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Mechanisms of Microglia Mediated Apolipoprotien E Neurotoxicity

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MECHANISMS OF MICROGLIA MEDIATED APOLIPOPROTIEN E NEUROTOXICITY

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Abstract

APOE4 is the strongest genetic risk factor for late-onset Alzheimer disease. ApoE4 increases brain amyloid-β pathology relative to other ApoE isoforms. However, whether APOE independently influences tau pathology or tau-mediated neurodegeneration, is not clear. Previous works using tau transgenic mice (P301S) on either a human ApoE knock-in (KI) or ApoE knockout (KO) background have shown that P301S/ApoE4 mice have significantly higher tau levels in the brain and microglia immune reactivity in vivo. The goal of this study was to characterize ApoE4 mediated neurodegeneration in vitro and screen Liver X receptor (LXR) agonist GW3965 as possible inducer of neuroprotection. Here we show ApoE4-expressing microglia in vitro exhibit altered expression of immune and lysosomal modulators at baseline. Co-culturing P301S tau-expressing neurons with ApoE4-expressing mixed glia results in markedly reduced neuronal viability compared with neuron/WT and neuron/ApoE3 co-cultures. Neurons cultured alone or with WT mixed glia showed the greatest viability. Neuron/ApoE4 viability was restored to WT levels after treatment with LXR agonist GW3965. This was accompanied by a significantly lower level of tumor-necrosis factor-α (TNF-α) secretion in addition to lower Cystatin F (CST7) and triggering receptor that is expressed on myeloid cells (TREM2) expression. In individuals with a sporadic primary tauopathy, the presence of an ε4 allele is associated with more severe neurodegeneration. In individuals who are positive for amyloid-β and tau pathology, ε4-carriers demonstrate greater rates of disease progression. Our results demonstrate that ApoE affects tau mediated neurodegeneration, neuroinflammation, and may potentially be exacerbated by lipid transport pathways in microglia.

Table of Contents

1. Introduction

Inheritance of the apolipoprotein E (ApoE) ε4 allele is the greatest genetic risk factor for developing late-onset Alzheimer's disease (AD). Early clinical observations elucidating the relationship between ApoE and AD revealed amyloid plaques co-localizing with ApoE in cortical brain regions (Namba et al., 1991). Amyloid plaque accumulation is one of the primary pathological hallmarks of AD. Since then, numerous epidemiological studies have revealed the frequency and incidence of human ApoE and its isoforms as the major genetic risk factor for AD. Single nucleotide polymorphisms (SNPs) in the ApoE gene result in changes in structure and function that alter the ApoE protein. The most common isoforms are ApoE2 (cys112/cys158), ApoE3 (cys112/cys158), and ApoE4 (cys112/cys158) (Mahey et al., 2006). Although these isoforms differ by only one or two amino acids, consequences of being a carrier of one of these genotypes may contribute to the incidence of AD in the human population. Compared to non-ε4 carriers, individuals with one ε4 allele are 3x more likely to develop AD while those with two alleles are 12x more likely. On the other hand, carriers of the ε2 allele are associated with a lower risk of developing AD (Michealson, 2014). These phenomena implicate ApoE as a genetic risk factor for AD that is both isoform and gene dosage dependent. While the vast majority of studies implicating ApoE in AD have focused on Aβ aggregation and clearance, this approach has yet to yield specific therapies that target ApoE. Astrocytes and microglia have recently emerged as disease modifying sources of ApoE and recent studies have identified unique microglial activation states and alterations in brain lysosomal homeostasis that may be a source of ApoE-associated neurotoxicity.

Aside from the liver, the brain is highest producer of ApoE (Elshourbagy et al., 1985). Under normal conditions, ApoE functions as a transporter of high density lipoproteins (HDL) containing phospholipids and cholesterols involved in CNS lipid homeostasis. The lipid components of lipoproteins are insoluble in water. ApoE, because of its detergent-like amphipathic properties, can surround HDL creating a lipoprotein particle that is water soluble and can be carried through water based CNS circulation. Like in the periphery, this is accomplished by ATP-binding cassette transporter (ABCA1) which facilitates the movement of insoluble cholesterols and phospholipids from cell membranes to its major protein acceptor in the CNS, ApoE, to form HDL (Mahley et al., 2006, 2016). This ApoE containing HDL is then transported to the LDL receptor or members of the LDL receptor family where ApoE binding mediates HDL endocytosis into neurons (Rensen et al., 2000). The exact role of ACBA1 and ApoE in CNS cholesterol homeostasis is not yet fully understood. In-vitro studies using mouse astrocytes with an ABCA1 knockout (KO) revealed cholesterol-poor ApoE and minimal HDL production (Hirsch- Reinshagen et al., 2004, Karasinska et al., 2009), while deletion of the LDL receptor has been shown to increase ApoE in cerebral spinal fluid (CSF) (Fryer et al., 2004). Together, these animal studies suggest that ABCA1 is critical for CNS ApoE acceptance of HDL, while LDL receptor endocytosis may preserve and recycle circulating ApoE. The importance of ABCA1 in the human CNS is also not completely clear. Studies with patients that have ABCA1 SNPs did not reveal a rate limiting interaction with HDL or ApoE nor an increased risk of AD (Wahrle et al., 2007), suggesting compensatory mechanisms for ABCA1 cholesterol transport or enhanced ApoE transport efficiency capabilities that have not been elucidated or specifically studied.

The amyloid hypothesis has been at the center of modern AD research and defines AD's principle characteristic as Aβ deposition in brain tissue during a postmortem examination. It is widely accepted that an imbalance of amyloid precursor protein (APP) cleavage by β- and γ-secretase results in deposition of Aβ42 which is more hydrophobic than its Aβ40 counterpart and more readily forms the fibrillogenic aggregates seen in postmortem brains. While the majority of research has focused on the clearance and aggregation of Aβ, multiple empirical observations in human patients have driven investigators to study how ApoE alters the presence of Aβ and if it can be used as a possible therapeutic intervention. A large-scale autopsy of 296 patients with AD assessed neuritic plaque and neurofibrillary tangle accumulation and whether this accumulation was associated with the presence of the ε4 allele. In this study, multiple brain structures were assessed including the hippocampus, midfrontal, inferior parietal, and superior tempora cortices. Strikingly, patients with two ε4 alleles had significantly more neuritic plaques and neurofibrillary tangles in all regions assessed as compared to those with either one or no ε4 alleles. Furthermore, carriers of the ε2 allele revealed decreased neuritic plaques compared to non-carriers suggesting a protective role (Tiraboschi et al., 2004). This confirmed two earlier studies that found 62% of late onset patients with sporadic AD possessed an ε4 allele and that this reduced the age of AD onset by approximately 10 years per allele (Rebeck et al. 1993; Corder et al. 1993). These findings have recently driven investigators to ask several questions regarding the effect of ApoE on Aβ. Analyzing the extent to which ApoE and its isoforms promote the aggregation and clearance of Aβ will ultimately help determine if targeting ApoE is a viable therapeutic option.

Animal studies using genetically modified mice that mimic Aβ deposition seen in humans have allowed for more direct questioning of the cognitive decline and neurodegeneration aspects of AD. With human data supporting that possession of the ApoE3 and ApoE4 alleles increases susceptibility to Aβ deposition, genetically modified mice expressing human ApoE isoforms have been developed to better explain how ApoE alters Aβ aggregation and clearance. Several studies have shown the gene dosage and isoform dependent effects of ApoE on Aβ. Using transgenic mice that increase the production of Aβ, as well as the ratio of Aβ42:Aβ40, several studies revealed that human ApoE accelerates Aβ deposition in mouse brains and that this acceleration was isoform dependent with ApoE4>ApoE3>ApoE2, suggesting a differential effect on Aβ clearance (Holtzman et al., 2000, Fagan et al., 2002). Indeed, in a follow up study animals expressing human ApoE4 revealed that Aβ was re-disturbed into cortical regions (Fryer et al., 2005). It was also recently shown that ApoE4 expressing mice exhibited a slower rate of Aβ clearance from the Interstitial fluid (ISF) than ApoE3 expressing mice. Furthermore, these ApoE isoforms do not appear to affect Aβ protein translation as seen through western blotting (Castellano et al., 2011; Huynh et al., 2017). With this data, it is reasonable to presume ApoE does not exert its effects by modifying Aβ gene transcription. Together, this data represents an ApoE isoform dependent sequestration of Aβ that could potentially be an artifact of inherent ApoE transport where lower ISF levels of ApoE4 from the brain allows for more substantial deposition of Aβ. While this may seem like a plausible hypothesis and promising point of therapeutic intervention, recent studies targeting ApoE have revealed that waiting until Aβ has deposited may not be an efficacious strategy. ApoE is produced in microglia and astrocytes (Xu et al., 2006). Exploring phenotypic changes in

these cells could allow for a different therapeutic intervention of ApoE induced neurotoxicity before Aβ deposition occurs.

While there are multiple competing prognoses that describe ApoE's role in AD pathogenesis, competing hypotheses are envisioned as plausible avenues for which therapeutic intervention can be achieved. As described, one such approach envisions ApoE as altering Aβ transport and clearance. However, there is a growing body of evidence implicating microglia and astrocytes as significant disease altering sources of ApoE. Indeed, recognizable Aβ deposition occurs relatively late in AD (Jack and Holtzman, 2013) and a recent study by David Holtzman and colleagues (2017) revealed that human ApoE4 suppression using antisense oligonucleotides was ineffective at reducing Aβ in APP/PS1 mice that were more than 6 weeks old. While in this study ApoE suppression did mildly reduce Aβ in mice that were <6 weeks old, this developmental window likely corresponds to a period in the AD pathological time course where Aβ monomers form oligomers (Jarrett and Lansbury, 1993) and consequences of possessing an ε4 allele have already taken hold. This data suggests that Aβ plaque growth is primarily driven by non-ApoE factors in older mice and that ApoE targeted therapy would need to start very early. Recent studies are underway investigating ApoE's modulatory effects on CNS immune responses and lipid homeostasis early in disease that may help trigger AD pathogenesis.

Another recent comprehensive study by David Holtzman and colleagues (2017) implicates ApoE as a significant driver of neurodegeneration that has powerful control of proinflammatory gene expression in microglia. Using the P301S model of tauopathy, which

overexpresses 1N4R human tau with the P301S mutation, a series of mice were generated on a human ApoE or ApoE KO background. Unlike Aβ, which poorly correlates with symptoms or tissue loss (Josephs et al., 2008), tauopathies correlate better with neurodegeneration (Williams et al., 2007). At 9 months, these tau/ApoE expressing mice demonstrated robust ApoE isoform dependent neurodegeneration with ApoE4>ApoE3>ApoE2. Indeed, significant tissue atrophy occurred at the hippocampus, entorhinal cortex and amygdala revealing powerful tau mediated neurodegeneration. Importantly, this phenomenon was mirrored in-vitro with P301S expressing neurons showing cell loss when co-cultured with human ApoE expressing glia. Under normal conditions, ApoE is expressed in ~75% of astrocytes. This is increased to ~85% when treated with the potent neurotoxin kainic acid. This was confirmed using a mouse line expressing green fluorescent protein (GFP) controlled by the endogenous ApoE locus. Furthermore, ~10% of microglia are shown to express ApoE, but only after neurotoxin treatment (Xu et al., 2006).

Despite relativity low ApoE expression, Holtzman and colleagues (2017) showed that ApoE3 and ApoE4 expressing microglia in AD mice have higher innate immune reactivity, with an upregulation of more than a dozen proinflammatory genes, while at the same time, a down regulation of homeostatic microglial genes. One such proinflammatory gene, triggering receptor that is expressed on myeloid cells (TREM2), has recently been under the spotlight as an ApoE receptor that is necessary for microglial activation and Aβ clearance (Jendresen et al., 2017, Keren-Shaul et al., 2017). Earlier studies illustrate TREM2 mediated microglial activation as necessary for Aβ clearance (Wang et al., 2015). Using 5xFAD model of AD, which express the

Swedish, Florida, London, and PSEN1 Aβ mutations (Goodwin et al., 2017), TREM2 KOs suppressed Aβ clearance while also reducing microglial immune reactivity visualized by IBA1. Indeed, TREM2KO mice show deficits in Aβ clearance and lack of co-localization of microglia around Aβ which, under normal conditions, would clear Aβ (Ransohoff and Cardona, 2010). This was confirmed in a separate study where instead of a TREM2 KO, its gene dosage was enhanced via gain-of-function overexpression of human TREM2 exclusively in microglia (Lee et al., 2018). Indeed, this study by Lee and colleagues validated TREM2 as a scalable controller of microglial immune reactivity by showing enhanced Aβ clearance in AD mice overexpressing TREM2. Furthermore, this was accompanied by fewer and less activated microglia per plaque while still, and arguably more efficiently, clearing Aβ.

It is not clear if TREM2 function is altered in carriers of ε3/ε4. Given the direct control TREM2 has over microglial activation, any ApoE3/4 induced alterations on TREM2 function may have a significant impact on microglia phagocytic capability. A study by Li-Huei Tsai and colleagues (2018), illustrates widespread and robust alterations in ApoE4 expressing microglia. In the most clinically relevant setting yet, with human stem cell derived microglia expressing ApoE4, this study revealed clear contrasts in microglial gene expression, morphology, and Aβ uptake. At the onset, these microglia displayed morphologically shorter ramifications indicative of a partially activated state with ~30% of upregulated genes being immune related. Shorter ramifications are known to be correlated with Aβ uptake (Sarlus and Heneka., 2017), however, once these microglia were treated with Aβ, they displayed an abnormally impaired/slower uptake capability. These observations indicate that ApoE4 expressing microglia have

impairments effectively sensing and responding to Aβ in their environments. Given the importance of microglia surveillance and response functions to brain health, such impairments could have considerable disease outcomes. Determining if the reduced uptake capability of ApoE4 expressing microglia can be restored by targeting TREM2
may be a new therapeutic option.

As described, ApoE is the principle cholesterol transporter in the brain. The brain contains 25% of all body cholesterol, 70% of which is in myelin and 20% in astrocytes and microglia (Dietschy et al., 2004). The rate of brain cholesterol synthesis declines with age and ApoE's effect in promoting cholesterol efflux and lipidation proceeds in an isoform dependent manner with ApoE2>ApoE3>ApoE4 (Minagawa et al., 2009). A study by Simons and colleagues (2018), revealed that the liver X receptor (LXR) agonist, GW3965, can promote re-myelination and reduce cholesterol based myelin accumulation in WT and ApoEKO microglia in mouse models of Multiple Sclerosis. These effects were impaired in mice lacking LXR and led to sustained microglial activation and myelin debris accumulation in lysosomes. LXR is a cholesterol sensor in the CNS that promotes genes directly responsible for cholesterol export such as ApoE and ABCA1. Follow up experiments revealed that microglia lacking ApoE demonstrated profound deficiencies in clearance capacities of cholesterol based cell debris and had pronounced intracellular buildup of cholesterol as indicated through fluoromyelin staining. Because the majority of ApoE is primarily produced in astrocytes, clearance capacity in these ApoE KO microglia were restored to WT levels when incubated with WT astrocyte conditioned media. That is, in the absence of ApoE, microglia appear to have an upper limit of clearance

capacity that is restored when treated with ApoE, WT astrocyte secreted factors or GW3965. Furthermore, other sterols and oxysterols have impaired synthesis in ApoE KO brains suggesting widespread impairments in CNS lipid processing (Eder Carlos Rocha Quintao et al., 2018). Based on this data, it appears that microglia clearance capacity can be enhanced and even restored when cholesterol synthesis and transport pathways are activated. Given that cholesterol clearance and lipidation by ApoE is isoform dependent, ApoE4's deficiencies in Aβ uptake might also be a consequence of isoform dependent clearance impairments. Thus, future experiments should interrogate microglia lipid sequestration as a modifier of ApoE4 mediated neurotoxicity.

In a separate comprehensive study by Heneka and colleagues (2011), microglia uptake of Aβ was stimulated in a dose-dependent manner by LXR agonist TO901317. Utilizing a similar paradigm as Simons and colleagues (2018), microglia were incubated with media collected from astrocytes which were treated with increasing concentrations of TO901317. This astrocyte conditioned media stimulated microglial uptake of Aβ as visualized with FAM-labeled Aβ and increased concentrations of ABCA1 and ApoE in astrocytes as seen through western blotting. Thus, LXR stimulation of astrocytes secretes products that positively regulates phagocytosis in microglia. In contrast, enhancements of Aβ uptake was nearly abolished when microglia were exposed to media from ApoE KO astrocytes. Thus, solidifying ApoE as essential for LXR mediated phagocytosis of Aβ. Additionally, Aβ:42 overexpressing mice treated with T0901317 for 7 weeks, demonstrated a decrease of Aβ and oligomers by as much as 80%. Further studies

confirmed GW3965 as reducing plaque formation by up to 50% and improving fear conditioning and object recognition in mice (Riddel et al., 2008).

Whether from the perspective of immune activation or lipid processing via LXR, microglia are playing a central role in AD. It is far from clear what is wrong with microglia in carriers of ε3 and ε4, but the transition of resting microglia to their activated state via TREM2 is thought to encompass all necessary immune-modulation to protect against CNS insult (Keren-Shaul et al., 2017., Krasemann et al., 2017, Friedman et al., 2018). It is in this transition where glia's transcriptional profile is altered leading to a drastic ApoE dependent hyper-inflammatory neurotoxicity (Shi et al., 2017). Whether this maladaptive transition is mediated by deficient lipid processing by ApoE of surrounding cell debris/Aβ in a degrading brain or impairments in microglial immune surveillance via TREM2 is unknown. It is imperative that ApoE4's maladaptive alterations to microglia be tied to a therapeutically targetable pathway prior to the emergence of hyper-inflammation.

Exploration of LXR's modulatory effects on ApoE4 expressing microglia and the necessity of TREM2 within LXR's influence of phagocytosis is unknown. Given that ApoE3/4 expressing microglia have deficient uptake capability, activation of LXR may be neuroprotective by promoting microglial responsiveness to cell debris and several questions are now at the forefront: How is microglial gene expression altered in ε3 and ε4 carriers? How does this change contribute to microglial activation in AD pathogenesis? Where and how can this dysfunction be targeted for therapeutic intervention? Thus, the goal of these experiments is (a)

to characterize ApoE3/4 microglial toxicity when cultured with tau P301S expressing neurons (b) to assess the neuroprotective capabilities of LXR stimulation on ApoE4 mediated neurotoxicity, (c) to examine alterations in TREM2 expression in microglia before and after LXR stimulation.

2. Method

2.1 Animals

PS19 transgenic mice were purchased from The Jackson Laboratory (Stock No: 008169) expressing human P301S 1N4R tau driven by mouse prion protein promoter. Homozygous ApoE4 and ApoE3 mice were purchased from Taconic (Model# 1548 and 1549). The APOE4 targeted replacement mouse was developed in the laboratory of Nobuya Maeda at the University of North Carolina. The model was created by targeting the murine ApoE gene for replacement with the human APOE4 allele in E14TG2a ES cells and injecting the targeted cells into blastocysts. Resultant chimeras were backcrossed to C57BL/6 for seven generations (N7). Taconic received stock in 2000. The mice were backcrossed once more (N8) and embryo transfer derived. The colony is maintained through mating of homozygotes.

2.2 Primary neuron culture

Primary neurons were obtained from E17 PS19 mouse fetuses (N=6-7). Entire fetal cerebral cortices were dissected in calcium and magnesium free Hank's Balanced Salt solution

(HBSS). Tissue was digested in 10ml HBSS containing 0.25% trypsin (ThermoFisher Cat# 25200056) and .2mg/ml DNase (Sigma Cat#D-4527) in at 37 °C water bath for 10 minutes and inverting the tube every 2-3 minutes. Neurobasal medium (1x B27 + 1x penicillin/streptomycin + 1x L-glutamine, ThermoFisher Cat# 21103049) was added to inactivate trypsin and was gently pipetted up and down 10-15 times to dissociate tissue then allowed to sit at room temperature for 5 minutes. Cells were filtered through 40µm strainer. Filtrate was centrifuged at 0.6 rcf for 10 minutes. Media was removed and pellet was re-suspended in 10ml neurobasal media. Cells were plated in 24-well tissue culture plates over glass coverslips coated with 100µg/ml poly-D-pysine (Sigma Cat# A-003-E) at a density of 200,000 cells per well with 1ml neurobasal medium. Neurons were kept for 1 week replacing 50% of neuronal media every 3 days with fresh media prior to addition of mixed glia. Diagram 1 depicts the basic flow of experimental timelines from isolating primary cells to executing biochemical assays.

2.3 Glia neuron co-culture

ApoE3 and ApoE4 glia were obtained from P1 pups (N=4). Entire pup cerebral cortices were dissected in calcium and magnesium free Hank's Balanced Salt solution (HBSS). Tissue was digested in 10ml HBSS containing 0.25% trypsin (ThermoFisher Cat# 25200056) and .2mg/ml DNase in at 37 °C water bath for 10 minutes inverting the tube every 2-3 minutes. Dulbecco's Modified Eagle Medium nutrition mixture F12 (DMEM/F12, ThermoFisher Cat# 10565018) supplemented with 10% fetal bovine serum and 1x penicillin/streptomycin was added to inactivate trypsin and was gently pipetted up and down 10-15 times to dissociate tissue then allowed to sit at room temperature for 5 mins. Cells were filtered through a 40µm

strainers. Filtrate was centrifuged at 1200 RPM for 12 minutes. Media was removed and pellet was re-suspended in 10ml DMEM/F12 media. Cells were plated in T75 flasks coated with poly-D-lysine (100µg /ml) at 3 -4 pups per flask. Media was replaced the next day with 10ml DMEM/12F then 50% was replaced 3 days later. After 7 days, cells were harvested by replacing the media with 10ml 0.25% trypsin and incubating at 37°C for 3-4 minutes. Sides of the flask were gently tapped to dissociate glia and trypsin was inactivated with 10ml DMEM/F12. Cells were then centrifuged at 1200 RPM for 12 minutes after which media was removed and pellet suspended with fresh 10ml DMEM/F12 and counted. Glia were plated directly on top of 7 day old neurons at 25,000 cells per well. *Microglia* were independently harvested from T75 mixed glia flasks by gently tapping each side 40 times and collecting the media. Media was then centrifuged at 2300 RPM for 10 minutes and pellet re-suspended in fresh DMEM/F12 and plated in 24-well tissue culture plates over glass coverslips coated with 50 µg poly-D-lysine at a density of 250,000 cells per well with 1ml DMEM/F12 media.

2.4 Treatment with LXR agonist, GW3965

GW3965 was purchased from Sigma (Cas number 405911-17-3) and dissolved Dimethyl sulfoxide (DMSO) to a stock concentration of 5mM. Co-cultures were treated with GW3965 at a final concentration of 1µM or DMSO at the same time glia were plated on top the neurons (day 1) and then again 3 days later during a media change.

2.5 Immunocytochemistry

Cells were washed in phosphate buffer solution (PBS) once followed by fixation with 4% paraformaldehyde in PBS for 15 minutes on a rocker at room temperature. After 3 washes with PBS, cells were permeabilized in in 0.1% trition in PBS for 15 minutes followed by blocking with 5% goat serum in 0.1% trition/PBS for 30 minutes at room temperature. Cells were incubated in primary antibodies: Microtubule associated protein 2 (MAP2 1:500), Glial fibrillary acidic protein (GFAP, 1:500), ionized calcium-binding adapter molecule 1 (IBA1 1:500), and/or cluster of differentiation 68 (CD68, 1:500) at 4°C overnight. The next day cells were washed with 0.1% triton in PBS 3 times and incubated with secondary antibodies (1:1000) for 1hr on a rocker covered in aluminum foil. Cells were then washed in 0.1% triton in PBS 3 times 10 minutes each, once more in PBS and mounted on slides with Flouromount-G with DAPI (Invitrogen ref# 00495952). Images were taken on a Carl Zeiss confocal microscope (LSM 800).

2.6 qRT-PCR

Cells were pipetted into new RNAse free tubes (ThermoFisher cat# AM12400) and 1ml of TRIzol (ThermoFisher cat# 15596026) was added to each tube. Cells were homogenized by pipetting up and down 15-20 times then allowed to sit on ice for 5 minutes. After which, 1ml of chloroform was added to each tube followed by rigorous shaking. After centrifuging the samples for 15 minutes (12g at 4°C), the upper aqueous phase containing the RNA was placed into new RNAse free tubes and further isolated with 1ml isopropanol to precipitate the RNA. Isopropanol was removed and RNA was washed with 75% ethanol and allowed to air dry before suspending in 50µl RNAse free water and quantified with a nanodrop spectrophotometer.

RNA was reverse transcribed to cDNA using SuperScript III First-Strand Synthesis kit (Invitrogen cat# 1172050). Primers were designed from the National Institute of Biotechnology Information (NCBI) and purchased from Eurofins Genomics. Quantitive PCR and analysis of data was performed on BioRad IQ5 software and MyiQ thermocycler.

2.7 Enzyme-Linked Immunosorbent Assay (ELISA)

After 3 days, all culture media was collected and centrifuged at 1,500 rpm for 10 minutes at 4°C followed by an ELISA (Cayman Chemical #589201) to detect Tumor Necrosis Factor-α (TNF-α) levels in the supernatant. TNF-α mediates immune cell activation and cytokine secretion in monocytes/macrophages.

2.8 Statistics

All cell culture data was shown as mean (M) +/- standard error of mean (SEM). RT-qPCR data was shown as unscaled expression relative to GAPDH +/- SEM. ELISA data shown as pg/ml relative to the standard curve. Differences between groups were done as individual independent samples t-tests on Microsoft Excel 2017 version 15.35.

2.9 ImageJ Analysis

Fiji version 1.52b (http://imagej.net/Fiji) was used to determine MAP2 staining intensity. Each image was taken at 20x magnification totaling an area of 3e06 pixels per image. Image conversion formulas are located at https://imagej.nih.gov/ij/docs/menus/analyze.html.

3 Results

3.1 Mixed Glia Co-cultures Induces ApoE Isoform Dependent Neurotoxicity.

Mean MAP2 staining intensity is summarized in figure 1. The detrimental effect of ApoE3 and ApoE4 on neurodegeneration recapitulated previously published works (Shi et al., 2017). MAP2 is an established neuron-specific cytoskeletal protein marker and is generally considered to be the earliest histological indicator of neuronal pathophysiology. Loss of MAP2 positive cells is known to correspond with loss of neurons (Posmantur et al., 1996). Pixel intensity of images containing MAP2 was quantified via imagej and used as a proxy for neuronal cell loss. Quantification of MAP2 staining intensity via imagej revealed ApoE4 (1.973 +/- 0.265) and ApoE3 (5.724 +/- 1.562) co-cultures to be significantly different (P=<.05) with ApoE3>ApoE4. P301S expressing neurons, when cultured alone (12.35 +/- 0.254), showed significant preservation of MAP2 staining intensity when compared to ApoE3 (P=<.05) and ApoE4 (P=<.05). Thus, the degree of neurotoxicity between ApoE3 and ApoE4 co-cultures mimicked clinical observations in that carriers of the ε4 allele show more neurodegeneration (Namba et al., 1991). Furthermore, co-staining with DAPI revealed nuclear fragmentation and condensation within ApoE3 and ApoE4 co-cultures, indicative of cellular apoptosis or necrosis (data not shown). Generally, under healthy conditions, DAPI stained nuclei are spherical with DNA evenly distributed within the nucleus. (Crowley et al., 2016). ApoE3 or ApoE4 expressing

glia can be interpreted as accelerating neurodegeneration when compared to P301S tau expressing neurons cultured alone at the same time point. When co-culturing P301S tau expressing neurons with mixed glial cells harvested from ApoE3 or ApoE4 expressing mice, substantial neuronal cell death occurred. Whereas, P301S tau expressing neurons cultured alone showed the least neurotoxicity as visualized by MAP2 immunofluorescence (Fig. 1A). Co-culturing P301S tau-expressing neurons with ApoE expressing mixed glia led to reduced neurite arborization and shrunken soma characteristic of dying neurons (Fig. 1B-C).

3.2 Mixed Glia Co-cultures treated with LXR agonist GW3965 Induces Neuroprotection

Mean MAP2 staining intensity is summarized in figure 3. Microglial uptake of Aβ and cholesterol based cell debris is promoted by LXR stimulation. This effect has been shown to be accompanied by fewer and less activated microglia per plaque and absent in ApoEKO glia (Deane et al., 2008, Terwel et al 2011., Cantuti-Castelvetri et al 2018). Here we report, for the first time, LXR stimulation inducing neuroprotection from ApoE4 mediated toxicity. Fluorescent intensity of MAP2 revealed ApoE4 co-cultures treated with vehicle (1.865 +/- 0.447) and WT co-cultures treated with vehicle (6.2696 +/- 0.469) to be significantly different (P=<.05) with WT>ApoE4. In contrast, ApoE4 co-cultures treated with GW3965 (5.546 +/- 0.392) restored MAP2 intensity to WT levels. Interestingly, GW3965 treatment also significantly enhanced WT co-cultures MAP2 intensity (9.6895 +/- 0.916) with WT treated with GW3865>WT treated with vehicle. Activation of LXR induces the expression of genes directly responsible for cholesterol efflux from cells, including ABCA1 and APOE. This promotes the transfer of excess intracellular cholesterol to extracellular acceptors such as ApoE, thereby facilitating reverse cholesterol

transport. Thus, LXR stimulation can be interpreted as partially limiting ApoE4 mediated neurotoxicity when cultured with P301S tau expressing neurons. Whereas culturing P301S tau expressing neurons with ApoE4 expressing glia without treatment leads to drastic neuronal cell death (Fig.3C) which did not occur with WT glia (Fig.3A). Treating ApoE4 glia cultured with P301S tau expressing neurons with GW3965 leads to enhanced neurite growth and elaborate axonal branching (Fig.3B).

3.3 Microglia Co-cultures treated with LXR agonist GW3965 secretes reduced levels of TNF-α

Secreted TNF-α (pg/ml) analyzed from cell culture media is summarized in figure 3F. Media collected from ApoE4 microglia cultured with P301S tau expressing neurons for 3 days showed significantly higher TNF-α levels (750.18 +/- 210.6) compared to cultures treated with GW3965 (125.95 +/- 37.1). Cytokines such as TNF-α are expressed myeloid cells, including microglia, and are well characterized makers of inflammation. Baseline physiological TNF-α has been reported to be between 10-100pg/ml (Damas et al.,1989, Turner et al., 2010). However, resting microglia have been shown to secrete ~100pg/ml TNF-α at baseline. This rises to more than 400pg/ml after LPS treatment (Guadagno et al., 2013). The significantly higher TNF-α levels at ~700pg/ml indicates ApoE4 mediated inflammatory signaling. This drops to ~100pg/ml after GW3965 treatment indicating a tempered inflammatory response that may play a role in the neuroprotection observed in figure 3B.

3.4 Disease Associated Microglia Genes are Differentially Expressed in Microglia

Average fold change in DAM associated genes at baseline is summarized in figure 2 and after GW3965 treatment in figure 4. Activated microglia are associated with higher immune reactivity in neurodegeneration and DAM associated genes have been shown to be present in microglia localized around Aβ plaques, demyelinated neurons, and in aged microglia (Keren-Shaul et al., 2017; Krasemann et al., 2017., Hammond et al 2018). While DAM genes have been shown to be upregulated and differentially expressed in ApoE3 and ApoE4 microglia, the nature of this differential gene expression and how this alters microglial function is unknown. Getting a sense of how these genes are expressed in isolation and after GW3965 treatment may glean microglia susceptibility to CNS injury and reveal altered gene expression. Fold change gene expression by RT-qPCR revealed TREM2 expression in ApoE3 (1.912 +/- .0159) and ApoE4 (0.829 +/- .0187) to be significantly different ($P<.05$) with ApoE3>ApoE4. Furthermore, fold change in TREM2 expression in WT microglia (3.521 +/- 0.839) was found to be significantly greater than both ApoE3 and ApoE4 ($P<.05$, Fig. 2). Further experiments revealed TREM2 expression in ApoE4 microglia cultured with P301S tau expressing neurons to be significantly attenuated when treated GW3965 (30.54 +/- 1.09) when compared to cultures treated with vehicle (46.33 +/- 1.66). This lower expression of TREM2 after GW3965 treatment, in addition to lower TNF-α secretion, suggests a net anti-inflammatory effect by GW3965. Thus, neurotoxicity observed in figure 1 may be facilitated by an inherently lower expression of TREM2. Indeed, TREM2 deficiencies have been shown to reduce microglial/macrophage cytokine secretion and phagocytic capability in AD mouse models and stroke models. (Seiber et al., 2013, Jay et al., 2015, Linnartz-Gerlach et al 2018).

Cystatin F is encoded by CST7 and is one of the most upregulated DAM genes. Microglia deficient of CST7 show robust phagocytic activity and cystatins are the endogenous inhibitors of cathespins (lysosomal proteases) that are co-localized in the lysosome (Deng et al., 2001, Hamilton et al., 2008, Kang et al., 2018). When compared to ApoE3 (1.825 +/- 0.234), ApoE4 has a significantly lower fold change in CST7 expression (0.5657 +/- .00156, $p<.05$). Intriguingly, however, WT CST7 expression was found to be relatively absent (.004118) and significantly less than both ApoE3 and ApoE4 ($P<.05$). Cystatin F was recently reported to accumulate in DAMs localized around Aβ and the exclusive expression of CST7 in resting ApoE4 microglia may degrade the endogenous cathepsins responsible for lysosomal degradative capacity. Indeed, ApoE4 expressing cells demonstrate diffuse co-localization of cathespins with lysosomes and cathespin KO macrophages demonstrate deficient neutrophil recruitment and IL-1β secretion when activated by cholesterols (Duewell.,et al 2010, Ofengeim et all.,2017; Lee et al., 2018). Thus, increased CST7 expression in resting ApoE4 microglia may cause lysosomal deficiencies that result in hyperinflammation. The net anti-inflammatory effect of GW3965 may represent a correction in lysosomal integrity that preserves degradative function.

Intriguingly, Clec7a, also known as dectin 1, localizes around Aβ plaques specifically on DAMs (Kraseman et al., 2017) and have an ApoE Isoform dependent expression pattern that is absent in an ApoEKO (Shi et al., 2017). Fold change expression of Clec7a by qRT-PCR revealed ApoE3 (1.869 +/- 0.099) and ApoE4 (0.619 +/- 0.250) to be significantly different ($p<.05$).

3.5 ApoE4 Expressing Glia Co-Cultured with Tau Expressing Neurons Induced Morphological Changes in Microglia.

Glia expressing ApoE4 co-cultured with tau expressing neurons revealed distinct morphological changes in microglia characteristic of a phagocytic phenotype. The schematic diagram in figure 5A is a simplified representation of the morphological change microglia undergo after activation. Microglia expressing ApoE4 exhibited shortened and rounder processes with an overall amoeboid shape, characteristic of reactive microglia (Fig.5B, 5C). Reactive microglia accumulate at the site of injury (Giordana et al., 1994) where they play a neuroprotective role phagocytosing damaged cells and debris. When co-cultured with WT neurons, however, microglia showed elongated and ramified processes (Fig. 5D,5E). Ramified microglia are considered to be inactive under physiological conditions, although they, also express phagocytic marker CD68 albeit at lower levels (Graeber et al., 1989; Booth & Thomas 1991; Thomas 1992; Slepko & Levi 1996).

4 Discussion

Microglia in AD are emerging as disease modifying sources of ApoE that, under pathological conditions, express a unique set of proinflammatory genes that could potentially differentiate its phagocytic capabilities as protective or detrimental. Recent studies have confirmed that DAMs co-localize around Aβ plaques and myelin debris. This transition from "homeostatic" to phagocytic is TREM2 dependent and thought to encompass all necessary transcriptional changes to protect the brain from insult. (Keren-Shaul et al., 2017; Krassemann et al., 2017). A subsequent study confirmed that neurodegeneration related DAM genes are

almost exclusively expressed in microglia in PS2App, 5XFAD, P301L and P301S models of AD (Friedman et al., 2018). It is not clear based on these studies the extent that ApoE4 corrupts microglial function however, loss of lysosomal integrity has been seen in both ApoE4 expressing and LXR deficient cells. The study presented here confirms that ApoE is capable of inducing isoform dependent neurodegeneration and reveals, for the first time, that LXR activation can partially attenuate ApoE4 mediated neurodegeneration.

Several studies have revealed DAM associated genes to be upregulated in pathological conditions (Shi et al., 2017; Keren-Shaul et al., 2017; Krassemann et al., 2017; Lee et al., 2018) and TREM2 expression as protective and necessary for Aβ clearance (Wang et al., 2015; Lee et al., 2018). The current experiments reveal, for the first time, baseline expression of TREM2 in ApoE3 and ApoE4 expressing microglia and after GW3965 treatment in pathological conditions. Interestingly, TREM2 expression was lower in an isoform dependent manner (ApoE3>ApoE4) at baseline. Considering increased TREM2 gene dosage clears Aβ more efficiently (Lee et al., 2018), these results suggest that ApoE3 and ApoE4 expressing microglia are already primed to have a weakened or slower response to Aβ or other damage. Indeed, a study by Li-Huei Tasi and colleagues (2018) confirmed that human induced pluripotent stem cell derived microglia expressing ApoE4 have slower uptake of Aβ thus may be a consequence of inherently lower TREM2 expression. On the other hand, TREM2 expression after GW3965 treatment may represent a normalized immune response considering the corresponding increase in neuronal survival and attenuated TNF-α secretion observed in figures 3D and 3F. Indeed, activated microglia have been reported to secrete ~400pg/ml TNF-α after LPS treatment (Guadagno et

al., 2013). In this study, ApoE4 expressing microglial co-cultures secreted >700pg/ml TNF-α which may correspond to hyperinflammation and the activated microglial morphology observed in figures 5C and 5E while untreated cultures secreted ~100pg/ml TNF-α.

LXR stimulation has been shown to promote re-myelination, increase phagocytosis and reduce inflammation (Koldamova et al 2004, Fitz et al., 2010, Cantuti-Castelvetri et al., 2018). The net anti-inflammatory effect of GW3965 here is the first to be shown in ApoE4 expressing glia. Microglia normally accumulate lipids during phagocytosis. LXR stimulation ensures lipid efflux from cells by promoting expression of ABCA1 and ApoE, while at same time, reducing lipid uptake by downregulating ApoE receptors such as LDLR on microglia (Zelcer et al 2009, Saijo & Glass., 2011). Indeed, lowering microglial lipid levels been shown to promote Aβ transport to lysosomes and LXRKO microglia have shown lipid accumulation and loss of lysosomal integrity. (Lee et al., 2011, Simon et al., 2018). Thus, the net-anti-inflammatory effect here may represent an LXR mediated sequestration of lipids via gain of function overexpression of ABCA1 and ApoE, while simultaneously, downregulation of lipid uptake via LDLR on microglia. Considering, LDLRs take up lipidated ApoE via endocytosis, down regulation of this receptor may allow microglial lysosomes to sufficiently process lipid based cells debris.

Unexpectedly however, CST7 was present in resting ApoE3 and ApoE4 expressing microglia (fig 2). CST7 is a highly upregulated DAM associated gene that strongly correlates with TREM2 and encodes for the known lysosomal protease inhibitor, cystatin F. (Hamilton et al., 2008, Kang et al., 2018, Lee et al., 2018). Importantly, cystatins are the endogenous inhibitors

of lysosomal degradative enzymes (cathespins) and are co-localized together in the lysosome (Deng et al., 2001). Consistent with previous findings, CST7 expression was absent in WT microglia, however, the presence of CST7 in ApoE3 and ApoE4 microglia is indicative of an activated state despite not being in any pathological condition. This finding would suggest that these microglia are aberrantly activated with cystatin F present in the lysosomes and studies have reported ApoE4 expressing microglia have a partially activated morphology while fully activated microglia accumulate cystatin F in the lysosomes. (Ofengeim et al., 2017, Lin et al., 2018). Its not fully understood if lysosomal cystatins are disruptive of microglial immune capacity but siRNA knockdown of CST7 has led to increased phagocytic activity in microglia, suggesting that cystatin F induction in microglia may contribute to decreased phagosomal/lysosomal integrity and lipid accumulation in lysosomes (Kang et al., 2018).

In addition to regulating lipid efflux by promoting expression of ABCA1 and ApoE, LXR exerts control of lipid uptake via LDLR. Upon ApoE binding, LDLR initiates endocytosis of lipoprotein particles where cholesterol is hydrolyzed to free cholesterol or transported to lysosomal cathespins which play multiple and essential roles in lysosomal degradation (Maxfield F. R. & Wüstner D., 2002, Maxfield & Mondal., 2006). It has been reported that GW3965 inhibits the binding and uptake of lipoproteins at LDLR and such reductions of cellular lipid uptake has been reported to promote lysosomal degradation of Aβ. Partially depleting microglial cholesterol content with the known cholesterol chelator, methyl-β-cyclodextrin (MβCD), was shown to promote Aβ transport to lysosomes. Further experiments promoted cholesterol accumulation by inhibiting trafficking of late endosomes to lysosomes with the

Niemann-Pick C-1 protein blocker U18666A. Live cell imaging of microglia primed with accumulated cholesterol revealed slower delivery of fluorescent Aβ to lysosomes. (Lee et al., 2012). Earlier work revealed that ApoE deficient macrophages accumulate lipids in lysosomes and, importantly, have reduced levels of lysosomal cathespins. Mice lacking ApoE fed with a high fat diet also demonstrated lipid accumulation in macrophages and reductions in lysosomal cathespins (Wu et al., 2007). Thus, it appears that lipid accumulation does impact lysosomal function. Given that 70% of all brain cholesterol is in myelin, lipid homeostasis in a degrading brain may be thwarted by loss of lysosomal degradative capacity or inhibition of lysosomal cathespins potentially by high expression of cystatins such as CST7. Indeed, the experiments here have significantly higher expression of CST7 in ApoE3 and ApoE4 microglia which is absent in WT and future experiments should interrogate cathespin content in ApoE4 microglial lysosomes (Dietschy et al., 2004, Leoni et al., 2010).

These experiments are the first to show that regulators of cholesterol homeostasis can drastically induce neuroprotection and regulate immune responses in ApoE4 expressing glial/neuronal co-cultures. The effects of LXR on cholesterol homeostasis may act on immune processes by minimizing cholesterol uptake to lysosomes that already have a cathepsin/cystatin imbalance. Cystatins and cathepsins are co-localized in lysosomes and cystatin F is a specific inhibitor of cathepsin C. Cystatin F overexpression has led to suppression of cathepsin C activity and cystatin F KOs have led to activated microglia and increased phagocytosis (Deng et al., 2001, Adkison et al., 2002; Hamilton et al., 2008). The high expression of cystatin F and low expression of TREM2 in figure 2 would suggest a decrease in phagocytic capability which would

be consistent with previous reports describing deficient ApoE4 microglial responses to Aβ and TREM2's control of microglial activation (Keren-Shaul et al., 2017; Krassemann et al., 2017, Lee et al., 2018, Lin et al., 2018). The neurodegeneration observed in figure 3 may represent dysfunctional microglial responses that result from lysosomal cathepsin/cystatin imbalance.

The importance of lysosomal cathespins in immune responses was underscored in experiments where macrophages lacking cathepsins had deficient IL-1β secretion and neutrophil recruitment after cholesterol treatment. Cells expressing ApoE4 have shown isoform specific lysosomal and cathepsin dysfunction. Neuro2a cells expressing ApoE4 have shown lysosomal activation and leakage and high rates of apoptosis after Aβ treatment. Further invitro studies revealed ApoE4 expressing neurons to have enlarged lysosomes with diffuse co-localization of cathepsins, and enhanced Aβ accumulation. (ji et al 2006, Belinson et al., 2008, Duewell.,et al 2010). The apoptotic consequences of lysosomal leakage are well characterized and are primarily carried out by lysosomal cathepsins that are translocated to the cytosol (Aits & Jäättelä 2013). Future experiments should determine if the neuroprotective effect of GW3965 is mediated by reduced LDLR uptake of lipoproteins bound for the lysosome. Further experiments should determine if the slow uptake by ApoE4 microglia is mediated by reduced cathepsin activity by cystatins and if this directly results in lysosomal enlargement or leakage.

Cytosolic antioxidants have anti-apoptotic properties and thioredoxin-1 (Trx1) is known to negativity regulate apoptosis by interacting with apoptosis signal regulating kinase 1 (ASK1).

Cathepsin D normally resides within lysosomes but can translocate to the cytoplasm under stress conditions where its known to degrade Trx1. (Saitoh et al., 1998, Zhang et al,.2018). Cells expressing ApoE4 have shown isoform specific lysosomal enlargement/leakage, lack of cathepsin co-localization and high expression of their inhibitors-cystatins (ji et al 2002, 2006, Belinson et al., 2008, Duewell.,et al 2010, Kang et al., 2018). A recent study confirmed that treating primary human neurons and SHSY-5Y cells with ApoE4 can induce lysosomal leakage of cathepsin D and, importantly, trigger apoptosis specifically when ApoE4 is present. Additionally, treatment with ApoE4 led to diffuse co-localization of cathepsin D and the classic lysosomal marker, LAMP2. Both SHSY-5Y cells and neurons demonstrated compromised lysosomal membrane integrity and diminished levels of Trx1 in the cytosol according to western blot. Importantly, this was not followed by a reduction is Trx1 RNA and inhibiting cathepsin D preserved Trx1 levels and neuronal survival. Human studies on brain tissue have shown cathepsin accumulation and decreased Trx1 levels (Cataldo et al., 1997, Persson et al., 2017).

Microglia are the principle responders to CNS damage and any alterations their protective capacity can have significant and detrimental disease outcomes. These experiments demonstrated that ApoE3 and ApoE4 glia are capable of inducing significant neurodegeneration that is attenuated with LXR stimulation. The microglia in these experiments demonstrated altered gene expression that indicates deficient response capacity and future experiments should determine the maladaptive consequences this altered gene expression and how it leads to microglial inflammation. Lysosomal rupture may result from accumulation of CNS debris caused by cystatin mediated inhibition of critical degradative cathepsins. ApoE4 mediated

neurodegeneration may result from spillage of these cathepins into the cytosol where they breakdown critical antioxidants resulting in microglial necrosis. Future experiments should determine if LXR stimulation attenuates uptake of lipoproteins destined for microglial lysosomes and if this acts to mitigate lysosomal debris accumulation and lysosomal rupture. Importantly, further studies should recapitulate the findings in this study in vivo by treating AD/ApoE mice with an LXR agonist and assessing the degree of brain volume loss, cognitive dysfunction, microglial activation, and microglial gene expression.

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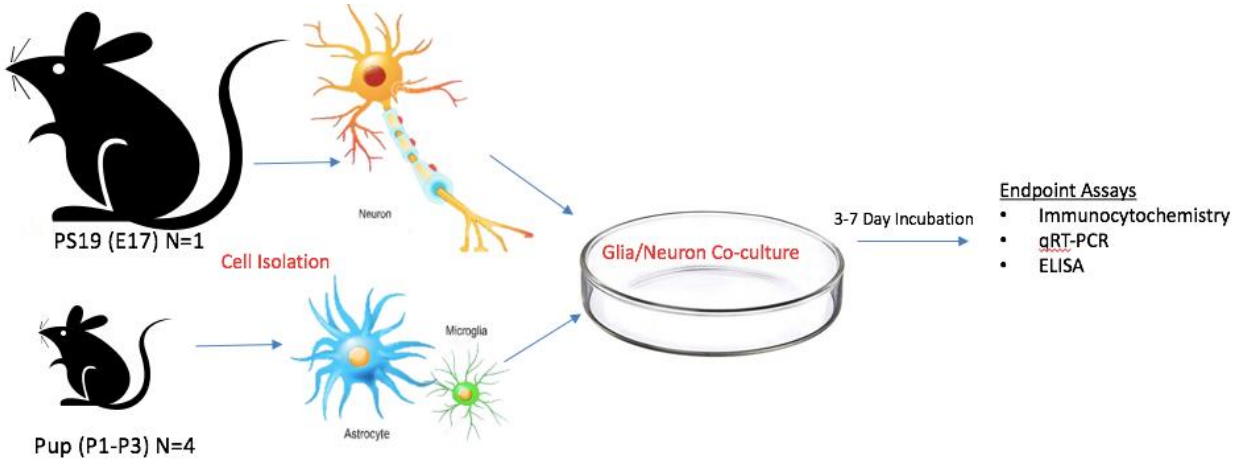

Diagram 1: Schematic diagram showing the isolation of primary neurons/glia from adults and pups respectively. Cells were then combined at a 1:8 ratio and incubated for 3-7 days followed by appropriate biochemical assays (ICC,qRT-PCR,ELISA).

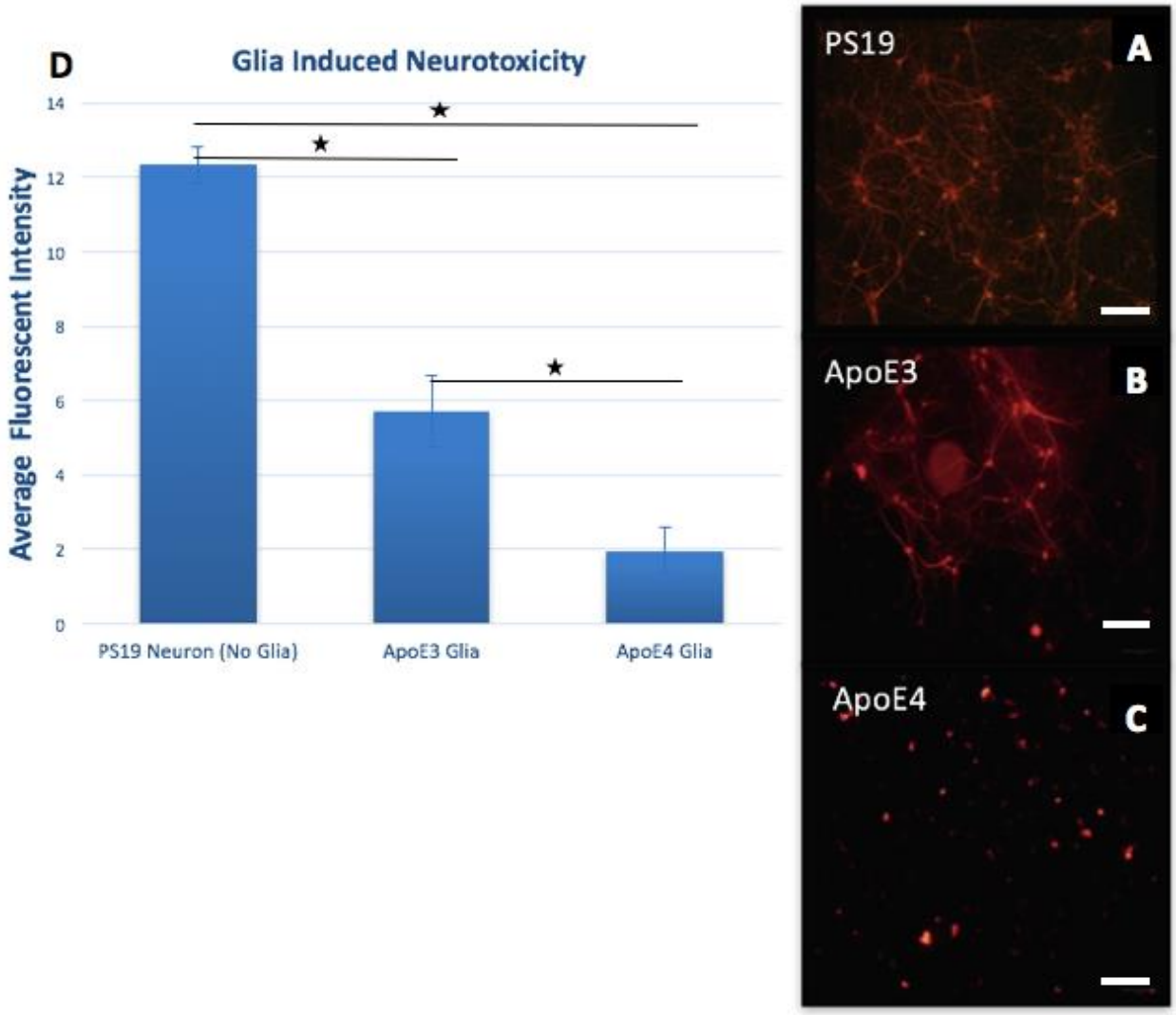

Figure 1: PS19 neurons collected from E17 fetuses co-cultured with ApoE3 and ApoE4 expressing mixed glia from P1pups leads to robust neuronal death. A-C: Representative images of primary P301S neurons (200,000) co-cultured alone (A) with ApoE3 (B) or ApoE4 (C) mixed glia (25,000) for 7 days. D: Quantification of MAP2 (red) staining intensity for co-cultures (80-90% astrocytes, 10-20% microglia). Scale bar=100μm.

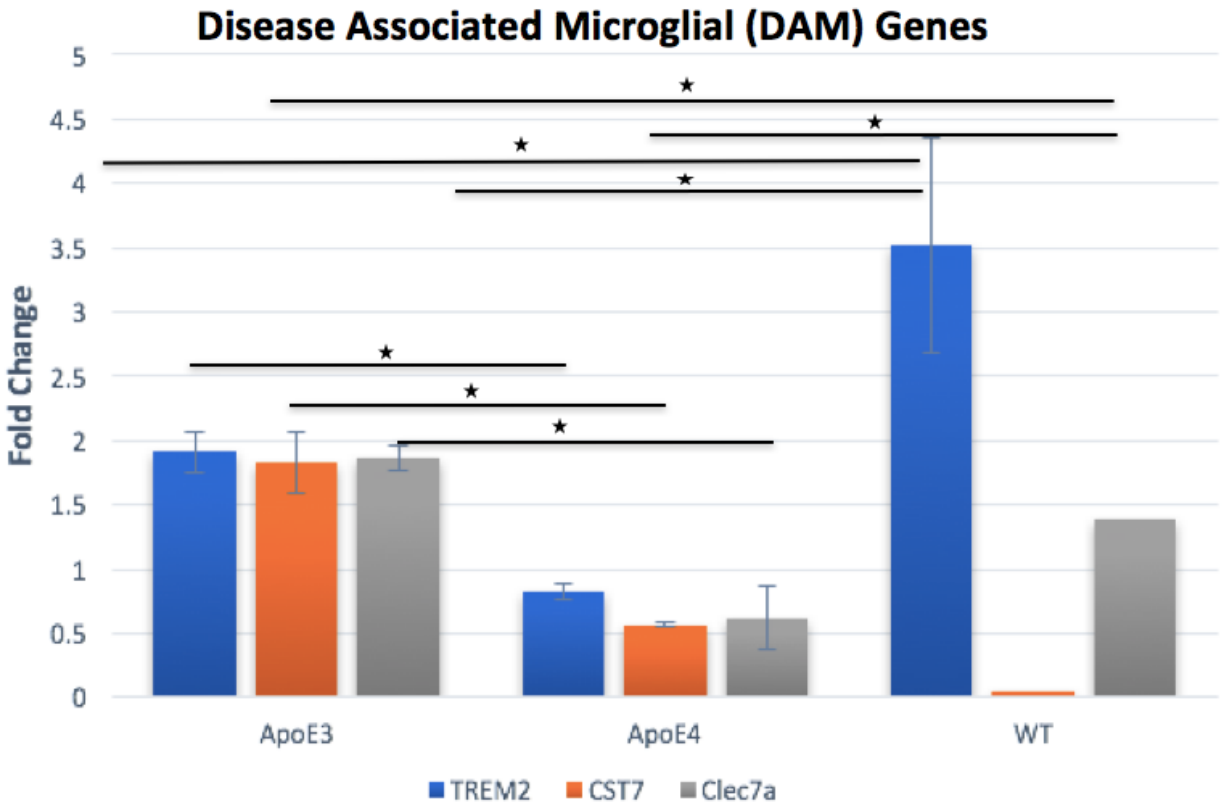

Figure 2: ApoE isoform dependent control of disease associated microglia (DAM) genes. RT-qPCR of selected DAM genes relative RNA (fold change) from primary microglia collected from P1 pups normalized to GAPDH (not shown).

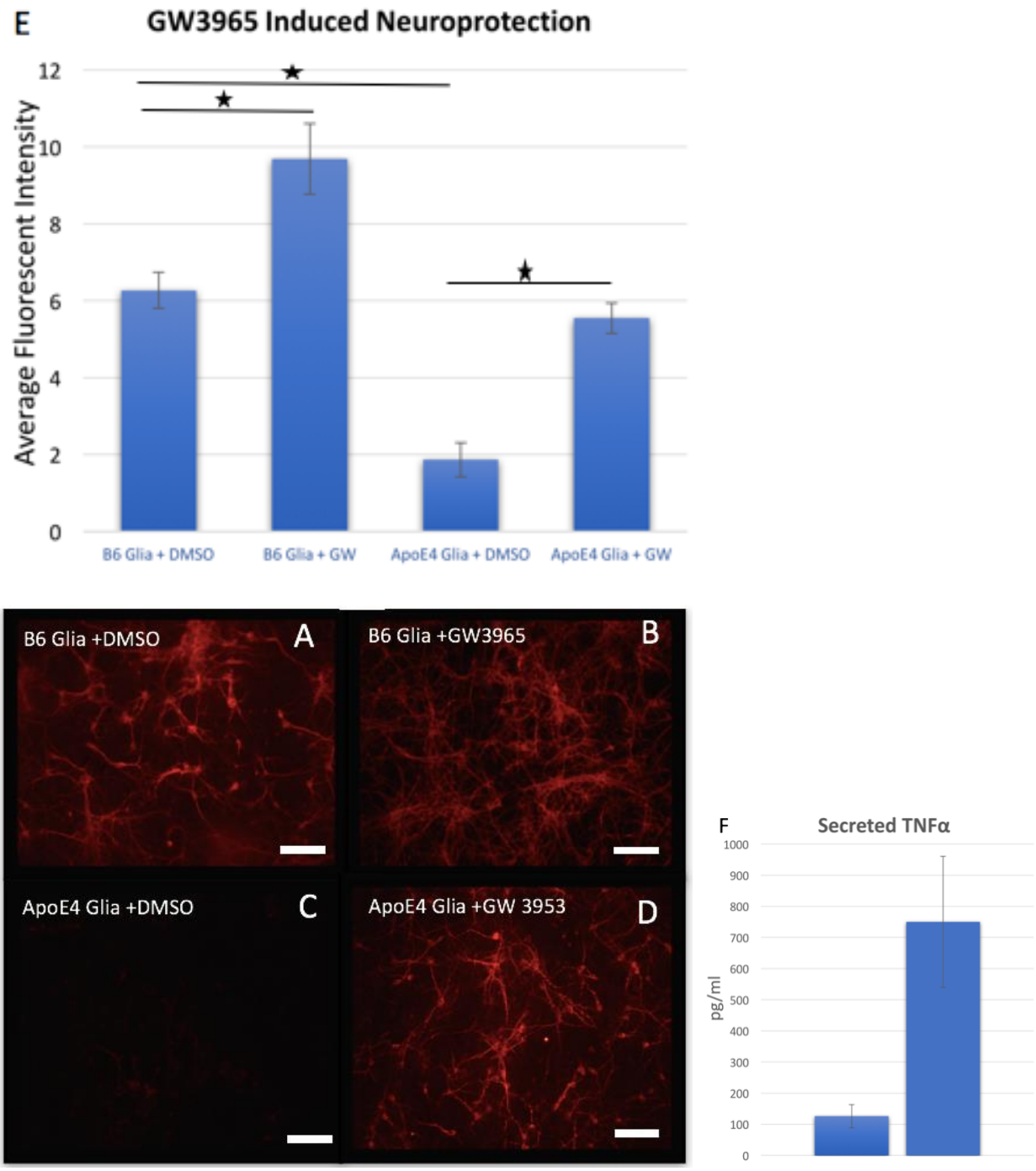

Figure 3: PS19 neurons collected from E17 fetuses co-cultured with ApoE4 expressing mixed glia treated with GW3965 (1μM) from P1pups leads to neuroprotection. A-D: Representative images of primary P301S neurons (200,000) co-cultured with WT mixed glia (25,000) treated with vehicle (A) with WT mixed glia treated with GW3965 (B) with ApoE4 mixed glia treated with vehicle (C) or with ApoE4 mixed glia treated with GW3965 (D) (25,000) for 3 days. Secreted Tumor Necrosis Factor-α collected from P301S neuron media co-cultured with ApoE4 microglia for 3 days (F). E: Quantification of MAP2 (red) staining intensity for co-cultures (80-90% astrocytes, 10-20% microglia). Scale bar=100μm.

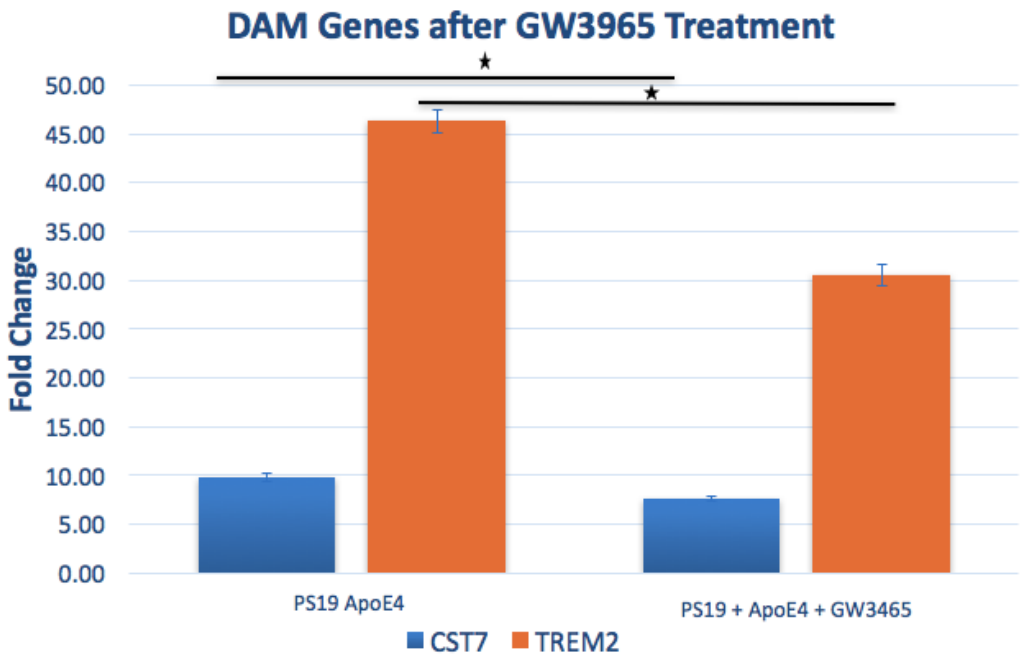

Figure 4 ApoE isoform dependent control of disease associated microglia (DAM) genes. RT-qPCR of selected DAM genes relative RNA (fold change) from primary microglia collected from P1 pups normalized to GAPDH (not shown).

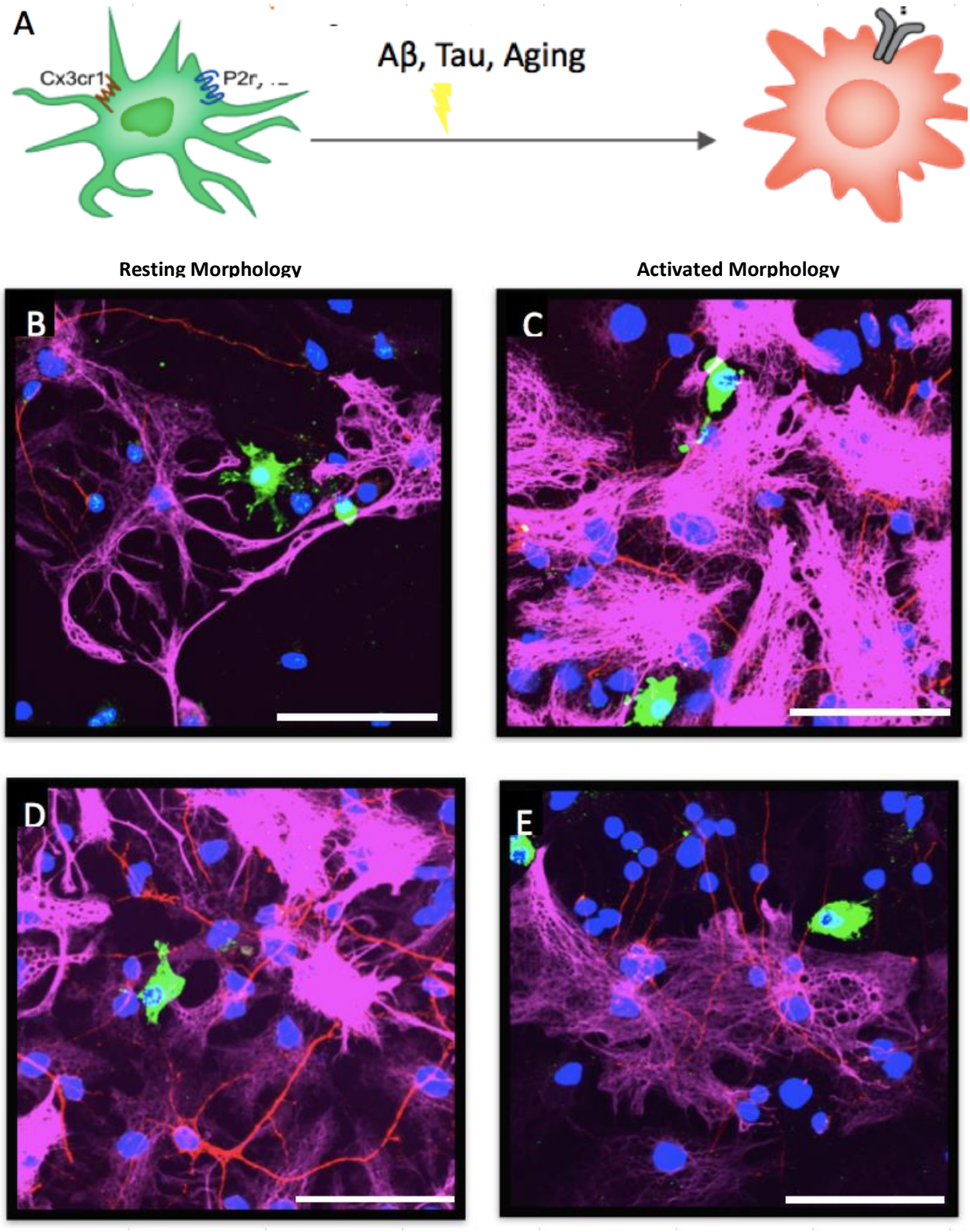

Figure 5: Altered microglia morphology in ApoE4 expressing microglia collected from P1 pups co-cultured with P301S expressing neurons from E17 fetuses, (B) Representative images of WT neurons (200,000) co-cultured with ApoE4 expressing glia (50,000) stained with IBA1 (green),

GFAP, (pink), MAP2 (red) for 3 days. (C) Representative images of PS19 neurons (200,000) co-cultured with ApoE4 expressing glia (50,000) stained with IBA1 (green), GFAP, (pink), MAP2 (red) for 3 days. (D) Representative images of WT neurons (200,000) co-cultured with ApoE4 expressing glia (50,000) stained with CD68 (green), GFAP, (pink), MAP2 (red) for 3 days. (E) Representative images of PS19 neurons (200,000) co-cultured with ApoE4 expressing glia (50,000) stained with CD68 (green), GFAP, (pink), MAP2 (red) for 3 days. Scale bar=30μm.