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UNIVERSITY OF CALIFORNIA SAN DIEGO

Human microglia from APOE4 and APOE3 patient iPSCs: energy and cholesterol metabolism

A Dissertation submitted in partial satisfaction of the requirements
for the degree Doctor of Philosophy

in

Biology

by

Jasmin Siri Revanna

Committee in charge:

Professor Fred Gage, Chair
Professor Nicola Allen
Professor Nicole Coufal
Professor Christopher Glass

2024

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University of California San Diego

2024

DEDICATION

I would like to dedicate this work to the individuals who donated their samples to allow this study to be conducted. To the late Dr. Eduardo Macagno, who served as my mentor, supporter, and evaluation head of my thesis committee. To my family and friends who have shaped who I am today, allowed me room to grow, and stayed close when I needed the support.

TABLE OF CONTENTS

DISSERTATION APPROVAL PAGE iii

DEDICATION iv

TABLE OF CONTENTS v

LIST OF FIGURES vi

LIST OF TABLES vii

LIST OF ABBREVIATIONS viii

ACKNOWLEDGEMENTS ix

VITA xi

ABSTRACT OF THE DISSERTATION xiii

INTRODUCTION 1

Chapter 1: APOE4 microglia exhibit metabolic impairments due to lysosomal cholesterol ester accumulation 7

Chapter 2: Modeling Alzheimer's Disease Using Human Cell Derived Brain Organoids and 3D Models 45

LIST OF FIGURES

Figure 1.1. Graphical Abstract……7

Figure 1.2. iMGs show heterogeneity in 2D culture similar to that of other *in vitro* and *in vivo* mouse and human microglia……13

Figure 1.3. APOE4 influences state shifts in microglia, reducing the DAM signature……16

Figure 1.4. APOE4 microglia exhibit an increased population of G2 arrested cells……...19

Figure 1.5. APOE44 microglia are more proinflammatory and shift to glycolysis…….....22

Figure 1.6. APOE44 microglia have an accumulation of ChEs in the lysosome……25

Figure 1.7. LXR agonist GW3965 partially rescues APOE44 phenotype.……....28

Figure 2.1. Schematic diagram illustrating the generation of brain organoid models of AD……82

Figure 2.2. Schematic diagram outlining AD related cellular pathology and possible readouts to measure pathological effects……....83

Figure 2.3. Healthy and diseased microglial processes implicated in AD which may be modeled in brain organoids. ……84

LIST OF TABLES

Table 2.1. Overview of primary literature using brain organoids to investigate AD and AD-related pathological features……79

LIST OF ABBREVIATIONS

AD	Alzheimer's disease
iMG	induced microglia
APOE	Apolipoprotein E
iPSC	induced pluripotent stem cell
scRNAseq	single cell RNA sequencing
DAM	disease associated microglia
LDAM	lipid droplet accumulation microglia
CRM	cytokine response microglia
RM	ribosomal response microglia
ARM	activated response microglia
HLA	human leukocyte antigens
MAM	mitochondria associate membrane
ER	endoplasmic reticulum
ROS	reactive oxygen species

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Chapter 2, in full, is a reprint of the material as it appears in Frontiers Neuroscience. Fernandes, S., Revanna, J., Pratt, J., Hayes, N., Marchetto, C., Gage, F. (2024). Modeling Alzheimer's Disease Using Human Cell Derived Brain Organoids and 3D Models. Front. Neurosci. 18:1434945. The dissertation author was the secondary investigator and author of this paper.

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ABSTRACT OF THE DISSERTATION

Human microglia from APOE4 and APOE3 patient iPSCs: energy and cholesterol metabolism

by

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Doctor of Philosophy in Biology

University of California San Diego, 2024

Professor Fred Gage, Chair

Microglia are known as the guardians of the brain, acting as either an angel or an assassin. These brain resident macrophages serve as the brains very own immune system, phagocytosing debris and pruning neuronal synapses. A major focus in studying neurodegenerative diseases has been placed on neuronal compartments, often deemphasizing immunological mechanisms. Much evidence points towards

microglia playing a role in these diseases, for example the number of GWAS (genome wide association studies) hits in Alzheimer's disease. One of these hits is the apolipoprotein E (APOE), with the ε4 allele correlated with the highest risk and the ε2 allele to reduced risk, and ε3 allele being neutral. Using human cells to model human disease is crucial, as studies have shown numerous microglial differences between species. Here, I utilize a protocol to convert patient derived induced pluripotent stem cells (iPSCs) into induced microglia (iMGs) from 22 individuals to study how the APOE4 haplotype impacts microglial function. By studying microglia *in vitro* without perturbations, we can begin uncovering the cell autonomous roles APOE4 plays in microglia. By performing single cell RNA sequencing (scRNAseq), we uncover how APOE4 shifts the transcriptomic state of microglia away from disease associated microglia (DAM) and to include a senescent-like phenotype. This is reflected in the cellular bioenergetics and the lipid content of APOE4 microglia. We find that lysosomes in APOE4 microglia have an accumulation of cholesterol esters and using a liver x receptor (LXR) agonist, we are able to rescue these phenotypes, by reducing cholesterol esters and inducing autophagy. This study highlights the importance of stratifying patient populations in Alzheimer's, as the mechanisms at play are different for APOE4 carrier. Finally, we highlight the importance of iPSC-based models in studying Alzheimer's in a review. We discuss the potential of using human brain organoids to study disease and specifically how incorporating microglia into these models could help further uncover how microglia impact other cell types.

INTRODUCTION

Alzheimer's disease (AD) is the most common type of dementia, accounting for 6.2 million cases worldwide. It is projected that by 2050, this number will double. The greatest known risk factor is advanced age, with the vast majority of those diagnosed being over the age of 65 years old ("2023 Alzheimer's disease facts and figures," 2023). In the field of neuroscience, neuronal cells have been the focus of understanding neurodegenerative diseases, such as AD. However, about half of the cells in the brain are glial cells – more specifically, ~10-15% of cells in the brain are microglia (Lawson et al., 1992). Microglia are specialized macrophages of the central nervous system that help to clear debris, pathogens, and neuronal synapses. Under homeostatic conditions, microglia provide support to neurons, while under pathological conditions, microglia often prune synapses excessively, increase inflammation, and reduce support to neurons (Wolf et al., 2017). In Alzheimer's disease (AD), microglia activation was thought to be triggered by amyloid deposits and dystrophic neurites, but recent genome-wide association studies have identified that many AD risk loci are found in or near genes unique or highly expressed in microglia (Verheijen and Sleegers, 2018). This leads to the notion that microglia may be critical in the early development of AD and could be potential targets for therapeutics. It is still unclear whether microglia activity in AD is detrimental or beneficial, or even both.

Apolipoprotein E (APOE) haplotype is the major genetic risk factor for the development of AD, with the ε4 allele correlated to increased risk and ε2 allele to reduced risk (Michaelson, 2014). In white individuals homozygous for APOE4, risk is increased by 15-fold and in East Asian population 33-fold; African Americans and

Hispanic populations exhibit lower risk ranging from 1 to 5 fold increases. Albeit the known association between APOE4 and AD, currently it is unknown how APOE4 haplotype influences AD onset and progression or how APOE2 provides protection. In the brain, APOE is predominantly expressed in glia cells and there is increasing evidence that APOE4 glia cells exhibit differences in comparison to their isogenic APOE3 counterpart (Tcw et al., 2022). Recently, Chen et al. showed that APOE4/Tau mice models experience detrimental brain atrophy compared to APOE3/Tau mice, and that depleting microglia and/or T cells robustly reduces this atrophy (Chen et al., 2023). It is unclear in this study whether the T cells or the microglia are the main contributors to this atrophy, or if there is a synergistic effect.

Microglia have a complex role in neurodegenerative diseases, like AD, partly due to their numerous activation pathways. Highly ramified microglia transform to the activate amoeboid-shaped form, while those lacking branching in aged brains have a difficult time surveilling their surroundings (Norden and Godbout, 2013). In AD, it's been shown that microglia that are close to amyloid plaques can change morphologically, whereas microglia plaque-distant showed minor changes overtime (Plescher et al., 2018). Transcriptomic studies in AD mouse models have revealed gene expression differences in microglia paralleling the progression of the disease (Keren-Shaul et al., 2017). While there is overlap between mouse and human microglia profiles, there is undoubtedly differences, such as an upregulation of CD58, ERAP2, GNLY and S100A12 in human microglia (Galatro et al., 2017; Keren-Shaul et al., 2017). Similar differences arise when analyzing the RNA profiles of AD human brains in comparison to AD mouse models (Friedman et al., 2018; Yin et al., 2017). Together, these findings

demand careful interpretation when analyzing RNA profiles from animal models, as well as bulk tissue. To overcome these obstacles, in Chapter 1, I employ an established protocol with slight modifications to generate induced microglia cells (iMGs) from AD patient iPSCs and age-matched controls with different APOE haplotypes (14 AD/8 CTLR; 5 APOE2/3, 6 APOE3/3, 7 APOE3/4, 4 APOE4/4) (Abud et al., 2017). Transcriptomic analysis of these cells helps us identify the cell autonomous effects APOE4 has on microglia function and disease progression.

We provide a transcriptomic data set of iPSC derived microglia from different APOE haplotypes, allowing us to investigate the impact APOE has on state shifts. We observed APOE44 microglia represent a smaller population of the DAM population compared to APOE33, suggesting DAMs are a protective population. We suggest DAMs have high TGs, while another population we have identified as G2-arrested are high in cholesterol esters. This senescent-like population is enriched in APOE4 microglia and contains high cholesterol ester content. Because of the downregulation of lysosome cholesterol transport genes, we believe APOE44 microglia accumulate cholesterol esters in their lysosomes, therefore reducing the amount of cholesterol transported to the endoplasmic reticulum (ER). Studies have shown that cholesterol is crucial at the mitochondria associated membrane (MAM) space, which tethers the ER and mitochondria (Fujimoto et al., 2012; Harned et al., 2023). It has been shown that increasing this MAM space leads to dysfunction mitochondria producing less ATP, and therefore shifting the system towards glycolysis and producing more lactate (Bassot et al., 2023). We observe APOE44 microglia have reduced ATP-linked respiration and a

shift towards glycolysis, which supports our idea that APOE44 microglia have ER stress due to a reduction of cholesterol being transported to the ER.

We show that an LXR agonist GW3965 rescues the defects seen in APOE44 microglia, including reducing lactate levels and increasing mitochondria membrane potential. It has been suggested that LXR activation is able to rescue APOE44 microglia because of its upregulation of cholesterol efflux regulatory proteins, like ABCA1 (Litvinchuk et al., 2024). It is unclear if ABCA1 regulation would impact the efflux of cholesterol esters, so it may be possible that the rescue we see is related to the upregulation of autophagy genes with GW3965 treatment. By increasing autophagy, we might be able to rid the microglia of the lipid accumulated lysosomes. We believe the effects of LXR agonism is microglia needs to be further investigated to understand the exact mechanism leading to rescue.

The findings from this study elucidate aspects of microglial contribution to AD, potentially offering new therapeutic avenues for treatment. Currently, there are no effective AD drugs on the market and the recently approved drugs Aducanumab and Lecanemab increased incidence of ARIA-E in individuals carrying APOE4 (Honig et al., 2023; Salloway et al., 2022). ARIA-A is characterized by amyloid-related imaging abnormalities involving the breakdown of the tight endothelial junctions of the blood-brain barrier and subsequent accumulation of fluid. Results like this convey the importance of understanding APOE4's contribution to AD better so that we can address AD from a mechanistic and individualistic point of view.

In Chapter 2, I summarize the potential of modeling AD and other diseases using iPSC derived brain organoids and the importance of incorporating microglia into these

systems. Microglia have been introduced into brain organoids, allowing us to capture a more homeostatic microglia state. In 2D culture, microglia are prone to activation and cannot be maintained long term, even in co-culture systems (Timmerman, Burm and Bajramovic, 2018). Abud et al. were the first to incorporate hiPSC-derived microglia into hiPSC 3D brain-organoids (BORGs), which contain neurons, astrocytes, and oligodendrocytes. Schafer et al. developed a different approach and found that infiltrating EMPs, generated using protocols from Abud et al., into forebrain organoids and then subsequently transplanting them to immunodeficient mice, allowed the EMPs to convert to microglia, taking on a more homeostatic morphology and transcriptional state when compared to *in vitro* microglia (Schafer *et al.*, 2023)(Abud et al. 2017). Most recently, Park et al. infiltrated iPSC-derived primitive macrophages (iMacs) into cerebral organoids. After infiltration, iMacs expressed microglia markers such as P2RY12, CX3CR1, and SALL1 at a higher level compared to in vitro iMacs (Park *et al.*, 2023). Notably, these iMacs (called iMicro once infiltrated) did not express microglia marker TMEM119, which urges a comparison to existing microglia protocols to ensure these iMacs do fully convert to microglia. This study concluded that iMicro are enriched with PLIN2+ lipid droplet that export cholesterol, which were taken up by neural progenitor cells (NPCs). They found that organoids with iMicros were reduced in size and cell number, due to reduced proliferation of NPCs, which they suggest is a consequence of iMicro protecting against excessive growth. This study highlights the importance of cholesterol transport in development, potentially via APOE. While the direct study of microglia in AD organoids is limited, Haney et al. recently showed that conditioned media from lipid droplet high APOE44 microglia, compared to lipid droplet low, induced

Tau phosphorylation and apoptosis in neurons (Haney *et al.*, 2024). Together, this prompts the field to investigate the role microglia plays in cholesterol transport to other cell types and how this pathway is altered in AD, which could be further elucidated with microglia-rich organoids.

Chapter 1: APOE4 microglia exhibit metabolic impairments due to lysosomal cholesterol ester accumulation

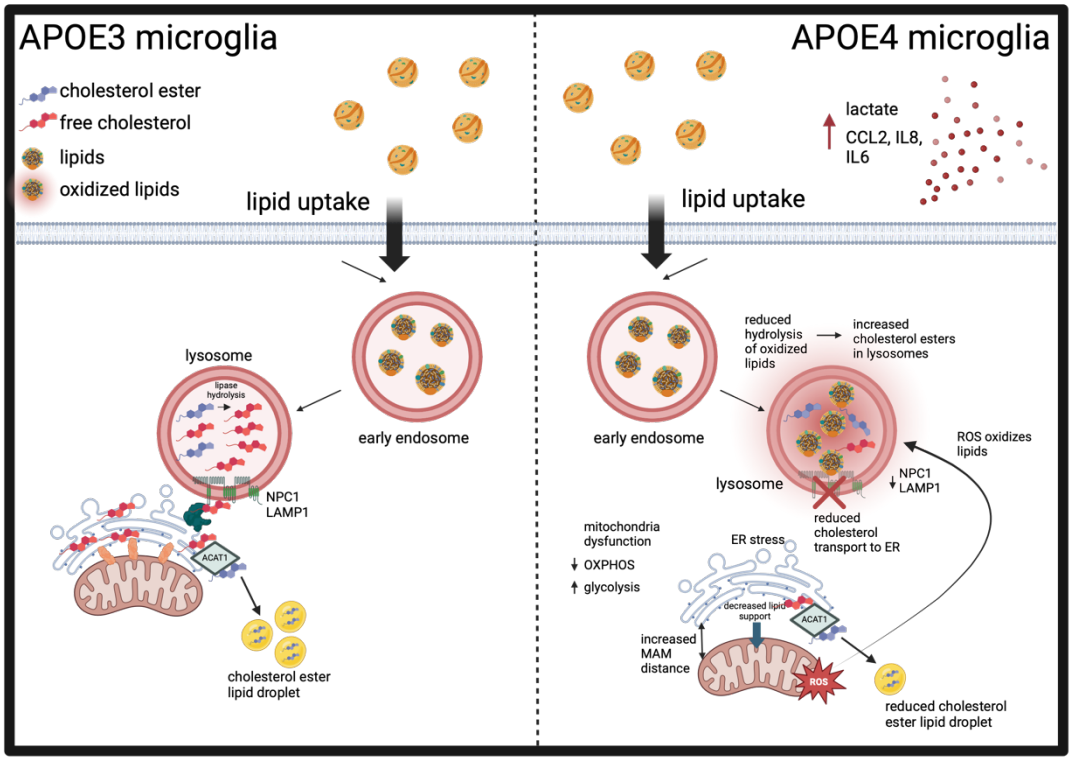


Figure 1.1. Graphical Abstract. The left depicts the canonical pathway in which lipids enter the cell and are hydrolyzed in the lysosomes, converting cholesterol esters into free cholesterol. This free cholesterol is transported to the ER through various transporters, such as NPC1 and LAMP1. Cholesterol is crucial at the ER for maintaining lipid support to the mitochondria. The right depicts the disruption in this pathway, for which we believe APOE4 is responsible. We have observed that APOE4 microglia release excess ROS, which we believe leads to an upregulation of lipid oxidation. This oxidation impacts the lysosome's ability to hydrolyze the lipoproteins, leading to an increase of cholesterol esters in the lysosomes, and decreasing the amount of free cholesterol at the ER site. This leads to ER stress and mitochondrial dysfunction, shifting APOE4 microglia to glycolysis. This excess lactate production may be responsible for the increase of cytokines and senescent-like cells we see in APOE4 microglia.

Abstract

Microglia are specialized resident macrophages of the central nervous system that help to clear debris, pathogens, and neuronal synapses through phagocytosis. These cells play a crucial role in neuroinflammation and disease (Li and Barres, 2018). However, its role in onset or progression of disease, particularly in Alzheimer's disease (AD) remains unclear. Glia express high levels of APOE, an important risk factor gene for AD (Michaelson, 2014). It has been shown that homozygote carriers for the APOE4 variant have an increased risk for AD, and recently a new study is suggesting that the APOE44 genotype is an autosomal dominant form of AD (Fortea et al., 2024),(Tsai et al., 1994). Microglia are important in the clearance of pathology in disease, therefore we sought to understand the impact APOE4 has on microglial function. Our data shows that APOE4 plays a role in transcriptional state shifts in microglia, reducing disease associated microglia and increasing a senescent like G2 cell cycle arrested state. Our study reveals APOE44 microglia are proinflammatory and shift to glycolysis, releasing excessive lactate. We believe these changes are related to dysfunctional cholesterol transport. Our study offers an insight into the impact APOE4 has on disease onset.

Introduction

Microglia are specialized resident macrophages of the central nervous system that help to clear debris, pathogens, and neuronal synapses through phagocytosis (Li and Barres, 2018). Under physiological conditions, microglia maintain plasticity by providing trophic support to neurons. Under pathological conditions, microglia often prune synapses excessively, increase pro-inflammatory cytokine release, and reduce trophic factor release. Currently, there is a limited understanding of the mechanistic role

of microglia in Alzheimer's disease (AD). The apolipoprotein E (APOE) haplotype is the major genetic risk factor for the development of AD, with the ε4 allele correlated to highest risk and the ε2 allele to reduced risk (Tsai et al., 1994). A recent study of homozygote carriers for APOE44 show that the penetrance of the disease is similar to that of autosomal dominant AD and should be treated as so (Fortea et al., 2024). By age 55, nearly all APOE44 carriers exhibited AD pathology, and at a higher level compared to APOE33 carriers. This stresses the importance of understanding the role of APOE4.

Recent studies have identified subclusters of exhausted cells in APOE4 microglia that may contribute to the detrimental effects seen in AD (LDAM and TIM)(Haney et al., 2023; Millet et al., 2024). Additionally, more evidence is emerging that microglia have the ability to senesce in AD mouse models and aged mouse models (Hu et al., 2021; Matsudaira et al., 2023; Rachmian et al., 2024). The phenomenon of replicative senescence was first described by Hayflick and Moorhead in 1961 (Hayflick and Moorhead, 1961). Later, senescent cells were found to secrete inflammatory cytokines (senescence associate secretory phenotype – SASPs) and impact surrounding cells (Young and Narita, 2009). Recently, our lab showed that fibroblast-induced neurons from AD individuals and AD pathology have an increase in senescent neurons and that the SASP can induce astrogliosis (Herdy et al., 2022). Microglial senescence is understudied, and it is unclear whether APOE4 microglia are more vulnerable to cell cycle arrest. Replicative-senescent cells are believed to arrest in G1 or G0 stage of the cell cycle. Interestingly, studies performed in the 1970s observed a population of replicative-senescent cells that contained a significant fraction of cells with 4N DNA

content (Grove and Mitchell, 1974; Yanishevsky et al., 1974). At the time, it was not possible to distinguish G2 cells from tetraploid G1 cells. G1/G0 arrest in senescent cells became a dogma and G2 arrest was understudied. Since then, papers have shown that up to 60% of senescent fibroblasts are G2 arrested, left in a tetraploid G1 phase (Mao et al., 2012). In normal, healthy conditions, microglia are in a G0 quiescent state and studies have shown that microglia proliferate when challenged (Füger et al., 2017; Lawson et al., 1992; Réu et al., 2017; Rossi and Lewis, 2018). It is believed that proliferation may be the catalyst for microglia to become senescent (Hu et al., 2021). It remains unclear if APOE4 influences this transition.

To investigate the transcriptional state of microglia in APOE4 carriers, we used established methods to convert induced pluripotent stem cells (iPSCs) from AD and healthy aged individuals with different APOE haplotypes into induced microglia (iMGs) and performed single cell RNA sequencing (scRNAseq)(Abud et al., 2017). Though *in vitro*, we observed distinct transcriptional states mimicking what has been observed *in vivo*. This allows us to investigate how the APOE4 genotype impacts microglial state shifts, and how these shifts may impact onset and progression of disease. We demonstrate that APOE4 microglia represent less of the disease associate microglia (DAMs) and an increase of cells in the G2 cell cycle. We discovered these cells in G2 are cell cycle arrested and have an increase of cholesterol esters. We also find that APOE4 contributes to a shift from oxidative phosphorylation to glycolysis, which then leads to an increase of lactate being secreted from the cells. APOE4 microglia are more proinflammatory, secreting more cytokines. We believe these detrimental changes are caused by a buildup of cholesterol in the lysosomes in APOE4, which we have

observed via electron microscopy and immunofluorescence. We believe this reduces the amount of cholesterol being transported to the ER, increasing the mitochondria associate membrane (MAM) space, therefore causing mitochondrial stress and the shift to glycolysis (Bassot et al., 2023; Harned et al., 2023). We show that a combination of the lactate and cytokines in the media contribute to degradation of neurons. This study helps us further understand the effect APOE4 has on microglial function and how it contributes to Alzheimer's disease.

Results

iMGs show heterogeneity in 2D culture, similar to that of other in vitro and in vivo mouse and human microglia

We utilized established methods, with slight modifications, to convert iPSCs from AD and healthy aged individuals with different APOE haplotypes into induced microglia (iMG) (Figure 1.2A)(Abud et al., 2017). This includes first converting iPSCs into induced hematopoietic progenitor cells, and then onto iMGs (Figure 1.2B). Microglia are known to have many different states with different roles, *in vivo* and even *in vitro* (Dolan et al., 2023; Olah et al., 2020). We employed single cell RNAseq to capture these different states and begin to understand state shifts in APOE4 iMGs. We captured high quality whole cell transcriptomes from 43,591 iMGs from 22 different patient lines (APOE23 n= 5, APOE33 n= 6, APOE34 n= 7, APOE44 n= 4), excluding low quality cells and doublets. We identified 9 distinct clusters which we annotated as Activated Response/Disease Associate (ARM or DAM), Ribosomal Response (RR), Antigen-presenting response (HLA-1 and HLA-2), Homeostatic (HM), SPP1, G2 cell cycle, G1 cell cycle and Cytokine Response (CRM) (Figure 1.2C). Using existing datasets, we

observe the ARM/DAM cluster overlaps well with both human and mouse DAM signatures (Figure 1.2D), expressing APOE, TREM2, FUCA1, C1QA, and CD68 (Figure 1.2E), highlighting this population's role in clearance (Dolan et al., 2023; Mancuso et al., 2019; Sala Frigerio et al., 2019). The SPP1 cluster also expresses TREM2 which may suggest this is a DAM like signature, as some studies have identified 2 DAM populations (Grubman et al., 2021; Keren-Shaul et al., 2017). In Keren-Shaul et al, the DAM-2 cluster expresses higher levels of SPP1 in comparison to DAM-1, suggesting our SPP1 cluster may reflect this DAM-2 population. Our ribosomal response cluster has high expression of many ribosomal genes, including RPL30, RPL19, RPL10, RPS8, and RPL18 (Figure 1.2E). Our HLA-1 cluster is defined by MHC class II genes such as HLA-DRB1, HLA-DRB5, HLA-DRA and our HLA-2 cluster is defined by both ribosomal and MHC class II genes, suggesting this is a transitioning cluster from HLA-1 to RR (Figure 1.2E). Our homeostatic microglia are enriched in P2RY12, CX3CR1, TGFBR1 and MERTK, known homeostatic markers (Butovsky and Weiner, 2018). Our cytokine response microglia are defined by CCL4, CCL3, IL1B, NFKB1 and SOD2 and make up a small, but distinct, proportion of the entire dataset (Figure 1.2E). Lastly, we have two clusters that express G1 cell cycle (PARP1, MCM5) and G2 cell cycle (TPX2, TUBB, H2AFX) markers, which are thought to be proliferating microglia (Figure 1.2C, 1.2E).

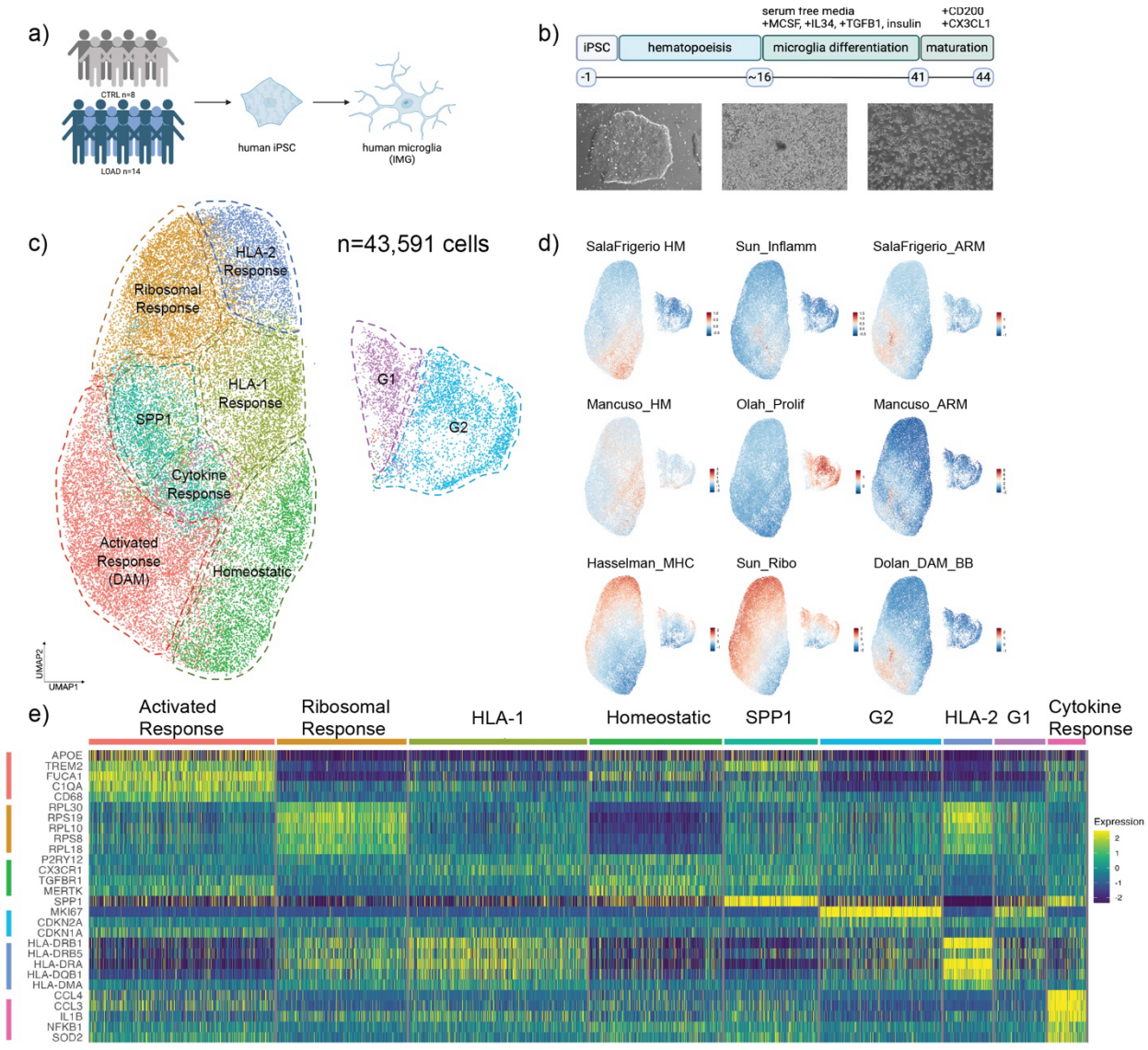


Figure 1.2. iMGs show heterogeneity in 2D culture, similar to that of other *in vitro* and *in vivo* mouse and human microglia. (A) Schematic of patient cohort. (B) Schematic of iPSC to iMG workflow. (C) UMAP representation of all cells (n=43,591) from scRNAseq, with transcription states annotated. Data has gone through quality control and bath correction. (D) Module scores of different transcriptional states from various microglia studies plotted onto our UMAP. (E) Heatmap with known gene markers of each state shown against our annotated states.

APOE4 influences state shifts in microglia, reducing the DAM signature

Next, we sought to understand how APOE4 influences state shifts in microglia (Figure 1.3A). Density plots aid in visualizing state shifts by APOE haplotype (Figure 1.3B). We found that APOE4 iMGs were less represented in the activated response state, also known as disease associate microglia (DAMs) (Figure 1.3C). DAMs were first identified through *in vivo* studies looking at AD mouse models where they observed microglia surrounding plaques have a different transcriptional state than those distant from the plaques (Kamphuis et al., 2016; Keren-Shaul et al., 2017). At the time, it was unclear whether this state shift was progressing the disease or responding to it in a beneficial manner. TREM2 KO studies have shown that TREM2 deficient microglia lack DAMs, and that this cluster is defined by clearance genes (Mancuso et al., 2024; McQuade et al., 2020). This is representative of our pseudobulk data on G0 cells, which reveals APOE44 microglia have reduced expression in lysosomal genes (GM2A, LAMP1, SCARB2, NCP1) (Figure 1.3F). We see an increasing trend of APOE4 microglia represented in the ribosomal response cluster, which is reflected by a significant increase in ribosomal genes (RPL8) in pseudobulk analysis (Figure 1.3C,1.3F). Additionally, we saw an increase of APOE4 microglia in cluster 5 (Figure 1.3C), which expresses markers of G2 cell cycle. The ‘proliferating’ clusters in most studies have been overlooked and often not considered in downstream analyses. To investigate whether metabolic pathways may be impaired in APOE4 iMGs, we performed Compass, an *in silico* approach to infer metabolic status of cells based on single-cell RNA sequencing (Wagner et al., 2021). Interestingly, many pathways are reduced in APOE44 iMGs compared to APOE33 iMGs, including fatty acid oxidation

and cholesterol metabolism (Figure 1.3E). Overall, we observe APOE4 iMGs are underrepresented in the activated response cluster, while they are overrepresented in the G2 cluster.

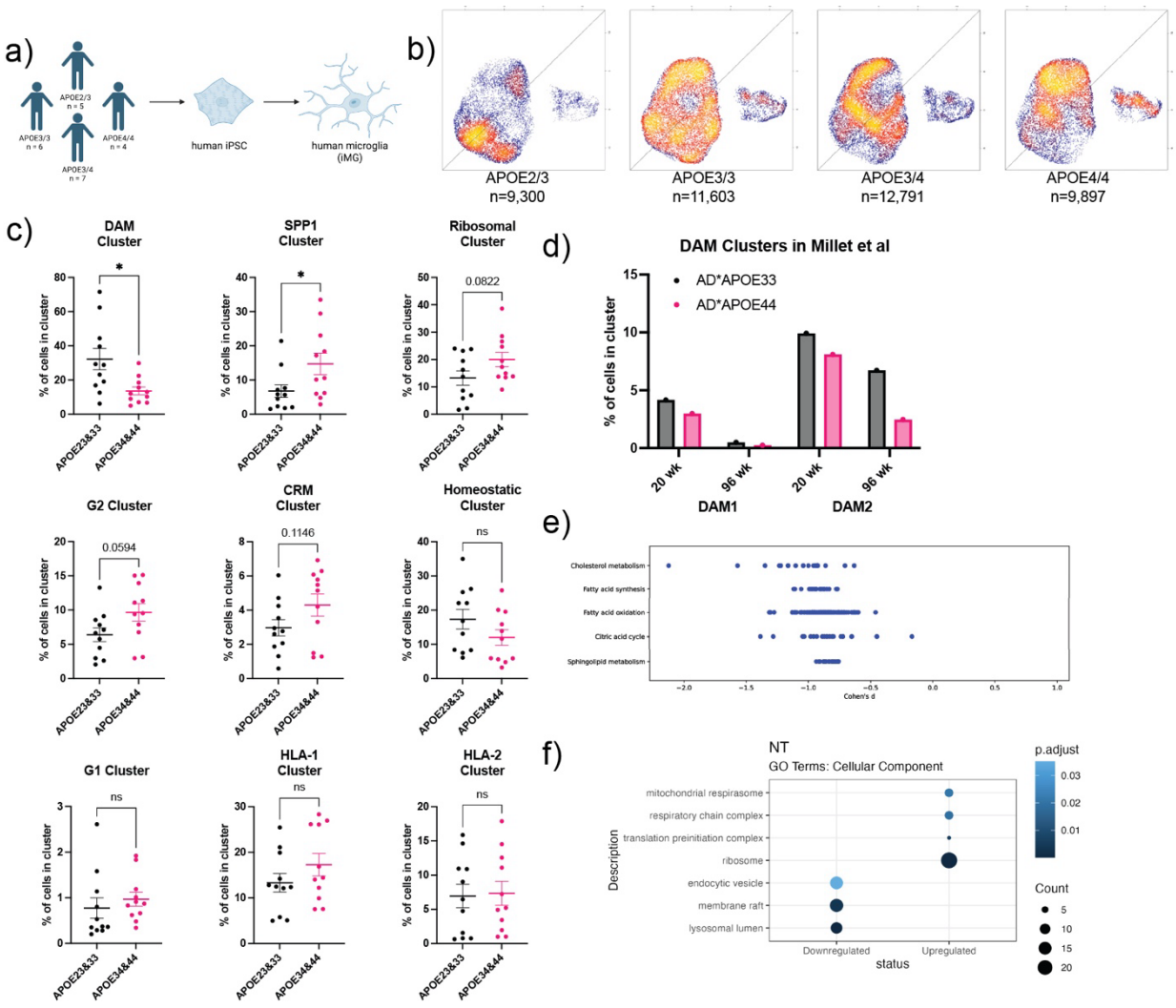


Figure 1.3. APOE4 influences state shifts in microglia, reducing the DAM signature. (A) Schematic of patients divided by APOE haplotypes (APOE2/3 n = 5, APOE3/3 n = 6, APOE3/4 n = 7, APOE4/4 n = 4). (B) Density plot of UMAP per APOE haplotype displaying distribution. (C) Distribution and proportion of cells across different cell states, separated by APOE4- and APOE4+. Each dot represents an individual line. Unpaired t-test, two-tailed, significance set to p<.05. Mean +- SEM. (D) Distribution and proportion of cells in DAM1 and DAM2 clusters in AD*APOE33 and AD*APOE44 mouse microglia snRNAseq, from Millet et al. (E) Compass analysis of metabolic pathways in APOE44 versus APOE33 lines in G0 phase cells. (F) Select top GO Terms for cellular component analysis, restricted to genes with a p<.01.

APOE4 microglia exhibit an increased population of G2 arrested cells

We performed cell cycle prediction with the Pegasus package to identify phase of the cells more accurately, and observed a decrease of APOE4 iMGs in G0, but an increase in G2 (Figure 1.4A). Interestingly, while comparing our clusters to existing datasets, we observed that the lipid droplet associated microglia (LDAMs) signature from Haney et al. overlapped with our cells in the G2 cell cycle phase (Figure 1.4B)(Haney et al., 2023). Haney et al. discuss senescence being a top GO Term in their lipid high microglia. In our data, the G2 cluster expresses not only their LDAM signature, but also known senescent markers CDKN2A (p16) and CDKN1A (p21) (Figure 1.4C)(Baker et al., 2008). APOE4 iMGs were over-represented in this state. Interestingly, this population also had increased expression of G2 cell cycle markers like KI67, TOP2A, and CENPF, and DNA damage/replication genes like RRM1, BUB1B, and H2AFX (Zhang et al., 2024). CDKN2A (P16) staining revealed that P16+ cells had increased nucleus size, a known feature of senescence (Figure 1.4D, 1.4E)(Cristofalo and Pignolo, 1993). We sought to understand if these cells had been arrested in a G2 phase and left in a senescent-like state. G2 cell cycle arrest leaves cells in a G1 tetraploid state, therefore, we sought to isolate this population for further analysis (Mao et al., 2012). Using the Vybrant DyeCycle dye with verapamil to avoid dye efflux, we isolated cells with 4N DNA content from APOE44 microglia using fluorescence-activated cell sorting (FACS) (Figure 1.4F). We plated these cells and after 72 hours, we reanalyzed the cells for DNA content and observed the G0 cells remained 2N, and the G2 cells remained 4N, confirming that these cells had not divided and were arrested in a tetraploid state (1.4G). To analyze whether these cells express a senescence

associated secretory phenotype (SASP), we collected conditioned supernatant from the G2 and G0 cells and utilized cytokine blots; we did not observe differences in cytokine secretion. To understand if lipid content is higher in this G2 arrested population, we performed lipidomics and observed that these cells had an increase of cholesterol esters (Figure 1.4H). These findings are supported by previous studies that find microglia in a Tau-APOE4 mouse model have higher cholesterol ester content when compared to the Tau-APOE3 microglia (Litvinchuk et al., 2024). We next sought to understand if the differences seen in lipid content could be impacting the bioenergetics of APOE44 microglia.

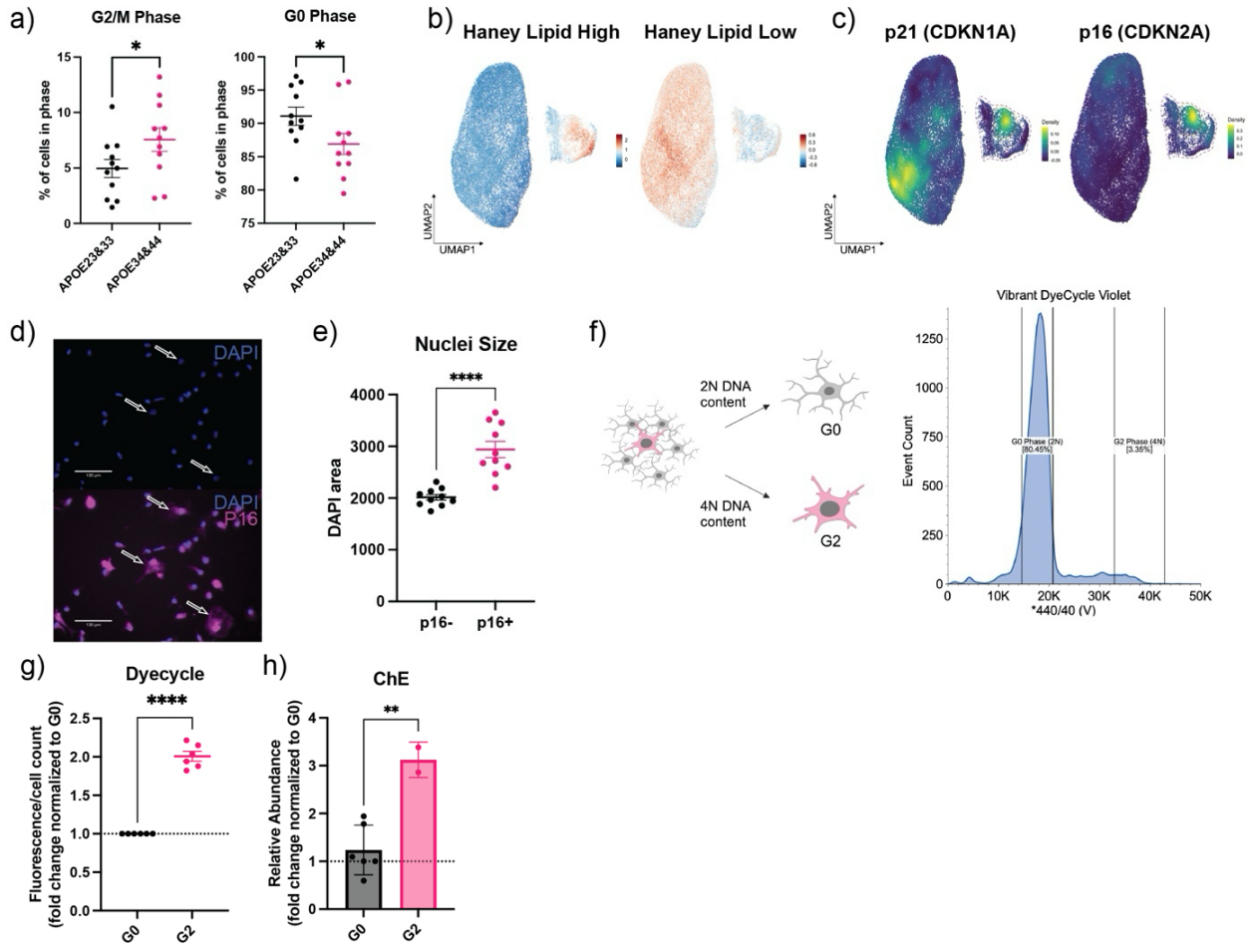


Figure 1.4. APOE4 microglia exhibit an increased population of G2 arrested cells. (A) Distribution and proportion of cells in G2/M and G0 phase, as predicted by the pegasus cell cycle prediction package, separated by APOE4- and APOE4+. Each dot represents an individual line. Unpaired t-test, two-tailed, significance set to p<.05. Mean +- SEM. (B) Module scores created using DEGs from Haney et al. Left represents upregulated genes, right represents downregulated genes. (C) Density UMAP plot of expression of CDKN1A (p21) and CDKN2A (p16). (D) Representative image of p16 staining with DAPI on iMGs. (E) ImageJ analysis of nuclei size measuring area of DAPI, p16- and p16+ cells. Unpaired t-test, two-tailed, significance set to p<.05. Mean +- SEM. (F) Schematic of using Vybrant DyeCycle Violet Dye with verapamil to stain live nuclei in APOE44 iMGs and collecting 2N (G0) and 4N (G2) cells. (G) Using a plate reader, G0 and G2 cells that were collected were stained again with DyeCycle and verapamil 72 hours after plating, and were read using an excitation of 365nm and an emission of 415-445nm. G2 is normalized to G0 and cell count. Unpaired t-test, two-tailed, significance set to p<.05. Mean +- SEM. (H) Relative abundance of cholesterol esters between G0 and G2 cells. Unpaired t-test, two-tailed, significance set to p<.05. Mean +- SEM.

APOE44 microglia are more proinflammatory and shift to glycolysis

To understand the direct role of the APOE44 genotype, we converted APOE33 and APOE44 isogenic iPSCs into iMGs, as previously described (Figure 1.5A) (Abud et al., 2017). We looked at cytokine secretion of CCL2, IL8 and IL6 and observed a significant increase of all three cytokines in APOE44 iMGs compared to APOE33 iMGs (Figure 1.5B). To understand the bioenergetics of APOE44 microglia, we performed the Mito Stress Test Kit on the Seahorse XFe96 Analyzer (Figure 1.5C). We observed that APOE44 microglia have a decrease in oxygen consumption rate at baseline, ATP linked respiration and max respiration (Figure 1.5D). Because of this reduction in ATP linked respiration and increase of LDHA gene expression levels, we were interested in whether APOE44 microglia shift their bioenergetics to glycolysis from OXPHOS. Using JC-1, we observed mitochondria membrane potential is reduced in APOE44, a sign of unhealthy mitochondria (Figure 1.5E) (Sivandzade et al., 2019). Treating cells with 13C-glucose, we quantified incorporation into the TCA cycle by GC-MS in APOE33 and APOE44 iMGs. We found that APOE44 microglia are producing significantly more lactate, as well as an increase of pyruvate (Figure 1.5F). The ratio of pyruvate to lactate, which represents a readout of mitochondrial oxidative metabolism to glycolysis, is reduced in APOE44, suggesting APOE44 are shifting to glycolysis (Figure 1.5G). We measured secreted lactate, as well, and observed an increase of lactate from the APOE44 microglia (Figure 1.5H). Studies have shown that lactate treatment can shift microglia into a senescence state via histone lactylation through the NFKB signaling pathway (Wei et al., 2023). Because of the stress on mitochondria, we measured reactive oxygen species (ROS), which measures at superoxides and hydroxyl radicals.

We observed that APOE44 microglia produce significantly more ROS compared to APOE33 microglia (Figure 1.5I). Together, these results illuminate an energetic shift in APOE44 microglia. Because of the increase in ROS, we sought to understand whether these free radicals were oxidizing lipids in APOE44 microglia, and how this may impact lipid metabolism.

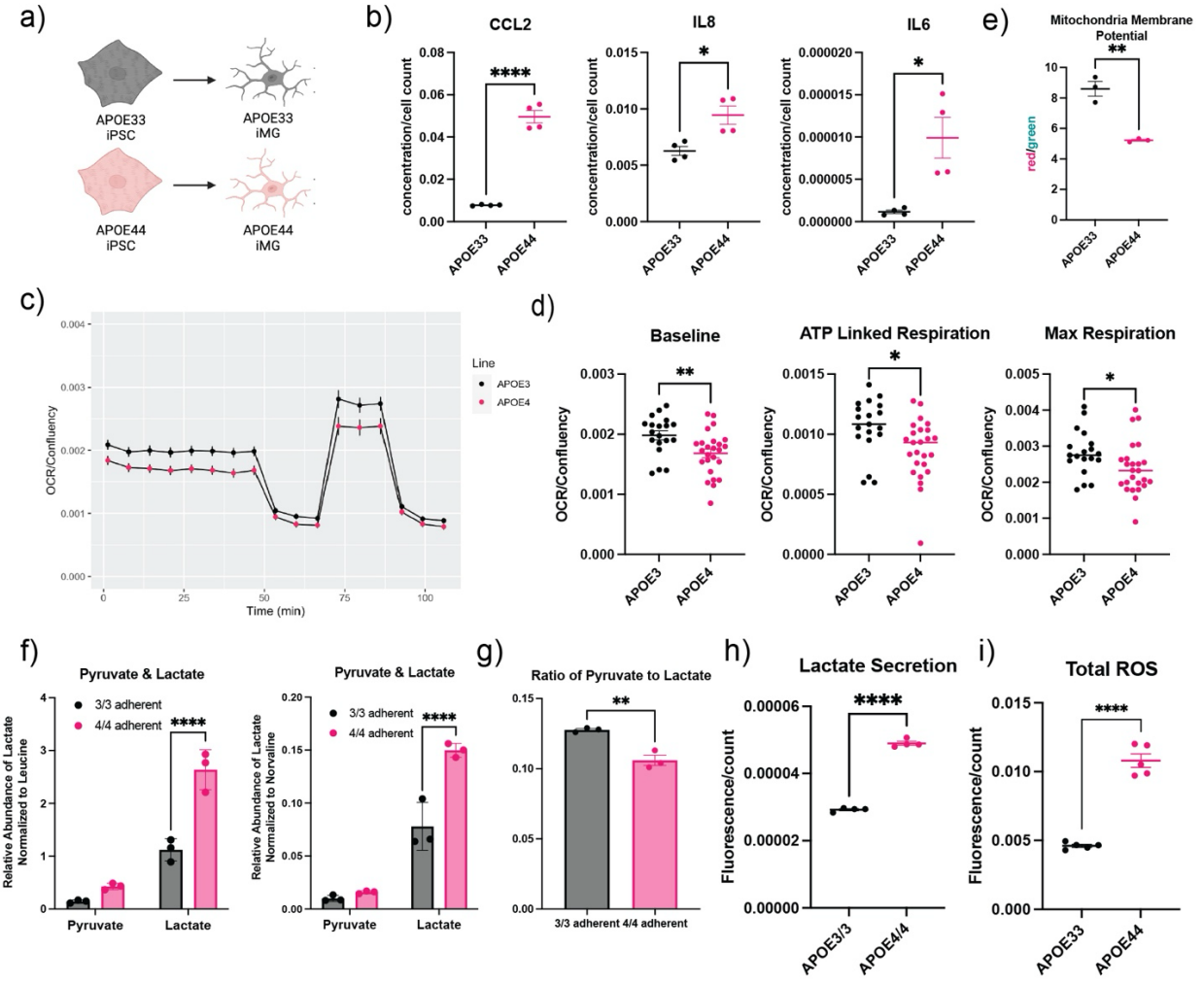


Figure 1.5. APOE44 microglia are more proinflammatory and shift to glycolysis. (A) Schematic of isogenic APOE33 and APOE44 iMGs. (B) Cytokine analysis of CCL2, IL8 and IL6 (Legendplex) from media collected from APOE33 and APOE44 iMGs, normalized to cell count. Unpaired t-test, two-tailed, significance set to p<.05. Mean +- SEM. (C) Seahorse XFe96 Mito Stress Test Kit of APOE33 and APOE44 iMGs. (D) Baseline oxygen consumption rate, ATP linked respiration, and max respiration normalized to confluency, comparing APOE33 and APOE44 iMGs. Unpaired t-test, two-tailed, significance set to p<.05. Mean +- SEM. (E) JC-1 dye measurement read on plate reader, comparing APOE33 and APOE44 iMGs. Red was measured at an excitation of 520nm and green at an excitation of 475nm. Unpaired t-test, two-tailed, significance set to p<.05. Mean +- SEM. (F) Ratio of labeled pyruvate to lactate after D-glucose13C tracing. Unpaired t-test, two-tailed, significance set to p<.05. Mean +- SEM. (G) Labeled pyruvate and lactate after D-glucose13C tracing normalized to leucine and norvaline. Unpaired t-test, two-tailed, significance set to p<.05. Mean +- SEM. (H) Measurement of secreted lactate from APOE33 and APOE44 iMGs normalized to cell count. Unpaired t-test, two-tailed, significance set to p<.05. Mean +- SEM. (I) Measurement of superoxides and hydroxyl radicals using the ROSBrite dye, normalized to cell count. Unpaired t-test, two-tailed, significance set to p<.05. Mean +- SEM.

APOE44 microglia have an accumulation of ChEs in the lysosome

LXR agonists have been used as rescue molecules in several APOE4 studies, so we were interested in how this drug would impact lipid metabolism (Litvinchuk et al., 2024; Tcw et al., 2022). Using the isogenic lines, we treated our APOE44 microglia with GW3965 for 24hrs and then measured cholesterol esters and triglycerides (Figure 1.6A). We found that at baseline, APOE44 microglia have decreased cholesterol esters and triglycerides (TGs) compared to APOE33 microglia, unlike previous studies (Haney et al., 2023). We find that GW3965 knocks down ChEs but does not impact TGs (Figure 1.6B). The decrease in TGs at baseline levels is complementary to our finding that APOE44 microglia make up less of the DAM population. Studies have suggested DAMs have increased TGs, as shown by an increase of lipids in amyloid beta treated microglia and in BHLHE40/41 KO microglia, which shifts microglia into DAMs (Haney et al., 2023; Podlesny-Drabiniok et al., 2023). Because we saw an increase in cholesterol esters in G2-arrested microglia, we sought to further understand why we would see a global decrease of ChEs in APOE44 microglia. ChEs are shuttled into cells via apolipoproteins, where they are hydrolyzed in the lysosomes and converted into free cholesterol (Goldstein and Brown, 1977). This free cholesterol is then shuttled to the ER where it provides lipid support to the mitochondria via the mitochondrial associated membrane (MAM) ((Gaigg et al., 1995; Vance, 1990). Excess free cholesterol is esterified and packaged into lipid droplets via ACAT1 (acetyl-CoA acetyltransferase 1) (Chang et al., 1998). To understand whether this decrease in cholesterol esters is due to an impairment in the lysosome or at the ER, we treated our cells with an ACAT1 inhibitor and then measured free cholesterol and ChEs. This would allow us to

understand whether there is a dysfunction of lipoprotein hydrolysis or of esterification at the ER (Figure 1.6D). We treated our APOE44 microglia with GW3965 and subsequently with an ACAT1 inhibitor. Analysis of the ratio of cholesterol esters (ChE) and free cholesterol to total cholesterol revealed that APOE44 microglia have a buildup of ChE in the lysosomes and that treatment with GW3965 reduces ChEs (Figure 1.6E, 1.6F). This finding suggests that there may be a dysfunction in hydrolysis of lipoproteins in the lysosomes in APOE44 microglia. Coupled with our finding that ROS is increased in APOE44 microglia, we were interested in whether the lipoproteins in APOE44 microglia are oxidized. Studies have shown that oxidized low density lipoproteins (oxLDL) have difficulties being hydrolyzed in the lysosome (Jerome et al., 2008; Schmitt et al., 1995). Our scRNAseq showed that ALOX5AP (arachidonate 5-lipoxygenase activating protein), a microglia specific dioxygenase that catalyzes the peroxidation of polyunsaturated fatty acids and promotes inflammation, is higher in our APOE44 patient iPSC-derived microglia (Figure 1.6G) (Bonham et al., 2019; Gaschler and Stockwell, 2017). To detect lipid peroxidation, we measured HNE (4-hydroxy 2-nonenal), a byproduct of lipid peroxidation, in APOE33 and APOE44 microglia (Esterbauer et al., 1991). We found that APOE44 microglia had higher levels of HNE compared to APOE33 microglia (Figure 1.6H). Using the scRNAseq data from brain tissue in Haney et. al, we observed that AD patients had a higher percentage of ALOX5AP+ microglia compared to age-matched healthy controls (Figure 1.6I). Taken together, these results point to dysregulation in lipid metabolism, specifically in the hydrolysis of ChEs in the lysosomes. Because LXR agonism decreases ChE, we sought to investigate whether GW3965 could rescue the other deficits we see in APOE44 microglia.

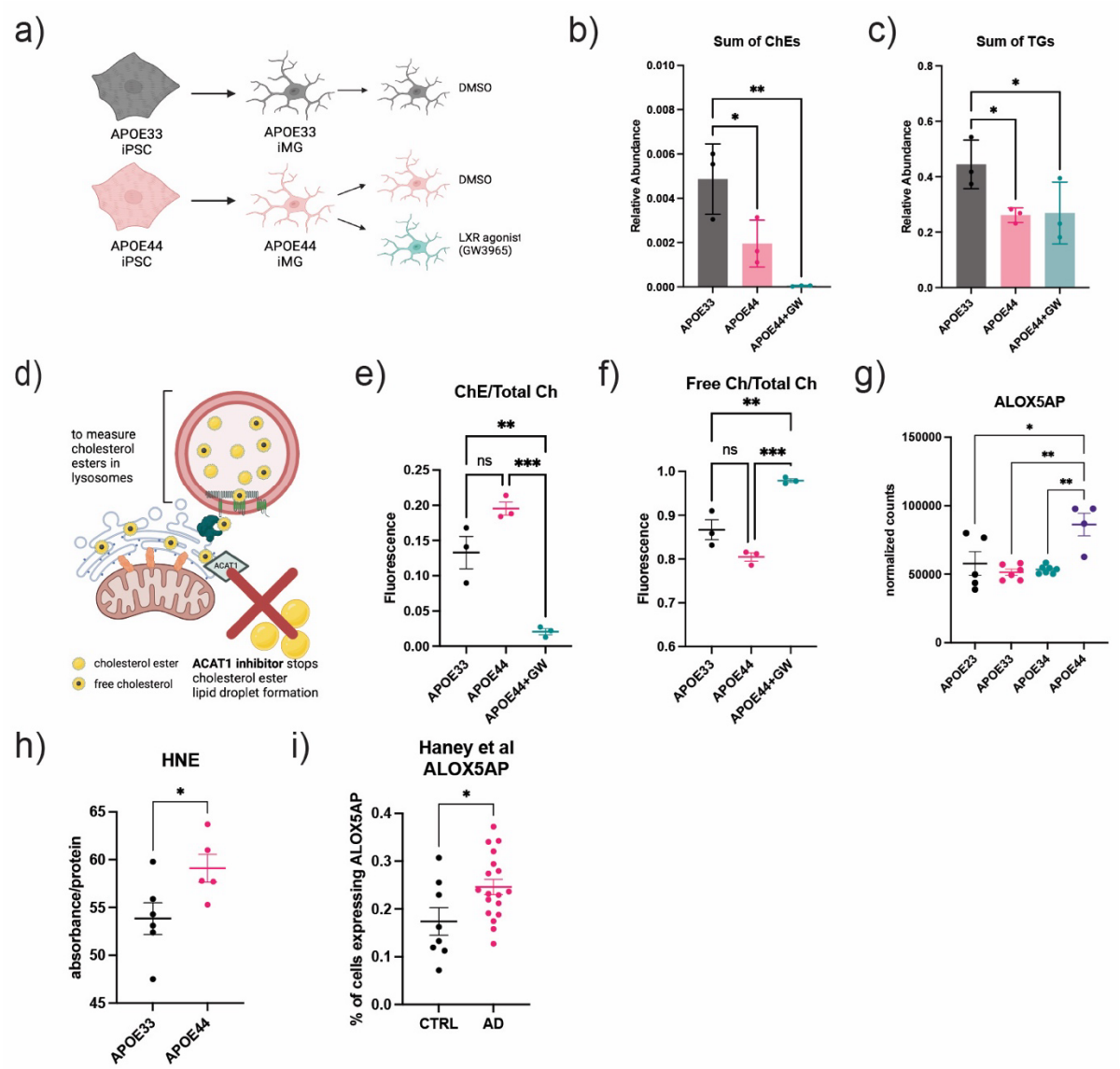


Figure 1.6. APOE44 microglia have an accumulation of ChEs in the lysosome. (A) Schematic of treatments on APOE3 and APOE4 isogenic iPSC-derived microglia. (B) Relative abundance of cholesterol esters, as measured by QE. One way ANOVA, significance set to p<.05. Mean +- SEM. (C) Relative abundance of triglycerides from, as measured by GCMS. One way ANOVA, significance set to p<.05. Mean +- SEM. (D) Schematic depicting ACAT1 inhibition to measure cholesterol in the lysosomes. (E) Ratio of ChE to total cholesterol. One way ANOVA, significance set to p<.05. Mean +- SEM. (F) Ratio of free cholesterol to total cholesterol. One way ANOVA, significance set to p<.05. Mean +- SEM. (G) ALOX5AP expression across different APOE haplotypes in patient iPSC-derived microglia. One way ANOVA, significance set to p<.05. Mean +- SEM. Each dot represents an individual line. (H) Measurement of HNE across APOE33 and APOE44 isogenic microglia. Unpaired t-test, two-tailed, significance set to p<.05. Mean +- SEM. (I) ALOX5AP expression between AD and control patient microglia. Unpaired t-test, two-tailed, significance set to p<.05. Mean +- SEM. Each dot represents an individual patient.

LXR agonist GW3965 partially rescues APOE44 microglia phenotype

We treated our APOE44 iMGs with the GW3965 LXR agonist for 24hrs and measured lactate secretion and mitochondria membrane potential. We observed an increase of mitochondria membrane potential in APOE44 iMGs treated with GW3965 (Figure 1.7A). Next, we measured cytokine secretion and found CCL2 and IL6 levels were decreased with treatment (Figure 1.7B), though IL8 was not rescued. We performed bulk RNA sequencing on APOE33 iMGs + DMSO, APOE44 iMGs + DMSO, and APOE44 iMGs + GW3965 and heatmap analysis reveals APOE44 treated with GW3965 is more closely related to APOE33 + DMSO than APOE44 + DMSO, further supporting that activating LXR partially rescues APOE44 (Figure 1.7C). We found that GW3965 treatment reduced cell cycle markers (CDKN2A) and inflammatory markers (CD53), while increasing autophagy markers (PIK3R2) (Figure 1.7D, 1.7E). To visualize this, we created module scores of genes that were upregulated and genes that were downregulated by at least 1.5 fold and plotted these signatures on our scRNAseq data to visualize how LXR impacts state shifts (Figure 1.7D, 1.7E). It has been suggested that LXR agonists are beneficial by upregulating cholesterol efflux genes (Litvinchuk et al., 2024). We found GW3965 increases ABCA1, a cholesterol efflux regulatory protein, by ~6 fold. We observe that ChE levels are significantly reduced in APOE44 microglia treated with GW3965, after treatment of an ACAT1 inhibitor, indicating a reduction of ChE in the lysosomes (Figure 1.7F,G). The ratio of FC to ChE is increased in the lysosomes of APOE44 microglia (Figure 1.7H). Though LXR agonism rescues several APOE44 phenotypes, when looking at the bioenergetics of GW3965 treated cells, there seems to be an opposite effect. We see that with LXR agonism, the flux of glucose to

citrate is reduced, pyruvate/lactate ratio is decreased, and lactate secretion is higher, indicating a further shift to glycolysis in APOE44 microglia (Figure 1.7I-K). Together, we observe that LXR agonism is able to rescue some of the deficits seen in APOE44 microglia, but that the mechanism behind LXR agonism needs to be further elucidated.

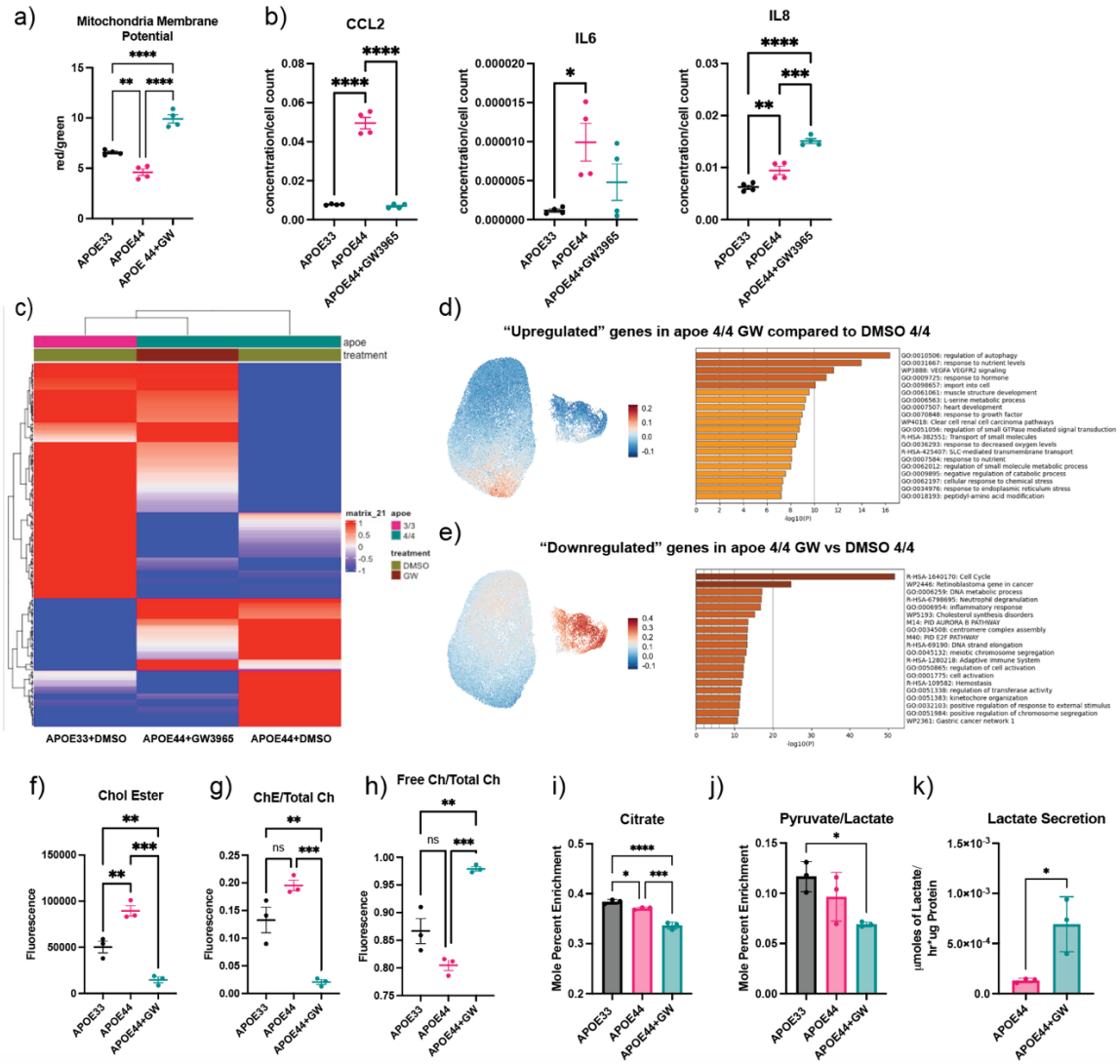


Figure 1.7. LXR agonist GW3965 rescues APOE44 phenotype. (A) JC-1 dye measurement read on plate reader, comparing APOE33(DMSO), APOE44(DMSO) and APOE44(GW) iMGs. Red was measured at an excitation of 520nm and green at an excitation of 475nm. One way ANOVA, significance set to p<.05. Mean +- SEM. (B) Cytokine analysis of CCL2, IL6, and IL8 (Legendplex) from media collected from APOE33(DMSO), APOE44(DMSO) and APOE44(GW) iMGs, normalized to cell count. One way ANOVA, significance set to p<.05. Mean +- SEM. (C) Heatmap of genes that have >1.5fold difference between APOE33(DMSO) and APOE44(DMSO) from bulk RNA sequencing data. (D) Module score of genes upregulated by >1.5fold in APOE44(GW) compared to APOE44(DMSO) displayed on our UMAP and associated GO Terms. (E) Module score of genes downregulated by >1.5fold in APOE44(GW) compared to APOE44(DMSO) displayed on our UMAP and associated GO Terms. (F-G) ChE and FC measured by cholesterol kit. One way ANOVA, significance set to p<.05. Mean +- SEM. (I-J) Citrate, pyruvate and lactate levels measured by GCMS. One way ANOVA, significance set to p<.05. Mean +- SEM. (K) Lactate secretion measured by YSI. Unpaired t-test, two-tailed, significance set to p<.05. Mean +- SEM.

Discussion

We provide a transcriptomic dataset of iPSC derived microglia from different APOE haplotypes, allowing us to investigate the impact APOE has on state shifts. APOE44 carriers have an increased risk of developing late onset Alzheimer's and a recent study suggests this is an autosomal dominant form of AD (Fortea et al., 2024). The APOE protein is highly expressed in glia and is a key transporter of cholesterol (De Chaves et al., 2008). By studying microglia *in vitro* without perturbations, we can begin uncovering the cell autonomous roles APOE44 plays in microglia. We observed APOE44 microglia represent a smaller population of the DAM population compared to APOE33, suggesting DAMs are a protective population. This is further supported by our finding that APOE44 iMGs contain less triglycerides compared to isogenic APOE33 iMGs. Triglycerides have been shown to be important in autophagy and we believe this population is crucial for responding to disease pathology (Shpilka et al., 2015; Soto-Avellaneda and Morrison, 2020). An existing dataset Millet et al supports our finding that APOE44 microglia struggle to shift to a DAM state, even in the presence of AD pathology (Millet et al., 2024). Understanding the lipid contents of microglia is crucial in identifying whether it is a beneficial or detrimental population. We suggest the DAMs have high TGs, while another population we have identified as senescent-like are high in cholesterol esters. While lipid dyes are helpful in identifying neutral lipids, they do not differentiate between lipids such as TGs and ChEs and therefore lipidomics must be performed. Additionally, identifying the location of these lipids, whether in droplets or accumulated in organelles, such as lysosomes, is crucial to understand the issue.

Studies have shown that microglia proliferate in response to injury and pathology, and this can lead to senescence (Füger et al., 2017; Lawson et al., 1992). On our study, we found a population of G2 cell cycle arrested microglia expressing senescent markers p21 and p16. This population was enriched in APOE4 microglia and contained high cholesterol ester content. Recently, a Tau-APOE4 mouse model was created and showed an increase of ChE in microglia in comparison to Tau-APOE3 microglia (Litvinchuk et al., 2024). Interestingly, at baseline, with an APOE33 and APOE44 mouse model, they observe ChEs are reduced in APOE44 microglia, which we also observe. But when we inhibit the formation of lipid droplets by ACAT1 and measure ChE, we find that APOE44 microglia accumulate cholesterol esters in their lysosomes, therefore likely reducing the amount of free cholesterol transported to the endoplasmic reticulum (ER). Cholesterol is esterified at the ER by ACAT1/SOAT1, which would explain the reduction of total cholesterol esters in APOE44 (Rudel et al., 2001). We hypothesize that APOE44 senescent-like microglia have even more cholesterol esters accumulated in lysosomes than that of the global population of APOE44 microglia, which is why we could detect an overall increase of cholesterol esters in the G2-arrested cells (Jerome et al., 2008).

Studies have shown that cholesterol is crucial at the mitochondria associated membrane (MAM) space, which tethers the ER and mitochondria (Fujimoto et al., 2012; Harned et al., 2023). It has been shown that increasing this MAM space leads to dysfunctional mitochondria producing less ATP, and therefore shifting the system towards glycolysis and producing more lactate (Bassot et al., 2023). We observe APOE44 microglia have reduced ATP-linked respiration and a shift towards glycolysis, which supports our idea that APOE44 microglia have ER stress due to a reduction of

free cholesterol being transported to the ER. It is this ER stress that may be leading to the increase of ROS we see.

Along with the increase in ROS, we also see HNE and ALOX5AP increased in APOE44 microglia, suggesting they have increased lipid peroxidation. All these components have been shown to be higher in AD, suggesting that APOE4 may be initiating or progressing the disease by increasing these factors (Bradley-Whitman and Lovell, 2015; Chen et al., 2020; Di Domenico et al., 2017; Manoharan et al., 2016).

We show that an LXR agonist GW3965 rescues the defects seen in APOE44 microglia, including reducing ChE levels, CCL2 and IL-6 levels, while increasing mitochondria membrane potential. GW3965 shifts APOE44 microglia closer to an APOE33 microglial transcriptional state, including reducing senescence associated genes like CDKN2A. It has been suggested that LXR activation is able to rescue APOE44 microglia because of its upregulation of cholesterol efflux regulatory proteins, like ABCA1 (Litvinchuk et al., 2024). It is unclear if ABCA1 regulation would impact the efflux of cholesterol esters, so it may be possible that the rescue we see is related to the upregulation of autophagy genes with GW3965 treatment. By increasing autophagy, we might be able to rid the microglia of the lipid accumulated lysosomes (Eriksson et al., 2020). However, we see that LXR agonism is also shifting the APOE44 microglia even further towards glycolysis. We believe the effects of LXR agonism in microglia needs to be further investigated to understand the exact mechanism leading to these findings.

In conclusion, our study helps uncover the role APOE4 plays in microglia and subsequently in Alzheimer's disease. The results stress the importance of stratifying patient populations by APOE haplotype when studying Alzheimer's disease. Our models

suggests that APOE44 carriers have different mechanisms leading to dysfunction, and therefore, the need for individualized prevention strategies. Our dataset provides a large patient derived population of human iPSC-derived microglia which helps the microglia field take a step closer to understanding the disease associated state-shifts in microglia.

Methods

iMG conversion from iPSCs and maintenance

Using an established protocol with minor modifications, iPSCs were converted into iMGs (Abud et al., 2017). iPSCs were first induced into hematopoietic progenitor cells (iHPCs) and then into microglia. Therefore, to isolate microglia, I will perform FACS sorting twice: first for iHPCs (CD43+) and subsequently for six microglia markers (CD64, CX3CR1, CD14, CD11B, HLADR, and CD45. iPSCs were dissociated using TrypLE (Invitrogen) and plated in a 15-cm tissue culture-treated plate (CytoOne USA Scientific) at a density of ~12million (full 6 well plate 90% confluent) cells per plate with 10 μM ROCK inhibitor (Stem Cell Technologies). The next day (day 0), cells were changed to hematopoietic differentiation media supplemented with FGF2 (50 ng mL−1, Joint Protein Central), BMP4 (50 ng mL−1, Proteintech) Activin A (12.5 ng mL−1, R&D) ROCK inhibitor (1 μM, Stem Cell Technologies) and LiCl (2mM, Sigma) and grown under hypoxic conditions (5% CO2, 5% O2). Hematopoietic differentiation media consisted of IMDM (50%, Thermo Fisher Scientific), DMEM/F12 (50%), ITSG-X, (2% v/v, Thermo Fisher Scientific), L-ascorbic acid 2-Phosphate magnesium (64 mg mL−1; Sigma), monothioglycerol (400 mM), PVA (10 mg mL−1; Sigma), Glutamax (1X, Thermo Fisher Scientific), chemically defined lipid concentrate (1X, Thermo Fisher Scientific), non-essential amino acids (NEAA, Thermo Fisher Scientific 1X), Penicillin/Streptomycin

(P/S Thermo Fisher Scientific, 1% V/V). On day 2, floating cells were collected, spun down, and media was replaced with hematopoietic differentiation media supplemented with FGF2 (50 ng mL−1, Joint Protein Central) and VEGF (50 ng mL−1, R&D) and returned to hypoxic conditions in the same plate. Day 2 media was equilibrated in hypoxia before use. On day 4, cells were collected and placed into normoxic conditions (5% CO2, 21% O2) and kept in hematopoietic differentiation media supplemented with FGF2 (50 ng mL−1, Joint Protein Central), VEGF (50 ng mL−1, R&D) TPO (50 ng mL−1, Proteintech), SCF (10 ng mL−1, Proteintech), IL-6 (50 ng mL−1, R&D) and IL-3 (10 ng mL−1, R&D) until colonies released hematopoietic progenitor cells (iHPCs) (usually between day 14 and 16). iHPCs were then isolated using Fluorescence-activated cell sorting (FACS) by gating on CD43-FITC (Biolegend, 315204)-positive cells. iHPCs were then cultured in microglia differentiation media supplemented with 25 ng ml−1 M-CSF (R&D), 100 ng mL−1 IL34 (R&D) and 50 ng mL−1 TGFβ1 (R&D) for 4 weeks. CD200 and CX3CL1 were added for 3 days before iMG collection. Microglia differentiation media consisted of DMEM/F12, ITS-G (2%v/v,Thermo Fisher Scientific), B27 (2% v/v), N2 (0.5%, v/v), monothioglycerol (200 μM), Glutamax (1X), NEAA (1X), and insulin (5 μg/ml; Sigma). iMGs were FAC sorted for microglia markers CD64, CX3CR1, CD14, CD11B, HLADR, and CD45 and plated for subsequent experiments or collected for scRNAseq.

Sequencing collection

To isolate for scRNAseq, iMGs were sorted for microglia markers CD64, CX3CR1, CD14, CD11B, HLADR, and CD45 and subsequently resuspened in BSA 0.04%. Cells from multiple lines were multiplexed for a total of 40,000 cells per reaction

and later separated *in silico* using demuxlet, as previously described (Kang et al., 2018). Droplet emulsions for the reverse transcription reaction were created using the 10X Genomics Chromium controller. Libraries were prepared following the standard protocol of 10X Single Cell 5′ v2 using v5.0 chemistry. Libraries were sequenced to a read depth of approximately 400M reads per reaction using NovaSeq S4.

scRNA-seq Quality control

Raw counts were mapped to hg38 genome using Cell Ranger and subsequently analyzed using the Seurat R package. We used the Scanpy package to discard cells with nCount_RNA < 2000 or more than 15% mitochondrial reads. Doublets were removed using Scrublet. Counts were then CPM scaled and log-normalized for downstream analysis. The SCTransfrom workflow was used and PCA computed as input for batch correction.

Microglia pseudobulk differential gene expression

The counts per individual from cells considered in the G0 phase were summed up to create the pseudobulk counts. We used DeSeq2 to normalize the bulk data and differential gene expression analysis was performed in R. We followed standard analysis steps with standard parameters. We used the likely hood ratio test, which utilizes Analysis of Deviance (ANODEV), to compare different APOE haplotypes (APOE23, APOE33, APOE34, APOE44) reduced by sex. DEGs were defined as those with a p-adj < .05.

Microglia clustering

Using the Seurat package, we used the first 15 principal components based on the top 2000 highly variable genes to conduct Louvain clustering and UMAP

visualization. We created module scores with the AddModuleScore function using existing gene signature sets from other microglia papers to characterize each of these clusters. Using the R package LSD which calculates kernel density, we have plotted cells by APOE haplotype to visualize density within each of these clusters.

iMG G2 isolation

To collect cells with 4N DNA content (G2) and 2N DNA content (G0), iMGs were treated with verapamil (50uM) to block efflux of Vybrant DyeCycle Violet (DCV; Thermo V35003) for 30 min at 37 C at a concentration of $1x10^6$ cells/ml. Subsequently, without washing the verapamil out, DCV was added at a concentration of 5uM for another 30 min at 37 C. Cells were sorted at room temperature immediately after treatment. Gates were created to collect 2N and 4N DNA content and plated at a density of 25K cells per well in a 96 well-plate for subsequent experiments, or 100K per well in a 24-well plate for lipid extraction.

Arrested-cell assay

To observed if 4N cells were arrested, after 72 hours of plating, verapamil and DCV were used again, and fluorescence of DCV was measured using a plate reader with an excitation of 405 nm and emission of 440 nm. Fluorescence was normalized to cell count, as measured by Incucyte.

JC-1

To measure mitochondria membrane potential, JC-1 dye was used at a concentration of 2uM in phenol free DMEM/F12 and measure after 30min on a plate reader. For the green fluorescence, the plate was read with an excitation of 480 nm and emission of 535 nm. For the red fluorescence, the plate was read with an excitation of

550 nm and emission of 600 nm. For membrane potential, we then divided the red by the green fluorescence.

Cytokine assay

Cells were plated at a density of 25K cells/well in a 96 well-plate. Media was replaced with 100ul DMEM/F12 and collected after 24 hrs. Supernatant was centrifuged to get rid of debris and immediately assayed with LegendPlex (IL6, CCL2, IL8 mix and match kit) using manufacturer instructions. Cells were imaged on Incucyte and normalized to cell count.

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Chapter 2: Modeling Alzheimer's Disease Using Human Cell Derived Brain Organoids and 3D Models

Abstract

Age-related neurodegenerative diseases, like Alzheimer's disease (AD), are challenging diseases for those affected with no cure and limited treatment options. Functional, human derived brain tissues that represent the diverse genetic background and cellular subtypes contributing to sporadic AD (sAD) are limited. Human stem cell derived brain organoids recapitulate some features of human brain cytoarchitecture and AD-like pathology, providing a tool for illuminating the relationship between AD pathology and neural cell dysregulation leading to cognitive decline. In this review, we explore current strategies for implementing brain organoids in the study of AD as well as the challenges associated with investigating age-related brain diseases using organoid models.

1. Introduction

Humans (*Homo sapiens*) are living longer than ever (Wheeler and Kim, 2011). While researchers are gaining insight into life prolonging interventions, we lack a mechanistic understanding of age associated brain diseases that diminish quality of life. Age-related brain diseases are challenging to model in vitro due to their complexity that includes contributions of multiple cell types, brain regions, and heterogenous pathological features, impacting both large-scale brain networks and local (small-world) cortical network organization responsible for high-level cognition (He, Chen and Evans, 2008)(Sanz-Arigita *et al.*, 2010)(Sun, Tong and Yang, 2012). AD is one such age associated brain disease characterized by the accumulation of extracellular amyloid-β

(Aβ) plaques and intracellular neurofibrillary tangles (NFTs), with NFTs demonstrating a stronger correlation to cognitive decline (Bennett *et al.*, 2004)(Josephs *et al.*, 2008)(Moloney, Lowe and Murray, 2021)(Coedert *et al.*, 1989). It is unclear how these pathologies are triggered or what temporal roles they might play in neuroinflammation and disease progression (Ahmad, Fatima and Mondal, 2019)(Akama and Van Eldik, 2000)(Cribbs *et al.*, 2012)(Sudduth *et al.*, 2013). Despite the greater association of NFTs with cognitive decline, cerebral spinal fluid (CSF) ratio of the 42-amino acid β-amyloid peptide (Aβ42) to the 40-amino acid β-amyloid peptide 40 (Aβ40) is a useful diagnostic tool of AD. Positron emission tomography (PET) has revealed that CSF Aβ42/40 ratio correlates better with AD pathology than Aβ42 alone (Lewczuk *et al.*, 2017)(Lewczuk *et al.*, 2014).

Familial AD (fAD) is an early onset form of AD caused by inherited genetic mutations in amyloid precursor protein gene (*APP*), presenilin 1 gene (*PSEN1*), or presenilin 2 gene (*PSEN2*) (Alzheimer's Association, 2006). Unlike fAD, the more prevalent sAD form of AD cannot be ascribed to specific genetic mutations. Despite the multifactorial risks associated with developing sAD, it is greatly influenced by an abundance of inherited genetic risk variants that may act in a concerted manner (Katsumata *et al.*, 2022). The *APOE* haplotype is one of the greatest influencing genetic risk factors for developing sAD, with the ε4 allele correlated with increased risk and the ε2 allele correlated with reduced risk (Michaelson, 2014)(Yamazaki *et al.*, 2019). In white individuals homozygous for *APOE4*, risk is increased by 15-fold and that risk increases to 33-fold for East Asian individuals. African Americans and Hispanic populations exhibit lower risk, ranging from 1- to 5-fold increases (Miyashita *et al.*,

2023). Despite the known association between *APOE4* and AD, how the *APOE4* haplotype influences AD onset and progression or how *APOE2* provides protection is still unknown. Furthermore, multitudes of environmental factors have been reported to contribute to the etiology and pathogenesis of age-related diseases; a challenging paradigm to recapitulate in vitro (Fuso *et al.*, 2005)(Smith *et al.*, 2021)(Vasanthakumar *et al.*, 2020)(Li *et al.*, 2021)(Masoud *et al.*, 2016). While aging is the greatest risk factor of developing age-related brain diseases like sAD (Qiu, Kivipelto and Von Strauss, 2009), whether aging causes sAD is debatable and beyond the scope of this review. However, it has been suggested that a gene or set of genes with a type of pleiotropy which confers beneficial or neutral effects in an individual during youth could have deleterious effects in the context of aging and, as a result, has evaded natural selection (Wheeler and Kim, 2011)(Williams, 1957).

Rodent models of AD have aided in the understanding of AD etiology and pathogenesis, albeit rodents do not inherently develop Aβ plaques or NFTs (Götz, Bodea and Goedert, 2018). The AD pathology observed in the majority of rodent models is induced via mutations in genes associated with fAD, including *APP* or *PSEN1*, which account for only about 5% of all AD cases (Alzheimer's Association, 2006). Mouse (*Mus musculus*) models exhibiting hyperphosphorylated tau in the somatodendritic domain have been established by expressing a mutation in familial FTLD *MAPT* under the control of the human promoter *THY1.2* (Götz *et al.*, 1995). More precise gene editing has been employed to humanize rodent models and achieve endogenous expression levels, yet these models often fail to recapitulate human specific responses to the induced pathological features, such as those by human

microglia or the neuroinflammatory response (Götz, Bodea and Goedert, 2018)(Sasaguri *et al.*, 2017). While stereotaxic injections of transgenic rodent or human AD brain lysates revealed complex amplification of proteopathic aggregates in mice, it is difficult to untangle the effects of the invasive injections and what could be the specific response of the mouse model (Bolmont *et al.*, 2007)(He *et al.*, 2018). Rodent models cannot replicate the genetic diversity of humans that may contribute to the overall risk of developing sAD (Götz, Bodea and Goedert, 2018). Behavioral differences between humans and rodents might also limit success of preclinical trials (Salloway *et al.*, 2014). Additionally, rodents are not a long-lived species and require genetic manipulation to mimic human susceptibility to age-related disease (Harkema, Youssef and de Bruin, 2016)(Cabral *et al.*, 2021). Nonhuman primates, like rhesus macaques (*Macaca mulatta*), could bridge the gap in lifespan between rodents and humans; however, these studies would be long, costly, and have high ethical considerations (Kennedy *et al.*, 2014). Rhesus macaques demonstrate spontaneous cerebral amyloidosis upon aging with features similar to human plaques, yet the age of onset and distribution of plaques could have important differences, plus observed tauopathies are minimal (Van Dam and De Deyn, 2017). Nonhuman animal models of AD are necessary for relating behavioral and cognitive changes to pathological features and in understanding safety of potential therapeutics; however, model systems that better recapitulate the human specific biology related to AD are required.

The advent of techniques for the reprogramming of donor somatic cells to human induced pluripotent stem cells (hiPSCs) and the subsequent differentiation of iPSCs to neural cell types, has provided researchers access to human specific cellular processes

of brain development and maturation (Takahashi *et al.*, 2007). hiPSCs maintain the genetic background of the donor from which they are derived while overwriting most of the epigenetic signatures an individual has acquired throughout one's life (Kim *et al.*, 2010)(Shutova *et al.*, 2016)(Marchetto *et al.*, 2009)(Hu *et al.*, 2010). This might allow for the disentanglement of genetic versus epigenetic influences in the context of age-related brain disease. hiPSCs can be employed to generate brain organoids that recapitulate some features of brain development in three-dimensional (3D) structures consisting of various subtypes of neuronal and glial cell populations (Matsui *et al.*, 2018)(Lancaster and Knoblich, 2014). These 3D models have increased tissue architecture when compared to 2D monolayer culture and both inhibitory and excitatory synapses that give rise to spontaneous neuronal activity suggesting functional maturity of neurons (Lancaster *et al.*, 2013)(Quadrato *et al.*, 2017)(Trujillo *et al.*, 2019a). Although brain organoids are currently limited in the amount of maturation that can be achieved, and sAD is a late onset disease, brain organoids could demonstrate sAD phenotypical differences apparent in neurodevelopment, exhibit greater cellular maturation and complexity compared to 2D monolayer cell culture, or represent physiological responses to aging simulating cellular stresses (Chiaradia and Lancaster, 2020). These features of brain organoids allow for the investigation of sAD pathogenesis in a human specific model and could accelerate the development of therapeutic or preventative strategies in sAD.

Most protocols for brain organoid derivation lack microglia since microglia develop from the mesodermal-derived yolk-sac and migrate into the brain during early development while other brain cell types comprising brain organoids originate from

ectoderm (Ginhoux *et al.*, 2010)(Utz *et al.*, 2020). Coculturing brain organoids with hiPSC or human embryonic stem cell (ESC) derived microglia, which are major cellular players in the etiology and pathogenesis of age-related brain diseases including AD, could untangle microglia influences on, or responses to, AD pathology (Plescher *et al.*, 2018). Microglia can be integrated into donor matched brain organoids allowing for the study of donor-specific genetic influence on microglia interactions during brain maturation (Schafer *et al.*, 2023). Throughout this review, we will consider whether brain organoids of increasing complexity could serve as models of age-related brain diseases either by representing developmental neurobiology that might foreshadow age-related brain diseases or via the perturbation of specific mechanisms, pathways, or homeostatic processes to induce age-related brain disease pathologies in organoids. We will discuss the application and challenges of brain organoids enriched with astrocytes, microglia, and oligodendrocytes to unravel cell-to-cell interactions and genetic contributions in age-related brain disorders with a focus on AD.

2. Generating brain organoid models of AD

Limited access to developing and diseased human brain tissues has motivated researchers to pursue in vitro models, providing a window into concealed processes of brain development and pathogenesis. Following the isolation of hESCs and establishment of successful hESC culture conditions (Thomson *et al.*, 2013), floating embryoid bodies (EBs) of aggregated hESCs were generated and plated to obtain neurons(Bain *et al.*, 1995). Lancaster et al. took the next step to generate cerebral organoids by embedding EBs in Matrigel and supporting a suspension culture in media with minimal influence on cell lineage specification. Cerebral organoids demonstrate

ventricular zone (VZ) and subventricular zone (SVZ)-like layering with differentiating neurons that migrate outwards to form heterogenous structures with forebrain, midbrain, and hindbrain identities (Lancaster and Knoblich, 2014)(Lancaster *et al.*, 2013). More recently, factors and small molecules have been incorporated into brain organoid generation protocols to direct cell fate and regional identity of brain organoids. Often guided approaches begin with dual SMAD inhibition (the inhibition of both transforming growth factor beta (TGF-β) and bone morphogenetic protein (BMP) pathways) to yield robust ectoderm (Chavali *et al.*, 2020), followed by dorsalizing factors to promote forebrain identity (Liem *et al.*, 1995)(Qian *et al.*, 2018), or exposure to WNTs and BMPs for dorsomedial identities including pallial hem with hippocampus (Sakaguchi *et al.*, 2015). Brain regions including the hippocampus, entorhinal cortex (EC), primary visual cortex, superior frontal gyrus (SFG), and middle temporal gyrus are histopathologically relevant to AD (Liang *et al.*, 2008). Although brain organoids do not result in the six distinct layers of the human cortex, discrete subregions of the adult cortex, or the complex cellular organization of the hippocampus, brain organoids that are more cortical or hippocampal in derivation could better represent cell types implicated in AD (Chen *et al.*, 2021). Brain organoids model some early cytoarchitectural features of brain development, including organization of SVZ and VZ-like layering, until approximately four months. Beyond four months of generation, brain organoids continue to recapitulate some developmental transcriptional programs, however SVZ and VZ-like layering begins to dissipate (Tanaka *et al.*, 2020)(Camp *et al.*, 2015)(Uzquiano *et al.*, 2022). Nevertheless, amyloid-β (Aβ) aggregates and NFT structures have been observed in brain organoids derived from fAD donor hiPSCs (Gonzalez *et al.*,

2018)(Raja *et al.*, 2016)(Park *et al.*, 2021). The complex extracellular environment of brain organoids could be conducive for Aβ protein aggregation (Chiaradia and Lancaster, 2020). Additionally, the lack of vasculature in brain organoids likely prevents physiological clearance of Aβ which would occur through the blood brain barrier (BBB), and the lack of microglia results in decreased uptake and degradation of Aβ (Qi and Ma, 2017). However, a key question remains outstanding; can brain organoids serve as models of AD and other age-related brain diseases? Here, we discuss strategies and challenges in elucidating age-related brain diseases using brain organoids (Table 2.1)(Figure 2.1).

2.1. Stem cell derived brain organoid models of sAD, fAD, and Down Syndrome related AD

Brain organoids are derived from either ESCs, isolated from the inner cell mass of a blastocyst (Thomson *et al.*, 2013), or iPSCs that are obtained through the reprogramming of donor somatic cells (Takahashi *et al.*, 2007). iPSCs experience a resetting of epigenetic and age-related signatures during the reprogramming process, providing iPSCs a pluripotency comparable to ESCs (Shutova *et al.*, 2016). However, it has been suggested that some of the epigenetic and age-related signatures of the somatic cells from which iPSCs are reprogrammed could be retained and influencing iPSC fate specification during differentiation (Kim *et al.*, 2010)(Marchetto *et al.*, 2009)(Hu *et al.*, 2010). If retention of somatic cell epigenetic signatures does occur during iPSC reprogramming, it could account for a portion of the high variability that has been observed in cerebral organoid models derived from different iPSC lines and challenges the ability to separate genetic versus epigenetic influence in AD. Hernandez

et al. developed cerebral organoids from the iPSCs of individuals with no diagnosis of dementia yet with *APOE-ε3/ε3* or *APOE-ε4/ε4* variants and from a clustered regularly interspaced short palindromic repeats (CRISPR)-Cas edited isogenic iPSC line of *APOE-ε4/ε4* to *APOE-ε3/ε3* to investigate the effects of *APOE* status related to late onset AD pathology. The researchers found no APOE dependent differences in APOE protein expression and amyloid-β secretion. Additionally, variations in phosphorylated tau (p-tau) could not be attributed to specific genotypes with the exception of a decrease in the phosphorylation of tau at S199 which was decreased in the isogeneic *APOE-ε3/ε3* organoids when compared to APOE-ε4/ε4(Hernández *et al.*, 2022). Conceivably, combinatorial effects of APOE status and additional sAD associated genetic variants influence ones' risk of developing sAD and accounts for the lack of effects observed (Lin *et al.*, 2018). Zhao et al. provided evidence supporting the hypothesis that *APOE4* contributes to sAD onset through a combinatorial effect with other sAD genetic risk variants. Similarly, Zhao et al. generated cerebral organoids using iPSC lines from *APOE-ε3/ε3* or *APOE-ε4/ε4* carriers, however five of the hiPSC lines were from cognitively unimpaired individuals carrying *APOE-ε3/ε3*, five were from cognitively unimpaired individuals carrying *APOE-ε4/ε4*, five were from sAD patients carrying *APOE-ε3/ε3* (AD-E3), and five were from sAD patients carrying *APOE-ε4/ε4* (AD-E4). The researchers found increased cleaved Caspase-3 (CASP3), indicating increased apoptosis, and decreased synaptophysin and PSD95, suggesting synaptic loss, in AD-E4 cerebral organoids at week 12 revealing a synergistic APOE4 and sAD status effect. Corroborating observations made by Hernandez et al., Zhao et al. found that Aβ40, Aβ42, and Aβ42/Aβ40 ratio were increased in sAD organoids independent of

APOE4 status. Lastly, the researchers found that both *APOE4* and AD status increase p-tau levels in cerebral organoids, although *APOE4* had a significant effect on p-tau in both AD and healthy derived organoids (Zhao *et al.*, 2020). This research highlights the importance of considering the broad range of genetic backgrounds contributing to an individual's risk of developing sAD when establishing physiologically relevant, human models of sAD.

It is estimated that approximately 50% of people with Down syndrome (DS) will develop AD as they age. This is caused by an extra copy of *APP* that people with DS carry on a third copy of chromosome 21. Gonsalez and colleagues performed experiments to determine whether fAD or DS patient derived cerebral organoids develop AD pathology when compared to control donors or Creutzfeldt-Jakob disease (CJD) derived cerebral organoids. CJD is thought to be caused by an abnormal isoform of a prion protein. The authors generated cerebral organoids from the hiPSCs of a patient with fAD carrying a mutation in *PSEN1*, a 5-month-old patient with DS, a 66-year-old control donor, and two patients with CJD. After analyzing 50 to 200 cerebral organoids at day 110, they found Aβ deposits in fAD and DS derived cerebral organoids and no Aβ deposits in control donor or CJD derived cerebral organoids. Enzyme-linked immunosorbent assay (ELISA) revealed an increased Aβ42/Aβ40 ratio in both fAD and DS donor derived cerebral organoids when compared to control donor derived cerebral organoids. Moreover, NFT structures and increased CASP3 were observed in fAD and DS derived cerebral organoids relative to control donor derived cerebral organoids (Gonzalez *et al.*, 2018). An additional study developed cerebral organoids from a donor carrying a point mutation in APP causing Dutch-type cerebral amyloid angiopathy (D-

CAA). No increase in the production of Aβ42 or Aβ40 in D-CAA iPSC derived neurons was found, however researchers observed an increase in Aβ deposition in D-CAA derived organoids relative to controls at both days 52 and 110, as well as an increase in transforming growth factor beta receptor 1 (TGFBR1), thought to promote APP and Aβ expression in astrocytes (Daoutsali *et al.*, 2023). The fact that only approximately 70% of individuals with DS develop dementia by age 60 has led researchers to hypothesize that additional genes located on chromosome 21 could be influencing the onset and progression of age-related dementia. B-site APP cleaving enzyme 2 (BACE2), a homologue of BACE1, is located on chromosome 21 and could have anti-amyloidogenic properties. When researchers used CRISPR-Cas9 to eliminate a copy of BACE2 (from three copies of BACE2 to one copy) in DS derived cerebral organoids, they saw a significant increase in Aβ plaque-like deposits by day 48 when compared to unedited DS derived cerebral organoids. Treatment of edited DS derived cerebral organoids with β and γ secretase inhibitors eliminated Aβ plaque-like deposits rescuing the observed effects(Alić *et al.*, 2021). These studies suggest that brain organoid models derived from DS patients are useful in elucidating mechanisms of APP processing related to Aβ-deposition, however they might not represent AD associated tau pathology or more complex interactions of genetic variants contributing to sAD pathogenesis.

2.2. Genetic modifications to investigate fAD and mitochondrial dysfunction

One of the earliest 3D models of AD pathology was described by Choi et al., although this model is unlike stem cell derived brain organoids in that it employs ReNcell VM human neural precursor (ReN) cells that are embedded as a cell suspension into Matrigel and differentiated into neurons with projections yet minimal

tissue architecture. Using lentiviral constructs, the ReN cells were transduced to overexpress human *APP* or *APP* and *PSEN1*, containing fAD mutations. fAD ReN cells showed extracellular amyloid-β deposits that were reduced upon treatment with β- or γ-secretase inhibitors. p-tau was increased in the neurites and neuronal cell bodies and, again, decreased following β- or γ-secretase inhibitors suggesting that increased APP processing resulted in elevated p-tau mislocalization (Choi *et al.*, 2014a). Although, in sAD, iPSC derived cortical spheroid models, β- or γ-secretase inhibitors were less effective at reducing Aβ42 levels when compared to iPSC derived, monolayer neural cells (H. K. Lee *et al.*, 2016). While a similar method to that used by Choi et al. could be applied to generate organoids from stem cells that overexpress fAD mutations, it is unclear if organoids generated from sAD iPSCs would recapitulate pathology to the same extent. However, a recent study of thousands of individuals found that almost all APOE4 homozygous carriers, the biggest genetic risk factor for sAD, exhibited high levels of Aβ42 in CSF (Fortea *et al.*, 2024). This supports the idea that fAD and sAD organoids could express AD pathology, and that investigating the cascade following Aβ accumulation is important in understanding AD pathogenesis.

More recently, researchers have investigated the effects of a missense loss of function mutation in pitrilysin metallopeptidase 1 (*PITR1*) causing mitochondrial dysfunction and an age-dependent, neurological syndrome with AD-like amyloidogenesis (Langer *et al.*, 2018). Using CRISPR-Cas9, the *PITRM1* gene of hiPSCs obtained from an 80-year-old, control donor was targeted to introduce knockout mutations. Cerebral organoids were generated from *PITR1*$^{-/-}$ iPSCs. At two months, western blot and immunohistochemistry (IHC) analyses of *PITR1*$^{-/-}$ organoids revealed

increased APP levels and tau hyperphosphorylation when compared to *PITR1*$^{+/+}$ organoids. Additionally, IHC demonstrated increased CASP3 while immunoassays showed higher Aβ40, Aβ42, and Aβ42/Aβ40 ratio in *PITR1*$^{-/-}$ organoids relative to controls. To determine whether enhanced mitochondrial mitophagy would decrease the observed AD pathology, *PITR1*$^{+/+}$ and *PITR1*$^{-/-}$ organoids were treated with the NAD+ precursor, nicotinamide mononucleotide (NMN). The researchers observed decreased Aβ42/Aβ40 ratio and p-tau/total tau ratio in addition to decreased CASP3 in *PITR1*$^{-/-}$ organoids following treatment with NMN (Pérez *et al.*, 2021). Further research applying gene editing tools, like CRISPR-Cas, to introduce either genetic or epigenetic modification associated with fAD or accelerated aging diseases could assist in elucidating specific processes associated with aging and AD. Nevertheless, it will be challenging to represent the genetic complexity of age-related brain diseases like sAD with gene editing alone.

2.3 .Exogenous induction of AD-like pathology and events of aging

Researchers have worked to overcome the challenges associated with phenotypically young characteristics of human brain organoids by exogenously introducing events to induce late-onset, disease-like pathology. To mimic BBB leakage, an effect that often occurs with aging and at increased rates in individuals harboring *APOE4* variants, Chen et al. treated brain organoids derived from hiPSCs with 10 percent human serum for 12 days. Control brain organoids were not treated with serum, and brain organoids were analyzed between days 90 and 110. The authors observed increased insoluble Aβ and increased extracellular Aβ aggregates in serum-treated brain organoids when compared to controls. BACE, an enzyme that drives the

production of Aβ, was also elevated in serum-treated brain organoids, and treatment of organoids with BACE inhibitor IV reduced Aβ levels in organoids even with serum exposure. Furthermore, the researchers observed synaptic loss measured by a decrease in synapsin-1 puncta, decreased calcium signaling identified with calcium imaging, and reduced neural network activity in the form of decreased number of spikes, bursts, mean firing rate, and synchrony index as measured by multi-electrode arrays (MEA) in brain organoids following serum exposure (Chen *et al.*, 2021). This research supports the hypothesis that exogenous factors could recapitulate events associated with aging and induce age-related brain disease-like pathology in brain organoid models. However, serum is heterogenous, containing various factors that could influence cell behavior, and differences in the composition of serum components across individuals could affect results. Determining which components are contributing to observed pathological effects is challenging when using complex substances to evoke disease pathogenesis.

A more precise approach to induce age-related disease pathology is the use of small molecules or factors to modulate cellular pathways implicated in the pathological feature of interest. Pavoni et al. targeted the production of Aβ42 in brain organoids generated from control donor derived iPSCs using a small molecule inducer of Aβ42 called Aftin-5. The authors observed an increase in Aβ42 and Aβ42/Aβ40 ratio, however there was no increase in p-tau or extracellular amyloid-β as indicated by IHC (Pavoni *et al.*, 2018). While the implementation of small molecules to induce AD pathology can teach us about the pathways related to the targeted pathological feature, potentially dysregulated cellular responses associated with a disease state would not be

represented in a control donor derived brain organoid. It would be informative to treat sAD patient derived and control donor derived brain organoids with AD pathology inducers like Aftin-5 to observe cellular responses with patient specific genetic backgrounds. Exogenous treatment of AD derived brain organoids with synthetic Aβ40 and Aβ42 could provide further insight into multicellular responses to Aβ pathology. Primary brain cell and stem cell derived neural cell types treated with Aβ showed increases in endogenous Aβ release resulting in neuronal dysfunction. Aβ is thought to cause neuronal dysregulation through the activation of receptors associated with neuroinflammation, increased oxidative stress, loss of synapses, and a dysregulation of neurotransmitters (Ferreira *et al.*, 2014)(De Felice *et al.*, 2007)(Izzo *et al.*, 2014)(Brito-Moreira *et al.*, 2011). The variety of cell types with complex interactions available in brain organoids may reveal cascading AD pathogenesis upon Aβ oligomer treatment (Fontana *et al.*, 2020).

2.4. **Limitations of Necrosis and Understanding the Capacity for Maturation**

The lack of vascularization in brain organoids prevents sufficient diffusion of oxygen beyond the initial approximately 600 μm depth and restricts nutrient penetration, leading some researchers to limit their analysis to a region of neuron-rich, CASP3-sparse tissues about 250 μm from the organoid surface (Raja *et al.*, 2016)(Magliaro, Rinaldo and Ahluwalia, 2019). Often, beyond the 250 μm depth into brain organoids, tissues abundant in apoptotic CASP3 are comprised of dead and dying cells with little to no protein expression forming a necrotic core. The restricted oxygen and nutrient diffusion limit is thought to inhibit cellular maturation, cellular subtype specification, and continued growth of brain organoids (Chiaradia and Lancaster, 2020)(Bhaduri *et al.*,

2020). Reports comparing single-cell RNA sequencing (scRNAseq) from developing human cortex and brain organoids found an increase in glycolysis and endoplasmic reticulum (ER) stress genes within brain organoids. The authors observed decreased cellular stress markers and increased cell subtype specification following transplantation of cells from dissociated, two-month-old organoids into mice suggesting that in vitro cellular stress was responsible for impairments in cellular subtype specification (Bhaduri *et al.*, 2020). ER stress leads to the unfolded protein response which occurs during early stages of tauopathies and is strongly correlated with p-tau accumulation in AD. Human neuroblastoma, SK-N-SH, cells treated with an inhibitor of glucose metabolism (2-deoxy glucose) demonstrated increased p-tau, although p-tau levels returned to prior levels upon removal and washout of the drug (Van Der Harg *et al.*, 2014). Furthermore, studies in mice suggest that metabolic stress associated oxidative stress increases brain inflammation and neuronal insulin resistance leading to Aβ deposition (Hahm *et al.*, 2020). The evidence linking elevated cellular stress with AD pathology, in addition to the increased cellular stress observed in brain organoids, can complicate the interpretation of observed Aβ and p-tau changes in these models. This highlights the importance of including relevant controls and minimizing cellular stress when modeling AD using brain organoids.

Early strategies to overcome the deficiencies attributed to a lack of vasculature in brain organoid culture include maintaining organoids in spinning bioreactors and the addition of neurotrophic factors, like brain derived neurotrophic factor (BDNF) or glial derived neurotrophic factor (GDNF), to support neuronal maturation and survival (Lancaster *et al.*, 2013)(Quadrato *et al.*, 2017)(Qian *et al.*, 2018). Additionally, slicing

organoids to establish a sliced neocortical organoid (SNO) model reduces the thickness of the organoid structure and increases access of cells to nutrients and oxygen. The SNO model has demonstrated improved neuronal survival and cortical layer formation, although it is unclear if the regular slicing of organoids could introduce cellular stress (Qian *et al.*, 2020). Implementing organoid generation protocols which promote more physiologically relevant proportions of microglia, astrocytes, and oligodendrocytes could better support the survival, maturation, and cortical layering of neurons during long term culture, nonetheless this will not improve oxygen diffusion limits within brain organoids (Madhavan *et al.*, 2018)(Cakir *et al.*, 2022)(Huang *et al.*, 2022). Perhaps the most advanced brain organoid cellular maturation and complete compilation of cell types has been achieved through the transplantation of brain organoids into immunodeficient mice which increases oxygen and nutrient delivery into the core as the mouse vasculature infiltrates the transplanted organoid (Wang *et al.*, 2024)(Mansour *et al.*, 2018). However, in the realm of disease modeling, it is difficult to untangle the influence of the rodent in vivo systems on the pathological features of the transplanted brain organoid.

Brain organoids are derived from embryonic-like cells and recapitulate several early fetal brain developmental programs across both guided and unguided brain organoid generation protocols (Tanaka *et al.*, 2020). Understanding the extent of cellular maturation and specification that can be achieved during long-term culture of brain organoids is essential in elucidating their capacity to model late-onset and age-related brain diseases like AD. A recent study identified subpopulations of cells in brain organoids that might deviate from human fetal cortex transcriptional paradigms. This included a more gradual decline in cell cycle, neural stem cell (NSC), and neural

progenitor cell (NPC) markers in brain organoids than what was observed in fetal cortex. Additionally, the transient expression patterns of outer radial glial (oRG) genes were observed in primary cortical development, however cortical brain organoids showed continuous upregulation of oRG genes. While the authors did observe some deviations in transcriptional programs of brain organoids relative to fetal cortex development, they found only fluctuations in the expression of glycolytic and ER-stress genes in both brain organoids and primary cortical development with no drastic increase at advanced time-points (Cheroni *et al.*, 2022). Uzquiano et al. corroborated these findings using a longitudinal single-cell atlas with eight timepoints across six months of brain organoid development and spatial transcriptomics of 10 organoids at one, two, and three months profiled by Slide-seqV2. The authors found that, during the first one to six months of organoid development, most differentiation transitions and cell type specification signatures matched those observed in vivo. Organoid NPCs, mostly apical radial glia (aRG), restricted to the center of organoids demonstrated an enrichment of glycolysis and hypoxia genes comparable to those observed in fetal tissue. This research suggests that the specification of most cortical cell types in organoids are unaffected by diverging metabolic states (Uzquiano *et al.*, 2022). Interestingly, studies of long-term human cortical organoid maturation revealed consistent levels of stress pathway genes rather than compounding cellular stress over time. The researchers also found that some postnatal maturation features were observed between days 250 and 300 of in vitro brain organoid culture, including ribonucleic acid (RNA) editing and a switch in NMDA receptor subunits characteristic of postnatal mammalian brain development (Gordon *et al.*, 2021). Tau filaments are abnormal, intracellular aggregates

of p-tau and their composition can vary greatly among neurodegenerative diseases (Goedert *et al.*, 1992). The tau filaments of AD contain all six isoforms of tau, however only the 0N3R isoform is present during fetal brain development with the additional five isoforms expressed perinatally and the four-repeat (4R) tau constituting the majority of tau isoform expression in the adult human brain (Hefti *et al.*, 2018)(Brion et al., 1993). iPSC-derived 3D neural models embedded in Matrigel-coated alginate capsules and cultured for 25 weeks with BrainPhys media were found to express all six adult tau isoforms, although not at the proportions observed in the adult brain (Miguel et al., 2019). Additionally, ReNcell VM human neural precursor cells with lentiviral overexpression of APP and PSEN1 fAD mutations were embedded in Matrigel to form a 3D cell suspension and showed increased expression of 4R adult tau isoforms in 3D compared to 2D (Choi *et al.*, 2014b). This suggests that long term culture of AD brain organoid models, and those cultured in maturation promoting medias, could express additional tau isoforms found in AD tau filaments although optimization is required to achieve adult-like proportions of the tau isoforms. Moreover, fetal tau is hyperphosphorylated and could resemble p-tau patterns observed during AD pathology (Brion et al. 1993)(Brion et al. 1993). While brain organoids, which model features of human brain development, may be helpful in elucidating mechanisms associated with AD related tau hyperphosphorylation, careful consideration is required when discerning a feature of fetal brain development from one related to AD pathogenesis. A brain organoid model with more postnatal and adult-like protein isoforms and neuronal features may better represent the effects of age-related brain disease etiology and

pathogenesis compared to shorter-term, 2D neural cell cultures. However, maintaining organoids in sterile culture for 300 days is technically challenging and costly.

As neuronal maturation occurs and complex cellular networks begin to form during brain development, neurons develop dendritic spine structures and become spontaneously electrophysiologically active. Much of what we understand about neuronal network maturation is limited to rodent and other nonhuman models due to the inaccessibility of human fetal brain development. Spontaneously active neuronal networks have been detected in eight-month-old cerebral organoids using high-density, penetrating microelectrodes (Quadrato *et al.*, 2017). Additionally, electron microscopy has revealed synapses and synaptic vesicles in cortical organoids by four months and these cortical organoids displayed increasing mean firing rate and burst frequency from two to 10 months measured via MEAs (Quadrato *et al.*, 2017)(Trujillo *et al.*, 2019a). The synchrony index of spontaneously active cortical organoids also increased from two to eight months, plateauing from eight to 10 months. Glutamate receptor antagonists and GABA receptor agonists reduced the number of spikes indicating that both excitatory and inhibitory neurons participate in spontaneous neuronal network modulation of cortical organoids (Trujillo *et al.*, 2019b). Since it has been shown that AD organoid models demonstrate AD pathology, observing neuronal network dynamics in brain organoid models of AD could provide insight into the influences of AD pathology on neuronal network dysregulation. Researchers have generated cerebral organoid models of fAD from one hiPSC line containing a *PSEN1* mutation, one hiPSC line with an *APP* mutation, and one isogenic control hiPSC line. At two months, they found increased Aβ42/Aβ40 ratio and total Aβ in *PSEN1* and *APP* mutant derived cerebral organoids,

respectively, and an increase in mean firing rate in both *PSEN1* and *APP* organoids compared to isogenic control derived cerebral organoids (Ghatak *et al.*, 2019). While brain organoids do not represent the adult human brain, research suggests that brain organoids can achieve neuronal maturation to the point of functional communication of neural networks and this level of maturation might recapitulate the effects of AD specific genetic background contributing to neuronal network dysregulation.

3. Cellular roles to model AD in brain organoids

Historically, neuronal cells have been the focus of understanding neurodegenerative diseases, such as AD. However, roughly half of the cells in a typical brain are glial cells and about 10 to 15% of glial cells are microglia, 20 to 40% are astrocytes, and approximately 75% are oligodendrocytes (Pelvig *et al.*, 2008). While understanding subtypes of neurons that show exceptional vulnerability to AD pathology is informative in elucidating disease progression, glial cells have critical roles in healthy brain homeostasis and could be dysregulated causing AD pathogenesis. In the brain, APOE is predominantly expressed by glial cells, and there is increasing evidence that *APOE4* glial cells exhibit detrimental differences in comparison to their isogenic *APOE3* counterparts (TCW *et al.*, 2022). Available brain organoid protocols generally produce astrocytes but amounts are often less than what is observed in vivo and maturation of astrocytes begins late, around 77 days of culture (Verkerke *et al.*, 2024). Brain organoids have been developed with an enrichment of astrocytes, microglia, and oligodendrocytes through the exogenous application of platelet-derived growth factor-AA (PDGF-AA) and additional factors (Wang *et al.*, 2024)(Madhavan *et al.*, 2018) or with the overexpression of the myeloid inducing transcription factor PU.1 to establish

microglia-like cells in cortical organoids (Cakir *et al.*, 2022). Moreover, microglia integrated into brain organoids at the erythrocyte myeloid progenitor cell (EMP) stage take on a more homeostatic morphology and transcriptional state as they convert to microglia in the brain organoid environment (Schafer *et al.*, 2023). Although the presence of multiple cell types at more physiological proportions likely improves the representation of AD pathogenesis in brain organoids, it is challenging to attribute an observed pathological feature to a specific cell type using these complex systems. Moreover, inter- and intra- batch variability observed in brain organoids emphasizes the need to normalize for cell type composition during analyses (Hernández *et al.*, 2022). Here, we discuss potential cell type specific contributions to AD-etiology and pathogenesis and what roles these cell types might play in brain organoid models of AD (Figure 2.2).

3.1. Neurons

Postmortem examinations of patients with AD have identified decreases in neuron number and tissue volume of the EC, layer II, and CA1 (JL *et al.*, 2001). Despite the substantial loss of neurons, the direct cause of neuronal death and synapse loss in AD is unclear. Soluble Aβ oligomers, hyperphosphorylated tau, neuroinflammation, and age-related mitochondrial damage have all been implicated, yet there is no consensus on how the interplay of these pathological features might lead to late-onset neurodegeneration (Knott *et al.*, 2008)(Mangalmurti and Lukens, 2022). Research has suggested that soluble Aβ oligomers are major contributors in AD pathogenesis and causative relationships between altered APP processing and increased p-tau have been investigated (Israel *et al.*, 2012). Even so, little is understood regarding the selective

vulnerability of subpopulations of neurons. While selectively vulnerable networks have been characterized in AD, characterization of selectively vulnerable neurons and mechanisms underlying the vulnerability of these neurons remain elusive (Schöll *et al.*, 2016)(Seeley *et al.*, 2009)(Leng *et al.*, 2021). Single-nucleus RNA sequencing (snRNA-seq) of postmortem brain tissue from patients with no, early, and late stages of neurofibrillary pathology in EC and SFG supported previous findings that excitatory neurons display a greater susceptibility than inhibitory neurons to AD pathology. More specifically, a population of RAR-related orphan receptor B (RORB) expressing excitatory neurons with exceptional vulnerability in AD was identified. IHC analysis showed a decrease in $RORB^+$ excitatory neurons associated with early disease stages and an increase in p-tau (CP13) in $RORB^+$ neurons when compared to $RORB^-$ excitatory neurons (Leng *et al.*, 2021). Brain organoids have been developed containing both excitatory and inhibitory neurons. Protocols to culture sliced cortical organoids with reduced necrosis demonstrated $RORB^+$ excitatory neurons with laminar expression patterns (Quadrato *et al.*, 2017)(Qian *et al.*, 2020). Although the maturation state of neurons in brain organoids might not represent that of an aged adult, brain organoids develop heterogenous populations of neurons and could be used to study vulnerable subtypes of neurons implicated in AD.

3.2. Astrocytes

Astrocytes are essential for maintaining healthy neuronal network communication by supporting the formation and function of synapses through homeostatic regulation and synaptic pruning (Chung *et al.*, no date)(Diniz *et al.*, 2012)(Clarke and Barres, 2013). Astrocyte activation is an early response to Aβ plaque and p-tau accumulation

that can upregulate nuclear factor kappa B (NFκB) signaling and increase the production of proinflammatory cytokines, including interleukin 6 (IL-6), interleukin 8 (IL-8), and C-C motif chemokine ligand 5 (CCL5) (Heneka, Kummer and Latz, 2014)(Carter *et al.*, 2012)(Hyvärinen *et al.*, 2019)(Lian *et al.*, 2015)(Liddelow *et al.*, 2017). However, it is unclear if the chronic activation of astrocytes is the cause of or a response to Aβ deposition and NFT accumulation which may lead to the dysregulation of neuronal networks and the death of neurons in sAD. It is thought that Aβ induced astrocyte NFκB activation results in the release of complement component 3 (C3) leading to calcium dysregulation at neuronal synapses and aberrant dendritic morphology (Lian *et al.*, 2015)(Mattson, 2007). Furthermore, research suggests that an Aβ induced upregulation of inflammatory cytokines could contribute to a dysregulation of neuronal networks in sAD. Injections of IL-1β have been shown to induce febrile seizures in mice, likely through the dysregulation of Glutamatergic and GABAergic signaling, while the treatment of cultured hippocampal neurons with TNF-α increased AMPA receptors causing a greater frequency of excitatory postsynaptic currents (Dubé *et al.*, 2005)(Knutson *et al.*, 2002)(Mosili *et al.*, 2020). Additionally, MCP-1 applied to rat substantia nigra slices increased cell excitability and dopamine release of dopaminergic neurons (Guyon *et al.*, 2009). Chemokines could modulate neuronal activity in several neuronal subtypes and through various mechanisms, possibly by increasing calcium transients, governing the release of neurotransmitters, or effecting voltage-dependent channels (Guyon and Nahon, 2007)(Graaf and Os, 2007).

Much of what is known about possible astrocyte reactivity and dysfunction in AD has been investigated in AD rodent models which represent AD pathology yet lack the

response of astrocytes with a human specific sAD genetic background. Although, a study using snRNA-seq to analyze postmortem EC samples of AD patients identified AD associated astrocyte subclusters with one such cluster demonstrating an enrichment for ribosomal, mitochondrial, neuron differentiation, and heat shock responses while another cluster showed downregulation of these processes and an increase in TGF-β signaling, immune responses, and C3 (Grubman *et al.*, 2019). Additionally, Mathys and colleagues performed scRNA-seq on prefrontal cortex (PFC) with high or low levels of Aβ and found an astrocyte population associated with AD that expressed GLUL and the AD risk factor CLU, demonstrated to be upregulated in reactive astrocytes (Mathys *et al.*, 2019). More recently, Leng et al. employed snRNA-seq and located astrocyte subpopulations in the EC and SFG corresponding to reactive astrocytes. They observed astrocyte subpopulations expressing high levels of GFAP and these GFAP high populations consistently expressed lower levels of genes associated with glutamate/GABA homeostasis and synaptic adhesion/maintenance (Leng *et al.*, 2021). While analyses of postmortem brain tissues using snRNA-seq provides a snapshot of human AD pathology, it lacks the ability to probe dynamic cellular mechanisms contributing to AD pathogenesis and loses some spatial information that might correlate transcriptional changes with cellular proximity to pathological features of AD.

Recent protocols describing the enrichment and accelerated induction of astrocytes in cortical organoids could fill in the gaps between nonhuman animal models and postmortem tissue studies. An accelerated astrocyte induction cortical organoid protocol achieved functional astrocytes by day 45 and advanced neuronal maturation. Applying the described protocol, the authors generated chimeric organoids with *APOE*

genotypes varying by cell type to investigate neuronal lipid droplet, cholesterol, and p-tau levels. The authors found that both astrocytic and neuronal *APOE4* genotypes were required to increase p-tau levels although Aβ levels were increased with astrocytic *APOE4* alone (Huang *et al.*, 2022). An additional study induced a gliogenic switch during early cortical organoid development and achieved approximately 25 to 31% astrocytes by about day 63. The researchers identified a reactive subpopulation of astrocytes that rapidly activated upon cytokine stimulation. Implementing a similar assay in AD derived organoids might reveal more or exacerbated AD astrocyte responses to proinflammatory stimuli. These studies allow for more physiological amounts of human astrocytes in AD organoids to better recapitulate AD pathogenesis (Wang *et al.*, 2024).

3.3. Oligodendrocytes

Oligodendrocytes are gaining increasing attention for their implications in AD, as their primary role is to generate myelin sheaths that act as electrical insulation of axons and wrap the axons of multiple neurons together forming white matter (WM) of the central nervous system (CNS). WM abnormalities and demyelination are well documented in early AD progression (S. Lee *et al.*, 2016)(Desai *et al.*, 2010)(Radanovic *et al.*, 2013). Oligodendrocytes are also vital in maintaining neuron homeostasis by mediating inflammation and providing metabolic support (Fünfschilling *et al.*, 2012)(Saab *et al.*, 2016). Like astrocytes and neurons, little is known about subtypes of oligodendrocytes that may arise during AD pathogenesis and even less is known about the molecular changes that emerge in cells related to proximity of AD pathological features. snRNA-seq of postmortem EC and SFG revealed that oligodendrocytes exhibit a discernable transcriptional response in AD with *LINGO1* as a top differentially

expressed gene in oligodendrocytes and excitatory neurons. *LINGO1* is a negative regulator of myelination, neuronal survival, axonal integrity, and oligodendrocyte differentiation (Leng *et al.*, 2021)(Grubman *et al.*, 2019)(Mathys *et al.*, 2019). Additionally, snRNA-seq and spatial transcriptomics (ST) of postmortem tissues recognized *CRYAB*, a myelin related gene, as dysregulated in oligodendrocytes within the proximity of amyloid deposition (Leng *et al.*, 2021)(Chen *et al.*, 2020). ST studies in a mouse model of AD further identified a gene co-expression network expressed mainly in oligodendrocytes that was enriched for genes related to myelination. This gene expression network was activated with mild amyloid stress yet decreased in the presence of high amyloid accumulation. When human orthologs within the identified mouse gene network were compared to human control and AD postmortem brain samples using ST, they found that many of the genes were also significantly changed in late-stage, human AD samples but not all the genes of the network were dysregulated or enriched only in oligodendrocytes (Chen *et al.*, 2020). ST analysis of brain organoids could advance our understanding of the cellular subtype specific responses to AD pathology and cellular mechanisms involved in AD etiology and pathogenesis. Cells with oligodendrocyte identity have been reported in unguided brain organoid protocols after long-term culture (Tanaka *et al.*, 2020). The implementation of patterning factors, including PDGF-AA, insulin-like growth factor 1 (IGF-1), and thyroid hormone (T3) during cortical organoid generation have increased myelinating oligodendrocyte populations in brain organoids as early as day 50, however myelinated organoids have yet to be used to investigate the role of oligodendrocytes in AD etiology and progression (Madhavan *et al.*, 2018)(Marton *et al.*, 2019)(Shaker *et al.*, 2021).

3.4. Microglia

Microglia are specialized macrophages of the CNS that clear debris, pathogens, and neuronal synapses. Under homeostatic conditions, microglia provide support to neurons, whereas under pathological conditions, microglia often prune synapses excessively, increase inflammation, and reduce support to neurons (Li and Barres, 2018). During AD pathological challenge, microglia are thought to contribute to neuroinflammation by increasing the secretion of pro-inflammatory cytokines (IL-1β and TNFR1) and chemokines, while reducing the amount of neurotrophic factors produced (DiSabato, Quan and Godbout, 2016). Acute activation of microglia can promote the clearance of pathogens and toxins, although chronic activation of microglia can lead to neuronal dysfunction (Heneka *et al.*, 2015)(Calsolaro and Edison, 2016). In addition to the pro-inflammatory cytokines produced, anti-inflammatory cytokines such as IL-1 receptor antagonist, IL-4, IL-10, and IL-11 are upregulated as what could be a mechanism to prevent excessive neuroinflammation (Calsolaro and Edison, 2016). Microglia activation was thought to be triggered by AD associated Aβ deposits and dystrophic neurites, however recent genome-wide association studies (GWAS) have identified several AD risk loci in or near genes associated with microglia suggesting that microglia are influential to the etiology of AD (Verheijen and Sleegers, 2018). Furthermore, neuroinflammation may be a key player in initializing AD pathology as microglia activation has been observed prior to plaque formation in animal models of AD (Hanzel *et al.*, 2014). This hypothesis was supported by research showing that Aβ injections alone did not induce AD amyloid pathology in primates, although injecting inflammation causing lipopolysaccharides with Aβ or injecting Aβ in animal models with

chronic, systemic inflammation resulted in the formation of Aβ plaques (Philippens *et al.*, 2016). Understanding which microglia responses are protective and which might be contributing to AD etiology could illuminate potential therapeutic targets.

Recently, Chen et al. demonstrated that APOE4/Tau mouse models experience detrimental brain atrophy compared to APOE3/Tau mice, and depleting microglia and/or T cells robustly reduced the observed atrophy (Chen *et al.*, 2023). It is unclear if T cells or microglia are the main contributors to brain atrophy or if there is a synergistic effect. Many APOE studies in glia have utilized isogenic models to study the impact of APOE4 which neglects genetic background that could influence AD pathogenesis. Studies have demonstrated that an individual's genetic heterogeneity can impact how APOE4 contributes to microglia dysfunction (TCW *et al.*, 2022). However, a recent study, surveying thousands of individuals, concluded that APOE4 homozygotes represent a genetic form of AD. They revealed that the age of dementia onset for APOE4 homozygotes is earlier than APOE3 homozygotes, and that nearly all APOE4 homozygotes exhibited AD pathology and at a higher level at the age of 55 compared to APOE3 homozygotes (Fortea *et al.*, 2024). This study emphasizes the importance of stratifying AD populations more strictly and considering separate mechanisms important in APOE4 homozygotes. Because APOE is highly expressed in glia, it would be important to parse out the role APOE4 plays in astrocytes and microglia. Microglia responses are complex, and the field has moved beyond the binary M1 and M2 microglia states. Several microglia states have now been characterized, including disease associate microglia (DAMs) and lipid droplet associate microglia (LDAMs) (Wei and Li, 2022). However, there is significant overlap among the defining features of these

two states that calls for further exploration into their distinction (Haney *et al.*, 2024a). It is unclear whether DAM and LDAM states are protective responses to disease pathology, although emerging systems to study microglia can begin to address these questions. Transcriptomic studies in AD mouse models have revealed gene expression differences in microglia paralleling the progression of the disease (Keren-Shaul *et al.*, 2017). While there is overlap between mouse and human microglia transcriptomic profiles, there is undoubtedly differences, such as an upregulation of CD58, ERAP2, GNLY and S100A12 in human microglia (Galatro *et al.*, 2017). Additionally, human microglia have species-specific metabolic reprogramming in response to inflammation, when compared to mouse microglia (Sabogal-Guáqueta *et al.*, 2023). The incorporation of human microglia into human brain organoids would allow for the investigation into influences of diseased microglia on healthy control organoids or vice versa to unravel human, cell type specific contributions in diseases like AD. Furthermore, characterizing the many disease-associated microglia states in proximity to AD disease pathology could shed light on the roles of these states. ST brings us closer to tackling this problem, though studies employing this method in brain organoids are limited (Norden and Godbout, 2013).

Microglia have been introduced into brain organoids, allowing us to capture a more homeostatic microglia state (Figure 2.3). In 2D culture, microglia are prone to activation and cannot be maintained long term, even in co-culture systems (Timmerman, Burm and Bajramovic, 2018). Abud et al. were the first to incorporate hiPSC-derived microglia into hiPSC 3D brain-organoids (BORGs), which contain neurons, astrocytes and oligodendrocytes. Microglia became ramified and responded to

injury of the BORG, however, this study did not investigate transcriptomic changes of these microglia after incorporation into BORGs or phagocytic capacity (Abud *et al.*, 2017a). Further analysis would be required to understand the extent to which the microglia are maturing within the organoid. Ormel et al. modified an unguided protocol to generate microglia containing organoids, however, the amount and distribution of microglia between organoids was variable (Ormel *et al.*, 2018). To overcome this issue, Cakir and colleagues overexpressed transcription factor PU.1 in cerebral organoids to generate microglia-like cells in a tunable manner (Cakir *et al.*, 2022). They went on to engraft these brain organoids into immunocompromised mouse brains to introduce vasculature into the brain organoid model. Still, limitations included variable distribution of microglia-like cells in the organoids, and limited conversion of some PU.1 expressing cells to mature microglia resulted in heterogenous microglia precursor populations. Additionally, this method requires the establishment of stable lines for each iPSC line of interest which is a timely effort when using large cohorts. Schafer et al. developed a different approach and found that infiltrating EMPs, generated using protocols from Abud et al., into forebrain organoids and then subsequently transplanting them to immunodeficient mice, allowed the EMPs to convert to microglia, taking on a more homeostatic morphology and transcriptional state when compared to *in vitro* microglia (Schafer *et al.*, 2023)(Abud et al. 2017). The introduction of EMPs into the organoids at five weeks mimicked the invasion of yolk sac derived EMPs into the fetal brain at this gestational period. Transplanting these microglia-enriched organoids into the cortex of immunocompromised mice advanced microglial maturation. The microglia became more ramified and demonstrated increased expression of P2RY12 and TMEM119,

features of homeostasis, when compared to in vitro microglia. However, it is unclear how the mouse host cells might be interacting and influencing the implanted brain organoid beyond increasing oxygen and nutrient penetration and delivery. Most recently, Park et al. infiltrated iPSC-derived primitive macrophages (iMacs) into cerebral organoids. After infiltration, iMacs expressed microglia markers such as P2RY12, CX3CR1, and SALL1 at a higher level compared to in vitro iMacs (Park *et al.*, 2023). Notably, these iMacs (called iMicro once infiltrated) did not express microglia marker TMEM119, which urges a comparison to existing microglia protocols to ensure these iMacs do fully convert to microglia. This study concluded that iMicro are enriched with PLIN2+ lipid droplet that export cholesterol, which were taken up by neural progenitor cells (NPCs). They found that organoids with iMicros were reduced in size and cell number, due to reduced proliferation of NPCs, which they suggest is a consequence of iMicro protecting against excessive growth. This study highlights the importance of cholesterol transport in development, potentially via APOE lipoproteins. While the direct study of microglia in AD organoids is limited, Haney et al. recently showed that conditioned media from lipid droplet high APOE44 microglia, compared to lipid droplet low, induced Tau phosphorylation and apoptosis in neurons (Haney *et al.*, 2024b). Together, this prompts the field to investigate the role microglia plays in cholesterol transport to other cell types and how this pathway is altered in AD, which could be further elucidated with microglia-rich organoids.

4. Challenges and future directions

Possibly the greatest challenge of implementing brain organoid technologies for the investigation of late-onset diseases like AD is the fact that brain organoids are

derived from embryonic-like cells. As brain organoids develop, they recapitulate features of early brain development and are deficient in some in vivo systems which might be limiting their capacity for cellular maturation. One important feature that is lacking in brain organoid models is vasculature. While the lack of vasculature might enhance some AD-related pathological features in brain organoids, such as Aβ aggregation, it limits oxygen and nutrient diffusion into the organoid causing cellular stress and necrosis. Aging has been defined as the time dependent accumulation of cellular damage (Kennedy *et al.*, 2014). While it is unrealistic to model every aging component influencing age-related diseases using brain organoids, it could be feasible that some phenotypic features of age-related brain diseases are driven by genetic background or cellular stress and may be apparent prior to symptom onset. Early phenotypic features of AD might be revealed in brain organoid models. Additionally, age-related signatures, including epigenetic aging markers, of a patient or non-affected donor can be retained via the transdifferentiation of somatic cells to neural cell types (Herdy *et al.*, 2022)(Gatto *et al.*, 2021)(Mertens *et al.*, 2021). The organization of transdifferentiated neural cell types into 3D cellular models could reveal the epigenetic and environmental contributions of multiple cell types to AD pathogenesis.

Recent advances in 3D cell biology, materials science, and bioengineering have produced models of increasing complexity with a systems wide approach. Assembloids consisting of fused brain organoids with various brain region specific identities have bene established and suggest that increased brain region specification of brain organoids models could allow for the study of dysregulated brain region connectivity in AD that could be implicated in cognitive impairment (Marton and Pașca, 2020).

Furthermore, the BBB protects the CNS from influencing blood-borne materials and, although some changes to the BBB may occur during healthy aging, its disruption is thought to cause neuronal dysfunction contributing to AD (Banks *et al.*, 2021). A BBB-like system might be incorporated into brain organoids to investigate the potential roles of endothelial cells (ECs), pericytes, microglia, astrocytes, and neurons contributing to vascular dysfunction in AD. Currently, BBB spheroids have been established through the spontaneous aggregation of primary human astrocytes, human brain vascular pericytes, and either primary brain microvascular ECs or immortalized human cerebral microvascular EC line D3. These BBB spheroids demonstrate tight junctions, efflux pumps, and transporters, albeit they lack the influence of microglia and neurons (Cho *et al.*, 2017). Additionally, organs-on-chips technology or microfluidic-chip-based systems containing compartments where cells can be co-cultured in either 2D or 3D architectures, recapitulating microenvironments of human tissues like BBB, might be employed (Li *et al.*, 2020). The gut-brain axis has been identified as an important relationship in homeostasis and pathophysiology. Organs-on-chips technology and iPSC derived gastrointestinal organoids might be combined with brain organoids to mimic the microbiome-gut-brain axis while maintaining patient specific genetic background and influence (Sato *et al.*, 2011)(Fujii *et al.*, 2018)(Min, Kim and Cho, 2020). Several potential AD drugs which showed promise during animal trials failed during phase III clinical trials (Salloway *et al.*, 2014)(Doody *et al.*, 2014). Transwell models, microfluidic systems, or organoid BBB models could one day allow for high-throughput screening of compounds for drug discovery but are currently challenging to scale up (Hajal *et al.*, 2022)(Urich *et al.*, 2013). Scaling up human brain organoid

technology, from brain organoid generation to systematic, high throughput drug screening and analyses, may accelerate AD drug development (Park *et al.*, 2021). Brain organoids provide access to some AD pathology, cellular interactions of multiple cell types implicated in AD, human specific genetic background influencing sAD etiology and pathogenesis, and AD-related cellular mechanisms that might be perturbed by extrinsic, modulating factors, all of which could reveal potential therapeutic targets. Even though human based cellular models considering multiple systems could advance our understanding of age-related brain diseases and drug development, they will not replace nonhuman animal models or postmortem human brain tissue in elucidating certain aspects of age-related brain diseases. Brain organoids are far from modeling the implications of cellular function on behavior and the effects of AD pathogenesis on aged, adult brain architecture.

Table 2.1. Overview of primary literature using brain organoids to investigate AD and AD-related pathological features. The list of model systems is based on findings and is not exhaustive. iPSC, induced pluripotent stem cells; AD, Alzheimer's disease; fAD, familial Alzheimer's diseases; sAD, sporadic Alzheimer's disease; D-CAA, Dutch-type cerebral amyloid angiopathy; DS, Down syndrome; CJD, Creutzfeldt-Jakob disease; ReN cells, ReNcell VM human neural precursor cells; NMN, nicotinamide mononucleotide; GSK3α/β, glycogen synthase kinase 3α/β; SYN1, synapsin-1; PiB, Pittsburg compound B; Fli, Flibanserin; Ripa, Repasudil.

Model system	Description of model including induced pathology/genetic background/mutated gene(s)	Age of organoids at readout	Findings	Rescue	Rescue phenotype	Reference
iPSC-derived cerebral organoids or 3D neuronal spheroids	iPSCs patient-derived cohort D-CAA (APP point mutation).	~ 4 months (110 days) and ~ 2 months (52 days)	Increased neuronal differentiation and maturation potential in D-CAA. Increased transcription of *vGLUT1*, *TGFBR1*, *AAQP4* and *GFAP* in D-CAA. Increased Aβ (4G8) deposition in D-CAA. No significant difference in p-tau.	CRISPR-edited isogenic	NA (Variability between biological triplicates warranted more isogenic lines)	Daoutsali et al., 2023
	iPSCs donor cohort (no diagnosis of dementia) APOE-ε3/ε3 and APOE- ε4/ε4. CRISPR-edited isogeneic APOE-ε4/ε4 to APOE—ε3/ε3.	6 months	No significant differences in APOE, Aβ42, Aβ40, or Aβ42/Aβ40 ratio, p-tau (S396 and T231) protein expression that could be attributed to *APOE* status	Isogenic *APOE-ε3/ε3*	Decrease in p-tau S199	Hernández et al., 2022
	iPSCs donor cohort APOE-ε3/ε3 and APOE-ε4/ε4 from control donors or patients diagnosed with sAD. CRISPR-edited isogeneic APOE-ε4/ε4 from sAD patient to APOE—ε3/ε3.	1, 2, and 3 months	Increased cleaved CASP3 in AD-E4 and decreased synaptophysin and PSD95 in AD at 12 weeks. Increased organoid maturation in AD around 4 weeks. Increased Aβ42, Aβ40, and Aβ42/Aβ40 in AD at 12 weeks. Increased p-tau (AT8) and soluble apoE in Con-E4, AD-E3, and AD-E4. Enrichment of gene sets involved in RNA metabolisms affected in AD	Isogenic *APOE-ε3/ε3*	Reduction of cleaved CASP3, Aβ42, Aβ40, p-tau, and G3BP (no change in Aβ42/Aβ40) at 12 weeks	Zhao et al., 2020
	iPSC-derived 3D neural models embedded in	25 weeks (~6.25	Expression of all 6 adult isoforms of tau (5 isoforms of tau were	NA	NA	Miguel et al., 2019

Table 2.1. Overview of primary literature using brain organoids to investigate AD and AD-related pathological features. (continued)

			2 months but no increased cell death was observed at later time points.			
	3D embedded cell suspension - ReN cells with *APOE-ε3/ε3 genotype* embedded in Matrigel. Lentiviral constructs used to overexpress *APP* or *APP* and *PSEN1* (fAD mutations) in ReN cells (fAD ReN cells).	~ 1 month (42 days)	Increase in the levels of 4R adult tau isoforms in 3D compared to 2D. Increased Aβ42, Aβ40, Aβ42/Aβ40 ratio, amyloid deposits, and p-tau (Ser199, Ser202, Thr205, and AT8) in fAD ReN cells.	β- and γ-secretase inhibitors. GSK3β inhibitors 1-azakenpaullone (1-AZA) and SB415286 (SB41).	β- and γ- secretase inhibitors decreased Aβ deposits, p-tau-positive cell numbers, and p-tau levels in neurites in fAD ReN cells. 1-AZA and SB41 decreased p-tau in fAD. APP and PSEN1 overexpressed ReN cells.	Choi et al., 2014
Exogenous induction of AD-like pathology in brain organoids	iPSC-derived brain organoids treated with 10% human serum for ~ 12 days.	~ 3 months (90 to 110 days)	Increased Aβ, Aβ aggregates, BACE protein, p-tau (Thr181), and phosphorylated/total GSK3α/β ratio after serum treatment. Decreased $SYN1^+$ synaptic puncta, calcium signaling, number of spikes, bursts, mean firing rate, and synchrony index after serum treatment.	BACE inhibitor IV (IV), GSK3α/β inhibitor CHIR99021 (CHIR)	IV reduced Aβ under serum exposure but not p-tau levels. CHIR reduced phosphorylated and total GSK3α/β and p-tau with serum exposure but not Aβ levels. Neither IV or CHIR increased $SYN1^+$ synaptic puncta.	Chen et al., 2021
	iPSC-derived brain organoids treated with Aβ42 inducer, Aftin-5.	1, 2, and 3 months	Increased Aβ42/Aβ40 ratio with Aftin-5 treatment. Increased APP and prion protein (PrP^C) between 1 and 3 months.	NA	NA	Pavoni et al., 2018
Network based, cerebral organoid drug screening platform	PiB-negative or PiB-positive (sAD) donor derived iPSCs used to generate cerebral organoids (iCOs). CRISPR edited APOE4 isogenic ($E4^{iso}$) iCOs (E3 parental line).	2 months (60 days)	PiB^+ iCOs secreted higher Aβ42, Aβ40, total tau, and p-tau with APOE4 carriers and $E4^{iso}$ iCOs secreting increased levels. PiB^+ and $E4^{iso}$ iCOs had increased calcium peaks and downregulated genes related to synaptic function and neurogenesis.	Silico perturbation analyses identified several drug candidates for targeted AD pathology like Eli and Ripa.	All identified candidate drugs reduced Aβ or tau deposition and enhanced or maintained cell viability to some extent.	Park et al., 2021

Table 2.1. Overview of primary literature using brain organoids to investigate AD and AD-related pathological features. (continued)

	Matrigel-coated alginate capsules and cultured with BrainPhys media	months or 175 days)	detected at week 25 in 3D models cultured without BrainPhys). Proportion of isoforms did not match that found in the adult human brain.			
	iPSCs donor cohort fAD, DS, CJD, and healthy controls.	~ 4 months (110 days)	Increased Aβ deposits, Aβ42, Aβ40, Aβ42/Aβ40, p-tau (AT8 and PHF-1), and NFT structures in fAD and DS. Increased CASP3 in DS.	NA	NA	Gonzalez et al., 2018
	3D neuronal spheroids - Patient cohort iPSCs from 5 patients with sAD diagnosis (some had PET scans).	~ 2 months (63 days)	sAD spheroids showed Aβ and p-tau (Thr181).	BACE1 and γ-secretase inhibitors.	Decreased Aβ40 and Aβ42. Clathrin and APP were reduced in BACE1 inhibitor responsive spheroids compared to a less responsive line. Neuronal spheroids showed less of a reduction in Aβ compared to 2D neurons derived from the sAD patients.	Lee et al., 2016
Gene edited cerebral organoids or 3D embedded cell suspension	iPSCs donor cohort DS (T21), isogenic iPSC clones with disomy 21 (D21) from same DS individual, non-DS individual with triplication of *APP* gene causing fAD (Dup*APP*), CRISPR eliminated *BACE2* in T21 (Δ7).	~ 3 to 4 months (100 to 137 days)	Increased Aβ19, Aβ20, and Aβ34 (Aβ degradation and preventing products) in T21. Decreased Aβ19, Aβ20, and Aβ34 and increased amyloid deposits and tau pathology (AT8 and TG3) in Δ7.	B- and γ-secretase inhibitors	Complete loss of amyloid deposits and decreased number of neurons expressing pathologically conformed tau (TG3) in Δ7.	Alić et al., 2021
	CRISPR *PITRM1* knockout iPSCs, from a non-affected control donor, (PITRM1$^{-/-}$) derived cerebral organoids.	1, 2, and 6 months	scRNA-seq revealed dysregulated mitochondrial function, oxidative phosphorylation, synaptogenesis, long-term potentiation, inflammatory pathways, and sirtuin in PITRM1$^{-/-}$. Increased APP, Aβ40, Aβ42, and Aβ42/Aβ40 ratio in PITRM1$^{-/-}$ at 2 months. Increased CASP3 positive cells at in in PITRM1$^{-/-}$ starting at	NAD+ precursor, NMN (enhances mitochondrial clearance)	Decreased Aβ42/Aβ40 ratio, p-tau/total tau ratio, and the number of CASP3 positive cells in PITRM1$^{-/-}$ organoids.	Pérez et al., 2021

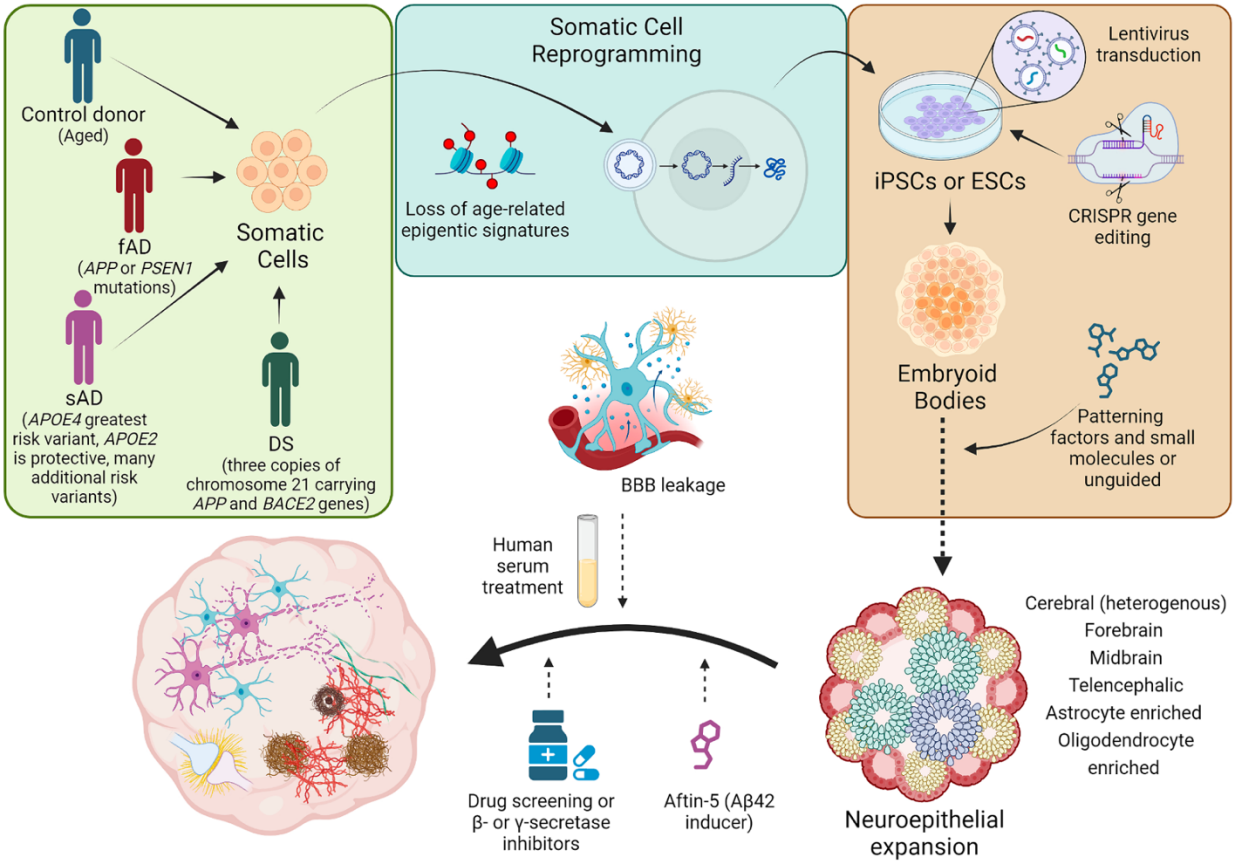


Figure 2.1. Schematic diagram illustrating the generation of brain organoid models of AD. Brain organoids generated from patient or control donor somatic cells will retain the genetic background of the patient or donor. Most, if not all, age-related epigenetic signatures from the donor will be lost during the reprogramming of somatic cells to iPSCs. Genetic modifications associated with AD can be introduced into iPSCs or ESCs using techniques including lentivirus overexpression or CRISPR induced point mutations and gene knockouts. Genome edited PSCs can then be employed in the generation of embryoid bodies which could be patterned to obtain more directed, brain region specific organoids or unguided approaches can be implemented to yield more heterogeneous cerebral organoids. Following organoid cellular maturation, brain organoids could be treated with factors to simulate age-related events like BBB leakage, to induce AD pathology, to observe the effects of potential pharmaceuticals on AD pathology, or to inhibit mechanisms hypothesized to effect AD pathogenesis. These strategies have successfully established brain organoids with some AD pathological features, including Aβ aggregates, NFTs, increased apoptosis, and neuronal network dysregulation. fAD, familial Alzheimer's disease; sAD, sporadic Alzheimer's disease; DS, Down syndrome; iPSCs, induced pluripotent stem cells; ESCs, embryonic stem cells; Aβ, amyloid-β; BBB, blood brain barrier. Not to scale.

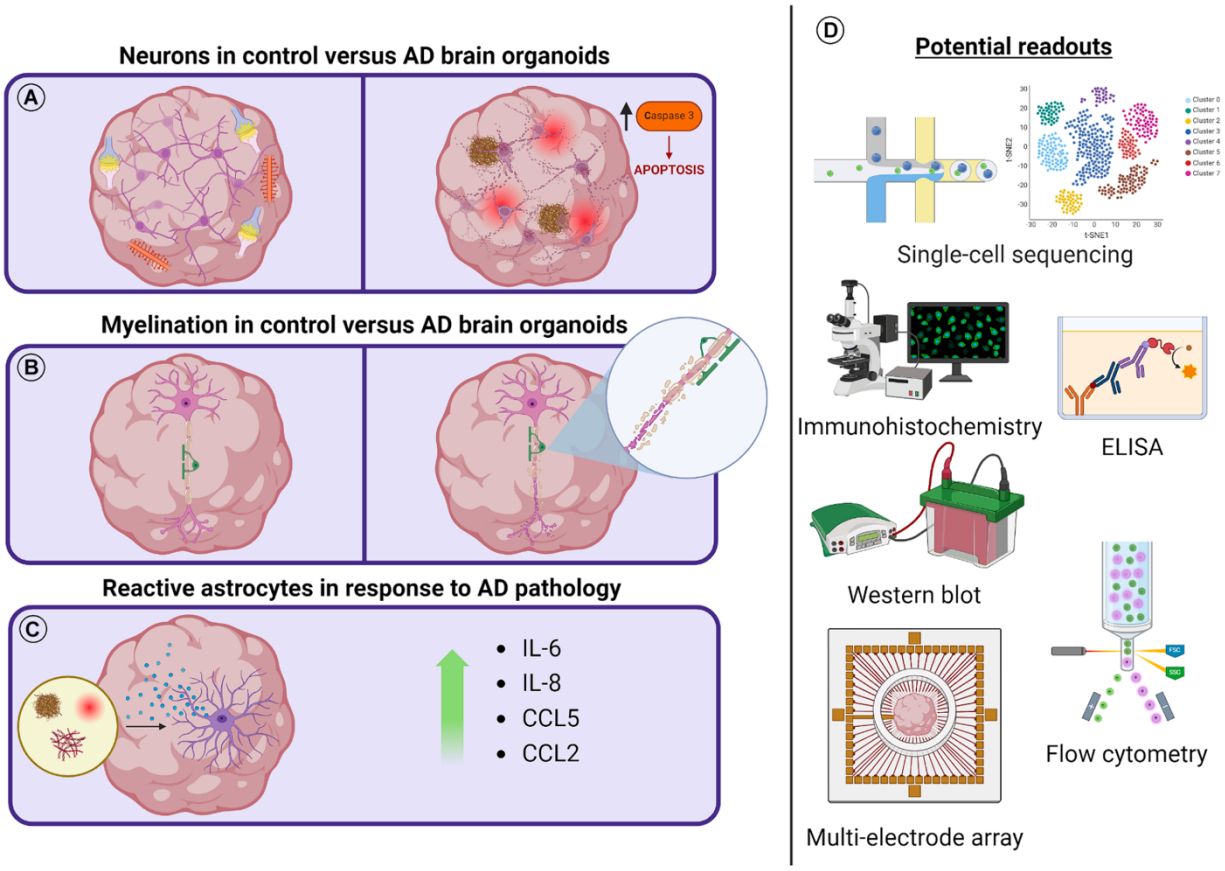


Figure 2.2. Schematic diagram outlining AD related cellular pathology and possible readouts to measure pathological effects. (A) As brain organoids mature, dendritic spine structures begin to form in neurons and functional synapses spontaneously fire. Aβ aggregates, increased p-tau and NFTs, decreased PSD95, and increased cleaved caspase-3 have all been observed in brain organoid models of AD. (B) Myelinating oligodendrocyte populations have been identified in unguided brain organoids after long-term culture or as early as 50 days using directed brain organoid protocols. It might be possible to observe the demyelination of axons and transcriptional or protein level regulation of this process in AD brain organoid models. (C) Astrocytes have been shown to increase inflammatory cytokine secretion in the presence of AD pathology. The reactivity of subpopulations of astrocytes in either unguided or astrocyte enriched brain organoid protocols can be studied in the presence of AD pathology. (D) Tools to observe cellular responses to AD pathology and the dysregulation of cellular processes in AD brain organoid models include scRNA-seq, ELISA, Western blot, flow cytometry, and multielectrode array technologies. Not to scale.

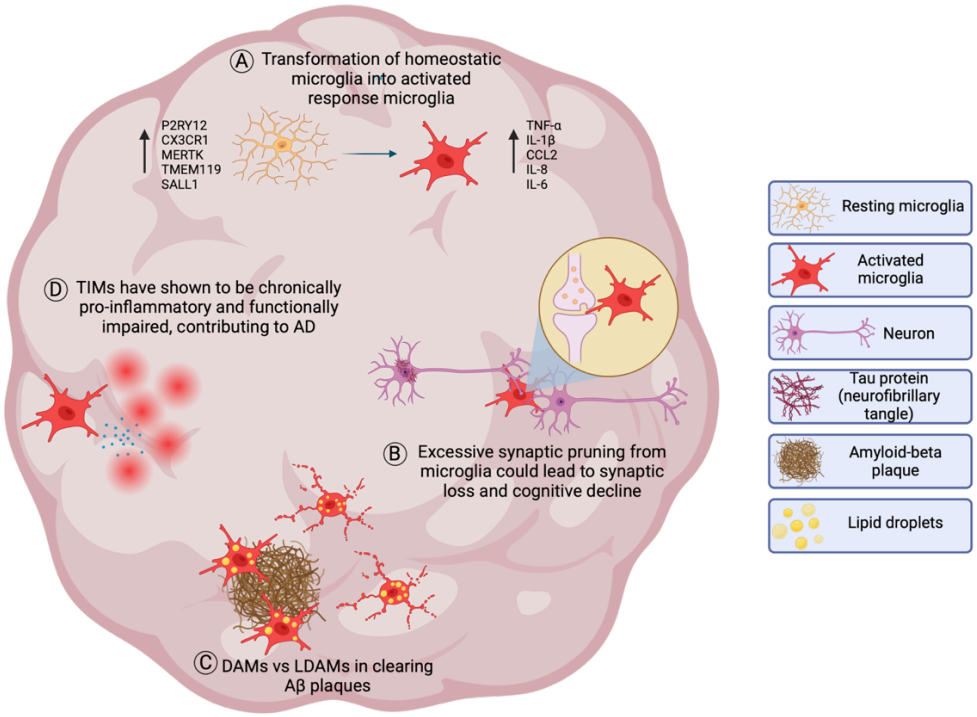


Figure 2.3. Healthy and diseased microglial processes implicated in AD which may be modeled in brain organoids. (A) Homeostatic microglia in a resting state in the healthy brain. Microglia participate in maintaining brain homeostasis, including synaptic pruning, surveillance of the microenvironment, and immune regulation. In response to stimuli or pathological conditions, homeostatic microglia transform into activation response microglia that have alterations in gene expression, morphology, and function. Microglial activation can promote an increase of cytokines such as tumor necrosis factor-alpha (TNF-α), interleukin-1 beta (IL-1β), chemokine ligand 2 (CCL2), interleukin-8 (IL-8) and interleukin-6 (IL-6). (B) Neuronal synaptic pruning. Microglia perform synaptic pruning by engulfing synaptic components to eliminate unnecessary or pathogenic synapses throughout development and disease. This process is essential for optimizing brain function. However, in AD, synaptic pruning may become dysregulated and could lead to excessive pruning leading to impairments in neuronal communication and contributing to cognitive decline. (C) DAMs vs LDAMs. Disease-associated microglia (DAMs) refer to microglia characterized by an upregulation of genes involved in phagocytosis, lipid metabolism, and immune response. They are thought to play a role in the clearance of amyloid beta (Aβ) plaques in AD. Lipid droplet accumulating microglia (LDAMs), on the other hand, refer to activated microglia associated with aging, late-stage disease progression, and the presence of lipid droplets. LDAMs may be senescent-like and could contribute to neurodegeneration due to dystrophic morphology and impaired phagocytic machinery reducing their ability to clear Aβ plaques. While both populations may have an increase of lipids, further investigation needs to be done to understand which lipids are accumulating (triglycerides, cholesterol esters, free cholesterol, etc.) (D) TIMs and inflammation. Terminally inflamed microglia (TIMs) may represent an exhausted state for inflammatory microglia that could contribute to AD risk and pathology. TIMs are senescent-like and are a terminal state for activated microglia, shown to be more inflammatory and functionally impaired. Not to scale.

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