeneration was assessed using hematoxylin and eosin-stained sections. Neuronal loss, astrogliosis and microvacuolation were semi-quantitatively graded as absent (0), mild (1), moderate (2) or severe (3). A similar scheme was used to rate Aβ deposits (neuritic plaques, diffuse plaques and vascular amyloid-beta deposits)[48], tau inclusions (neurofibrillary tangles, Pick bodies, globose tangles, neuronal cytoplasmic inclusions (other than those listed previously), tufted astrocytes, thorny astrocytes, astrocytic plaques, neuropil threads, and other glial cytoplasmic inclusions), α-synuclein inclusions (Lewy bodies and Lewy neurites), and TDP-43 inclusions (neuronal intranuclear inclusions, neuronal cytoplasmic inclusions, dystrophic neurites, glial cytoplasmic inclusions). Composite scores of neurodegeneration (ND score) [(microvacuolation + astrogliosis) / 2) + neuronal loss] and for tau, Aβ, α-synuclein, and TDP-43 were generated for each region by summing scores across inclusion types for each pathological protein.

We limited analyses to regions that had been stained for all four pathological proteins. This approach yielded a total of 609 regional assessments across the 79 patients, with an average of 8 regions per patient. Hippocampal CA1-4, subiculum, dentate gyrus and entorhinal cortex were the most commonly assessed regions and available in 72 of 79 patients. Additional regions

available from no less than 15 patients included midbrain subregions (oculomotor nucleus, tectum, periaqueductal grey, dorsal raphe and substantia nigra); anterior cingulate cortex; and middle frontal gyrus (Table 2.1). A linear mixed effects model conducted at the brain regional level was used to determine the influence of multiple predictors on composite neurodegeneration scores. APOE ε4 carrier status was used as the binary predictor of interest; additional fixed effects in the model included tau, Aβ, TDP-43, and α-synuclein composite scores; sex; age; pathological diagnosis; rater; and brain region. Patient identity was modeled as a random effect. The primary hypothesis was that possession of an APOE E4 allele would be associated with greater regional neurodegeneration after controlling for tau pathological burden and other relevant predictors.

Analysis of clinical disease progression in human AD patients. Participants from this study are part from two different longitudinal studies, the Knight Alzheimer's Disease Research Center (ADRC) at Washington University and the Alzheimer's Disease Neuroimaging Initiative (ADNI*). All procedures were approved by the Washington University Human Research Protection Office, and written informed consent was obtained from each participant.

The recruitment, assessment, and exclusion criteria methods for the Knight ADRC study has been published previously[49,50]. In the case of the ADNI study, further information was previously described[51]. Knight ADRC individuals were evaluated by Clinical Core personnel at Washington University and in the case of ADNI, each local site was in charge for the evaluation of local participants[51,52]. Briefly, cases received a clinical diagnosis of AD in accordance with the standard criteria[53] and presence or absence of dementia. The severity of dementia was staged with the Clinical Dementia Rating (CDR) where 0 indicates cognitive normality, 0.5 is defined as very mild dementia, 1 is mild dementia, 2 is moderate dementia, and 3 is severe dementia[54].

The scores in each of the six cognitive and functional domains surveyed by the CDR are summed to yield a Sum of Box scores (CDR-SB) ranging from 0 (no impairment) to 18 (maximal impairment)[55]. Sample selection was done as follow: only participants that had an AD diagnosis at the last visit and had a CSF profile compatible with AD were included in our analyses. Non-AD dementia was excluded, as well as individuals in which the CDR at last assessment was equal to 0. Individuals with diagnoses of neurological diseases other than AD were excluded. Not informative longitudinally measured CDR-SB was removed for each participant. After removing the non-informative data points only individuals with at least 1.5 year of follow-up were included. A total of 592 participants were included in the analyses.

Statistical analysis was carried out using R statistical software and the packages nlme was used for linear mixed model. A linear mixed-model repeated measure framework was used to account for correlation between repeated measures in the same individual. Disease progression was modeled as follows:

$$(\mathbf{Y}) = \boldsymbol{\beta_1}[\mathbf{SNP} * \mathbf{Time}] + \boldsymbol{\beta_2} * \mathbf{CDR_{baseline}} + \boldsymbol{\beta_3}\mathbf{AGE_{baseline}} + \boldsymbol{\beta_4}\mathbf{Gender} + \boldsymbol{\beta_5}\mathbf{Educacion} + \boldsymbol{\beta_6}\mathbf{SNP} + \boldsymbol{\beta_7}\mathbf{Time} + \boldsymbol{\beta_8}\mathbf{PC1} + \boldsymbol{\beta_9}\mathbf{PC2}$$

Where: Y was CDR-SB, the change in CDR Sum of Boxes per year baseline CDR, baseline Age, Gender, follow-up time, level of education, and, to avoid the possibility of spurious association due to population substructure, the two first principal components scores were included as covariates.

* A portion of the human AD data were obtained from the Alzheimer's Disease Neuroimaging Initiative database (http://adniloni.usc.edu). The ADNI was launched in 2003 as a public-private partnership, led by Principal Investigator Michael W. Weiner, MD. The primary goal of ADNI has been to test whether serial magnetic resonance imaging (MRI), biological

markers, and clinical and neuropsychological assessment can be combined to measure the progression of mild cognitive impairment (MCI) and early AD. For up-to-date information, see www.adni-info.org.

Statistical analysis. Unless explicitly stated, all mouse and cell culture data were shown as mean ± SEM. Differences between groups were evaluated by one-way analysis of variance tests with post hoc Tukey's multiple comparisons tests. For all experiments, data normality was analyzed using D'Agostino-Pearson omnibus normality test. For data not following normal distribution, Kruskal-Wallis test with Dunn's multiple comparisons test was performed for statistical analysis, and was stated in the figure legend. GraphPad Prism version 6.00 for Windows (GraphPad Software, La Jolla, CA) was used for these analyses and creation of the plots. For statistical analysis of the p-tau staining pattern distribution, two-sided Fisher's exact tests were performed using the "fisher.test" function in R version 3.4.0 (The R Foundation for Statistical Computing) for all four TE groups together as well as for individual comparisons between each two groups.

2.3 Results

2.3.1 APOE potently modulates neurodegeneration in P301S mice

To determine whether the presence of APOE or human APOE isoforms affect tau-mediated neurodegeneration, we performed brain volumetric analysis in 9-10 months old P301S mice on either a human APOE KI or KO background, designated as TE (Tau/APOE) mice. We found that the presence of APOE, regardless of isoforms, led to various degrees of brain atrophy in the hippocampus, piriform/entorhinal cortex, and amygdala, brain regions where tau pathology accumulates in this model, and was accompanied by significant lateral ventricular enlargement (Fig. 2.1). The atrophy was significantly exacerbated in P301S E4/E4 (TE4) mice compared to

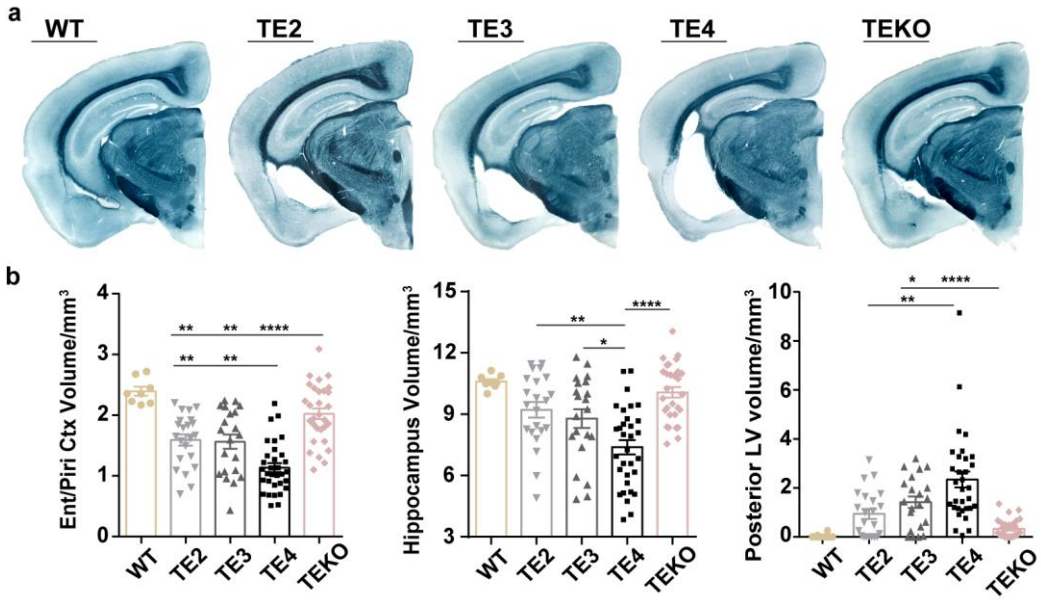

Figure 2.1 | APOE4 exacerbates neurodegeneration in aged P301S mice whereas genetic ablation of APOE is protective. a, Representative images of 9-10 months old wild type (WT) and TE mouse brain sections stained with Sudan black **b,** Volumes of the piriform /entorhinal cortex, hippocampus, and posterior lateral ventricle in 9-10 months old WT and TE mice (WT: n=8, TE2: n=22, TE3: n=21, TE4: n=32, TEKO: n=30). Data expressed as mean ± SEM, One-way ANOVA with Tukey's post hoc test (two-sided) was used for all statistical analyses except Kruskal-Wallis test with Dunn's multiple comparisons test (two-sided) was used for posterior LV volume analysis. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Ent: Entorhinal cortex, Piri: Piriform cortex, LV: Lateral ventricle.

P301S E2/E2 (TE2) and P301S E3/E3 (TE3) mice (Fig. 2.1). Strikingly, the absence of APOE in P301S mice (TEKO) largely attenuated the damage compared to P301S mice expressing human APOE, and almost completely abolished ventricular dilatation (Fig. 2.1), imposing a strong protective effect.

The granule cell layer in the dentate gyrus (DG) (Fig. 2.2 a) and the pyramidal cell layer in the CA1 region (Fig. 2.2 b) were noticeably and significantly thinner in TE4 mice, and the thickness correlated highly with hippocampal volume (Fig. 2.2 c, d), indicating that the brain atrophy is likely mediated by neuronal loss. Importantly, no brain atrophy or brain volume difference among APOE genotypes was observed in 3-month old TE mice (Fig. 2.3) prior to overt tau pathology onset or in 9-month old non-tau transgenic APOE KI mice (Fig. 2.3),

indicating that tau pathology is required to induce neurodegeneration and to reveal APOE genotype effect on neurodegeneration.

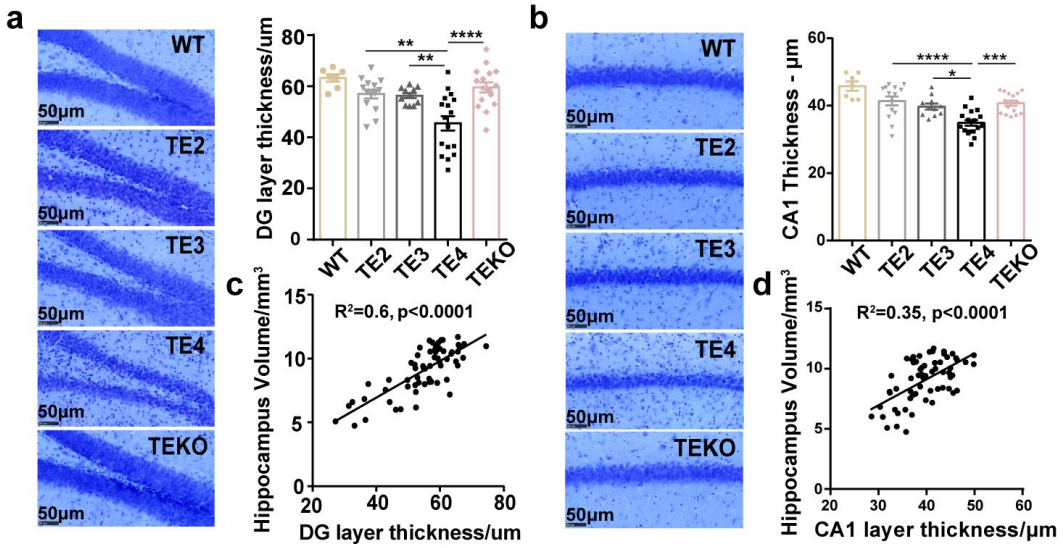

Figure 2.2 | Degrees of hippocampal neuronal loss correlate with hippocampal volume.
a, b, Thickness of the granule cell layer in the dentate gyrus (DG) and the pyramidal cell layer in CA1 respectively in 9-month old WT and TE mice with cresyl violet staining (WT: n=7, TE2: n=14, TE3: n=11, TE4: n=17, TEKO: n=16). Data expressed as mean ± SEM, One-way ANOVA with Tukey's post hoc test (two-sided). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. **c, d,** Correlation of DG/CA1 neuronal layer thickness with hippocampal volume, n=62 biologically independent animals. Pearson correlation analysis, two sided.

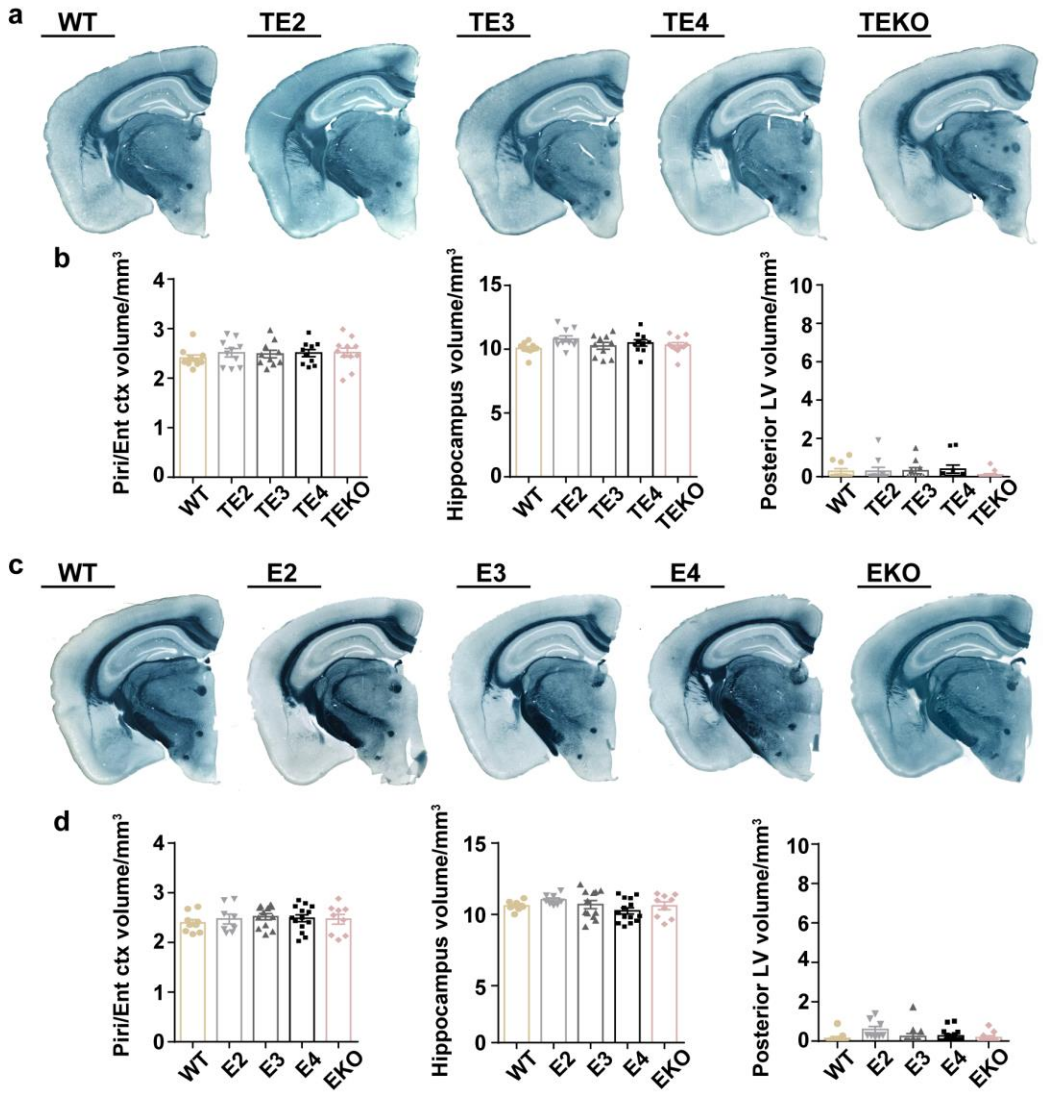

Figure 2.3| No brain atrophy or brain volume differences in 3-month old TE mice or 9-month old non-tau transgenic mice. a, Representative images of 3-month old TE mouse brains (WT: n=10, TE2: n=10, TE3: n=10, TE4: n=10, TEKO: n=11) **b,** Quantification of the piriform/entorhinal cortex, hippocampus, posterior lateral ventricle volume in 3-month old TE mice. **c,** Representative images of 9-month old non-tau transgenic mouse brains (WT: n=9, E2: n=8, E3: n=12, E4: n=14, EKO: n=9). **d,** Quantification of the piriform/entorhinal cortex, hippocampus, posterior lateral ventricle volume in 9-month old non-tau transgenic mice. Data expressed as mean ± SEM, One-way ANOVA with Tukey's post hoc test (two-sided) was used for statistical analysis. Kruskal-Wallis test with Dunn's multiple comparisons test was performed for posterior LV volume analysis.

2.3.2 APOE4 exacerbates regional neurodegeneration in human primary tauopathies.

To investigate whether the pro-neurodegenerative effect of APOE4 also manifests in human primary tauopathies, we assessed post-mortem regional neurodegeneration severity in relation to APOE genotype in individuals with a primary diagnosis of corticobasal degeneration (CBD), Pick's disease, and progressive supranuclear palsy (PSP), the three most prevalent sporadic primary tauopathies. Possession of an ε4 allele was associated with more severe regional neurodegeneration (linear mixed effects model, estimate 0.31, p=0.035, 95% CI [0.02, 0.59]) after controlling for age, sex, diagnosis, rater, Aβ deposition, Lewy body disease, tau burden, and TDP-43 inclusion burden (Table 2.1). In addition, we demonstrated greater regional neurodegeneration as a function of tau burden (estimate 0.17, p<0.001. 95% CI [0.14, 0.20]) and TDP-43 proteopathy (estimate 0.22, p=0.003, 95%CI [0.07, 0.37]). Aβ deposition, in contrast, was associated with less severe neurodegeneration (estimate -0.08, p=0.044, 95%CI [-0.15, -0.002]), whereas Lewy body disease, which was relatively uncommon in this cohort, showed no effect (estimate -0.01, p=0.87, 95% CI [-0.17, 0.15]) (Table 2.1).

2.3.3 APOE4 accelerates disease progression in AD subjects

We additionally analyzed disease progression in relation to APOE allele status in a cohort of CSF biomarker confirmed individuals with symptomatic AD, who invariably possess Aβ deposition and usually have tau pathology. We found possession of the ε4 allele was associated with significant higher rates of clinical disease progression (p=0.02) in a dose-dependent manner. Individuals with one ε4 allele progressed 14% faster than non-carriers and those with two ε4 alleles progressed 23% faster (Fig. 2.4). Overall, these findings provide converging evidence in humans that possession of ε4 allele worsens neurodegeneration in the setting of a tauopathy.

a

	N	Sex (M)	Mean Age Of Onset	SD	Mean Age At Death	SD	Mean Disease Duration	SD	Mean ND Score	SD	Mean Tau Score	SD	Mean Aβ Score	SD	Mean TDP-43 Score	SD	Mean α-Synuclein Score	SD
CBD	29	15	61	7.1	69	6.4	7.2	3.4	0.74	1.1	7.77	4.3	0.6	1.4	0.28	1	0.12	0.6
E4	6	4	60	4.2	67	5.7	6.5	2.1	0.76	1.1	7.75	4.6	1.77	2.1	0.55	1.4	0	0
no E4	23	11	62	7.7	69	6.6	7.3	3.6	0.73	1.1	7.78	4.2	0.32	0.9	0.2	0.8	0.16	0.7
Pick's	17	8	59	6.3	68	6.5	9.6	2.6	1.07	1.6	6.08	4.5	0.69	1.6	0.01	0.1	0.1	0.6
E4	5	2	55	6.4	65	6.2	10.2	2.9	1.09	1.6	5.88	4.1	1.2	1.7	0	0	0.18	0.7
no E4	12	6	60	5.9	70	6.4	9.4	2.6	1.06	1.7	6.16	4.6	0.51	1.6	0.01	0.1	0.07	0.6
PSP	33	13	64	7.5	72	6.9	8.2	3.1	0.53	1	7.08	4	1.07	1.9	0.07	0.4	0.25	0.8
E4	8	1	68	5.7	75	5.1	7.1	2.9	0.65	1	6.66	3.6	2.22	2.3	0.26	0.7	0.16	0.7
no E4	25	12	62	7.5	71	7.1	8.5	3.1	0.5	1	7.23	4.1	0.67	1.5	0.01	0.2	0.27	0.9
All Dx	79	36	62	7.3	70	6.8	8.1	3.2	0.72	1.2	7.1	4.2	0.82	1.7	0.13	0.7	0.17	0.7
E4	19	7	62	7.7	70	7.1	7.7	3	0.79	1.2	6.77	4.1	1.84	2.1	0.27	0.9	0.11	0.6
no E4	60	29	62	7.2	70	6.7	8.25	3.3	0.7	1.2	7.2	4.3	0.5	1.3	0.08	0.5	0.19	0.8

ND score: neurodegeneration score, determined by [(microvacuolation + astrogliosis) / 2) + neuronal loss]

b

Region	Total times region is used in analysis
3rd nucleus	28
Anterior cingulate cortex	51
CA1/subiculum	76
CA2	72
CA3-4	75
Dentate gyrus	73
Dorsal raphe	23
Entorhinal cortex	76
Mid front gyrus	15
Periaqueductal grey	40
Substatntia nigra	40
Tectum	40

Table 2.1| Possession of an *ε4* allele exacerbates regional neurodegeneration in human primary tauopathies. a, Demographics and relevant variables of patients. Brain regional neurodegeneration was analyzed using post mortem neuropathological data from a collection of 79 human primary tauopathy patients with sporadic FTLD-tau: 29 with corticobasal degeneration (CBD), 17 with Pick's disease, and 33 with progressive supranuclear palsy (PSP). Among these patients, 19 were *ε4* carriers. **b,** Brain regions used for analysis.

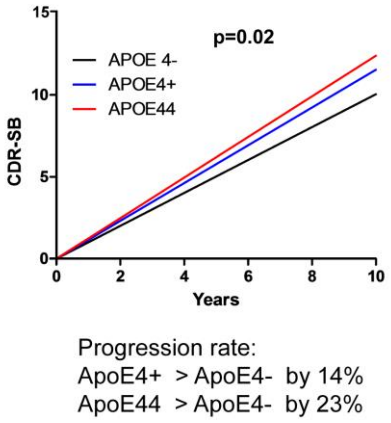

Figure 2.4| Possession of ε4 allele accelerates the rate of disease progression in AD patients. Disease progression rate in a cohort of 592 CSF biomarker confirmed individuals with symptomatic AD from two different longitudinal studies, the Knight Alzheimer's Disease Research Center (ADRC) at Washington University and the Alzheimer's Disease Neuroimaging Initiative (ADNI). Data generated based on the Clinical Dementia Rating Sum of Boxes (CDR-SB) scores.

2.4 Discussion

Despite extensive animal research investigating roles of APOE in Aβ pathogenesis, effects of APOE on neurodegeneration, one of the most important readouts in AD, has rarely been assessed, and has never been clearly demonstrated, likely due to lack of prominent neurodegeneration in amyloid-depositing mice. We found APOE strongly affects neurodegeneration in the setting of tauopathy in the absence of Aβ pathology, suggesting that the effect of APOE on AD may be partially mediated by APOE modulating tau-mediated neurodegeneration independent of Aβ. Importantly, the effect of APOE4 on exacerbating neurodegeneration recapitulates in primary tauopathy patients, providing strong supports to the animal study.

The protective effect of APOE-deficiency on neurodegeneration in the setting of tauopathy is unexpected, given that APOE serves nutritional purposes for delivering lipids/cholesterols as building bricks for neurons. This effect is likely due to APOE affecting tau pathogenesis, which further influences neurodegeneration. On the other hand, pathological tau accumulation triggers chronic neuroinflammation, which is another hallmark of AD in addition to Aβ and tau pathologies. As a common feature for many neurodegenerative diseases, chronic neuroinflammation is not only a consequence of neurodegeneration, but also actively contributes to neuronal death. Intriguingly, APOE itself is a potent regulator of immune response. Therefore, APOE regulating neuroinflammation may constitute another pathway through which it affects neurodegeneration. Effects of APOE on tau pathogenesis and neuroinflammation will be comprehensively investigated and discussed in the following chapters.

Chapter 3: APOE directly regulates tau pathogenesis independent of Aβ

This chapter is adapted from the following manuscript

Shi, Y. et al. APOE4 markedly exacerbates tau-mediated neurodegeneration in a mouse model of tauopathy. *Nature* 549, 523-527, doi:10.1038/nature24016 (2017).

3.1 Introduction

Early studies show that APOE isolated from human plasma binds to recombinant tau in an isoform-specific fashion in vitro. APOE3, but not APOE4, forms SDS-stable complexes with tau[39,40]. In addition, APOE immunoreactivity is found to colocalize with neurofibrillary tangles[19]. However, few studies haven followed up to investigate the APOE-tau connection. One group showed that artificial overexpression of APOE in neurons induced APOE fragmentation, leading to the generation of neurotoxic C-terminal truncated APOE fragments that induce tau phosphorylation by activating the extracellular signal-regulated kinase (Erk) [41,42]. APOE4 overexpression was found to result in a significantly higher level of APOE fragmentation and greater tau phosphorylation compared with APOE3[41,42]. Other data shows enhanced murine tau phosphorylation in APOE4 KI mice that develop Aβ pathology compared to their APOE3-expressing counterparts[56]. The effect is likely linked to exacerbated Aβ pathology in the presence of APOE4, as Aβ pathology has been reported to induce tau pathology[57]. However, whether physiologically expressed APOE affects human tau pathogenesis independent of Aβ is still not clear. Recent GWAS studies showing *APOE* association with CSF tau and ptau independent of Aβ[34,35] and the linkage of APOE with primary tauopathies[17,36-38] suggest that APOE may directly regulate tau pathogenesis.

The development of neurofibrillary tangles in the AD brain follows a characteristic progression pattern permitting the differentiation of tau pathology into six stages: starting from the trans-entorhinal cortex, going through the hippocampus and association cortices, and finally proceeding into the primary neocortex[58,59]. In the brain, tau is primarily an intracellular neuronal protein, but is normally secreted into the interstitial fluid (ISF) of the brain[3] and is believed to transmit tau pathology in a prion-like manner[60]. The prion hypothesis posits that tau aggregates

escape the cell of origin and enter adjacent and synaptically connected cells, where they seed new tau aggregation. In vitro experiments demonstrate aggregated tau can transfer between co-cultured cells[61-63] and seed tau aggregation in the newly accessed cells[47,49]. In vivo, restricted P301L human tau expression in the entorhinal cortex of WT mice induced tau pathology in synaptically connected brain regions[64,65]. Injection of brain homogenates from AD patients[66] as well as from P301S human tau Tg mice[67] into the brain of wild-type human tau-expressing mice induced assembly of human tau into filaments and spreading of pathology. Similar effects were observed when injecting recombinant full-length P301S tau fibrils or truncated P301L tau fibrils into P301S human tau Tg mice, which caused rapid induction of NFT-like inclusions that propagated from injected sites to connected brain regions in a time-dependent manner[68].

The prion-like property of pathological tau to propagate in brain structures in a hierarchical fashion provides a potential mechanism through which APOE may affect tau pathology. Cellular entry of aggregated tau is mediated by heparan sulfate proteoglycans (HSPG)-mediated macropinocytosis[69]. Importantly, HSPG is also a major receptor for APOE. It is universally expressed on cell surfaces along with other principal APOE receptors including low-density lipoprotein receptor (LDLR) and LDLR-related protein 1 (LRP1) to mediate APOE binding and cellular uptake. The abundance of APOE in the ISF and its potential interaction with tau may alter the behavior of tau aggregates in the extracellular space. APOE may bind to tau aggregates and affect tau uptake by neurons and other brain cell types via interaction with APOE receptors. Depending on the cellular routes of tau entry and the targeting cell types, both tau seeding and tau clearance may be affected. In addition, APOE binding to tau may alter the kinetics of tau aggregation and tau seeding capacity. Given the possibilities of APOE to be involved in

regulating tau pathogenesis, I assessed tau pathology in the TE mice via a combination of biochemical and immunohistochemical approaches.

3.2 Experimental procedures

Immunohistochemistry. The left hemi-brain of each mouse was sectioned coronally at 50 µm using a freezing sliding microtome. Three sections from each mouse (300 um apart), corresponding approximately to bregma coordinates -1.4, -1.7, and -2.0 mm respectively, were used for p-tau staining described by Yanamandra et al[70]. Briefly, brain sections were washed in Tris-buffered saline (TBS) buffer for 3 times followed by incubation in 0.3% hydrogen peroxide in TBS for 10 min at RT. After three washes in TBS, sections were blocked with 3% milk in 0.25% TBS-X (Triton X-100) for 0.5 h followed by incubation at 4°C overnight with biotinylated AT8 antibody (Thermo Scientific, MN1020B, 1:500). The next day, the slices were developed using the VECTASTAIN Elite ABC HRP Kit (Vector laboratories) following manufacturer's instructions. Stained sections were imaged by the NanoZoomer digital pathology system and pathology was quantified using Image J.

Brain extraction. Mouse cortex was processed in RAB, RIPA and 70% FA buffer sequentially as described previously[70] with modifications. Briefly, the tissue was weighed and homogenized using a pestle with 10ul buffer/1mg tissue in RAB buffer (100mM MES, 1mM EGTA, 0.5mM MgSO4, 750mM NaCl, 20mM NaF, 1mM Na3VO4, pH=7.0, supplemented by protease inhibitors (Complete, Roche) and phosphatase inhibitors (PhosSTOP, Roche)). After centrifugation at 50,000 × g for 20 min, the supernatant was taken as the RAB-soluble fraction and the pellet was dissolved in RIPA buffer (150mM NaCl, 50mM Tris, 0.5% deoxycholic acid, 1% Triton X-100, 0.1% SDS, 5mM EDTA, 20mM NaF, 1mM Na3VO4, pH 8.0, supplemented

by Complete and PhosSTOP) at 10ul buffer/1mg tissue by sonication. After centrifugation at 50,000 × g for 20 min, the supernatant was taken as the RIPA-soluble fraction. The pellet was sonicated in 70% formic acid at 10ul buffer/1mg tissue, and centrifuged at 50,000 × g for 20 min. The supernatant was taken as the FA-soluble fraction. All fractions were stored in -80°C until analyzed.

ELISA. Human tau and APOE ELISAs were performed as described previously[70]. For coating antibodies, Tau5 (gift from L. Binder, Northwestern University, Chicago, IL) was used for human tau ELISA. For detection antibodies, biotinylated HT7 (Thermo Fisher Scientific, MN1000B) was used for tau ELISA.

RT-qPCR. RNAs were isolated from mouse cortex using RNeasy Mini Kit (QIAGEN,74104), and reverse transcribed to cDNA using the high capacity RNA-to-cDNA kit (Applied Biosystems, #4387406). Quantitative PCR were performed using Taqman primers and probes with Taqman universal PCR master mix (Applied Biosystems, #4304437).

Analysis of tau seeding activity in brain lysates. Mouse cortex was homogenized in DPBS supplemented by Complete and PhosSTOP. Lysates were cleared by centrifugation at 12000rpm for 10min and protein concentration was measured by BCA assay. Seeding with the lysates was performed using a CFP/YFP-based biosensor HEK cell system kindly provided by Dr. Marc Diamond (UT Southwestern) as previously described[71]. Briefly, the pro-aggregating tau repeat domain (RD) carrying the P301S mutation was fused to either CFP (RD-CFP) or YFP (RD-YFP), and were transduced into HEK cells to generate stable HEK cell lines expressing either RD-CFP, RD-YFP or RD-CFP/YFP. RD-CFP/YFP HEK cells were transfected with brain lysates containing 1ug protein using lipofectamine 2000 (Invitrogen, #11668027) and cultured

for 24h. Cells were then collected and run through flow cytometry to quantify FRET signals generated. RD-YFP and RD-CFP HEK cells were used for channel compensation.

Coimmunoprecipitation. Mouse cortex was homogenized on ice using a pestle with 30ul buffer/1mg tissue in DPBS supplemented with 1mM EDTA, 1x protease inhibitors (Complete, Roche) and 1x phosphatase inhibitors (PhosSTOP, Roche). The samples were spun down at 1000g for 10min, and the supernatant was used for experiments. For human APOE coimmunoprecipitated with tau, HJ8.5 antibody (in house human tau antibody) was incubated with protein G dynabeads (Thermo Fisher Scientific, 10003D) overnight at 4 degree. The next day, the beads were washed with PBS for three times. The samples were precleared and incubated with HJ8.5-coated dynabeads overnight at 4 degree. After incubation, the beads were collected and washed in PBS for three times. Samples were then eluted from the beads with 1x western blot loading buffer and boiled for 10min before loading for western blot. HT7B (Thermo Fisher Scientific, MN1000B), anti-α-tubulin antibody (Sigma, T5168), and anti-human APOE antibody (Abcam, Cat# ab24139) were used to detect human tau, α-tubulin, and human APOE respectively. For tau coimmunoprecipitated with human APOE, HJ15.7 (in house human APOE antibody) was used as the pulling antibody, and the same set of antibodies were used for detection. For interaction between murine APOE and human tau, HJ6.3 (in house murine APOE antibody) was used for pulling APOE and HJ6.8B (in house murine APOE antibody) was used for APOE detection. All other procedures were the same.

3.3 Results

3.3.1 APOE affects human tau levels in P301S mouse brain

Human tau levels in brain lysates were analyzed via quantitative ELISA following sequential biochemical extraction in RAB (salt buffer), RIPA (detergent buffer) and 70% Formic Acid (FA), which contains soluble tau, less soluble tau, and highly insoluble tau respectively. At 3

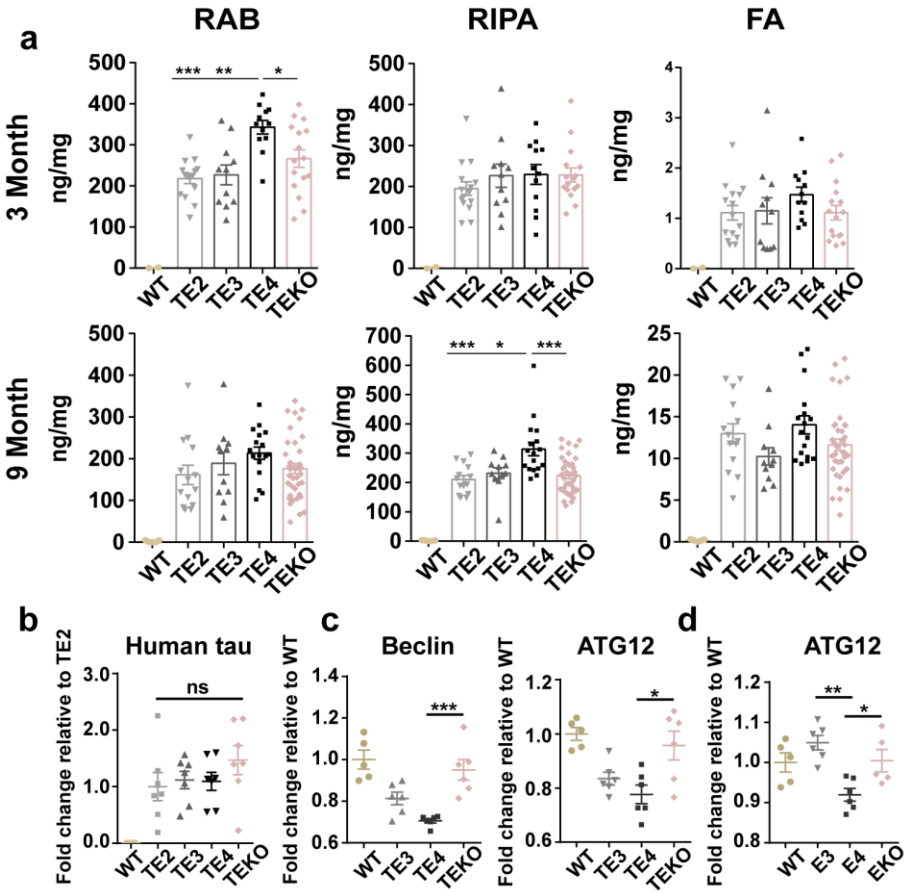

Figure 3.1 | APOE4 increases brain tau levels likely by affecting tau clearance via the autophagy pathway. a, Human P301S tau levels in TE mice were measured by ELISA in RAB, RIPA and 70% FA fractions respectively at two time points: 3 months (WT: n=2, TE2: n=15, TE3: n=11, TE4: n=12, TEKO: n=16) and 9 months (WT: n=5, TE2: n=14, TE3: n=11, TE4: n=17, TEKO: n=38). **b,** qPCR for human tau in 9month TE mouse cortex (WT: n=5, TE2, TE3, TE4, TEKO: n=7). **c, d** Nanostring analysis for autophagy-related gene expression in (c) 9-month old TE mouse hippocampus and (d) 9-month old non-tau transgenic APOE KI or APOE KO mouse hippocampus (n=5-6 per group). Data expressed as mean ± SEM, One-way ANOVA with Tukey's post hoc test (two-sided) was used for statistical analysis. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

months of age, prior to overt tau pathology onset, TE4 mice had significantly higher tau levels in the RAB fraction than other APOE genotypes (Fig. 3.1a). At 9 months of age, when large amounts of tau pathology have developed, TE4 mice still had significantly higher tau levels, but the elevated tau had changed from being in the RAB to the RIPA fraction (Fig. 3.1a), indicating a greater amount of tau in a more insoluble fraction. The higher tau accumulation in TE4 mice was not due to differences in tau synthesis assessed by qPCR (Fig. 3.1b). However, we found significant changes of autophagy-related gene expression associated with APOE4 in the presence or absence of tau pathology (Fig. 3.1c, d), suggesting APOE4 may affect autophagy-mediated tau clearance. This is consistent with a previous report of impaired autophagy associated with APOE4[72].

3.3.2 APOE regulates tau pathogenesis in P301S mice

Hyperphosphorylated tau, identified by p-tau staining with the AT8 antibody, revealed a greater p-tau covered area in 3-month old TE4 mouse hippocampus (Fig. 3.2a, b). While the pathological p-tau signal first appeared in the mossy fibers, axons of DG granule cells in the hippocampus, TE4 mice also showed more intense DG cell body staining (Fig. 3.2a, c), indicating a greater redistribution of pathological p-tau from axons to cell bodies at an early age. At 9 months of age, the differences in overall p-tau immunoreactivity between TE mice decreased (Fig. 3.3a), with the emergence of four major p-tau staining patterns, designated as types 1-4 (Fig. 3.3b). These staining patterns strongly correlated with the level of brain atrophy, with type 1 associated with the most preserved brain and type 4 associated with the most atrophied brain (Fig. 3.3c), suggesting potential differential toxicity associated with different p-tau patterns. The distribution of p-tau patterns differed across APOE genotypes. Type 1 and type

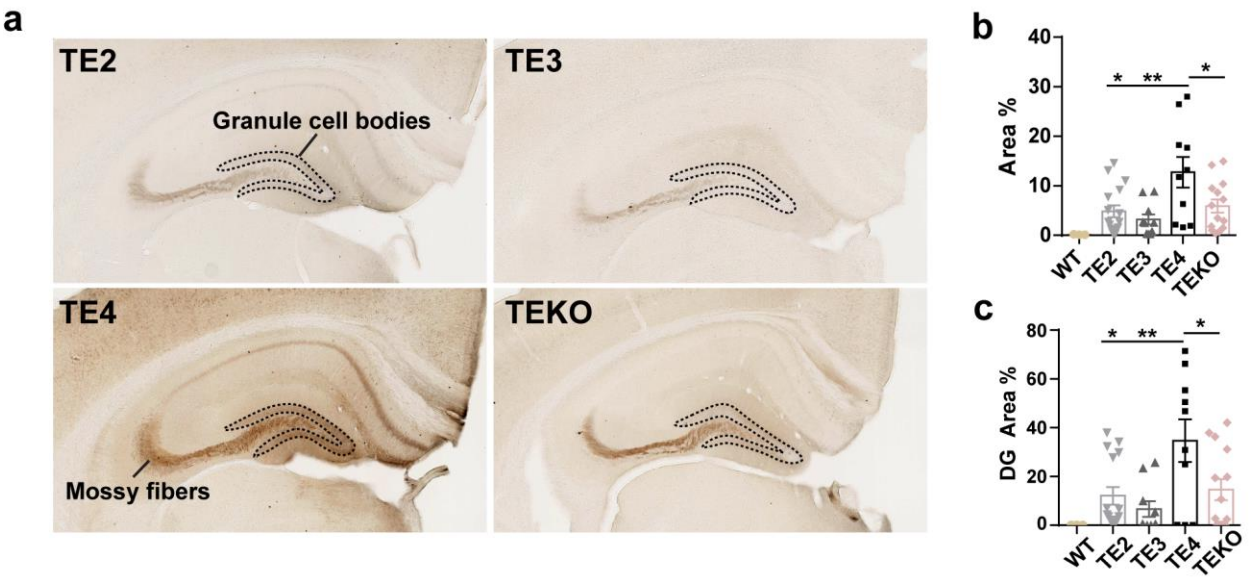

Figure 3.2| APOE4 increases p-tau pathology and promotes pathological tau redistribution from axons to cell bodies in P301S mice at an early age. a, AT8 staining for phosphor-tau in 3-month old TE mouse hippocampus. Dotted outline highlights the dentate gyrus (DG) granule cell bodies. **b, c,** Quantification of AT8 covered area in the hippocampus (b) and in the DG neuronal cell layer (c) (TE2: n=16, TE3: n=10, TE4: n=10, TEKO: n=14). Data expressed as mean ± SEM, One-way ANOVA with Tukey's post hoc test (two-sided). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

2 were primarily enriched in TEKO mice whereas type 3 and type 4 were enriched in P301S mice harboring human APOE. In particular, type 4 was enriched in TE4 mice. (Fig. 3.3d). The featured distribution of these p-tau staining patterns, which either represent different tau structures, or progressively more advanced pathological tau stages, indicate APOE affects either tau conformation, or tau pathology progression.

To further explore whether the p-tau patterns are associated with different tau structures, we assessed the tau seeding activity from frozen brain tissues using a biosensor HEK293T cell line that co-expresses CFP and YFP fused to the aggregation-prone repeat domain (RD) of tau, which can generate a FRET signal when tau aggregation is induce by exogenously applied tau seeds. There was a dramatic increase of tau seeding activity associated with type 2 staining relative to other staining patterns (Fig. 3.3e), although no difference was observed between

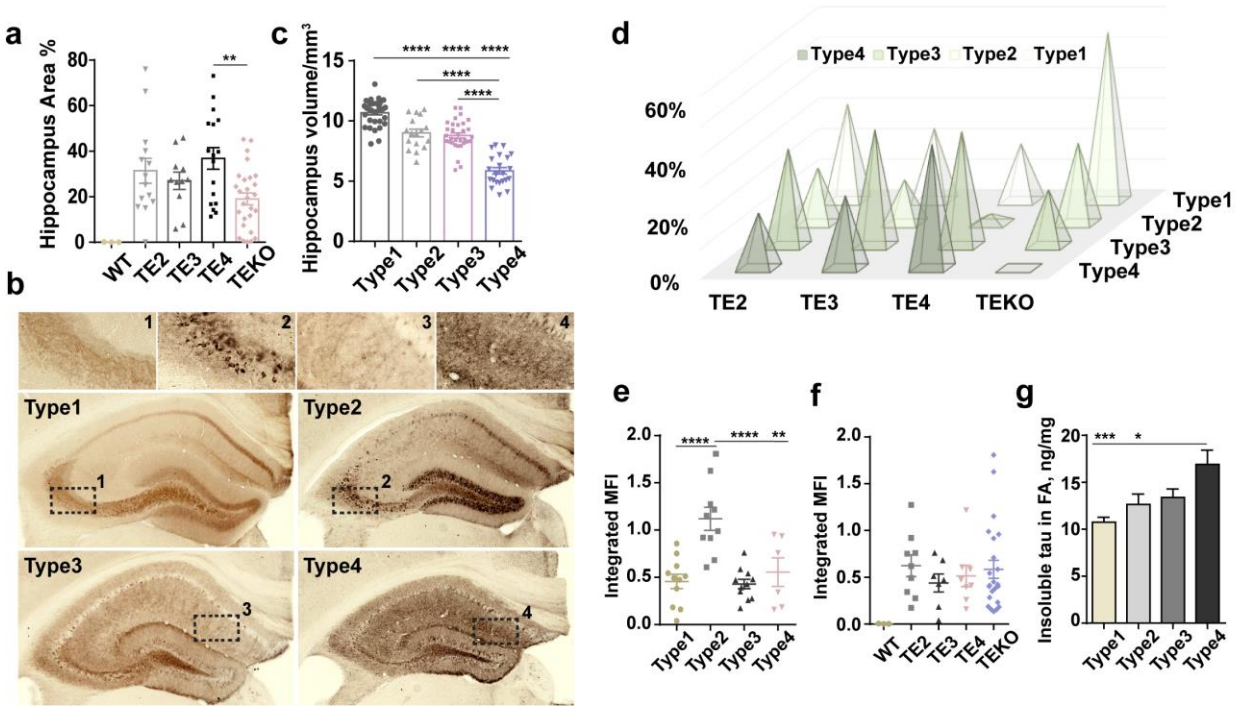

Figure 3.3| APOE affects hippocampal ptau staining patterns in 9-month old P301S mice. a, Quantification of ptau (AT8) covered area in 9-month old TE mouse hippocampus (WT: n=5, TE2: n=14, TE3: n=11, TE4: n=17, TEKO: n=38). **b,** Four distinct p-tau staining patterns were identified based on hippocampal staining features. Type 1 has intense mossy fiber staining as well as diffuse cell body staining in the dentate gyrus granule cell layer and CA1 pyramidal cell layer; type 2 has compact and dense tangle-like cell body staining primarily in the dentate gyrus granule cells and CA3 pyramidal cells, but also has sparse staining in the CA1 region; type 3 has staining primarily in the neuropil of the stratum radiatum of the CA region with clear staining of dendrites from pyramidal neurons and only some staining in the neuronal cell bodies; type 4 has dense staining over the entire hippocampus, unlike other staining patterns, type 4 staining is fragmented, dotted, and grainy. **c,** P-tau staining patterns were associated with different degrees of brain atrophy, n=104 biologically independent animals. **d,** Distribution of the four p-tau staining types in 9-10 months old TE mice (TE2: n=22, TE3: n=21, TE4: n=32, TEKO: n=38). Fisher's exact test, two-sided (All groups: p=3.4e-05, TE2 vs. TEKO: p=0.021, TE3 vs. TEKO: p=0.0016, TE4 vs. TEKO: p=1.9e-07). **e.** P-tau staining patterns were associated with distinct tau seeding activities. **f.** Tau seeding activities do not differ between APOE genotypes. **g.** Type1-type4 ptau staining patterns are associated with progressively more insoluble tau in the formic acid fraction. Data expressed as mean ± SEM, One-way ANOVA with Tukey's post hoc test (two-sided). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

APOE genotypes (Fig. 3.3f). Interestingly, despite harboring a higher seeding capacity, type 2 tau is relatively soluble (Fig. 3.3g), consistent with previous studies showing that tau seeds are present in the soluble fraction[62]. The association of enhanced tau seeding activity with a ptau

staining pattern that correlates with less brain atrophy indicates that tau aggregation to some extent may be less toxic, likely by trapping diffuse tau oligomers that could potentially be more toxic. This is consistent with previous findings showing that neurofibrillary tangles are not associated with acute neuronal death[73,74]. Alternatively, tau seeding may represent an early event in tau pathogenesis. Aggregated tau may undergo conformational change, lose its seeding capacity, and become more insoluble with higher toxicity over time as disease progresses.

3.3.3 APOE interacts with a specific form of tau, likely hyperphosphorylated tau.

Interestingly, I found that APOE specifically interacts with a particular form of tau in 9month old P301S mouse brain lysates. In P301S mice, when neurodegeneration occurs, an additional tau band stands out in western blot, which locates on top of the major tau band. This upper tau band barely or weakly shows up in brains harboring type1 tau, but is increasingly accumulated in brains harboring type2, type3 and type4 tau, with the highest amount present in type4 tau-containing brains that demonstrate the highest degree of brain atrophy (Fig. 3.4).

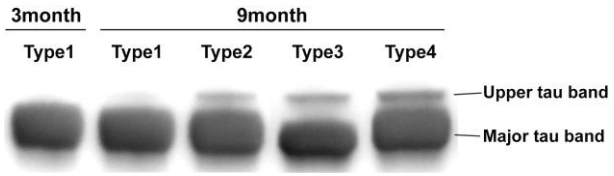

Figure 3.4 | An additional tau band shows up in western blot in diseased P301S mouse brains. Western blot for human tau detected by HT7B antibody in 3month old TE mouse brain that contains only type1 tau and in 9month old TE mouse brains containing type1-type4 tau respectively.

As tau hyperphosphorylation is a hallmark of tau pathology and is exacerbated during disease progression, the upper tau band likely represents a hyperphosphorylated form of tau. Intriguingly, APOE specifically interacted with the upper tau band in TE mouse brain lysates

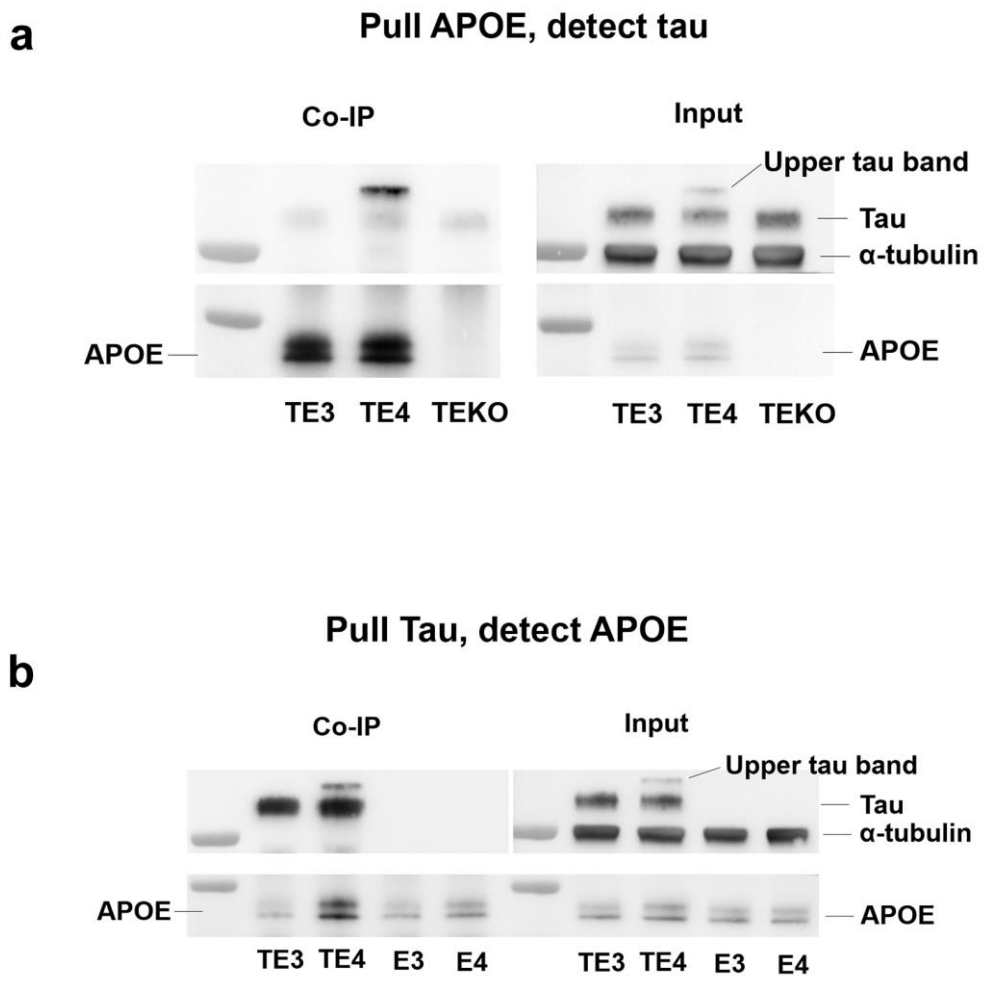

Figure 3.5 | Human APOE specifically interacts with the upper tau band in 9-month TE mouse brain lysates. a, Co-IP between human APOE and human tau. Brain lysates of 9-month old TE3, TE4 and TEKO mice were pulled with a human tau antibody HJ8.5. The eluted samples were detected by western blot with the human tau antibody HT7B and a polyclone human APOE antibody. α-tubulin was used as a loading control. **b,** Brain lysates of 9-month old TE3, TE4 mice and non-tau transgenic E3, E4 mice were pulled with a human APOE antibody HJ15.7. Eluted samples were detected by the human tau antibody HT7B and a polyclone human APOE antibody.

(Fig. 3.5) independent of APOE isoforms (data not shown). In APOE-tau coimmunoprecipitation (Co-IP) experiments, when APOE was pulled from the lysates, only the upper tau band was coimmunoprecipitated with APOE (Fig. 3.5a). Vice versa, when tau was pulled from the lysates, only when the upper tau band was present did more APOE coimmunoprecipitated with tau (Fig 3.5b). The specific interaction between APOE and the upper

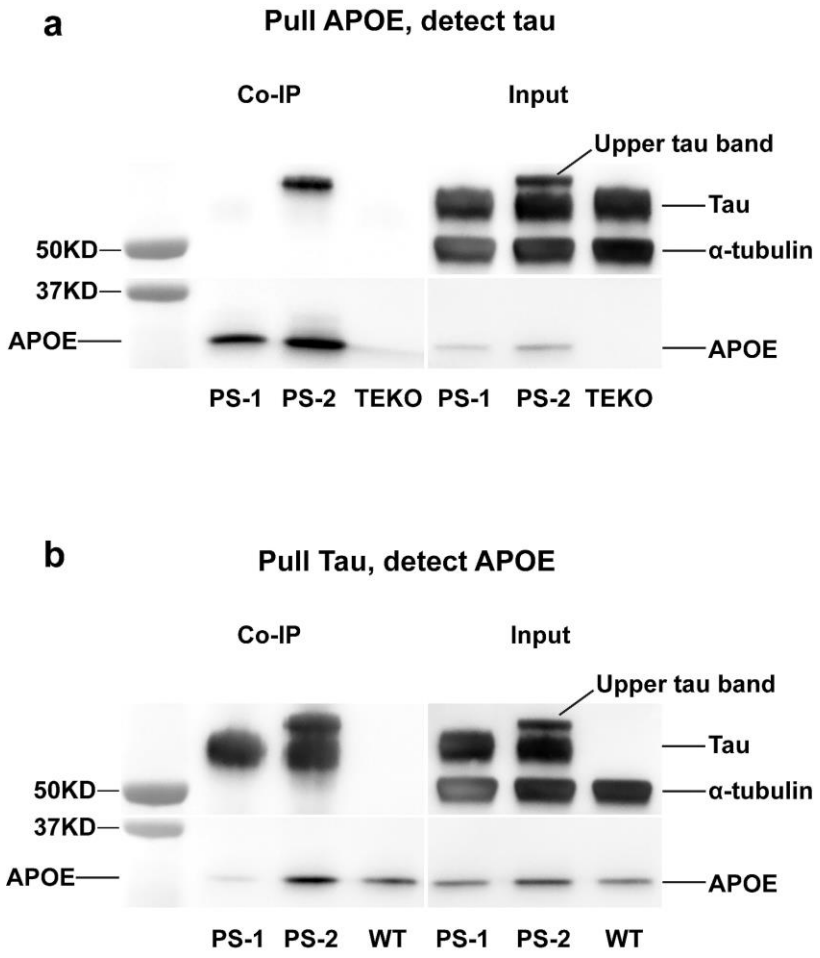

Figure 3.6 | Murine APOE specifically interacts with the upper tau band in 9-month P301S mouse brain lysates. a, Co-IP between mouse APOE and human tau. Brain lysates of 3-month old (PS1, type1 tau) 9-month old P301S mice (PS-2, type4 tau) that express endogenous murine APOE and 3-month old TEKO mice were pulled with a human tau antibody HJ8.5. The eluted samples were detected by western blot with the human tau antibody HT7B and a mouse APOE antibody HJ6.8. α-tubulin was used as a loading control. **b,** Brain lysates of 9-month old TE3, TE4 mice and non-tau transgenic E3, E4 mice were pulled with a human APOE antibody HJ15.7. Eluted samples were detected by the human tau antibody HT7B and a polyclone human APOE antibody.

tau band was observed in P301S mice that express endogenous murine APOE as well (Fig. 3.6). These results indicate that APOE may be able to regulate tau pathogenesis through direct APOE-tau interaction. Intraneuronal accumulation of APOE, either derived from APOE retention from endocytosis or de novo APOE synthesis inside neurons in stressed conditions, may bind to newly formed hyperphosphorylated tau in early disease and promote tau aggregation and insoluble tau

formation, which may in turn cause more cellular stress that induce more hyperphosphorylation of tau. Interestingly, impaired APOE4 recycling to the cell surface leading to intracellular accumulation of APOE relative to APOE3 has been reported[75]. Therefore, it's likely that increased level of intraneuronal APOE4 interacts with hyperphosphorylated tau to promote tau pathogenesis. On the other hand, high-molecular-weight (HMW), hyperphosphorylated tau derived from human AD brains and tauopathy mouse models has been reported to possess high seeding capacity and constitutes the tau species that mediates intercellular tau transmission and tau seeding within neurons[62]. Neurons were found to specifically take up this type of tau over other low-molecular-weight tau species and be effectively seeded by this HMW hyperphosphorylated tau[62]. This particular tau species likely represents the upper tau band seen in the diseased brain. Importantly, the HMW tau is present in the ISF of tauopathy mouse brains and in the CSF of human AD patients[62,76]. Given the abundance of APOE in the extracellular space, it is likely that APOE binds to these HMW hyperphosphorylated tau in the ISF and affects their uptake by neurons through interaction with APOE receptors. Tau-bound APOE may also alter the seeding activity of this tau species by altering its conformation. These aspects may collectively influence the spreading of tau pathology and disease progression. Given the faster turnover rate of APOE4 relative to other APOE isoforms, it may facilitate tau uptake into neurons via APOE receptor-mediated mechanisms and promote pathological tau spreading.

3.4 Discussion

Here we clearly demonstrated that physiologically expressed APOE directly regulates human tau pathogenesis independent of Aβ. In general, the presence of human APOE, particularly APOE4, worsens tau pathology relative to the absence of APOE. The effects of APOE on tau pathology

strongly correlates with its effects on neurodegeneration, indicating that APOE affects neurodegeneration in part by modulating tau pathology that confers neuronal toxicity in a cell-autonomous manner. The specific interaction between APOE and potentially hyperphosphorylated tau is intriguing and suggests that APOE may regulate tau pathogenesis through direct interaction with pathological tau. Binding of APOE to hyperphosphorylated tau appears to facilitate conversion of tau aggregates from a soluble state to a more insoluble state, during which course tau loses its seeding capacity. This may be achieved by APOE scaffolding and compacting hyperphosphorylated tau to form dense insoluble structures, likely in an APOE isoform-dependent manner. Although being less seeding-competent, these insoluble tau may possess stronger neurotoxicity. In addition, potential interaction between APOE and hyperphosphorylated tau in the ISF may influence tau uptake by neurons and regulates pathological tau propagation in an isoform-specific fashion.

To further understand the role of APOE in regulating pathological tau behaviors, I performed a full set of experiments assessing the effect of APOE and APOE isoforms on the binding, uptake, seeding, spreading, and degradation of aggregated tau both in vitro and in vivo. The initial highly replicable observation that APOE markedly promoted cellular uptake of tau aggregates eventually turned out to be an experimental artifact due to the rapid and robust binding of tau protein to laboratory containers. Experimental designs involving preincubation of tau aggregates with APOE in eppendorf tubes led to a drastic loss of tau protein in the absence of APOE as a result of tau binding to the tube surface. Surprisingly, the presence of APOE in the solution completely abolished tau association with the tube, likely due to APOE coating the tube surface to prevent tau binding, or APOE forming a complex with tau aggregates to retain them in the solution. This resulted in different amounts of starting tau material left in the tube prior to

cell or animal treatment, leading to the experimental artifacts. Although no clear conclusions were reached from these studies, the observation that APOE efficiently prevented tau aggregates from binding to container surfaces is novel. All proteins to certain degrees bind to container surfaces, and this is particularly true for sticky proteins like Aβ, tau, and α-synuclein. Similar effects of APOE on synuclein binding to eppendorf tubes were subsequently recapitulated in the lab after my initial finding. It's highly likely that similar effects of APOE also apply to other pathological proteins such as Aβ. Interestingly, this APOE effect has never been reported in previously literatures although numerous studies have utilized similar experimental settings to study APOE functions on pathological proteins, the results of which may need to be interpreted with caution.

In addition to a direct impact on tau pathology, APOE may also regulate tau pathogenesis via modulating neuroinflammation. It has been well documented that neuroinflammation influences tau pathology. In P301S mice, administering an immunosuppressing drug FK506 from an early age drastically reduces tau pathology[77]. In 3xTg-AD mice, chronic intraperitoneal administration of lipopolysaccharides (LPS) for 6-weeks is shown to induce marked microglial activation and promote site-specific tau hyperphosphorylation[78]. Similarly, deleting the microglia-specific homeostatic gene CX3CR1 in htau mice leads to amplified microglial activation and enhanced tau hyperphosphorylation /aggregation[79,80]; whereas overexpressing a soluble form of CX3CL1, the ligand for CX3CR1, reduces microglial activation and tau pathology in rTg4510 mice[81]. IL-1 signaling was identified to play a key role in neuroinflammation-induced tau phosphorylation by enhancing the activity of tau kinases including CDK5/p25, GSK-3β, and p38–MAPK[82,83]. As briefly mentioned earlier, APOE plays

an essential role in immune regulation. Effects of APOE on neuroinflammation may constitute another mechanism to influence tau pathogenesis as well as neurodegeneration.

Chapter 4: Immunomodulatory functions of APOE in neurodegeneration

This chapter is adapted from the following manuscripts

Shi, Y. et al. APOE4 markedly exacerbates tau-mediated neurodegeneration in a mouse model of tauopathy. *Nature* 549, 523-527, doi:10.1038/nature24016 (2017).

Shi Y, Holtzman DM. Interplay between innate immunity and Alzheimer's disease: APOE and TREM2 in the spotlight. *Nat Rev Immunol.* (Accepted)

4.1 Introduction

While APOE regulates tau pathology, which may subsequently affect neurodegeneration through tau-mediated neurotoxicity in a cell autonomous fashion, the accumulation of pathological tau along with degenerating neurons also induce chronic neuroinflammation that constitutes another major mechanism of neuronal loss. To date, more than 30 Alzheimer's disease-risk loci have been identified via a combination of genetic linkage, candidate gene, genome-wide association studies (GWAS), and whole genome/exome sequencing (WGS/WES) studies[84]. Of these risk loci, over 50% of gene variants validated by functional genomics are implicated in microglial and innate immune cell function, including the top two risk genes, *APOE* and *TREM2*. In addition, epigenomic analysis has shown that Alzheimer's disease GWAS loci are preferentially enriched in enhancer sequences implicated in innate immune processes[85]. These findings indicate an essential role of the innate immune response in Alzheimer's disease pathogenesis.

In the brain, neuroinflammation heavily relies on innate immune responses mediated primarily by CNS-resident microglia and also by astrocytes. Prolonged inflammation may induce neuronal injury and death via multiple ways. First, activated glia produce toxic substances such as reactive oxygen species (ROS) and nitric oxide (NO) that can directly damage or kill neurons; second, inflammatory stress and sub-lethal pathological insults can induce stressed-but-viable neurons to expose reversible 'eat-me' signals such as phosphatidylserine or calreticulin, and be phagocytosed and killed by activated microglia via a process called phagoptosis[86]; third, activation of the complement system promotes membrane attack complexes (MAC) formation, creating pores on cell membranes to induce cell lysis; fourth, activated microglia can crosstalk with astrocytes, inducing reactive astrocytes to rapidly kill neurons via secreted neurotoxic factors and loss of neurotrophic function[87], and fifth, neuronal NLRP1 (NACHT, LRR and PYD

domains-containing protein 1) inflammasome and caspase-1 activation can directly induce neuronal injury and death[88,89]. In addition to a direct effect on neuronal viability, neuroinflammation also affects tau pathogenesis, which in turn can influence neurodegeneration. Therefore, glia-mediated innate immunity plays a critical role in neurodegeneration and may constitute a driving force for disease progression.

Intriguingly, APOE itself has been found to be a potent modulator of immune responses. The association of APOE4 with a higher innate immune reactivity has long been observed. In healthy humans challenged with intravenous LPS infusion, ε4-carriers demonstrate significantly higher elevation of body temperature and plasma TNF levels compared to ε4 non-carriers[90]. Similarly, when whole blood isolates from human subjects were stimulated ex vivo with TLR ligands, enhanced production of a wide panel of cytokines and chemokines was observed in blood from ε4+ donors compared with ε4- donors[90]. A greater immune response associated with the ε4 allele compared to other APOE alleles is also observed in human APOE-targeted replacement (APOE-TR) mice and in cultured microglia/macrophages upon LPS stimulation[90-92]. This evidence suggests that the effects of APOE on neurodegeneration may be in part mediated by its immunomodulatory function. We therefore investigated this hypothesis in this chapter.

4.2 Experimental procedures

Primary microglia culture, LPS stimulation and cytokine measurement. Mixed glia culture from APOE2, APOE3 and APOE4 KI mice were prepared as described above in DMEM/F12 medium supplemented with 10% FBS and 5ng/ml GM-CSF. Loosely attached microglia were harvested at DIV12 by shaking for 2 hours at 400 rpm and then seeded onto 12-well tissue culture plates coated with PLL at a density of 1×10^6 cells per well. The next day, seeded

microglia were washed in serum-free medium (SFM-DMEM/F12 + 0.2% BSA +1x pen/strep) and placed in 450 µl SFM. Cells were then treated with 1 ng/ml LPS (Sigma, #L5293) for 24h. Medium were collected and spun down at 10,000g for 10 min. Supernatants were collected and assayed for mouse TNFα, IL-1α and IL-1β using single cytokine ELISAs (Bon Opus Biosciences) according to the manufacturer's instructions. All cytokine levels were normalized to microglial protein levels determined by BCA assay.

Nanostring gene expression assay. RNAs were isolated from mouse hippocampus using RNeasy Mini Kit (QIAGEN,74104). We performed nCounter multiplexed target profiling of 534 microglial transcripts with a custom designed microglial gene chip (MG550) using the Nanostring nCounter technology **(nanostring.com)**, which allows analysis of up to 800 genes from a single sample. Selection of genes was based on genes and proteins specifically or highly expressed in adult homeostatic microglia[93] plus 150 inflammation-related genes which were significantly affected in EAE, APP-PS1 and SOD1 mice[94]. Using this signature, we generated a new version (v3) of Nanostring-based microglia chip termed MG550. For each sample, 100ng RNAs were used for hybridization, and all data were normalized to 6 housekeeping genes: Cltc, Gapdh, Gusb, Hprt1, Pgk1, and Tubb5.

Immunofluorescence staining. Two sections (bregma -1.7mm and -2.0mm for CD68, bregma -2.3mm and -2.6mm for GFAP) from each mouse were used for staining. The sections were washed in TBS 3 times, and blocked with 3% BSA in 0.25% TBS-X for 0.5h at RT, followed by overnight incubation at 4°C with primary antibodies (CD68: AbD SeroTec, MCA1957, 1:500; GFAP: EMD Millipore, MAB3402, 1:2000). The next day, the sections were washed in TBS and incubated with fluorescence-labeled secondary antibodies (Molecular probes, 1:500) for 2h at RT. The slices were then washed and mounted in prolong gold antifade mounting media

(Molecular probes, P36931). Images were taken with an epi-fluorescence microscope at 4x magnification and quantified using MetaMorph.

Western Blot. Mouse cortex was homogenized by sonication in RIPA buffer supplemented with Complete and PhosSTOP. Samples were separated by 4–12% NuPAGE (Invitrogen) gel using MOPS buffer and transferred to nitrocellulose membranes. Primary antibodies anti-GFAP (Millipore, MAB 3402), anti-GAPDH (Abcam, ab9484), anti-α tubulin (Sigma, T5168) and HRP-conjugated secondary antibodies (Santa Cruz) were used for detection. Membranes were developed using Lumigen TMA6 (GE Healthcare).

Microfluidic qRT-PCR for astrocytic genes. Total RNA was extracted from mouse cortex using the RNeasy Plus kit (Qiagen) and microfluidic qRT-PCR was performed as described[87].

Primary neuron culture and recombinant APOE treatment. Primary neurons were obtained from E17 WT mouse (Charles River, #022) fetuses. Hippocampi were dissected in calcium- and magnesium-free Hanks' Balanced Salt solution (HBSS) with careful strip of meninges. Tissue was digested in HBSS containing 0.25% trypsin (GIBCO #15090-046) and 0.2mg/ml DNase (Sigma #DN-25) at 37 °C for 10 min, and was dissociated in HBSS containing 0.4mg/ml DNase using a fire-polished Pasteur glass pipette and filtered through a 70-μm nylon mesh. Filtered material was pelleted at 1000 g for 5 min, washed with neurobasal medium (Neurobasal + 1x B27 + 1x pen/strep + 1x L-glutamine) once, and infected with AAV2/8-synapsin-P301S tau virus for 3h on ice. Cells were then pelleted, washed, and plated onto 24-well tissue culture plates over glass cover slips that had been coated with 10 μg/ml Poly-L-lysine (PLL, Sigma, #P2636) at a density of 20,000 cells/well in neurobasal medium. For all cell culture experiments, only the central wells of the plates were used with the peripheral wells filled with 1ml autoclaved ddH2O to avoid different rates of medium evaporation during the culture. Recombinant APOE2,

APOE3, and APOE4 (Leinco, #A215, A218, A219) were added to the medium (10ug/ml) at the time of plating. Neurons were kept for 3 weeks with addition of 200 ul fresh recombinant APOE-containing medium (10 µg/ml) each well every week, and were then stained with MAP2 antibody (Thermo Fisher Scientific, #OSM00030W) for analysis and with HT7B (Thermo Fisher Scientific, #MN1000B) antibody to confirm tau expression.

Glia-neuron co-culture. E2, E3, E4 and EKO glia were obtained from P2 pups from the respective human APOE KI or APOE KO mice. Cortex was dissected and dissociated in the same way as with neuron isolation. Cells were plated in glial medium (DMEM + 10% FBS + 1x Pen/strep + 1x Glutamax). Once monolayers were confluent, cells were replated in 24-well plates over glass cover slips coated with geltrex (Gibco, #A1413201) at a density of 75,000 cells /well, and allowed to grow for 2 days in glia medium. The glia were then washed with 1 ml neurobasal medium (Neurobasal + 2% B27 + 1x pen/strep + 1x L-glutamine) and placed in 400 µl neurobasal medium before use. Primary neurons expressing P301S tau were prepared as described above, and were directly plated on top of the glia at a density of 20,000 cells/well. The co-cultures were kept for 3 weeks with addition of 200 µl fresh neurobasal medium each well every week. Neurons were then stained with MAP2 antibody (Thermo Fisher Scientific, #OSM00030W) for analysis and with HT7B (Thermo Fisher Scientific, #MN1000B) antibody to confirm tau expression.

Immunocytochemistry. Cells were fixed in DPBS containing 4% PFA and 4% sucrose at RT for 10 min and permeabilized with 0.3% PBST for 10min. After blocking in 0.1% PBS-Triton X-100 containing 3% BSA and 3% goat serum for 30min at RT, cells were incubated in primary antibodies overnight at 4°C. The next day, cells were washed with 0.1% PBST for 3 times and incubated in secondary antibodies for 1h at RT. Cells were then washed 3 times in 0.1% PBST

and mounted in prolong gold antifade mounting media (Molecular probes, P36931). Images were taken with an epi-fluorescence microscope at 10x magnification and quantified using MetaMorph.

Microglial phagocytosis of injured neurons. Primary microglia were isolated from mix glial cultures derived from neonatal WT, APOE KO, APOE2 KI, APOE3KI and APOE4 KI mice. Floating microglia were gently flushed off astrocytes with pipet are plated in 24-well plates at a density of 200000 cells/well. Isolated microglia were cultured in serum-free medium (DMEM/F12 + 1x GlutaMAX + 1x NEAA + 2% ITS-G + 2% B-27 Supplement minus vitamin A + 0.5% N2 Supplement + 200uM 1-Thioglycerol + 5ug/ml Insulin + 50 ng/mL TGF-β + 100 ng/mL IL-34 + 25 ng/mL MCSF). After cultured for 2 days, the medium was replaced with fresh serum-free medium supplemented with two additional maturation factors (100 ng/mL CX3CL1 + 100 ng/mL CD200) and cultured for 2 days prior to treatment with neurons. Primary neurons were cultured separately in 6 well plates at a density of 300000 cells/well. At DIV7, neurons were lifted from the plate by pipetting and were injured during this process. Collected neurons with labeled with Dil (Thermo Fisher Scientific, #D3911) at 37 degree for 20 min. Labeled neurons were washed with neurobasal medium for 3 times and resuspended in microglia serum-free medium. The neurons were then added to the cultured microglia medium and incubated with microglia for 3h or 12h. After incubation, the medium containing injured neurons were removed and microglia were washed with PBS for three times before collected with trypsin. Microglia were then run through flow cytometry to quantify the amount of intracellular Dil signals. Dil-labeled neurons were run separately as a control. Small areas of overlapped microglia and neuronal cell population were excluded from gating to avoid neuronal contamination in the microglial cell population.

4.3 Results

4.3.1 APOE modulates innate immune responses in cultured microglia and P301S mice

To demonstrate that APOE affects microglia-mediated innate immune response, we cultured primary microglia isolated from human APOE KI (E2, E3, E4) mice, and stimulated them with LPS. We measured the amount of proinflammatory cytokines in the culture medium and normalized it to protein concentration of microglia cell lysates. We observed significantly higher levels of TNF, IL-1α, and IL-1β produced by APOE4 microglia compared to APOE2 and APOE3 microglia (Fig. 4.1a), confirming that APOE4 is associated with an inherently higher immune capacity. We then assessed microglial gene expression profiles in 9-month old TE3, TE4 and TEKO mice as well as in 9-month old non-tau transgenic APOE KI and APOE KO mice using a customized MG550 microglial gene chip. We observed a marked upregulation of proinflammatory genes (cluster 1) and a concomitant downregulation of genes involved in normal cell function (cluster 2) in TE4 mice, whereas microglia in TEKO mice largely remained in a homeostatic state (Fig. 4.1b, c, d). Consistent with this, CD68-positive microglial staining, which represents an activated state of microglia, was significantly elevated in TE4 mice, and was drastically reduced in TEKO mice (Fig. 4.1e, f). Notably, no pro-inflammatory gene activation and no or minimal gene expression differences were observed in 9-month old APOE KI and APOE KO mice in the absence of P301S tau (Fig. 4.1b, d) or in 3-month old TE mice (Fig. 4.2), indicating the necessity of tau pathology to initiate microglial activation and to enable the APOE genotype-dependent effect on microglial activation. Interestingly, p-tau staining patterns were highly associated with the microglial activation profile, with type 1 staining displaying the lowest, type 2 and type 3 intermediate, and type 4 the strongest microglial activation (Fig. 4.3),

suggesting either distinct intrinsic capacities of different p-tau patterns to induce neuroinflammation, or distinct toxicities associated with p-tau

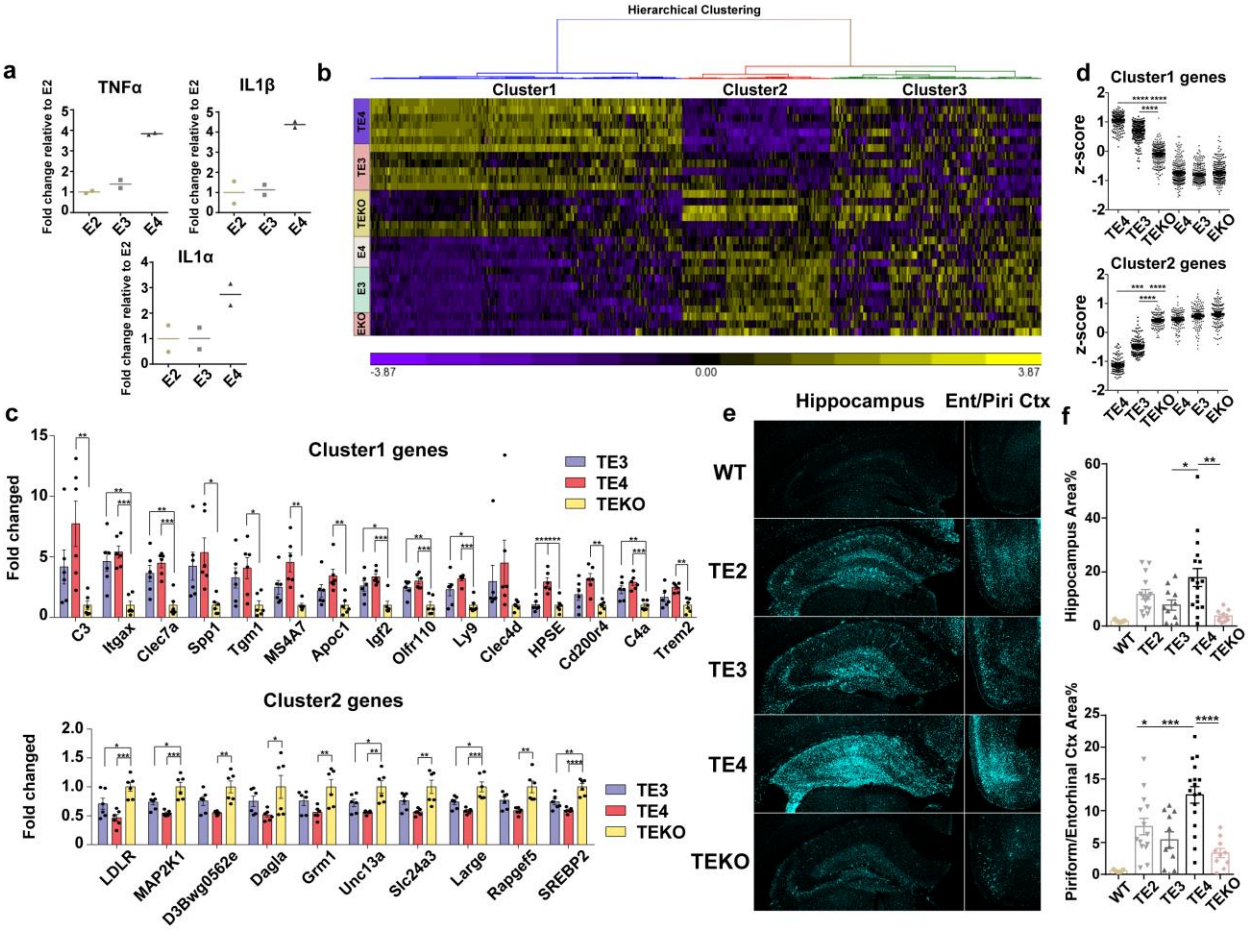

Figure 4.1 | APOE strongly modulates microglial activation. a, Cultured primary microglia derived from human APOE KI (E2, E3, E4) mouse brains were treated with 1 ng/ml LPS for 24h, cytokine levels in the media were measured by ELISA (2 wells/genotype) and were normalized to total microglial protein levels, experiment replicated 3 times. **b,** Nanostring analysis for microglial gene expression in 9-month old TE or EKI/EKO mouse hippocampus. Heatmap generated by hierarchical gene clustering based on genotypes (Horizontal: 534 microglial genes, vertical: individual mouse samples; TE3, TE4, TEKO, E3: n=6, E4: n=4, EKO: n=3). **c,** Top differentially expressed cluster1 and cluster2 genes from the heatmap (criteria: fold change TE4 vs. TEKO high to low, p-value <0.01; fold change TE4 vs. TE3>1.2). Cluster1: proinflammatory genes, Cluster2: cellular function-related genes (metabolism, signaling, transcription, etc.), Cluster3: homeostatic genes/genes below detection. **d,** z-score of genes from cluster 1 or cluster 2 for all groups. Kruskal-Wallis test with Dunn's multiple comparisons test (two-sided) was performed for statistical analysis. **e, f,** CD68 (activated microglia) staining and quantification in 9-month old TE mice (WT: n=5, TE2: n=14, TE3: n=11, TE4: n=17, TEKO: n=10). Data expressed as mean ± SEM, One-way ANOVA with Tukey's post hoc test, two-sided. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

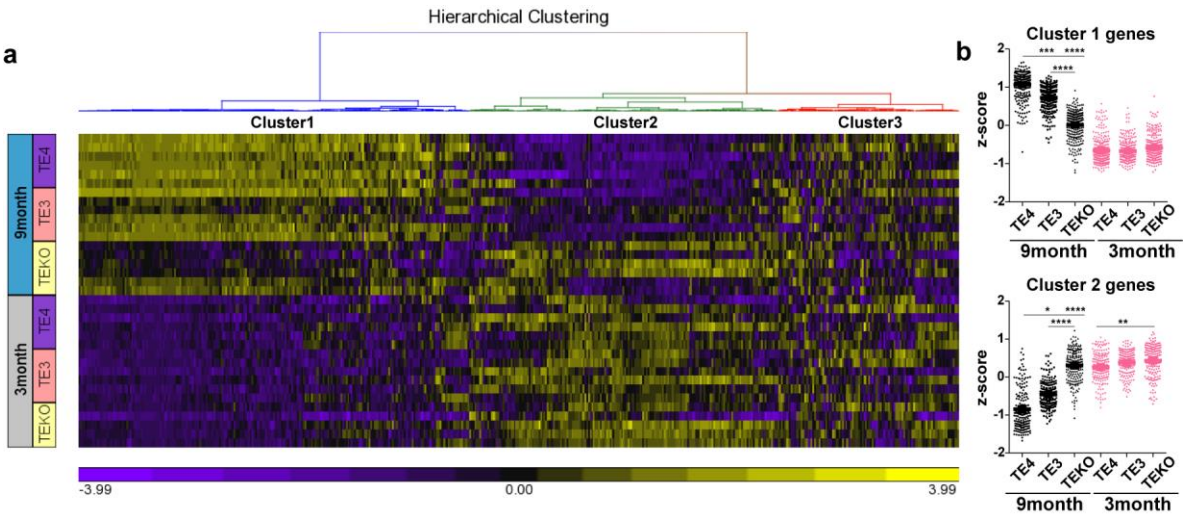

Figure 4.2 | No or minimal change of microglial gene expression in 3-month old TE mice despite significant changes in 9-month old TE mice. a, Nanostring analysis for microglial gene expression in 9-month old TE4 mice and 3-month old TE mice (n=5-6)**.** Heatmap generated by hierarchical gene clustering based on genotypes (Horizontal: 534 microglial genes, vertical: individual mouse samples) **b,** Z-score of genes from cluster 1 or cluster 2 category. Kruskal-Wallis test with Dunn's multiple comparisons test was performed for statistical analysis. Data expressed as mean ± SEM. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

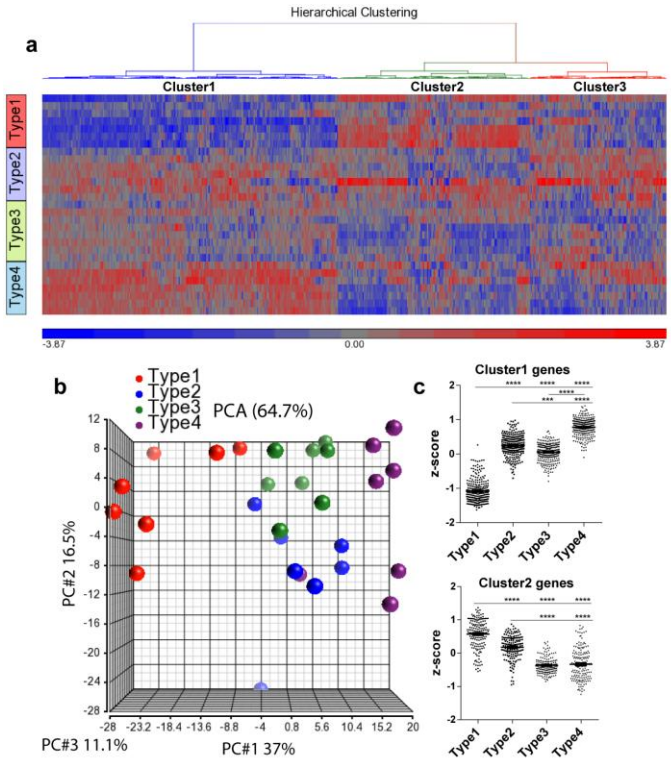

Figure 4.3 | P-tau staining patterns are associated with distinct microglial activation profiles. a, Heatmap generated by hierarchical gene clustering based on p-tau staining types for 9-month old TE mice (n=7-8). **b.** Principle components analysis (PCA) of microglial gene expression profile for p-tau staining types. **c.** Z-score of genes from cluster 1 or cluster 2 category. Kruskal-Wallis test with Dunn's multiple comparisons test was performed for statistical analysis. Data expressed as mean ± SEM. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

patterns that cause different levels of neurodegeneration accompanied by different degrees of neuroinflammation.

Another key cell type in the brain, astrocytes, also play an essential role in neuroinflammation. Unlike microglia that are derived from yolk sac precursors, astrocytes resemble neurons in that they originate from ectodermal precursors and are not conventional immune cells. However, astrocytes are immune-competent. They respond to a wide range of brain insults and are activated in a wide range of neurodegenerative diseases including Alzheimer's disease[87]. Recent studies have identified two distinct types of reactive astrocytes in mice depending on the initiating injury: "A1" induced by LPS-elicited inflammation, and "A2" induced by ischemia[95]. These reactive astrocytes are defined by three cassettes of genes: A1-specifc (inflammation only), A2-specific (ischemia only), and PAN reactive (upregulated in both injuries)[95]. Recent data show that inflammation-induced reactive microglia produce specific

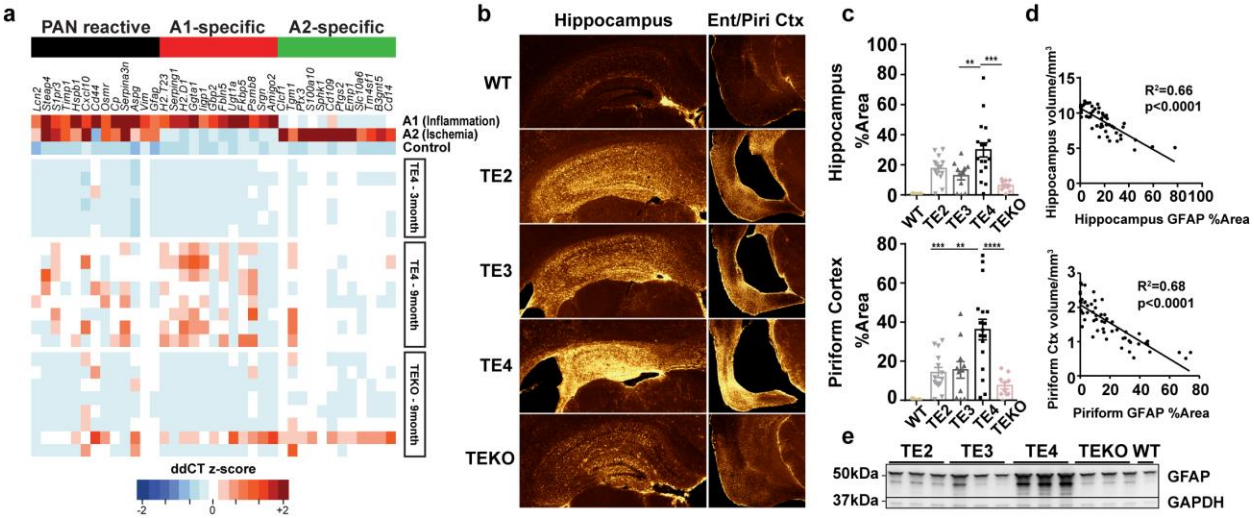

Figure 4.4 | APOE4 leads to robust astrocyte activation whereas the absence of APOE attenuates astrocyte activation. a, Microfluidic RT-qPCR for activated astrocytic genes in 9-month old TE4 (n=8), TEKO (n=8), and 3-month old TE4 mice (n=6). A1-specific: genes activated only by LPS; A2-specific: genes activated only by ischemia; PAN reactive: genes activated by either LPS or ischemia. **b,** GFAP staining in 9-month old TE mice (WT: n=5, TE2: n=14, TE3: n=11, TE4 n=17, TEKO: n=10). **c,** Quantification of area covered by GFAP signal. **d,** Correlation between GFAP signal and brain volume. N=57 biologically independent animals. Pearson correlation analysis (two-sided). Hippocampus: r^2=0.66, p<0.0001; Piriform cortex: r^2=0.68, p<0.0001. **e,** Western blot for GFAP in 9-month old TE mice (n=3).

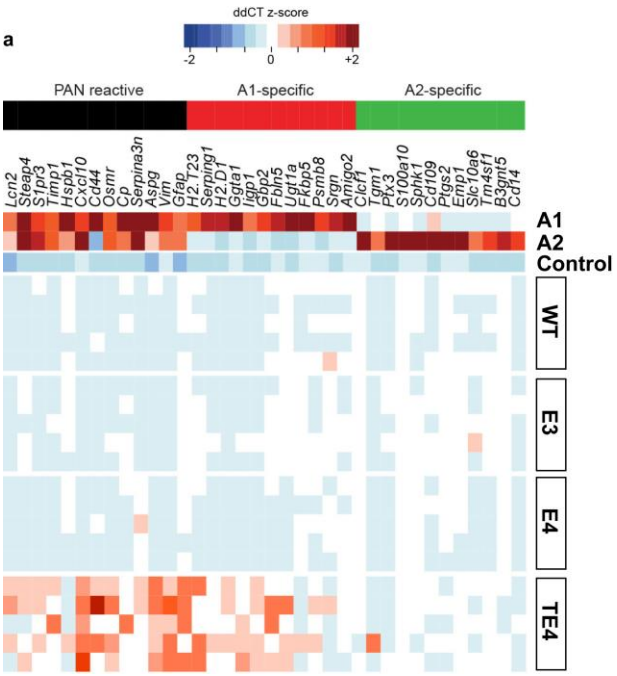

Figure 4.5 | No activation of A1 astrocytic genes in 9-month old non-tau transgenic mice. a, Microfluidic RT-qPCR for activated astrocytic genes in 9-month old TE4 mice and 9-month old non-tau transgenic WT and human APOE KI mice (n=5). A1-specific: genes activated only by LPS; A2-specific: genes activated only by ischemia; PAN reactive: genes activated by either LPS or ischemia.

cytokines (TNF, IL1α, C1q) that can activate "A1" astrocytes, which lose many normal astrocytic functions, and secret toxic factors able to rapidly induce neuronal death[87]. We observed strong activation of "A1" astrocytic genes in 9-month TE4 mice, but not in 9-month TEKO mice or 3-month TE4 mice (Fig. 4.4a), nor in 9-month old WT and non-tau transgenic APOE KI mice (Fig. 4.5). In addition, GFAP signal was drastically elevated in 9-month old TE4 mice, but not in TEKO mice (Fig. 4.4b, c, e). The amount of GFAP signal highly correlated with brain volume (Fig. 4.4d), suggesting a detrimental role of reactive astrocytes in neurodegeneration.

4.3.2 APOE regulates glia-mediated neuronal loss in vitro

Despite the observation that TE4 mice bear significantly higher levels of innate immune activation whereas TEKO mice show drastically reduced neuroinflammation correlates well with the degree of neurodegeneration seen in these mice, this correlation does not prove that the different degrees of neurodegeneration are directly caused by APOE-dependent glial activation.

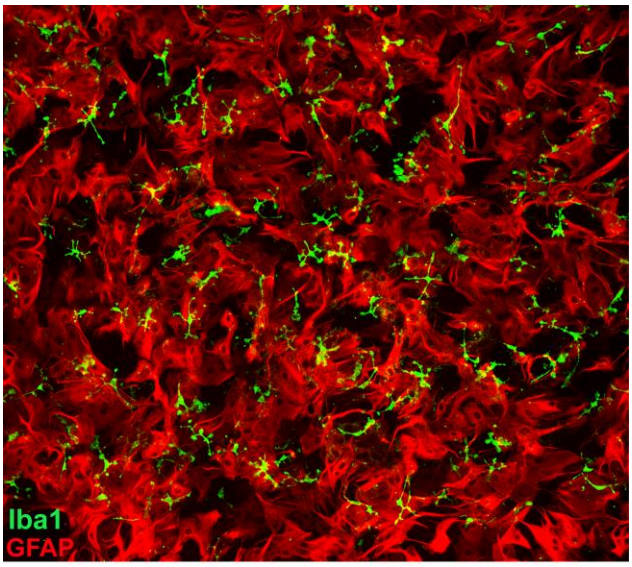

Figure 4.6 | Mixed glia culture. Primary mixed glia culture isolated from human APOE KI or APOE KO mice consisting of 80-90% astrocytes (GFAP+, red) and 10-20% microglia (Iba1+, green).

To further investigate this possibility, I performed in vitro cell culture experiments. I co-cultured P301S tau-expressing neurons with mixed glia cells consisting of 80-90% astrocytes and 10-20% microglia (Fig. 4.6) derived from APOE KI or APOE KO mice, where the neurons were plated directly on top of the glia so that the two cell populations are in direct contact with each other. Remarkably, the detrimental effect of APOE4 on neurodegeneration and the protective effect associated with the absence of APOE were robustly recapitulated in the co-culture system. Drastic neuronal loss occurred in the neuron/E4 glia co-culture, whereas neurons co-cultured with EKO glia showed the least injury (Fig. 4.7a, b). Concomitantly, a significantly higher level of TNFα was detected in the neuron/E4 co-culture medium whereas minimal TNFα levels were detected in the neuron/EKO co-culture medium (Fig. 4.7c), indicating activated glia likely played an important role in inducing neuronal death. Interestingly, when P301S tau-expressing neurons are cultured in the absence of glia cells and treated with recombinant APOE, a mild reduction of neuronal cell number and neurite arborization is observed in APOE-treated neurons, particularly in APOE4-treated neurons (Fig. 4.7d, e). But the degrees of neuronal damage and neuronal loss were much milder compared with the neuron-glia co-cultures, suggesting glia-mediated neuroinflammation indeed plays an essential role in exacerbating neurodegeneration. The observation that APOE itself is sufficient to induce mild neuronal

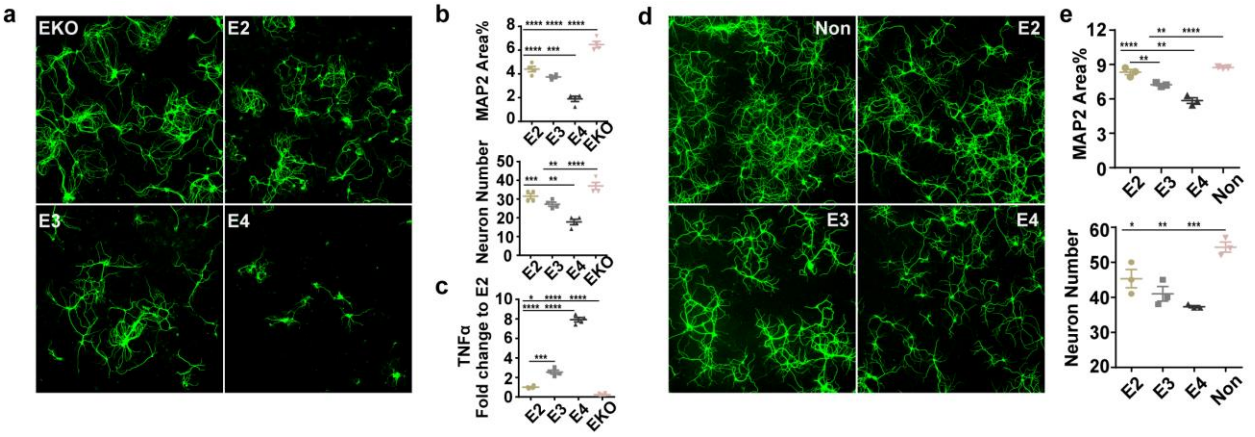

Figure 4.7 | APOE regulates neuronal viability in vitro largely through glia. a, Representative images of primary WT neurons infected with AAV2/8-synapsin-P301S human tau co-cultured with mixed glia cells (80-90% astrocyte, 10-20% microglia) derived from human APOE KI (E2, E3, E4) and APOE KO mouse brain for 3 weeks. Experiment replicated 5 times **b,** Quantification of neuron number and area covered by MAP2 immunostaining signals for co-cultured neurons (4 wells/genotype, 8 random images taken/well). **c,** TNFα levels in the co-culture medium measured by ELISA. **d,** Representative images of primary WT neurons infected with AAV2/8-synapsin-P301S human tau treated with 10μg/ml recombinant human APOE for 3 weeks. Experiment replicated twice. **e,** Quantification of neuron number and area covered by MAP2 staining for APOE-treated neurons (3 wells/treatment, 8 random images/well). Data expressed as mean ± SEM, One-way ANOVA with Tukey's post hoc test, two-sided. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

damage that is potentiated by APOE4, indicates that APOE may be directly involved in inducing neurotoxicity in P301S tau-expressing susceptible neurons. Interestingly, APOE has been shown to accumulate in damaged neurons of Pick's Disease[96] and in neurofibrillary tangle-bearing neurons of AD[97], suggesting a potential role of APOE in mediating neurotoxicity under pathological conditions, which is an interesting aspect to be further explored in future studies.

4.3.3 The effect of APOE on glia-mediated neuronal loss requires direct contact between neurons and glia

While APOE potently impacts neuronal viability by modulating glial functions, how it does it is still not clear. It is likely that APOE, particularly APOE4, enhances microglial activation by P301S tau-injured neurons or by the inherently stressful cell culture environment in vitro,

resulting in generation of higher levels of neurotoxic substances such as ROS and NO that could damage or kill neurons. Activated microglia may further activate the A1-type astrocytes, which potentiate the neuronal loss by secreting neurotoxic factors. To assess whether the toxicity comes from secreted factors produced by glial cells, I utilized a different neuron-glia co-culture approach called the 'Banker' method, where the P301S tau-expressing neurons were spatially separated from glia in the same culture dish (Fig. 4.8a). Surprisingly, under this condition, the effect of APOE on neuronal viability was completely abolished. No apparent neuronal death was observed in any of the co-culture groups (Fig. 4.8b). A similar result was obtained when the P301S tau-expressing neurons were cultured in glia-conditioned medium (E2, E3, E4, EKO) in

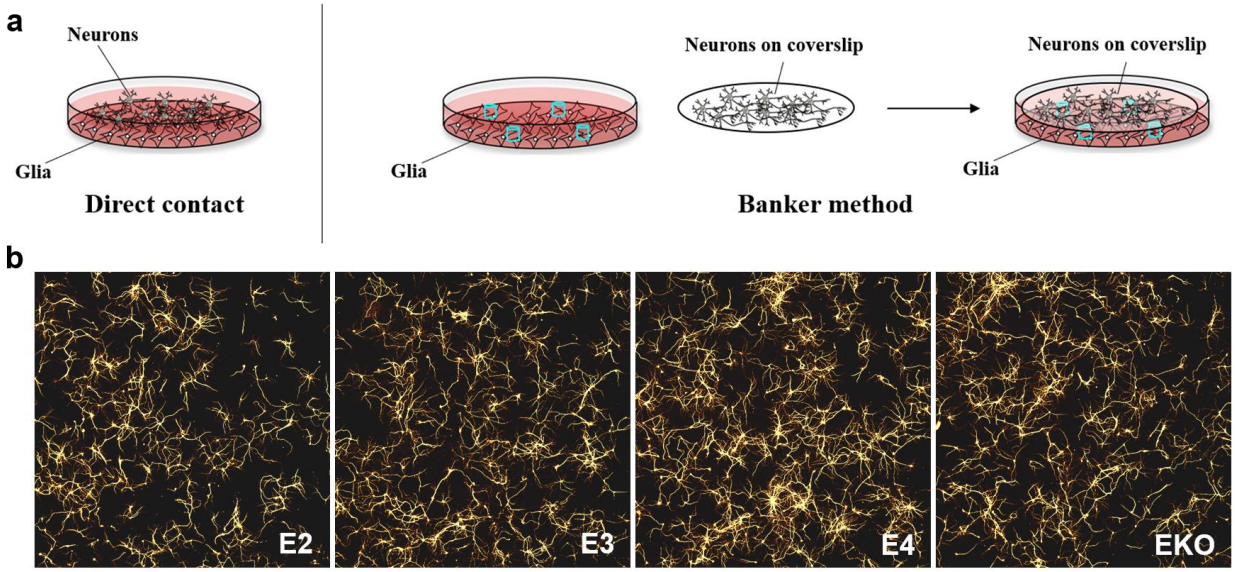

Figure 4.8 | Effects of APOE on glia-mediated neuronal loss require direct neuron-glia contact. a, Experimental schemes of different co-culture approaches. Left: Direct contact method. Primary neurons are plated directly on top of glia so that neurons are in direct contact with glial cells. Right: Banker method. Glia are plated into a dish where four wax dots were fixed to the bottom of the dish ahead of time. At the same time, primary neurons are cultured on a separate coverslip. Upon attaching to the coverslip, neurons are transferred to the glia dish with the coverslip sitting on top of the wax dots. In this scenario, neurons are immersed in the conditioned medium of glia and are exposed to all the secreting factors produced by glia, but are not in direct contact with glial cells. **b,** Representative images of primary WT neurons infected with AAV2/8-synapsin-P301S human tau co-cultured with mixed glia cells (80-90% astrocyte, 10-20% microglia) derived from human APOE KI (E2, E3, E4) and APOE KO mouse brain for 3 weeks using the Banker method.

the absence of glial cells (data not shown), indicating that the effect of APOE on glia-mediated neuronal loss requires direct contact between neurons and glial cells.

4.3.4 Hypothetic APOE function in glia-mediated neuronal loss

How does APOE modulate glial function through direct glia-neuron contact that ultimately impacts neuron viability? One essential immune function of glial cells leading to neuronal death that involves direct neuron-glia contact is phagoptosis, an immune process mediated by microglia targeting stressed-but-viable neurons that expose 'eat-me' signals on cell surfaces for phagocytosis, the process of which kills neurons. Given a protective effect in the absence of APOE, I propose a hypothesis that APOE may serve as an opsonin coating the surface of injured or dead neurons to promote neuronal phagoptosis by microglia. Neurons express a large number of APOE receptors, such as LRP1, HSPGs, and LDLR on cell membranes. Under normal conditions, APOE lipoproteins bind to their receptors and deliver cholesterols and lipids to neurons via endocytosis. In pathological conditions, such as pathological tau accumulation within neurons, neurons may be injured or killed. These jeopardized neurons may have impaired cell uptake function, which leads to accumulation of APOE on cell surfaces. Surface-accumulated APOE may recruit microglia by binding to the triggering receptor expressed on myeloid cells 2 (TREM2) expressed by microglia, which is the second strongest AD risk factor that has recently been demonstrated to be an APOE receptor[98-101]. These recruited microglia may subsequently phagocytose and kill stressed neurons by recognizing the 'eat-me' signals such as phosphatidylserine exhibited by these neurons, which exacerbates neuronal loss. Based on this hypothesis, microglia will phagocytose more neurons in the presence of APOE and APOE4 may serve as a more efficient opsonin compared to APOE2 and APOE3.

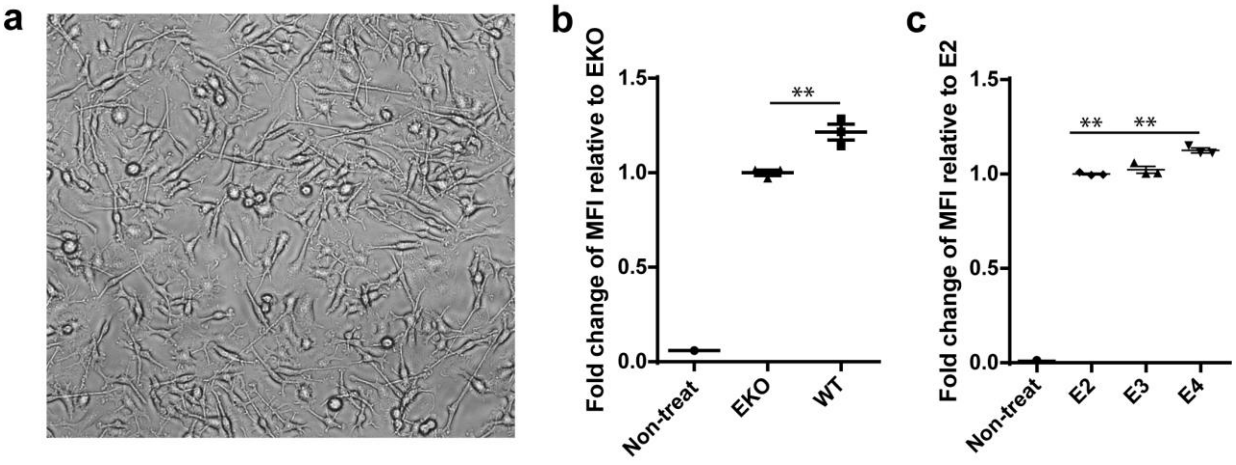

Figure 4.9 | APOE regulates microglial phagocytosis of injured neurons. a, A representative image of primary microglial culture. **b,** Primary microglia isolated from WT and APOE KO mice were cultured in serum-free medium and incubated with Dil-labeled injured neurons for 3h (3 wells/genotype) The amount of phagocytosed neurons in microglia was quantified through flow cytometry. **c,** Primary microglia isolated from human APOE KI mice (E2, E3, E4) were incubated with Dil-labeled injured neurons for 12h (3 wells/genotype). The amount of phagocytosed neurons in microglia was quantified through flow cytometry. Data expressed as mean ± SEM, One-way ANOVA with Tukey's post hoc test, two-sided. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

To test this hypothesis, I performed a preliminary experiment where primary microglia (Fig. 4.9a) isolated from WT and APOE KO mice were cultured in serum-free medium and incubated with injured neurons that were labeled with a fluorescent dye - 'Dil'. Dil is highly lipophilic and can readily incorporates into membranes. The incorporation turns it from being weakly fluorescent to a strongly fluorescent dye. After a three-hour incubation, the medium containing apoptotic neurons were removed and microglia were washed and collected with trypsinization. The amount of fluorescent signals within microglia was then quantified through flow cytometry to indicate the level of phagocytosed neurons. Interestingly, WT microglia exhibit a higher fluorescent signal compared to APOE KO microglia (Fig. 4.9b), indicating WT microglia phagocytose more neurons. In a separate experiment utilizing the same approach to analyze the APOE isoform effect, where primary microglia isolated from human APOE KI mice (E2, E3, E4) were incubated with Dil-labeled injured neurons for 12h, the E4 microglia

displayed a higher intracellular fluorescent signal compared to E2 and E3 microglia (Fig. 4.9c), indicating APOE4 enhances neuronal phagocytosis by microglia. While these are very preliminary data that need to be further verified with replicating experiments and optimized assay conditions, they support the hypothesis that APOE serves as an opsonin in mediating microglial immune function.

Intriguingly, evidence from previous literature also supports the APOE opsonin hypothesis. One study showed that APOE binding to cell surface was significantly enhanced in apoptotic *N2a* cells, a murine neuroblastoma cell line, compared to healthy cells, and cultured wildtype microglia phagocytosed significantly more apoptotic *N2a* cells with APOE added in the medium[98]. In contrast, TREM2$^{-/-}$ microglia showed impaired phagocytosis of apoptotic *N2a* cells[98], supporting an APOE-TREM2 axis in mediating microglial phagocytotic function.

4.3.5 Other immunomodulatory functions of APOE in neurodegeneration

The protective effect of APOE deficiency in neurodegenerative conditions is not restricted to tauopathy, but also applies to other neurodegenerative or neuroinflammatory settings[102-104]. In a mouse model of Parkinson's disease featuring synucleinopathy, APOE deletion significantly attenuates the loss of spinal cord motor neurons, delays disease onset, and improves survival[103]. In mouse models of experimental autoimmune encephalomyelitis (EAE), a neuroinflammatory disease characterized by infiltration of peripheral immune cells into the CNS that attack myelin and cause demyelination and neurodegeneration, APOE ablation attenuates inflammation and significantly reduces disease severity[102]. These evidence suggests a prevalent function of APOE in neurodegenerative conditions based on a potential common mechanism in addition to its specific effects on individual pathologies. As neuroinflammation is a common feature in

neurodegenerative conditions, this shared mechanism is likely linked to APOE's immunomodulatory function. The hypothesis that APOE opsonizes damaged/dead neurons for microglial phagoptosis may be a common principle to exacerbate neurodegeneration in neurodegenerative conditions. On the other hand, recent progresses on microglial research indicates another possibility.

Initial characterization of microglial cell polarization into pro-inflammatory 'M1' and reparative 'M2' phenotypes was based on the M1/M2 macrophage classification[105]. Macrophages acquire an M1 phenotype in response to interferon γ (IFNγ) and synthesize toxic substances, such ROS, NO, and lysosomal enzymes to kill microbes. By contrast, M2 macrophages are induced by interleukin-4 (IL-4) and express enzymes for collagen synthesis and fibrosis to promote tissue repair. Likewise, activated microglia are divided into an M1 phenotype, characterized by upregulation of inflammatory genes such as *TNF* and *IL1β,* and an M2 phenotype, characterized by the expression of pro-repair genes such as arginase 1 (*Arg1*) and chitinase-like 3 (*Chi3l3*, also known as *Ym1*)[106]. However, this nomenclature is now deemed inaccurate as accumulating evidence has revealed a broad diversity of macrophage activation statuses and shown that macrophages show remarkable plasticity to change phenotypes in response to environmental cues[107,108].

In neurodegenerative diseases, the precise gene signatures of microglia that mediate pathology-and neurodegeneration-associated sterile inflammation have not been clearly elucidated. Recent single-cell RNA-seq data identified a two-stage microglial cell activation process that occurs during disease progression in an Aβ-depositing mouse model, where the transition from the first to the second stage is determined by TREM2[109]. The end-stage activated microglia appear to display a conserved core-expression profile harbouring certain degrees of

diversity across individual disease phenotypes[104,109]. The core expression profile is likely induced by neuronal cell death or damage, whereas distinct pathologies may contribute to the diversity. The common disease-associated microglial cell signature was defined by different groups as the 'microglial neurodegenerative' (MGnD) phenotype[104] or the 'disease-associated microglia' (DAM) phenotype[109]. This signature is characterized by a suppression of microglial homeostatic genes, and highlighted by an induction of pro-inflammatory genes as well as genes involved in microglial phagocytotic function targeting degenerated/injured neurons and amyloid plaques[104,109]. The MGnD phenotype can largely be recapitulated by injecting apoptotic neurons into mouse brain parenchyma, where the subset of microglia that are activated to phagocytose apoptotic neurons show a similar molecular phenotype to the MGnD phenotype[104]. The MGnD microglia can also be activated via intracerebral administration of kainic acid that induces neuronal cell death[104], supporting the idea that neuronal death and damage determines the central microglial cell gene signature in neurodegenerative diseases. Importantly, although the microglia that phagocytose apoptotic neurons share many molecular signatures with lipopolysaccharide (LPS) and IFNγ-stimulated M1 microglia, they also exhibit significant differences, such as contrasting gene expression patterns of secreted phosphoprotein 1(*Ssp1*), CC-chemokine ligand 3 (*Ccl3*), and early growth response 1(*Egr1*). But the most distinguishing difference is that the expression of *Apoe* — the hallmark gene of the MGnD phenotype that has been found to be upregulated in microglia in all currently-assessed neurodegenerative diseases — is suppressed in M1 microglia. In addition, certain classical M2 microglial cell markers, such as *Arg1* and *Ym1*, are also induced in microglia that phagocytose apoptotic neurons, indicating a unique microglial activation profile in neurodegenerative diseases that is distinct from the M1/M2 classification[104] (Table 4.1).

Crucially, APOE deletion is found to prevent full acquisition of the microglial neurodegenerative phenotype with suppression of key MGnD genes and restoration of homeostatic microglial genes[104]. This may prohibit disease-associated microglia from being fully developed to carry out their pro-inflammatory and phagocytotic functions, and hinder their capacity to kill and damage neurons via phagoptosis or inflammatory mechanisms, which may underlie the protective effect of APOE deficiency in various neurodegenerative settings. Supporting this view, in a facial nerve axotomy model, global APOE deletion or conditional ablation of APOE in microglia reduces neuronal loss in the facial motor nucleus after axotomy of the facial nerve[104]. However, how APOE deficiency dampens MGnD microglial activation is still not clear and is an intriguing direction for future studies.

	M1 microglia	DAM/MGnD microglia	M2 microglia	
Stimulus	IFNγ, TNFα/LPS	Disease-associated pathologies, neuronal death / damage	IL-4, IL-13 (Alternative activation)	TGFβ, IL-10 (Acquired deactivation)
Marker genes	*IL1b, TNF, IL-6, IL-23, iNOS, ROS-producing genes, MHC-II?**	*Apoe, Trem2, Clec7a, Lpl, Itgax, Axl, Csf1, Lilrb4, Spp1, Ccl2, Cst7*	*Arg1, Ym1, MRC1, FIZZ1 Clec7a*	*IL-10, TGFβ*
Functions	Oxidative stress; Kill microbes or host cells; Phagocytosis; Antigen presentation?*	Phagocytose dead/injured neurons; Trim Aβ plaques; Inflammasome/ASC formation, promote plaque seeding; Inflammatory stress/damage	Extracellular matrix production/remodeling for tissue repair/wound healing; Phagocytosis; Immunosuppression	
Genes regulated in the same direction	*IL1b↑, Cybb↑, Lilrb4↑, Ccl2↑, Axl↑, Itgax↑, Tspo↑, P2ry12↓, Tmem119↓ Olfml3↓, Tgfbr1↓, Csf1r↓, Sall1↓, Hexb↓, Rhob↓ …*		*Arg1↑, Ym1↑, Clec7a↑…*	
Genes regulated in opposite directions	*Apoe, Trem2, Erg1, Spp1…*		Many genes…	

Table 4.1 | Comparison of different microglial cell phenotypes. * Inconclusive

4.3.6 The APOE-TREM2 axis in modulating microglial immune function: A hypothetic model

TREM2 is another risk gene for late-onset Alzheimer's disease and is second only to *APOE* in terms of the magnitude of its effects. The most common *TREM2* variant R47H increases Alzheimer's disease risk by 2- to 3-fold[110,111]. TREM2 is a cell surface receptor of the immunoglobulin superfamily that is expressed by tissue macrophages, such as bone osteoclasts, CNS microglia, alveolar macrophages, peritoneal macrophages and intestinal macrophages[112]. Upon activation, TREM2 signals through an adaptor protein DNAX-activation protein 12 (DAP12) to promotes cell anabolic metabolism[113], survival[113,114], proliferation[115] and phagocytosis[115,116].

In the CNS, TREM2 critically regulates microglial functions in disease. Similar to APOE that determines MGnD microglial activation, TREM2 is also required for acquisition of the neurodegenerative microglial phenotype. TREM2 deletion in APPPS1-21 or superoxide dismutase 1 (SOD1) mice results in APOE downregulation and a similar suppression of core inflammatory MGnD genes and restoration of homeostatic genes[104]. Targeting TREM2 signaling in the facial nerve axotomy model ameliorates neuronal loss[104], and ablation of TREM2 in tauopathy mice attenuates neuroinflammation and neurodegeneration[117]. These phenotypes closely resembling the effects of APOE deletion suggest that APOE likely shares the same pathways as TREM2 in regulating microglial function in neurodegeneration. APOE may activate the neurodegenerative microglial phenotype through interaction with TREM2 that triggers TREM2 signaling in microglia, it may also serve as an opsonin coating cell surfaces of damaged/dead neurons that recruit microglia for neuronal phagoptosis by binding to TREM2. The working model for the immunomodulatory function of APOE in regulating neurodegeneration is summarized in Fig. 4.10. While this model features tauopathy, the principles may also apply to other neurodegenerative diseases.

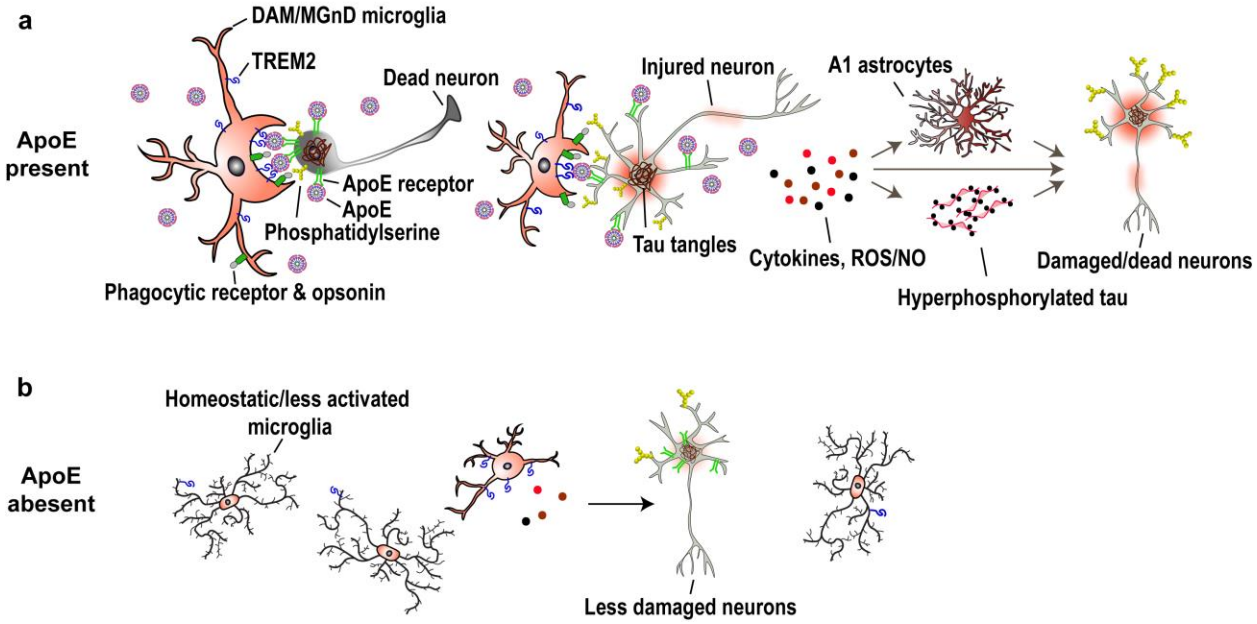

Figure 4.10 | A working model for the immunomodulatory function of APOE in regulating neurodegeneration in tauopathy. a, In Alzheimer's disease or tauopathy, intracellular accumulation of pathological tau in neurons causes neuronal injury or neuronal death. These jeopardized neurons may have impaired cell uptake function, which results in accumulation of APOE on neuronal cell surfaces. Surface-coated APOE may serve as an opsonin to recruit microglia for neuronal phagoptosis by binding to TREM2 expressed by microglia, thus exacerbating neurodegeneration. In addition, the presence of APOE enables full activation of neurodegenerative microglia (DAM/MGnD) that harbor a proinflammatory profile in addition to a high phagocytotic activity. These activated microglia produce inflammatory cytokines that induce A1 astrocytes, which kill/damage neurons via secreting neurotoxic factors and loss of neurotrophic functions. The large amounts of ROS/NO generated during microglia activation can also induce oxidative stresses that directly injure/kill neurons. Furthermore, the inflammatory milieu can also induce tau hyperphosphorylation by activating tau kinases through IL-1 signaling, thus exacerbating tau pathology that further aggregate neurodegeneration. **b,** In the absence of APOE, microglia largely stay in a more homeostatic status, generating less neurotoxic substances, which results in less neuron damage or death. In addition, loss of APOE as a potential opsonin for microglia-mediated neuronal phagoptosis may also contribute to preservation of intact neurons.

4.3.7 Effect of microglial depletion on neurodegeneration in the tauopathy mouse model

To provide concrete evidence for microglial function in neurodegeneration and in mediating

APOE's effects on neurodegeneration, I decided to deplete microglia in TE4 and TEKO mice to

assess how microglial deficiency affects neurodegeneration in the presence and absence of APOE. Current microglial depletion techniques are based on either genetic manipulation or pharmacological treatment. A common genetic approach utilizes transgenic mice that express herpes simplex virus thymidine kinase (*HSVTK*) under the CD11b promoter[118]. *HSVTK* encodes a suicide protein that phosphorylate ganciclovir (GCV), a guanosine analog, to a monophosphorylated form, which is further converted by host kinases to toxic GCV-triphosphate that induces premature DNA chain termination and cell apoptosis. Therefore, GCV administration in CD11b-HSVTK transgenic mice results in depletion of cells of the myeloid origin, including microglia. However, this approach has multiple disadvantages. First, systemic GCV treatment results in low efficiency of microglia depletion and induces lethal myelotoxicity that need to be resolved by bone marrow transplantation[119]; second, direct intracerebral delivery of GCV achieves higher efficiency of microglial depletion, but the surgery can cause brain damage and the treatment cannot go longer than 4 weeks due to lethal microhemorrhages induced by GCV[119]; third, GCV treatment damages the blood brain barrier[120] which may generate experimental artifacts. In contrast, pharmacological treatment through oral delivery of drugs that block the colony-stimulating factor 1 receptor (CSF1R) signaling constitutes a noninvasive and a highly effective approach to deplete microglia. CSF1R is expressed primarily by macrophages, microglia, and osteoclasts, and its signaling is critical for microglial proliferation, differentiation, and survival. PLX3397, a small molecule that potently and selectively inhibit CSF1R and c-Kit, was reported to be able to deplete 90% microglia within 7 days when administered as a supplement in mouse chow[121]. Importantly, long-term treatment of PLX3397 does not induce notable toxicity in mice[122]. Therefore, I utilized this approach and treated TE4 and TEKO mice as well as their age-matched non-tau transgenic counterparts with

PLX3397-supplemented chow from 186 days to 285 days, a critical time window for the development of neurodegeneration in this mouse model.

Preliminary characterization showed that PLX3397 treatment via mouse chow potently depleted microglia in the mouse brain. Seven-day treatment resulted in ~90% reduction of microglia whereas by twenty-one days, microglia were almost completely eliminated (Fig. 4.11). A bonus finding on top of this study is that the ingredients of the base chow strongly affect the

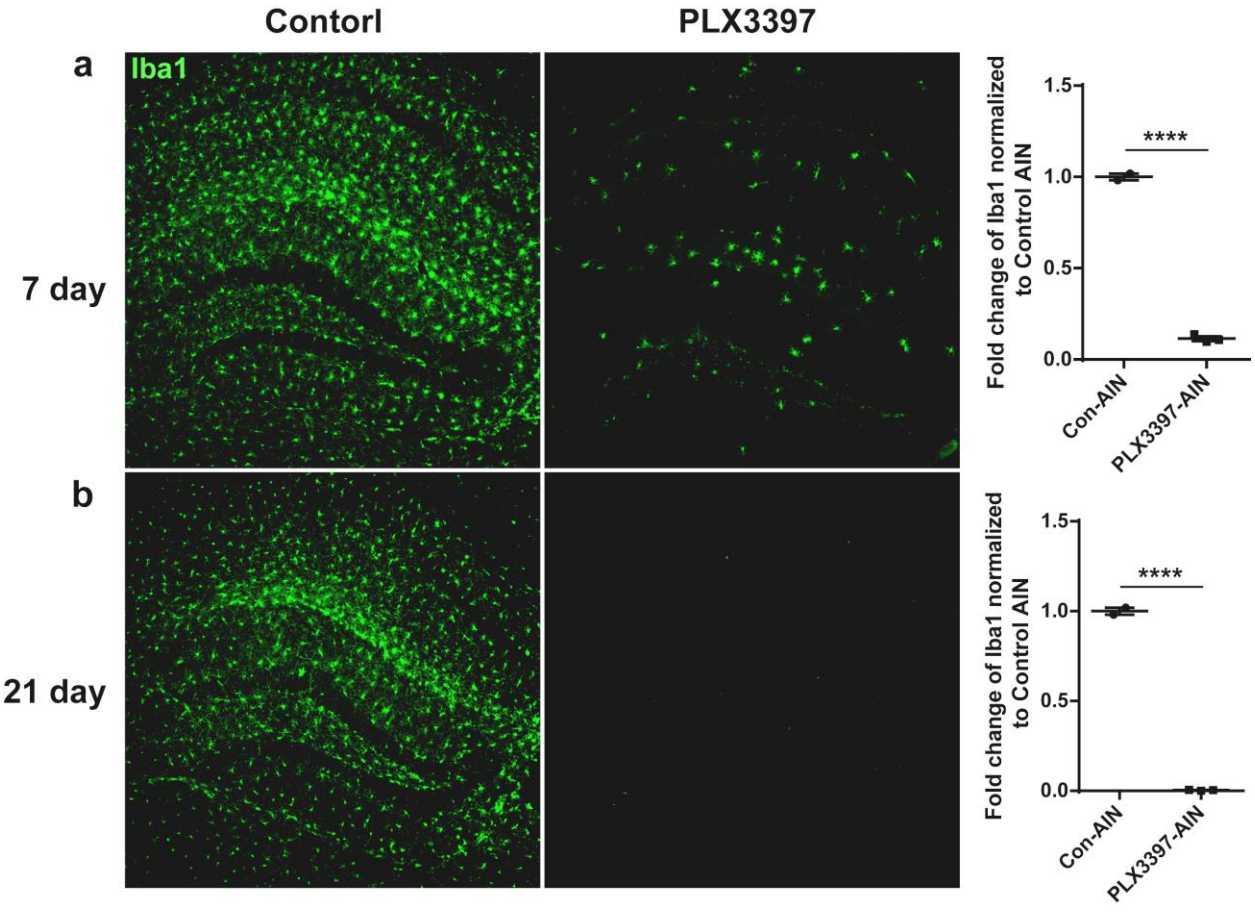

Figure 4.11 | PLX3397 treatment eliminates microglia in the brain. a, b, Representative images and quantification of Iba1-labeled microglia at 7 days after treatment with PLX3397-supplemented AIN chow (400mg drug/kg chow) in 4.5-month APOE4 KI male mice. n=2-3 mice per group. **b,** Representative images and quantification of Iba1-labeled microglia at 21 days after treatment with PLX3397-supplemented AIN chow (400mg drug/kg chow) in 4.5-month male APOE4 KI mice. n=2-3 mice per group Quantification of area covered by Iba1 signal. Data expressed as mean ± SEM, One-way ANOVA with Tukey's post hoc test (two-sided). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

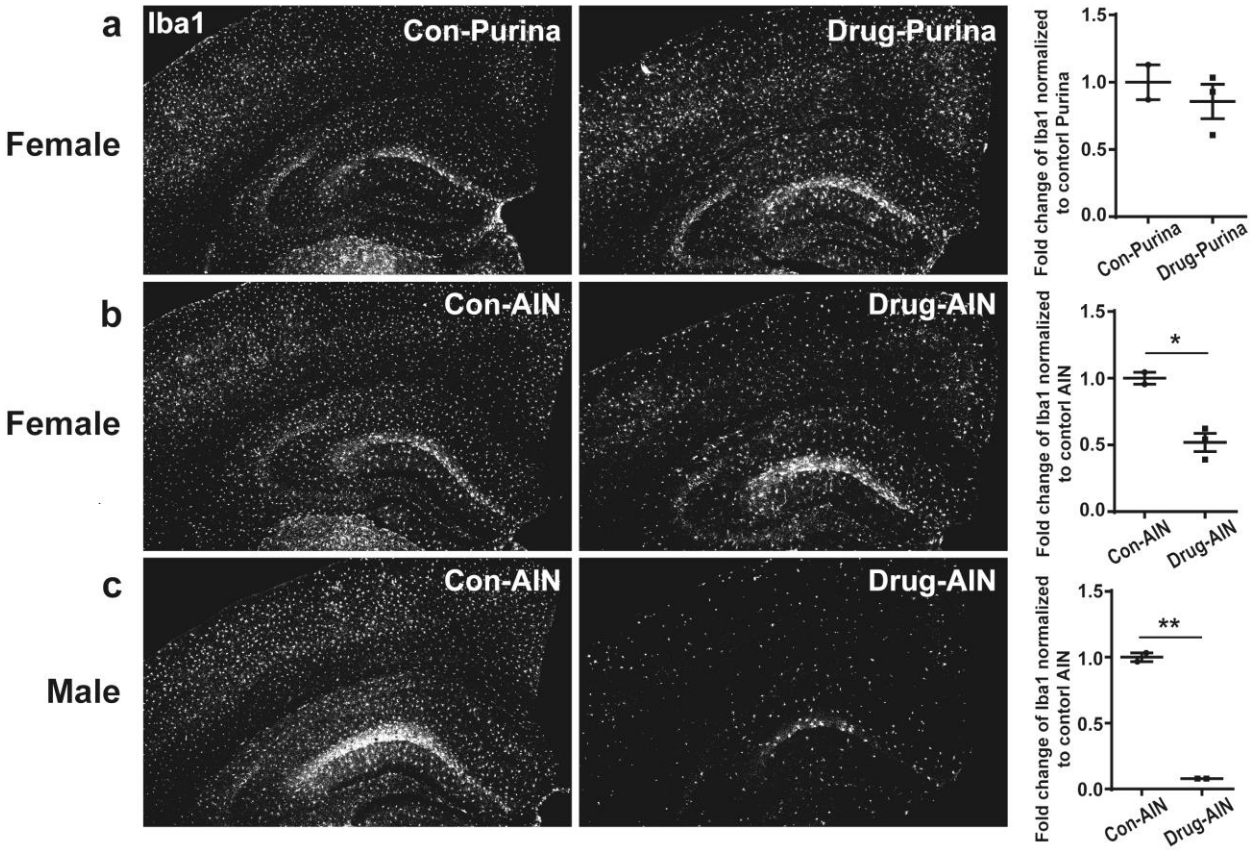

Figure 4.12 | Both chow ingredients and mouse genders affect PLX3397-mediated microglial depletion. a, b, Three to four months old female TE4 mice were treated with either PLX3397-AIN chow (300mg drug/kg chow) or PLX3397-Purina chow (300mg drug/kg chow) together with their respective control chows for 7 days (n=2-3). The brains were stained with Iba1 and quantified for Iba1 covered area. **b, c,** Three to four months old female and male TE4 mice were treated with PLX3397-AIN chow and control AIN chow for 7 days (n=2-3). Microglia were quantified through Iba1 staining. Data expressed as mean ± SEM, One-way ANOVA with Tukey's post hoc test (two-sided). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

drug efficiency. PLX3397 supplemented in a grain-based chow (Purina 5053) showed markedly reduced microglial-depletion efficiency compared with PLX3397 supplemented in a purified ingredient chow (AIN-76A) (Fig. 4.12a, b, Table 4.2a). This effect was in part due to a poorer capacity of the grain-based chow to assimilate the drug during chow manufacturing (Table 4.2a), leading to a slightly lower level of drug uptake by the mice compared to the AIN chow (20% difference, Table 4.2a). However, plasma PLX3397 concentration, which highly correlates with microglial depletion efficiency, was drastically reduced in mouse treated with PLX3397-Purina

chow compared to mice treated with PLX3397-AIN chow (55% difference, Table 4.2a), indicating the chow ingredients impact PLX3397 absorption or clearance. Interestingly, there also appeared to be a gender effect, with male mice accumulated twice as much PLX3397 in the plasma compared to females when treated with PLX3397-AIN chow, even though their drug uptake is similar (Table 4.2b). Correspondingly, male mice demonstrated a significantly higher level of microglial elimination than female mice (Fig. 4.12b, c, Table 4.2b). This result suggests that females may have a higher capacity to resist immune disturbances and to maintain a homeostatic immune status. This trait may underlie the protective effect observed in female P301S mice, which show delayed disease onset compared to male mice. Given this observation

a

Female mice	Purina	AIN	Purina/AIN (%)
Drug incorporation rate in diet (%)	70	90	78
Average drug uptake per day ug (drug) / g (mouse weight)	23.7	29.6	80
Average plasma drug concentration (ug/ml)	134.3	299.7	45
Microglial depletion rate (%)	14	48	30

b

AIN chow	Female	Male	Female/Male (%)
Average drug uptake per day ug (drug) / g (mouse weight)	29.6	32	93
Average plasma drug concentration (ug/ml)	299.7	580	52
Microglial depletion rate (%)	48	92	52

Table 4.2 | Effects of base chow and gender on microglial depletion parameters. a, Comparison of base chow ingredients in affecting drug incorporation rate in diet, drug uptake, plasma drug concentration and microglial depletion rate in 3-4 month old TE4 female mice treated with PLX3397-AIN chow (300mg drug/kg chow) or PLX3397-Purina chow (300mg drug/kg chow) for 7 days. **b,** Comparison of gender effects on drug uptake, plasma drug concentration and microglial depletion rate in 3-4 month old TE4 male and female mice treated with PLX3397-AIN chow (300mg drug/kg chow for 7 days.

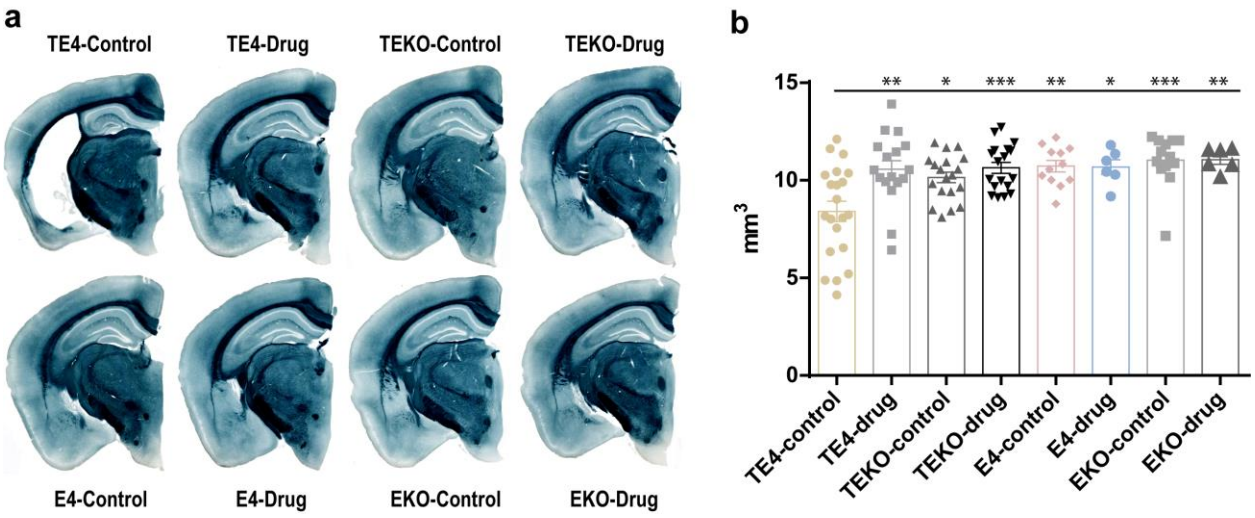

Figure 4.13 | Microglial depletion in tauopathy mice rescues neurodegeneration in an APOE-dependent manner. a, Representative brain sections of 285 days old TE4, TEKO, E4, and EKO male mice treated with control AIN chow or PLX3397-supplemented AIN chow (400mg drug/kg chow) from day 186 to day 285. **b,** Quantification of hippocampal volume for these mice (TE4-control: n=21, TE4-drug: n=17, TEKO-control: n=19, TEKO-drug: n=18, E4-control: n=12, E4-drug: n=6, EKO-control: n=13, EKO-drug: n=6). Data expressed as mean ± SEM, One-way ANOVA with Tukey's post hoc test (two-sided). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

and an early disease onset in male P301S mice, only male mice were used for this study.

Consistent with our previous observation, by 285 days, TE4 mice showed marked brain atrophy whereas TEKO mice showed much more preserved brain (Fig. 4.13). Impressively, microglial depletion in TE4 mice rescued brain atrophy to the level of a normal non-tau transgenic mouse brain (Fig. 4.13), indicating that microglia-mediated neuroinflammation plays a fundamental role in driving neurodegeneration. Intriguingly, the rescuing effect of microglial depletion on neurodegeneration occurred only in the presence of APOE (i.e., only in TE4 mice, but not in TEKO mice), and in the absence of microglia, TE4 and TEKO mice showed the same brain volume independent of APOE status (Fig. 4.13), indicating that the effect of APOE on neurodegeneration in the tauopathy mouse model primarily lies in APOE's immunomodulatory function in affecting microglia-mediated neuroinflammation. As discussed in previous sessions, APOE controls MGnD microglial activation, and may opsonize activated microglia for their

phagoptosis of injured/dead neurons, which may be key mechanisms connecting APOE to microglia-mediated neurodegeneration. Additional investigations to prove the "APOE opsonin" hypothesis and to elucidate how APOE regulates MGnD microglial activation are ongoing to further understand the mechanisms. In addition, we are also addressing important questions including the direct effect of APOE on tau pathogenesis in the absence of microglia-mediated neuroinflammation, the status and function of astrocytes in neurodegeneration independent of microglia, effects of APOE and PLX3397 on peripheral immune cells in the blood circulation that may indirectly impact neurodegeneration via affecting neuroinflammation, etc. These analyses will lead us towards a comprehensive understanding of how APOE, tau, and neuroinflammation interacts with each other to cause neurodegeneration.

4.4 Discussion

4.4.1 Differential immunomodulatory functions of APOE in infectious and neurodegenerative conditions

Neuroinflammation has been increasingly recognized to play an essential role in neurodegenerative diseases. The association of APOE4 with enhanced immunoreactivity has been consistently documented in infectious diseases or upon direct LPS stimulation[90-92,123]. How APOE4 achieves this is not well understood. Evidence shows that lipid rafts play an essential role in immune activation by serving as platforms for signaling complex assembly (Fig. 4.14); hence minor intervention on membrane lipids such as cholesterol loading or efflux is sufficient to induce membrane remodeling and subsequent signal transduction[124]. Higher retention of cholesterol in lipid rafts due to lack of the cholesterol efflux transporters ABCA1 or ABCG1, enhances TLR signaling in macrophages[124] and induces leukocytosis under hypercholesterolemia

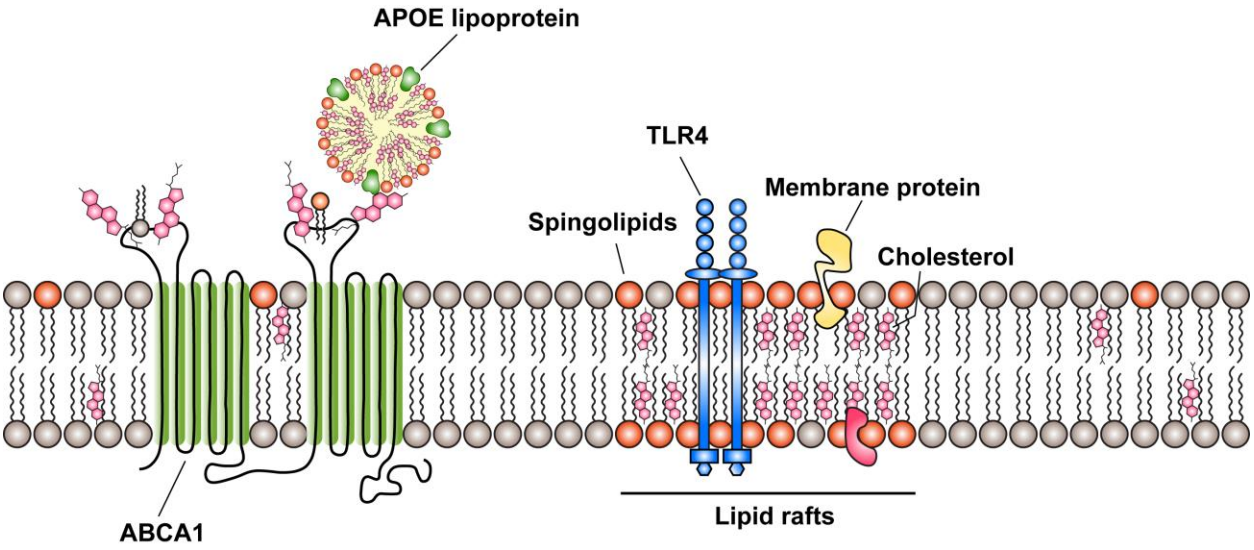

Figure 4.14 | APOE-mediated cholesterol efflux affects immune cell activation by regulating cell membrane lipid contents. Lipid rafts that contain high concentrations of cholesterol and glycosphingolipids serve as platforms for the assembly of immune signaling complexes on cell membranes, and therefore are enriched with immune receptors such as TLR4. The cholesterol content on cell membrane critically regulates lipid raft formation, which would further affect immune activation. APOE lipoprotein can induce cholesterol efflux from cell membrane via the ABCA1 transporter, therefore reducing cholesterol levels on the plasma membrane, which inhibit lipid raft formation and immune activation.

conditions[125], whereas myeloid cell surface-bound APOE promotes cholesterol efflux in a cell-autonomous fashion (Fig. 4.14) to inhibit myelopoiesis[126]. APOE4 is reported to be less effective than APOE3 in inducing cholesterol efflux from macrophages [127], which causes cholesterol accumulation on cell membranes[90,127]. This mechanism has been proposed to explain the higher immune reactivity associated with APOE4. Alternatively, other research shows that as a lipid carrier, APOE can directly bind to LPS, which also contains a lipid component, and redirect it to liver for uptake and clearance by liver parenchyma cells, hence reducing the amount of circulating LPS and attenuates LPS-induced immune responses[128]. Given that domain interaction in APOE4 affects the lipid binding function of APOE, it is likely that APOE4 has impaired LPS-binding and neutralizing capacity compared with other APOE isoforms, resulting in a higher level of immune responses. Regardless of the mechanism, multiple evidence supports an

immune-suppressing role of APOE in LPS stimulation or bacterial infection. For example, APOE-deficient mice are shown to behave similarly to APOE4-targeted replacement (APOE4-TR) mice in response to intracerebroventricular LPS stimulation with enhanced gliosis and cytokine production compared with APOE2-TR and APOE3-TR mice[92]. APOE-deficient mice are also more susceptible to infection- and LPS-induced lethality with amplified TNF production in the serum[129-131].

Contrary to LPS stimulation where APOE deletion amplifies the immune response, under neurodegenerative or neuroinflammatory conditions, APOE deficiency attenuates inflammation[18,23,102,104] and disease severity[18,102,103]. As mentioned in early sections, APOE ablation protects against neurodegeneration in mouse models of tauopathy, Parkinson's disease, EAE, and facial nerve axotomy, indicating multidomain immunomodulatory functions of APOE in regulating infectious diseases and neurodegenerative diseases. These functional differences are likely caused by different mechanisms. On the one hand, APOE may reduce the excitability of immune cells towards LPS stimulation by modulating lipid raft content on cell membranes, or through direct APOE-LPS interactions to neutralize LPS toxicity; on the other hand, under neurodegenerative conditions, APOE is required for intact functionality of MGnD microglia. APOE deletion prevents full acquisition of the microglial neurodegenerative phenotype with suppression of key MGnD genes[104], and therefore may prohibit disease-associated microglia from being fully developed to carry out their pro-inflammatory and phagocytotic functions. In addition, APOE may serve as an opsonin to promote neuronal phagoptosis by MGnD microglia through interaction with TREM2, therefore exacerbating neurodegeneration.

Our observation that microglia depletion in tauopathy mice almost fully restored brain integrity provides concrete evidence for a dominating role of microglia-mediated

neuroinflammation in inducing neurodegeneration. In the absence of microglia, the effect of APOE on neurodegeneration is gone, indicating that APOE regulates neurodegeneration primarily by modulating microglial functions. Mechanistically, how APOE triggers microglia to depolarize into the neurodegenerative phenotype distinct from conventional M1/M2 microglia is a central question to be solved. Opsonization may be another major mechanism through which APOE regulates microglia-mediated neurodegeneration, but needs to be clearly demonstrated.

4.4.2 The APOE-TREM2 axis in modulating Aβ pathology in addition to its role in neurodegeneration

The APOE-TREM2 axis that is essential for regulating neurodegeneration also appears be involved in the regulation of Aβ pathogenesis. Microglia have long been observed to cluster around Aβ plaques in postmortem AD brains and in Aβ-depositing mouse models. Unlike non-plaque-associated microglia that retain a more homeostatic phenotype[109,132], plaque-associated microglia show major alterations in their molecular signatures and constitute the population of 'disease-associated microglia' in the setting of Aβ-deposition[109]. TREM2 expression is specifically upregulated in these plaque-associated microglia during plaque deposition, and was essential for promoting microglial cell association with plaques[133-138]. TREM2 haplodeficiency, ablation, or the *TREM2*^{R47H} mutation leads to a significant reduction of plaque-associated microglia compared to their TREM2-sufficient counterparts[133-138]. This is likely in part due to TREM2 being as an Aβ receptor that facilitate microglial association with plaques[139]. Corresponding to a loss of microglial enclosure around plaques, TREM2-deficient Aβ-depositing mice develop significantly enlarged and less compact plaques containing longer and diffuse star-shaped amyloid fibrils that project from loosely packed cores[137,138] (Fig. 4.15a). Such morphological changes in plaques are frequently associated with a greater degree of neuritic

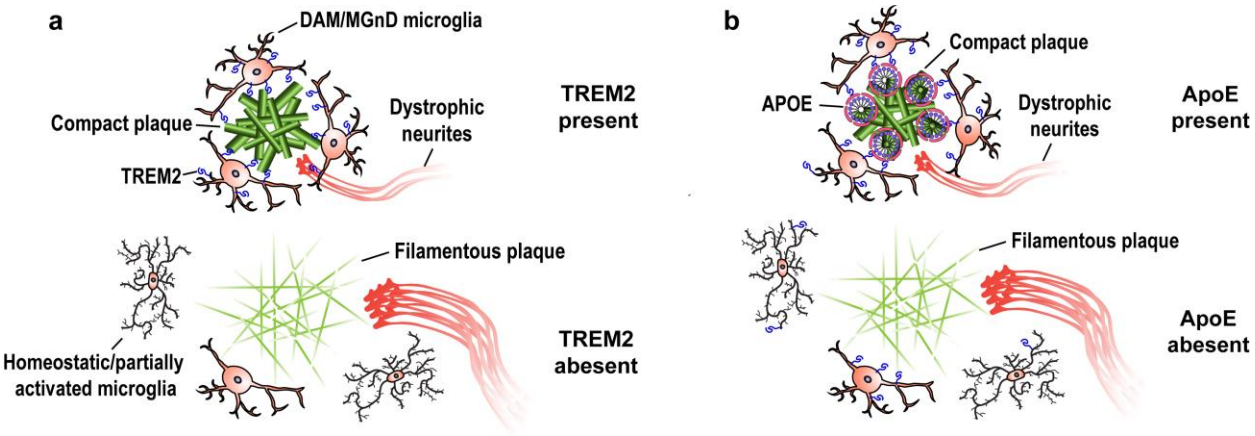

Figure 4.15 | Deficiency of APOE or TREM2 leads to similar amyloid plaque phenotypes. a, During Aβ plaque deposition, the presence of TREM2 enables activation of the DAM/MGnD microglia and allows them to associate with amyloid plaques and trim Aβ fibrils on the edge to compact the plaque. Plaque-associated microglia also serve as a barrier between the plaque and surrounding tissues to reduce Aβ-induced toxicity to neurites. TREM2 deficiency hinders full acquisition of the DAM/MGnD microglial phenotype and significantly reduces the number of plaque-associated microglia. This results in enlarged amyloid-β plaques with wispy fibre-like structures projecting from loosely packed cores and greater neuritic dystrophy in close vicinity. **b,** Microglial clustering around Aβ plaques for plaque trimming and insulation is also strictly regulated by APOE. The presence of APOE activates DAM/MGnD microglia and promotes microglial association with plaques to perform plaque-trimming and -compacting functions. In addition, APOE is abundantly present in amyloid plaques and may opsonize plaques for microglial phagocytosis through APOE-TREM2 interactions. The absence of APOE prevents DAM/MGnD microglial activation and loses its opsonization function, leading to reduction of plaque-associated microglia and formation of diffuse plaques accompanied by enhanced neuritic dystrophy, similar to the phenotype of TREM2 deficiency.

dystrophy in close vicinity[137,138] (Fig. 4.15a), indicating that plaque-associated microglia perform plaque trimming and compacting functions that help to restrict plaque growth, and that these cells serve as a barrier to limit plaque-induced neuronal toxicity. Indeed, plaque-associated microglia have been shown to contain plaque-derived materials[109,138], indicating their capability to phagocytose plaque components. Importantly, APOE ablation in $APP_{Swe}PSEN1_{dE9}$ or APPPS1-21 mice also impairs microglial association with plaques and leads to enlarged plaques exhibiting wispy fibre-like structures projecting from loosely packed cores that are accompanied by significantly elevated neuritic dystrophy in the vicinity[140], which recapitulates the phenotype observed in TREM2-deficient Aβ-depositing mice[137,138] (Fig. 4.15). The shared phenotypes

between APOE deficiency and TREM2 deficiency on Aβ pathology in addition to their common effects on neurodegeneration strongly suggest an APOE–TREM2 axis linking APOE to microglial function. APOE may function upstream of TREM2 to activate the MGnD microglia that possess a high phagocytotic capacity targeting either damaged/dead neurons or plaques, likely by binding to TREM2 and inducing TREM2 signaling. In addition, APOE may serve as an opsonin by binding to TREM2 to enhance microglial phagocytosis of amyloid plaques in addition to targeting injured/dead neurons for phagoptosis, as APOE is abundantly present in amyloid plaques as a significant plaque component[19,20]. This intriguing model needs to be demonstrated concretely in future studies.

Chapter 5: Targeting APOE as a therapeutic approach in treating tauopathy

5.1 Introduction

The protective effect of APOE deficiency on neurodegeneration in the setting of tauopathy leads to the possibility of targeting APOE as a therapeutic approach for treating tauopathy. While multiple approaches can be utilized to reduce APOE levels at either transcriptional or translational levels, one convenient way to target APOE with high efficiency is to manipulate APOE receptors to enhance APOE uptake and degradation. APOE binds primarily to a group of receptors known as the low density lipoprotein receptor (LDLR) family, which contains over 10 receptors including LDLR, LRP1, apolipoprotein receptor 2 (APOER2), very low density lipoprotein receptor (VLDLR), and others[4]. All these receptors contain a single transmembrane domain with a short cytoplasmic tail. The extracellular regions of these receptors contain three characteristic modules: ligand-binding repeats (also called complement-type repeats), epidermal growth factor (EGF) repeats and YWTD-containing β-propeller domains[4]. LDLR is the smallest receptor and the prototype of this family. It together with LRP1 constitute the two major types of APOE metabolic receptors in the brain. LDLR is predominantly expressed in glia whereas LRP1 is primarily expressed in neurons and to a lesser degree in glia. Systemic ablation of *Ldlr*[128] or conditional *Lrp1* knockout in mouse forebrain neurons[141] increases APOE levels in the brain, whereas overexpression of LDLR[142] markedly reduces brain APOE levels, indicating these receptors regulate APOE metabolism and can be utilized to modulate APOE levels. The large size of LRP1 makes it difficult to be manipulated for experimental purposes. Although functional LRP1 minireceptors have been generated[143], overexpressing mLPR2 - the minireceptor that preserves the ligand binding and internalization capacity of LRP1- does not alter APOE levels[144]. Therefore, LDLR serves as an appropriate target for APOE modulation.

LDLR transgenic mice have been generated previously. These mice overexpress over five-fold of murine LDLR in both neurons and astrocytes, leading to a ten-fold reduction of soluble APOE in the brain[142]. By crossing LDLR transgenic mice to P301S mice, I generated $P301S^{+}LDLR^{+}$ and $P301S^{+}LDLR^{-}$ littermates and assessed them at 9 months of age.

5.2 Experimental procedures

Animals. LDLR transgenic mice on a B6/CBA background were generated in house previously[142]. P301S tau transgenic mice (The Jackson Laboratory, #008169) on a B6 background expressing human P301S 1N4R tau driven by PrP promoter were crossed to LDLR transgenic mice to generate $P301S^{+}LDLR^{+}$ and $P301S^{+}LDLR^{-}$ littermates. APOE knockout mice (C57BL/6) were purchased from the Jackson Laboratory (#002052) and were crossed to P301S mice to generate P301S mE/- mice, which were further crossed to APOE knockout mice or wildtype mice (C57BL/6, Charles River Laboratories, #027) to generate P301S mE/mE, P301S mE/- and P301S APOEKO mice. Only male mice were used for this study.

Volumetric analysis, immunohistochemistry, and brain extraction were performed as described in chapter 2 and chapter 3.

ELISA. Human tau, ptau and murine APOE ELISAs were performed as described previously[70]. For coating antibodies, Tau5 (gift from L. Binder, Northwestern University, Chicago, IL) was used for human tau ELISA. HJ14.5 (in house) was used for ptau ELISA and HJ6.2 (in house) was used for murine APOE ELISA. For detection antibodies, biotinylated HT7 (Thermo Fisher Scientific, MN1000B) was used for human tau ELISA. Biotinylated AT8 (Thermo Fisher Scientific, MN1020B) was used to ptau ELISA, and HJ6.8B (in house) was used for murine APOE ELISA.

5.3 Results

5.3.1 LDLR overexpression in P301S mice attenuates neurodegeneration and tau pathology

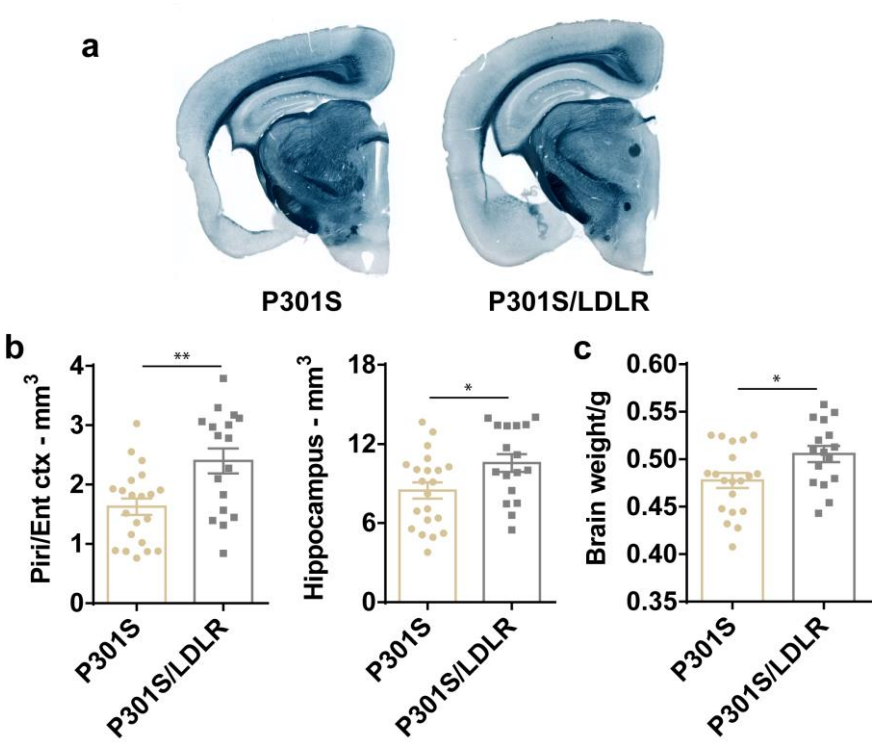

Figure 5.1 | LDLR overexpression in P301S mice attenuates neurodegeneration. a, Representative images of 9-month old P301S and P301S/LDLR mouse brain. **b, c,** Quantification of brain weight and brain volume for these mice. (P301S: n=21, P301S/LDLR: n=17). Data expressed as mean ± SEM, One-way ANOVA with Tukey's post hoc test (two-sided). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Ent: Entorhinal cortex, Piri: Piriform cortex.

Volumetric analysis of 9-month old P301S and P301S/LDLR mice showed that LDLR overexpression significantly attenuated neurodegeneration in the piriform/entorhinal cortex and hippocampus (Fig. 5.1a, b). In addition, brain weight was significantly increased in P301S/LDLR mice compared to P301S mice (Fig. 5.1c). Subsequent assessment of tau pathology in these mice revealed that LDLR overexpression shifted ptau staining patterns from

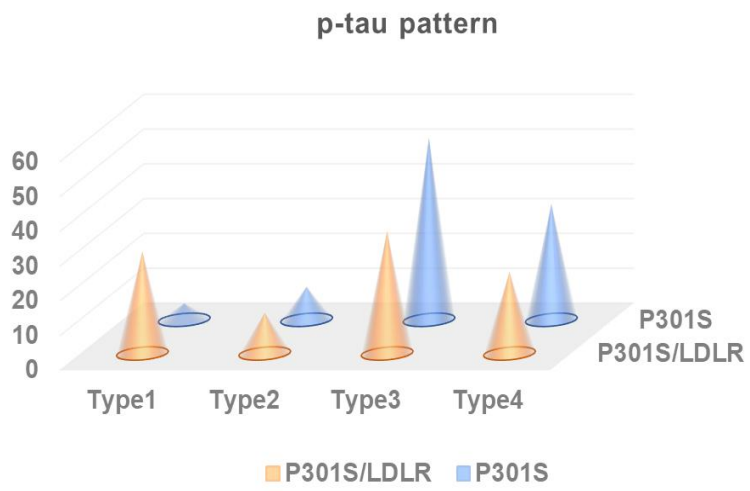

Figure 5.2 | LDLR overexpression in P301S mice shifts ptau staining patterns towards the ones associated with less brain damage. Distribution of p-tau staining patterns between 9-month old P301S and P301S/LDLR mice

type3 and type4 - patterns that are associated with more severe brain damage - towards type1, an early disease pattern associated with the most preserved brain (Fig. 5.2).

Biochemical analysis confirmed a 10-fold reduction of soluble APOE in the RAB fraction and also some reduction of insoluble APOE in the formic acid fraction in 9-month P301S/LDLR mice compared to P301S mice (Fig. 5.3a). Despite the marked APOE reduction, human tau levels were not altered in any fractions (Fig. 5.3b). However, notable reduction of ptau levels were observed in RAB and RIPA fraction upon LDLR overexpression (Fig. 5.3c). Interestingly, although no change of absolute human tau levels was observed, APOE level correlated with less soluble tau and a higher level of insoluble tau (Fig. 5.3d). In addition, APOE level strongly correlated with ptau levels in all fractions (Fig. 5.3e), indicating that APOE may promote ptau generation, which may further lead to insoluble tau formation.

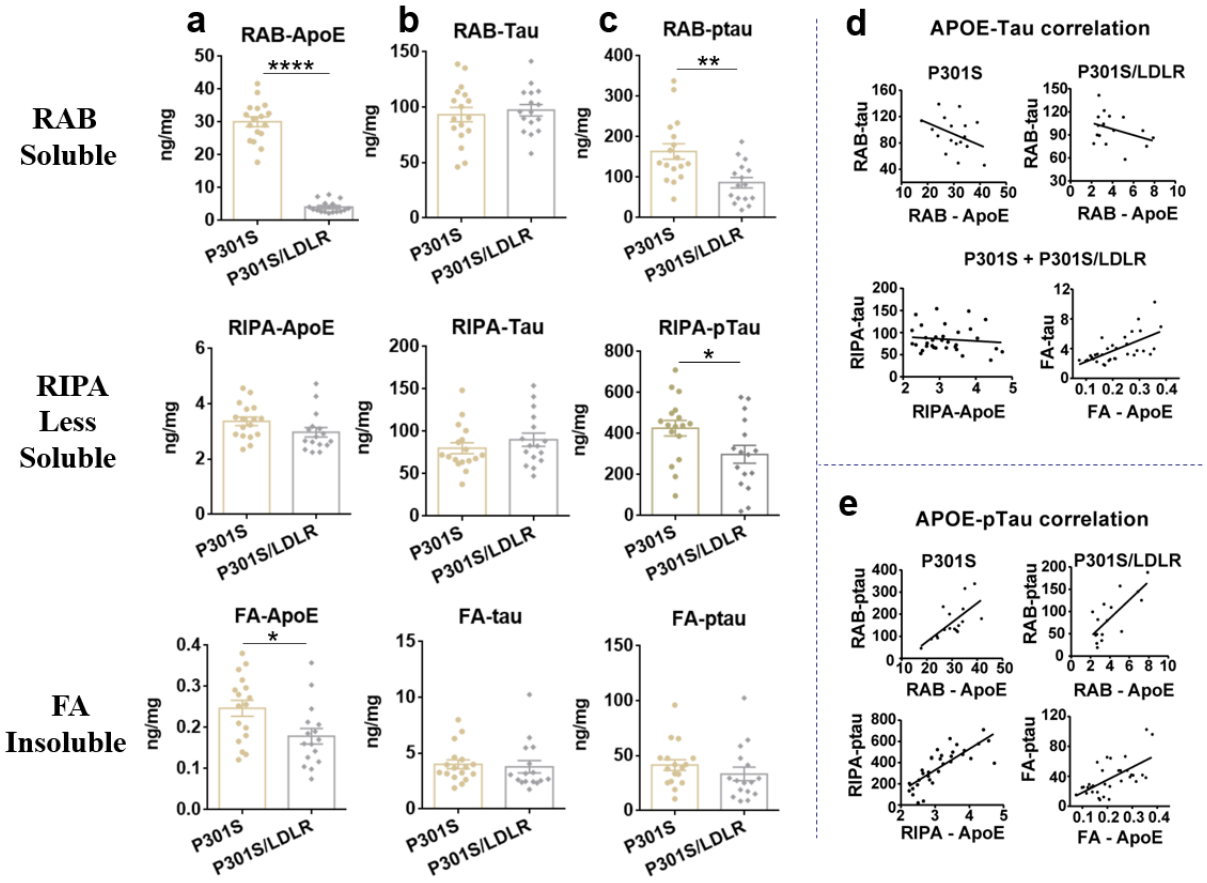

Figure 5.3 | LDLR overexpression in P301S mice reduces ptau level without altering tau levels. a-c, Human P301S tau, ptau, and murine APOE levels in 9-month P301S and P301S/LDLR mouse brains were measured by ELISA in RAB, RIPA and 70% FA fractions respectively (P301S: n=17, P301S/LDLR: n=16). **d,** Correlation between APOE and human tau levels in RAB, RIPA and FA fractions. **e,** Correlation between APOE and ptau levels in RAB, RIPA and FA fractions. Data expressed as mean ± SEM, One-way ANOVA with Tukey's post hoc test (two-sided) was used for statistical analysis. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

To confirm that the change of ptau levels in P301S/LDLR mice was mediated by APOE, but not an artifact due to LDLR overexpression, we generated a separate cohort of P301S mice expressing two alleles, one allele, or no murine APOE by crossing P301S mice to APOE knockout mice. We observed exactly the same pattern of ptau reduction in RAB and RIPA fractions with in P301S mice expressing one allele or no APOE (Fig. 5.4), supporting the role of APOE in mediating ptau pathogenesis. How APOE promotes ptau generation is not clear. APOE may enhance tau hyperphosphorylation through direct interaction with tau that alters pathological tau conformation and toxicity, which may further boost cellular stress that induces tau phosphorylation. On the other hand, given an essential role of neuroinflammation in inducing tau phosphorylation, the immunomodulatory function of APOE may also underlie its effect on ptau generation.

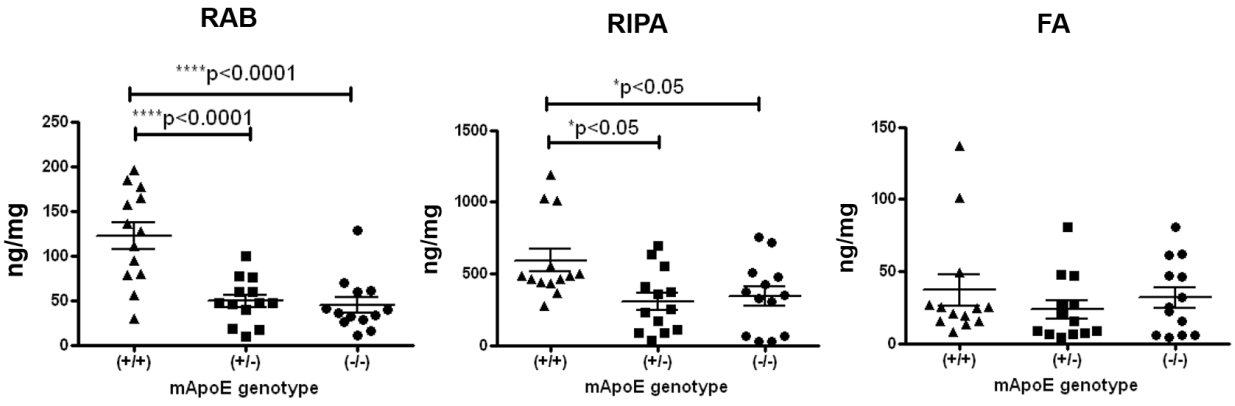

Figure 5.4 | APOE deficiency in P301S mice results in ptau reduction similar to the effect of LDLR overexpression. Ptau levels in 9-month old P301S mE/mE, P301S mE/-, and P301S -/- mouse brains were measured by ELISA (P301S mE/mE: n=13, P301S mE/-: n=13, P301S -/-: n=13). Data expressed as mean ± SEM, One-way ANOVA with Tukey's post hoc test (two-sided) was used for statistical analysis. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

5.4 Discussion

As a proof-of-concept study, lowering APOE levels by overexpressing its metabolic receptor LDLR attenuates neurodegeneration and tau pathology, indicating that APOE-based therapy may constitute an effective way to treat tauopathy. Interestingly, although LDLR overexpression reduces APOE levels, it bears fundamental differences from APOE-deficient mice in that LDLR^{+} mice still produce APOE so that any cell-autonomous APOE functions are preserved. The observation that LDLR overexpression confer similar protective effects on neurodegeneration as APOE ablation indicates that the extracellular pool of APOE plays an essential role in neurodegeneration. This is in line with the APOE opsonin hypothesis where extracellular APOE serves as an opsonin to enhance microglial phagoptosis of stressed neurons. On the other hand, it's not clear how APOE controls MGnD microglial activation. It's likely that extracellular APOE stimulates MGnD microglial activation by interacting with TREM2 and initiates microglial signaling, yet an alternative possibility is that microglia-synthesized APOE activates MGnD microglia in a cell-autonomous fashion, in which case lowering extracellular APOE may not prevent MGnD microglial activation. Preliminary analysis of our single nuclei RNAseq data from P301S and WT mice shows that in the diseased brain, APOE expression is drastically upregulated in microglia, but only shows minor changes in astrocytes, indicating that microglia-derived APOE, which has key differences from astrocyte-derived APOE in their lipid contents, is the one that is involved in neurodegeneration. In fact, the population of APOE serving as opsonins may exclusively come from microglia-derived APOE, in which case the specific composition of APOE particles may serve as unique landmarks to facilitate microglial recruitment. This specific APOE may also act as a signaling molecule to notify microglia for disease onset and to prepare them for immune functioning by activating the MGnD signature.

Interestingly, although LDLR overexpression attenuates neurodegeneration in P301S mice, the pattern of protection is different from that of TEKO mice. While not a single case of severe neurodegeneration harboring type4 tau was observed in approximately 40 TEKO mice that were assessed, a portion of P301S /LDLR mice still develop severe neurodegeneration with type4 tau, although the ratio is lower with LDLR overexpression. This could be simply due to a partial rescue effect as a result of remaining APOE in the brain, or the MGnD microglia was still activated in P301S/LDLR mice in a cell-autonomous manner independent of extracellular APOE reduction, which exacerbated neurodegeneration compared to TEKO mice. Still it could be a gain of toxic function from LDLR overexpression. Therefore, a cleaner approach to reduce APOE levels would be to directly target APOE mRNAs. This would reduce APOE levels without introducing additional confounding factors. One commonly adopted and clinically relevant approach to target mRNAs is to use antisense oligonucleotides (ASOs), which are short pieces of synthetic, chemically modified DNA sequences designed to bind complementary RNA targets to facilitate their degradation. Our ongoing experiments with APOE ASO treatment in TE4 mice would be an important supplement to the LDLR overexpression scheme.

5.5 Concluding remarks and future directions

APOE, the strongest genetic risk factor for Alzheimer's disease, has long been considered to affect AD through modulating Aβ pathology. However, this mechanism alone is insufficient to explain APOE's robust impact on AD given the poor correlation between Aβ pathology and AD-associated neurodegeneration and cognitive impairment. In this work, we uncovered a significant function of APOE in regulating neurodegeneration in the setting of tauopathy independent of APOE's effect on Aβ pathogenesis. We found APOE4 significantly exacerbates tau-mediated

neurodegeneration compared to APOE2 and APOE3, whereas APOE deficiency is strongly neuroprotective. The effects of APOE on neurodegeneration are likely derived from two aspects: a direct role of APOE in regulating tau pathogenesis, which induces neurotoxicity in a cell autonomous manner; and an immunomodulatory function of APOE to regulate neuronal viability by modulating neuroinflammation in a non-cell autonomous fashion. Although our microglia-depletion experiment suggests that regulation of microglia-mediated neuroinflammation by APOE plays a dominant role in APOE-dependent neurodegeneration, the effects of APOE on tau pathogenesis cannot be neglected, as pathological tau and tau-induced neuronal injury likely serve as the initial trigger of neuroinflammation. We found that APOE specifically interacts with a high-molecular-weight form of tau. This interaction may enable APOE to regulate tau pathogenesis, for instance, by affecting tau structure, modification, aggregation, seeding, or clearance. We found APOE promotes tau phosphorylation and correlates with higher levels of insoluble tau. In addition, APOE4 leads to higher levels of tau accumulation compared to APOE2, APOE3 and APOE deficiency, likely by affecting tau autophagy. In characterizing tau pathology in the P301S mouse model, we established a new pathological tau categorization system based on hippocampal ptau staining patterns. These patterns serve as sensitive indicators of neurodegeneration, and may also represent distinct tau species. The fact that APOE affects ptau patterns also points to a likelihood that APOE may participate in shaping tau conformation and regulate tau species formation.

On the other hand, once neuroinflammation is initiated by pathological tau accumulation and associated neural injury, it becomes a driving force of neurodegeneration. APOE has previously been implicated in systemic inflammation during infection. APOE4 is associated with higher levels of immune responses upon conventional immune stimulation (such as LPS

stimulation) compared to APOE2 and APOE3, and the ε4 allele has been hypothesized to be acquired during human evolution to confer initial advantages in pathogen resistance. Here we introduced the concept of APOE's immunomodulatory function implicated in infectious diseases to the field of neurodegeneration. We found TE4 mice demonstrated significantly higher levels of microglial and astrocytic activation compared to TE2 and TE3 mice, whereas APOE deficiency reduced glial activation relative to the presence of APOE. Through in vitro glia-neuron co-culture, we demonstrated that glia cells directly contributed to neuronal death. We found that glia-mediated neuronal loss requires direct cell-cell contact between glia and neurons, and this process is strongly regulated by APOE. The presence of APOE, particularly APOE4, significantly exacerbates neuronal loss. In vivo, depleting microglia rescued neurodegeneration in TE4 mice, but showed no impact on TEKO mice, highlighting an APOE-dependent microglial function in neurodegeneration. We proposed an "APOE opsonin" hypothesis where APOE serves as an opsonin coating the surfaces of sick neurons that show impaired APOE endocytosis, and recruits activated microglia by binding to TREM2 expressed on microglia to facilitate microglia-mediated neuronal phagoptosis. This hypothesis is supported by our preliminary in vitro observation that microglia phagocytose more dead neurons in the presence of APOE compared to the absence of APOE, but needs to be proved with further experiments. In addition to coating jeopardized neurons, APOE may also serve as an opsonin binding to amyloid plaques, which regulates microglia-mediated plaque trimming and phagocytosis. Aside from hypothetic opsonization functions, APOE has lately been shown to be a critical determinant of microglial polarization into the neurodegenerative phenotype under neurodegenerative conditions. APOE ablation leads to incomplete microglial activation, yielding semi-activated microglia that fail to acquire the neurotoxic MGnD phenotype featuring inflammatory and phagocytotic microglial

functions. APOE-dependent regulation of microglial reactivity likely relies on APOE's capacity to modulate lipid contents on microglial cell membranes, which controls lipid raft remodeling that plays a critical role in immune cell activation. However, detailed mechanisms beneath APOE-controlled microglial activation still await elucidation. We think the immunomodulatory function of APOE constitutes a common mechanism regulating neurodegeneration in a variety of neurodegenerative disorders. Our findings, together with emerging evidence from other groups, suggest a re-evaluation of the role of APOE in AD and other neurodegenerative diseases.

Given an essential role of APOE in regulating tau-mediated neurodegeneration, we targeted APOE as a therapeutic approach to treat tauopathy. We found a protective effect on neurodegeneration with LDLR overexpression, which lowered soluble APOE by 10-fold and most likely targets the extracellular APOE pool, suggesting extracellular APOE plays a role in neurodegeneration. However, the rescue effect is much milder than that of APOE ablation, suggesting that intracellular APOE, particularly microglia-derived APOE that shoots up sharply in disease, may cell-autonomously regulate microglial immune function that is crucial in neuroinflammation-induced neurodegeneration. Emerging evidence suggests that APOE particles produced in various cell types are composed of different lipid contents and may have distinct functions. Yet the detailed composition and function of APOE lipoprotein coming from different sources still await elucidation.

Our findings that APOE directly regulates neurodegeneration, neuroinflammation and tau pathogenesis independent of its well-known role in Aβ pathogenesis, add new perspective to AD pathogenesis and open up new directions in AD research. Here I summarize the important questions to be solved in future studies.

1. Tau, instead of Aβ, is directly associated with neurodegeneration. However, how pathological tau induces neurotoxicity is still unclear. Distinct tau strains have been identified in AD and other primary tauopathy patients. How tau conformation in relate to its function is linked to different aspects of tau neurotoxicity adds another layer of complexity to the question. Traditionally it was believed that tau seeding is detrimental. However, we found that compared to certain forms of tau, the tau species that harbor higher seeding activity is associated with less neurodegeneration, indicating that under certain conditions, tau seeding may be less detrimental, likely by trapping more toxic forms of tau. Importantly, we found that tau seeding is not equivalent to insoluble tau formation, the concept of which have been confused in the past. The tau species harboring higher seeding capacity is in fact associated with relatively high solubility, whereas certain tau species showing low seeding capacities turn out to be more insoluble and are associated with more severe brain damage. We think tau solubility may be a better indicator of brain damage than tau seeding activity. Interestingly, we found that tau seeding capacity strongly correlates with thioflavin S signal (data not shown), a marker for β-sheet structure. The tau species possessing high seeding activity stains highly positive for ThioS, whereas other tau species harboring low seeding capacities stains low or negative for ThioS, indicating that β-sheet structure is a determinant for tau seeding activity. ThioS has been commonly used to stain for tau pathology. However, our findings indicate that ThioS staining may only reveal the pathological tau species that present high seeding activity, but may skip other tau species that are more insoluble with higher neurotoxicity. Therefore, a detailed characterization of tau species and the type of neurotoxicity they confer would be critical in understanding the mechanism of tau-mediated neurodegeneration. On top of this, understanding how APOE affects tau pathology and tau-mediated neurotoxicity, either through direct APOE-tau interactions, or via indirect

mechanisms, would provide important insight on APOE's function in neurodegeneration in the setting of tauopathy.

2. Neuroinflammation plays a critical role in driving neurodegeneration. Although multiple hypotheses have been proposed as to how neuroinflammation causes neurodegeneration, none of them have been evidently proved. To date, it's still unclear how neurons die under inflammatory milieu, either through apoptosis, necrosis, pyroptosis or other pathways. It would be critical to understand the concrete neuronal pathways being affected by neuroinflammation that lead to neuronal death in order to design targeted therapies to prevent neuronal loss.

3. Microglia and astrocytes constitute the two major cell populations in the brain mediating neuroinflammation in neurodegenerative diseases. However, their respective roles in inducing neurodegeneration are not well understood. Microglia are conventional immune cells reserving all innate immune cell properties and may play a dominant role in neuroinflammation including pathology sensing, cytokine secretion, and phagocytosis, etc. Unlike microglia that are derived from yolk sac myeloid precursors, astrocytes resemble neurons in that they originate from ectodermal precursors and are not conventional immune cells. In physiological conditions, the predominant functions of astrocytes are to provide nutritional and growth support to neurons to maintain their survival and function, and to regulate extracellular ion and neurotransmitter concentrations to maintain environmental homeostasis. Under pathological conditions, astrocytes are immune-competent and can secret inflammatory cytokines upon activation. They can also crosstalk with microglia and produce neurotoxic factors. However, in general, the neurotoxic effects of reactive astrocytes may principally lie in their loss of neural support functions in addition to their gain of neurotoxic function with unique secreted factors, which mechanistically could be different from microglia-mediated neurodegeneration. Dissecting out the respective

roles of microglia and astrocytes in neurodegeneration would provide critical mechanistic insights into understanding neuroinflammation-induced neurodegeneration. Our ongoing microglia-ablation experiment in tauopathy mice for the first time clearly demonstrated a critical role of microglia in driving neurodegeneration. However, how microglia achieve this, either solely by itself though its innate immune functions independent of astrocytes, or via crosstalk with astrocytes is not clear. Specific functions of astrocytes in neurodegeneration are even less understood. These questions need to be comprehensively addressed in future studies.

4. Despite the resemblance of CNS microglia to macrophages in peripheral tissues in a variety of aspects, microglial activation signature in neurodegenerative diseases bears unique features and is different from macrophage activation profiles in systemic inflammation. Studies in this area have just begun. Deeper characterization of disease-associated microglia signatures and functions with cutting-edge technologies such as single cell RNAseq and mass cytometry will be key to understanding the role of microglia-mediated neuroinflammation in neurodegeneration, and to directing neuroinflammation-based therapies. While chronic neuroinflammation can exacerbate neurodegeneration, initial microglial activation at an early disease stage may help clear pathological proteins and prevent their accumulation. Specific reprogramming of microglia to increase the clearance of pathological proteins without amplifying inflammatory stress or targeting neurons are promising fields in future research.

5. The potent immunomodulatory function of APOE may be a principle mechanism through which APOE regulates neurodegeneration in various neurodegenerative diseases. Based on our findings and inference from literature, we proposed two mechanisms in respect to how APOE may modulate microglial functions: 1) by modulating microglial cell membrane lipid content to affect microglial immune reactivity 2) by serving as an opsonin regulating microglial

phagocytotic functions. Yet additional evidence is needed to support these hypotheses. Future studies to further investigate mechanisms underlying APOE's immunomodulatory function would be essential to understand APOE's role in neurodegeneration. Research can focus on connecting APOE-dependent lipid profiles with microglial immune activation signatures, and further investigation of the APOE-TREM2 axis in regulating microglial functions.

6. Our single nuclear RNAseq data show that under neurodegenerative conditions, brain APOE expression goes up in general, but uniquely surge in microglia many folds over other cell types, suggesting cell-type dependent APOE function in disease. As lipids perform both nutritional and immunoregulatory functions, APOE lipoprotein produced in different cell types may carry distinct lipid contents and serve different functions. Future studies investigating the composition and function of APOE derived from different cellular source, such as microglia, astrocytes, neurons and peripheral tissues, via APOE conditional knockout or overexpression in these cell types, would be critical to fundamentally understand the multifaceted roles of APOE in neurodegeneration.

7. The blood-brain barrier separates the brain parenchyma from the blood circulation. Therefore, neuroinflammation in neurodegenerative diseases is predominantly characterized of innate immunity mediated by the brain-resident glia cells. However, accumulating evidence suggests crosstalk between the CNS and the periphery that may play an essential role in brain pathogenesis. In particular, recent studies showing connections of gut microbiota with AD[145-147] and PD[148] pathogenesis and microglial functions[145,149] strongly suggest signaling exchange between the periphery and the CNS, likely mediated by soluble factors that can cross the blood-brain barrier. The periphery-CNS immune connection and its role in brain pathogenesis and neurodegeneration is a promising field that should be investigated in depth in future studies. On

the other hand, many neurodegenerative diseases begin with disruption of proteostasis. This process, once initiated, cannot currently be self-resolved and will inevitably lead to pathological protein accumulation that eventually progresses to neurodegeneration. One reason for the failure of the brain to effectively maintain proteostasis is likely due to the lack of adaptive immunity in brain parenchyma compared with peripheral tissues, which jeopardize the brain's clearance machinery. Boosting immune activity in the brain to increase waste clearance under homeostatic or pathological status without damaging neurons by introducing adaptive immunity into the brain, such as pathology-directed antibody treatment or engineered T cell-based strategies may be promising fields that can be worked out with caution to avoid potential side effects in the future.

8. Therapeutic studies should follow up new mechanistic discoveries to treat neurodegenerative diseases. Traditionally, immunotherapies targeting pathological proteins such as Aβ, and lately tau, have been widely adopted. However, the results are not very satisfactory. Given a dominating effect of microglia-mediated neuroinflammation in driving neurodegeneration in the presence of APOE, future attention should be focused on targeting neuroinflammation or APOE. PLX3397, the CSF1R inhibitor that deplete microglia, may serve as an effective drug to prevent neurodegeneration and slow down disease progression. With better mechanistic insights in the future, targeted drugs designed to inhibit neurodegenerative microglial activation may be an optimal choice. APOE-based therapies to lower down APOE mRNA or protein levels, such as APOE immunotherapy or ASO treatment, are other promising options to work out in the future.

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