THE ROLE OF LIPOPROTEINS ON SELECTED FUNCTIONS RELEVANT TO ALZHEIMER'S DISEASE IN HUMAN BRAIN PERIVASCULAR CELLS

by

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Abstract

Alzheimer's Disease (AD) is a progressive neurodegenerative disease that affects millions of people world-wide. It is characterized by amyloid plaques and neurofibrillary tangles in the brain. Many AD patients also show loss of cerebrovascular integrity, which is thought to lead to decreased capillary flow, neuronal injury and impaired clearance of amyloid beta. Since apolipoprotein E (apoE) and high density lipoprotein (HDL) show many beneficial effects in the vasculature in the body, we aimed to test the effects of these lipoproteins on primary human perivascular cells in the brain. We found that pharmacologically increasing apoE levels with GW3965 in a scratch-wound assay was not associated with changes in pericyte migration. Interestingly, we found that Axl inhibitor A1 slowed pericyte migration without showing changes in secreted apoE levels, suggesting an apoE-independent pathway for pericyte migration. We also tested whether HDL can attenuate the CypA-NFκB-MMP9 inflammatory pathway associated with apoE4 pericytes, but failed to observe the activation of this inflammatory pathway in our pericytes. Lastly, we found that when macrophages were treated with HDL, Aβ phagocytosis was not changed. Moreover, there were donor differences in the inflammatory response of macrophages to Aβ, making consistent observations difficult. Taken together, we did not show beneficial effects of lipoproteins on perivascular cell function in the context of AD.

Lay Summary

Alzheimer's Disease (AD) is a neurodegenerative disorder that is characterized by build-up of a protein called amyloid beta in the brain, which is thought to cause damage. We aim to test the beneficial effects of lipoproteins apolipoprotein E (apoE) and high density lipoprotein (HDL) on cells that surround the blood vessels in the brain since they are shown to be beneficial to blood vessels in the body. This is especially important because in AD, inflammation in these blood vessels cause damage to the protective barrier in the brain. Moreover, migration of these cells away from this barrier makes it vulnerable to damage. We found that increasing apoE levels do not affect migration of cells that surround the barrier, and that HDL does not increase clearance of amyloid beta by immune cells. These results suggest that these lipoproteins do not provide beneficial effects for these specific cells and their functions.

Preface

All experiments were conceived by Sonja Soo, Jerome Robert, and Cheryl Wellington. All data collection and analysis was performed by Sonja Soo, under the guidance of Dr. Jerome Robert, with the exception of Figures 2.2 and 2.3, which were collected and analyzed by Dr. Jianjia Fan; and Figure 4.1 and Figure 4.2, which were collected and analyzed with the help of Guilaine Boyce.

All apoE-modulating compounds in Chapter 2 were provided by AstraZeneca.

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List of Abbreviations

Aβ	Amyloid beta
ABCA1	ATP-binding cassette protein 1
AD	Alzheimer's Disease
AJ	adherens junctions
ANOVA	analysis of variance
apoA-I	apolipoprotein A-I
apoE	apolipoprotein E
APP	Amyloid Precursor Protein
ATP	adenosine triphosphate
BBB	blood brain barrier
BCA	bicinchoninic acid
BLI	bio layer interferometry
BM	basement membrane
BSA	bovine serum albumin
CAA	cerebral amyloid angiopathy
CCR2	C-C chemokine receptor type 2
CETP	cholesteryl ester transfer protein
CBF	cerebral blood flow
cDNA	complimentary deoxynucleic acid
CNS	central nervous system
CSF	cerebral spinal fluid
CypA	cyclophilin A

DMSO	dimethyl sulfoxide
ECM	extracellular matrix
ELISA	enzyme-linked immunosorbent assay
EMT	epithelial mesenchymal transition
FDA	Food and Drug Administration
FDG	fluorodeoxyglucose
FITC	fluorescein isothiocyanate
fMRI	Functional Magnetic Resonance Imaging
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
G-CSF	granulocyte-colony stimulating factor
GFP	green fluorescent protein
GLUT1	glucose transporter 1
GM-CSF	granulocyte macrophage colony stimulating factor
GTP	guanosine triphosphate
HDL	high density lipoprotein
HDL-C	high density lipoprotein cholesterol
Iba-1	ionized calcium-binding adaptor molecule 1
ICAM-1	intracellular adhesion molecule 1
IDL	intermediate density lipoprotein
IgG	immunoglobin G
IL-1β	interleukin 1β
IL-6	interleukin 6
IL-9	interleukin 9

IL-10	interleukin 10
IL-12	interleukin 12
ISF	interstitial fluid
ITAM	immunoreceptor tyrosine-based activation motifs
LDL	Low density lipoprotein
LRP1	lipoprotein-related protein 1
LTP	long-term potentiation
LXR	liver X receptor
MAPK	mitogen-activated protein kinase
MCI	Mild Cognitive Impairment
M-CSF	macrophage colony stimulating factor
MGM	meningeal macrophages
MMP-2	matrix metalloproteinase 2
MMP-9	matrix metalloproteinase 9
MMSE	Mini Mental State Examination
MRI	Magnetic Resonance Imaging
mRNA	messenger ribonucleic acid
NFT	Neurofibrillary tangles
NO	nitric oxide
PAGE	polyacrylamide gel electrophoresis
PBMC	peripheral blood mononuclear cells
PDGFRβ	platelet derived growth factor beta
PET	positron emission tomography

PFA	paraformaldehyde
PI3K	phosphoinositide 3-kinase
PiB	Pittsburgh Compound B
PICALM	phosphatidylinositol binding clathrin assembly
PVM	perivascular macrophages
PVDF	polyvinylidene difluoride
RAGE	receptor for advanced glycation end products
RCF	relative centrifugal force
ROS	reactive oxygen species
RXR	retinoid X receptor
sAXL	soluble AXL
SD	standard deviation
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel
SREPB-11	sterol regulatory element-binding protein 1
SR-B1	scavenger receptor class B member 1
sLRP	soluble LRP1
TBI	traumatic brain injury
TEER	trans-endothelial cell electrical resistance
TIMP	tissue inhibitors of metalloproteinases
TNFα	tumor necrosis factor α
TLR	toll-like receptors
TLR-4	toll-like receptor 4
TJ	tight junctions

TR	targeted replacement
TREM2	triggering receptor expressed on myeloid cells 2
VaD	vascular dementia
VCAM-1	vascular cell adhesion molecule 1
VLDL	very low density lipoproteins
VLDLR	very low density lipoprotein receptor
WGA	wheat germ agglutinin

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Lastly, to my family – Mom, Dad, Kevin, Shana – thank you for your unconditional love and support. I love you.

Dedication

To my family.

Chapter 1: Introduction

1.1 Alzheimer's Disease (AD)

1.1.1 AD: Symptoms, progression, epidemiology

As we age, we experience changes not only to our bodies, but also to our brains. We may notice our cognitive abilities declining; faces, names and places become harder to remember and recall. This age-related cognitive decline occurs as part of the normal aging process. However, for a certain population of aging adults, learning and memory is more severely impaired than normal, such that everyday functions are impaired. The umbrella term "dementia" is used to describe the set of symptoms that relate to severe cognitive decline, which can be due to many diseases and disorders. The symptoms of dementia are difficulties with memory, language, problem-solving and other cognitive functions that impair daily living. The most common form of dementia is Alzheimer's Disease (AD), named after the German psychiatrist Dr. Alois Alzheimer who was the first to characterize the distinct aggregations of amyloid plaques and neurofibrillary tangles that together comprise the neuropathological hallmarks of AD in the early 1900's.

Today, AD is the most common neurodegenerative disorder and affects over 47 million people worldwide. AD is believed to account for 60 – 80% of overall dementia cases[1]. Early symptoms of AD are often memory loss, which includes difficulty remembering names, events and conversations. Planning and solving problems become more difficult as well; AD patients may have difficulty with keep track of finances, counting change and concentrating on tasks. Problems also arise with words when speaking and writing. AD patients often exhibit decreased or poor judgment. These changes are believed to be due to neuronal death in the brain areas

involved in cognition. Mood and personality changes can also occur; a person with AD may have confusion, suspicion, anxiety and depression. Finally, when neurons from other parts of the brain start to degenerate, late-stage symptoms appear, which include difficulty speaking, swallowing and walking. In this stage of the disease, constant care and assistance with daily basic tasks are required. Moreover, since these patients have difficulty with mobility, they become more vulnerable to infections like pneumonia, and since they have difficulty swallowing, they are more prone to dehydration and malnutrition. AD is thus a fatal disease that has caused many deaths worldwide. In Canada from 2004 to 2011, there were over 80,000 AD-related deaths, which made up 4.3% of deaths during that period[2].

As a fatal, irreversible disease that progresses in its degeneration, AD affects a significant proportion of the population and has no effective cure. There is thus a tremendous emotional and economic burden on society. It is thus important to understand the etiology and pathogenesis of this disease, in order to find ways to prevent and treat the disease.

1.1.2 Mild Cognitive Impairment

The path leading up to AD symptoms is preceded by a stage called Mild Cognitive Impairment (MCI)[3]. In this stage, the individual has mild cognitive deficits as seen in AD, but these deficits do not interfere with daily living. Individuals with MCI may not necessarily develop AD, but are more likely to develop AD or other dementias[4]. Studies report a progression rate from MCI to dementia to be 20 - 40%[5].

1.1.3 Brain changes associated with AD

Neuroimaging tools such as magnetic resonance imaging (MRI) have shown that brain matter atrophy in AD happens first in medial temporal lobe[6], starting from the entorhinal cortex and eventually spreading to the hippocampus, amygdala and parahippocampus[7,8], which are areas of the brain that are involved in memory and cognition. More specifically, atrophy is seen predominately in grey matter, which consists of cell bodies of the neurons and glial cells, as well as synapses and dendrites. As the disease progresses, degeneration spreads to the parietal lobe as well as the frontal lobe[9], areas involved in visuospatial abilities and executive functions, respectively. Early studies using functional MRI (fMRI) to study AD patients find consistently decreased hippocampal activity during memory encoding tasks[10,11]. Some studies also report increased activity in the prefrontal cortex[12–14], suggesting a compensatory mechanism for deficits in hippocampal functions.

1.1.4 Treatment for AD

Despite decades of research on AD, there is no effective cure. Currently, there are 6 drugs approved by the U. S. Food and Drug Administration (FDA) for the treatment of AD, though these pharmacological agents are only able to temporarily improve symptoms but do not slow down or stop the disease. There are also non-pharmacological treatments available such as memory training programs, music therapy, exercise, and sleep-targeted therapies. These therapies aim to maintain and improve cognition and quality of life, and may also serve to decrease behavioural symptoms such as depression and apathy. Of the numerous non-pharmacological approaches evaluated, aerobic exercise and a combination of aerobic and non-aerobic exercise seem to be associated with slower rate of cognitive decline and improvement of

symptoms[15]. Interventions focusing on cognitive stimulation have also been reported to be effective[16]. These cognitive stimulation therapies include Reality Orientation, which presents orienting information such as time, place and people.

1.1.5 The Amyloid Hypothesis

How AD starts and progresses and how it relates to the accumulation of amyloid plaques and neurofibrillary tangles (NFT) remain unanswered to this day. The first clue into AD pathology started with Dr. Alois Alzheimer's examination of a brain afflicted with AD. His histological description of what is now known as amyloid plaques was believed for a long time to contribute to the development of AD. Amyloid plaques are formed from aggregations of amyloid beta (Aβ) peptides. Aβ is not a product made specifically during AD pathogenesis, but is produced constitutively in brains of healthy subjects. Aβ is produced under normal physiological circumstances through successive cleavage of the transmembrane protein amyloid precursor protein (APP) by β- and γ- secretases[17]. Aggregation of Aβ results in formation of dimers, trimers and oligomers. Eventually fibrils and plaques form, which give AD brains their characteristic neuropathological hallmarks.

Several interesting observations suggest that Aβ accumulation contributes to the pathology of the disease. Firstly, patients with a mutation in the APP gene, which influences the production of Aβ, are more likely to develop familial AD[18]. Secondly, genetic variants in the APP gene that reduces Aβ production protects against AD pathogenesis and reduces risk of developing AD[19]. Lastly, Aβ oligomers are shown to be toxic to neuronal cultures, and inhibited hippocampal long-term potentiation[20]. Taken together, these findings support the "amyloid

hypothesis", which posits that Aβ accumulation triggers a sequence of events that eventually lead to AD dementia.

However, despite long-held beliefs that amyloid plaques were the main culprit in AD pathogenesis, it was shown that they did not correlate well with synaptic loss, neuronal death or cognitive dysfunction in AD patients[21]. Amyloid plaques can also be present in individuals with normal cognitive abilities[22]. Rather, evidence points towards the soluble, oligomeric form of Aβ as the more detrimental characteristic of this disease[23]. Indeed, early studies found that soluble Aβ oligomers are toxic to neuronal cultures, inhibit hippocampal long-term potentiation (LTP)[20], contribute to memory impairments[24] and correlate with severity of neurodegeneration in brains of AD patients[25].

Only a relatively small number of AD patients (< 2%) are familial, early-onset cases, in which there is an overproduction of Aβ in the brain due to mutations in the APP gene or presenilin genes [26,27]. Rather, most cases are sporadic and late-onset, marked by disruptions in Aβ clearance rather than overproduction, which was demonstrated through metabolic labeling of AD patients[28].

1.1.6 Clearance of Aβ

Several distinct mechanisms mediate Aβ clearance. One such clearance mechanism is through enzyme-mediated degradation[29,30]. Many Aβ-degrading enzymes have been identified, belonging to various protein classes such as zinc metalloendopeptidase, serine proteases, cysteine proteases[31]. Aβ is also cleared from the brain through phagocytotic uptake and degradation by microglia[32,33]. Another clearance pathway is transport across the blood brain barrier (BBB) [34–36]. Scavenger receptors such as lipoprotein-related protein 1 (LRP1) and

scavenger receptor class B member 1 (SR-B1) expressed on astrocytes and endothelial cells are found to bind and internalize Aβ to be transported into the blood [37].

1.2 Cerebrovascular contributions to AD

Many epidemiological studies find the association between risk factors for cardiovascular diseases and late-onset AD[38,39]. Vascular conditions such as cardiovascular disease, type 2 diabetes mellitus, hypertension, obesity, and stroke have been observed to be comorbid in AD patients[40–42]. Indeed, most AD cases exhibit vascular pathologies[43], and it is believed vascular diseases play a role in AD pathogenesis[44,45].

Post-mortem studies also show vascular abnormalities such as tortuous vessels and reduced microvascular density in AD brain tissues[46]. BBB breakdown is also observed, in which blood-derived proteins accumulate in the hippocampus and cortex, which is coupled with degeneration of pericytes, muscular cells that are highly abundant in brain capillaries[47–50]. Microbleeds and iron accumulation are also observed[51]. AD patients also exhibit degenerating pericytes that are characterized by their abnormal mitochondria, large lipid granules, and pinocytic vessels[46].

Other studies have found reduced glucose utilization in the cerebrovasculature of AD patients. Glucose transporter 1 (GLUT1) is decreased in cerebral microvessels in AD brains[52]. Moreover, individuals that later develop AD already show reduced glucose utilization in the hippocampus as measured by fluorodeoxyglucose (FDG)-Positron Emission Tomography (PET)[53].

Clinical studies also show reduced cerebral blood flow (CBF) in AD patients, with the severity of CBF reduction correlating with AD severity[54–56]. These CBF changes have been

detected before symptoms of cognitive decline, neuronal degeneration and amyloid accumulation appear [57–59].

Deposition of Aβ in the walls of the cerebrovasculature may be driving the degeneration of vessels seen in AD. Indeed, cerebral amyloid angiopathy (CAA), which is characterized by amyloid deposits in the brain vessels, is prevalent in 80 – 90% of AD cases[60,61]. CAA leads to vascular injuries such ischemic lesions, hemorrhages, and impaired CBF[62]. Vascular dementia (VaD) also causes vascular injuries such as hemorrhagic injuries and hypoperfusion which directly leads to cognitive impairment[63]. VaD and AD share many overlapping features in terms of their risk factors[64] and clinical presentation[65]. It is thus possible that vascular dysfunction interacts with other neurodegenerative pathways to promote dementia.

1.2.1 The Two-Hit Hypothesis

The association between cerebral vascular pathology and AD led to the idea that vascular damage may be contributing to the AD pathogenesis. According to the "Two Hit Hypothesis", vascular changes combined with accumulating Aβ contribute to the development of AD[66]. In this paradigm, Hit 1 consists of vascular damage involving brain hypoperfusion and/or blood brain barrier dysfunction. This can be due to cerebrovascular disorders (such as ischemia and stroke) and/or vascular risk factors (such as hypertension, diabetes). These vascular changes leads to increased Aβ levels (Hit 2), which may be due to faulty clearance across the BBB and/or increased Aβ production by APP. Elevated Aβ levels are neurotoxic and cause subsequent damage such as neuronal dysfunction and neuronal loss, culminating in dementia.

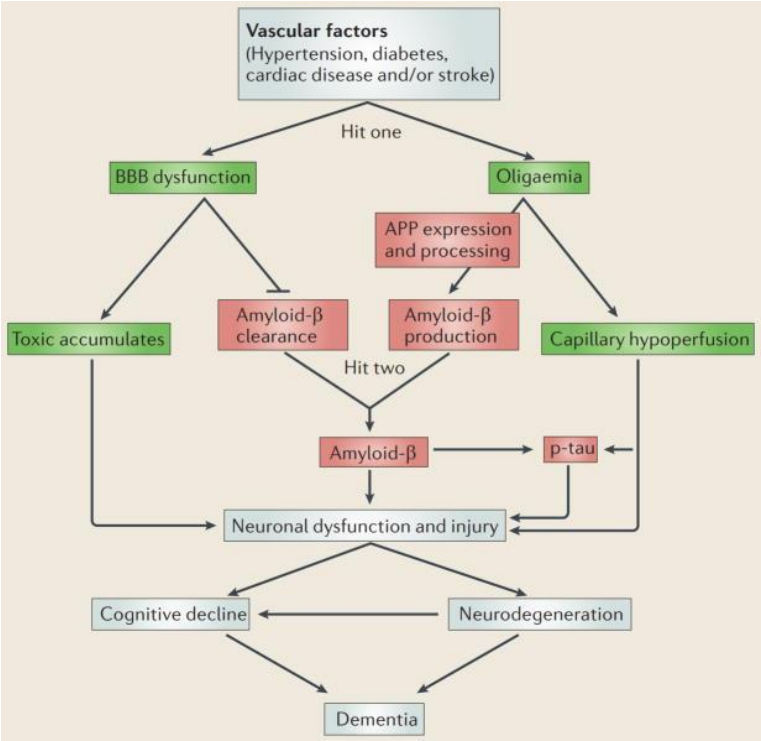

Figure 1.1 The two-hit vascular hypothesis for AD

The two-hit vascular hypothesis for AD posits that cerebrovascular damage (Hit 1) initiates neuronal injury and neurodegeneration, but can also promote Aβ accumulation in the brain (Hit 2). Figure reprinted with permission[66].

1.3 The Blood Brain Barrier (BBB)

The BBB is a specialized endothelial cell membrane that line the cerebrovasculature and functions to regulate entry of blood plasma components into the brain parenchyma and export waste from the brain to the blood[67]. Moreover, the BBB allows for delivery of crucial energy metabolites and nutrients to the brain. The endothelial barrier is enriched in tight junctions (TJ) and adherens junctions (AJ) that aid in reducing the permeability of the intracellular space[68]. TJs of the brain endothelial consists of integral membrane proteins such as occludin, claudin and

junctional adhesion molecules[69]. These TJs are not only involved in intercellular contacts but are also in contact with scaffolding proteins in the cytoplasm such as zonula occludens proteins and actin cytoskeleton[69].

1.3.1 Clearance of Aβ across the BBB

Transvascular clearance through the BBB accounts for 70 – 85% of Aβ cleared from the brain[70–72]. LRP1 is the primary receptor that mediates transcytosis of Aβ across the BBB, and is mostly found on the abluminal side of the BBB[70,72,73]. Aβ binds to LRP1 on the endothelial cells and is internalized by phosphatidylinositol binding clathrin assembly (PICALM)[74]. PICALM mediates PICALM/clathrin-dependent endocytosis and guides trafficking of the endocytic vesicle containing Aβ towards exocytosis[74]. Soluble LRP1 (sLRP1) is also known to bind and sequester free Aβ40 and Aβ42 and transport them via the blood to the liver and kidney[75].

1.3.2 BBB changes in AD

Various post-mortem studies find BBB breakdown in AD patients. In early studies, BBB breakdown in AD was histologically observed by the accumulation of blood-derived proteins such as immunoglobin G (IgG), fibrinogen, thrombin and plasminogen in the hippocampus and cortex[47,48,76]. More modern neuroimaging studies also detect microbleeds through MRI[51,77], which can even be observed in the preclinical AD stage[78].

Leakage of serum proteins into the brain due to BBB dysfunction can have detrimental effects. Presence of immunoglobin and albumin in the brain parenchyma can cause brain edema and decreased blood capillary flow, while thrombin can lead to neurotoxicity and memory impairment[79]. Elevated fibrin levels can accelerate neurovascular damage[80], and plasmin can

accelerate degradation of laminin and subsequently cause neuronal death[81]. This leakage in the BBB is believed to lead to decreased capillary blood flow, neuronal injury, and impaired Aβ clearance, ultimately contributing to AD pathology[66].

Changes to BBB receptors and function are also observed. In aging and in AD, LRP1 is decreased in the brain endothelial cells[82] as well as in cerebral vascular smooth muscle cells[83]. This decrease in LRP1 expression is associated with increased Aβ in the brain, since there are fewer receptors to aid its clearance[82]. Moreover, oxidation of cell surface LRP1 and soluble LRP1 prevents its binding of Aβ and subsequently decreases Aβ clearance from the brain[84]. Receptor for advanced glycation end products (RAGE), in contrast, is a receptor that mediates Aβ influx from the blood to the brain[85]. Normally, RAGE is expressed in low levels at the BBB, but its expression increases in aging and in AD brains, which ultimately increases accumulation of Aβ in the brain[85–87].

1.4 Pericytes

Pericytes are mesenchymal cells that surround blood vessel at the capillaries and venules. They are characterized by their small nucleus, small cytoplasm and elongated processes that wrap around endothelial cells[88]. Generally, pericytes are separated from the endothelial cells by the basement membrane, but at certain points, they make direct contact with endothelial cells, which is thought to play an anchoring role. One such interaction is through peg-and-socket connections in which the cytoplasmic processes of the pericytes (pegs) are inserted into the endothelial invaginations (sockets)[88]. Other close contacts between pericytes and endothelial cells have been described, such as through adhesion plaques and gap junction-like connections, which is thought to facilitate exchange of ions and molecules[89]. Thus, pericytes not only

structurally support endothelial cells, they also communicate with them through important physical contact and paracrine signaling.

1.4.1 Pericytes and immune function

Pericytes may also play an underappreciated role in immunity. A study with primary mouse brain capillary pericytes finds that pericytes secrete interleukins, cytokines and chemokines both under basal conditions and also when challenged with inflammatory stimuli[90]. Basally, pericytes were found to secrete interleukins such as interleukin 9 (IL-9), interleukin 10 (IL-10), and interleukin 12 (IL-12), as well as tumor necrosis factor α (TNFα) and granulocyte-colony stimulating factor (G-CSF)[90]. When challenged with a pro-inflammatory stimuli, pericytes can upregulate their expression of interleukin 6 (IL-6), interleukin-1β (IL-1β), TNFα, reactive oxygen species (ROS), nitric oxide (NO), and matrix metalloproteinases (MMP-2 and MMP-9), contributing to their detachment and migration[90,91]. Pericytes can also express vascular cell adhesion molecule 1 (VCAM-1) and intracellular adhesion molecule 1 (ICAM-1), which are important for recruiting immune cells such as monocytes and leukocytes from the blood and across the vessel walls[92,93], initiating an inflammatory process. More recent studies with human brain pericytes confirmed the expression of various chemokines and cytokines, and reported the expression of toll-like receptor 4 (TLR-4) in response to pro-inflammatory stimuli[94,95].

1.4.2 Pericytes in the brain and contributions to the BBB

Pericytes are very abundant in the brain. The central nervous system (CNS) has a higher density of pericytes in the vasculature compared to the periphery; an early study estimates a 1:1 – 1:3 pericyte-to-endothelial ratio in the CNS versus a 1:10 – 1:100 ratio in striated muscles[96]. In

the CNS, approximately 70 – 80% of the capillary network is covered with pericyte processes[49,97,98].

Animal studies have demonstrated that pericytes are crucial in forming and maintaining the BBB. During embryogenesis, pericytes are found to be recruited to nascent vessels when the barrier is being formed[97]. Mice completely lacking in pericytes (*Pdgfrb-/-*) are embryonically lethal as they have a leaky BBB[97], while mice that are deficient in pericytes (*Pdgfrb+/-)* show increased BBB permeability[99]. Moreover, endothelial tight junction barriers are tighter when pericytes are present, as measured by trans-endothelial cell electrical resistance (TEER)[97]. Although pericytes do not induce the expression of genes specific to the BBB in endothelial cells, they inhibit the expression of molecules that increase vascular permeability, such as Angiopoetin-2 (*Angpt2*)[97], thus contributing to the BBB integrity. Pericytes also induce polarization of astrocytic endfeet, which is important since astrocytic endfeet carry channels and transporters to control homeostasis at the vessels[99].

1.4.3 Pericytes and AD

Post-mortem studies show pericyte loss and degeneration in AD patients, especially in the hippocampus and the cortex[46,49] . Pericyte loss is accompanied by capillary reductions and tortuous vessels[46]. Other notable changes to pericytes include large lipid granules, large pinocytotic vessels and abnormalities in the mitochondria[46]. In studies with AD patients, decreased pericyte coverage in the brain is correlated with BBB leakage, as seen by increased deposits of IgG and fibrin into the brain[49]. How pericyte loss in AD occurs is unclear, though Aβ may be a contributing factor. It is observed that in AD patients, Aβ accumulates around pericytes and capillaries during later stages of AD[100,101]. Another study found that Aβ load in the

hippocampus was correlated with less pericyte coverage[49]. Some *in vitro* studies support the causal link between Aβ and pericyte death. For example, at high concentrations, Aβ can induce pericyte death[102].

The contributions of pericyte degeneration to AD pathology have also been studied in experimental models. Pericyte-deficient mice (*Pdgfrb*$^{+/-}$) show increased BBB permeability, which is thought to be due to upregulated endothelial transcytosis[99]. This increased vascular permeability is thought to lead to accumulation of blood-derived toxins in the brain. Chronic leakage of the BBB due to pericyte loss or degeneration is thought to damage the neurons, decrease CBF, and ultimately cause tissue hypoxia, neuronal dysfunction and degeneration[98]. Another study showed that in mice overexpressing APP (APPS$^{w/0}$), pericyte loss increased levels of soluble Aβ40 and Aβ42 in the brain[103]. This study further delineated the role of pericytes in clearing Aβ from the brain interstitial fluid (ISF)[103]. These *APPS*$^{w/0}$*;Pdgfrb*$^{+/-}$ mice also showed exacerbated neuronal loss and behavioural impairment such as burrowing, nest construction and novel object location tasks[103].

1.5 Brain resident myeloid cells

Microglia cells are the most prominent immune cells in the brain. As immune cells, they are involved in responding to tissue damage and presence of pathogens. Studies have also implicated other roles for microglia in the CNS such as interacting with neurons at the level of the synapse[104], aiding in the development of neural circuits in development[105], and mediating synapse loss during neurodegeneration[106]. Other macrophages that reside in the brain include perivascular macrophages (PVM), meningeal macrophages and macrophages in the choroid plexus[107].

1.5.1 Perivascular macrophages

PVM are macrophages that are located in the perivascular space and line the walls of blood vessels. The perivascular space is the space between the vascular basement membrane on the abluminal side of the vessel and the glial basement membrane on the parenchymal side (**Fig 1.2**).

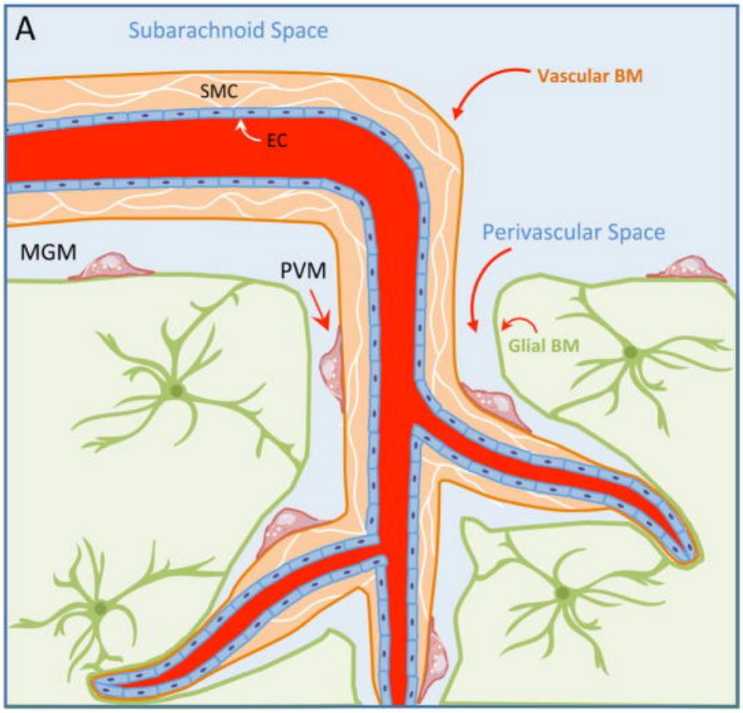

Figure 1.2 Perivascular macrophages in the perivascular space.

PVM are located in the perivascular space, which surrounds arteries and veins that penetrate into the brain parenchyma. The perivascular space consists of the space between vascular basement membrane (Vascular BM) on the abluminal side of the vessel and the glial limitans basement membrane (Glial BM) on the parenchymal side. The location of meningeal macrophages (MGM) is also shown. Figure adapted with permission[108].

PVM are more similar to blood-derived macrophages than microglia in terms of their immunophenotype and morphology[109], though recent studies suggest that they are more transcriptionally related to microglia[107]. PVM were previously thought to be replenished by circulating monocytes rather than being self-renewed in the brain, which largely comes from older studies using cell transplantation experiments[110,111]. Another study replaced the bone marrow cells of adult mice with green-fluorescent-protein (GFP)-transfected bone marrow cells and found that 2 weeks after the transplant, GFP-positive cells were found in the perivascular space, suggesting that PVM may come from monocytes[112]. However, irradiation was used to generate bone-marrow chimeras, a technique that damages the BBB and allows serum proteins to infiltrate the brain parenchyma[113]. A recent study using a fate-mapping approach found that CNS macrophages are derived from yolk sac precursors during development, suggesting their self-renewing abilities within the CNS[107]. Currently, it is believed that macrophage migration across the BBB, especially at the level of the post-capillary venules, only occurs during inflammatory conditions[114].

PVM express scavenger receptors, and are known to survey their environment and sample debris and dying cells[115]. Both microglia and macrophages can be polarized into M1 or M2 phenotype, though the classifications of which are ambiguous due to the overlapping classical markers and functional characteristics[116]. Broadly, M1 macrophages are involved in infections by microbes and viruses, and produce high levels of pro-inflammatory cytokines as well as nitrogen and oxygen intermediates; M2 macrophages are involved in parasite containment and tissue remodeling[117]. However, it is important to note that these phenotypes represent a spectrum, along which M1 and M2 states are but two points. Lastly, there is also

research showing that PVM are associated with capillaries as well, to the same frequency as pericytes, and may promote capillary stability and contribute to BBB integrity[115,118].

1.5.2 Macrophages and AD

PVM are believed to remove aggregates of Aβ through phagocytosis, and aid in the clearance of Aβ effluxed from the parenchyma into circulation since they are located near the vessels. PVM are believed to contribute to the clearance of waste products from the brain parenchyma since they express type I and type II scavenger receptors[119]. In one study with AD mice, depletion of PVM resulted in an increased deposition of amyloid plaques in the cerebral vessels while stimulation of PVM turnover showed decreased plaque accumulation[120]. In a recent study, C-C chemokine receptor type 2 (CCR2)- expressing myeloid cell population were preferentially recruited to amyloid deposits in three different AD mice models, and it was found that when PVM were deficient in CCR2, Aβ clearance was impaired and vascular Aβ showed increase deposition[121]. This suggests that PVM remove Aβ from the brain, and require CCR2 to do so.

Macrophages express various surface receptors that are known to bind to Aβ, such as scavenger receptors and Toll-like receptors (TLRs)[122]. Scavenger receptor class B type I (SRB1), which in particular, is known to be involved in Aβ clearance; deletion of SR-B1 in AD mice results in increased parenchymal and vascular Aβ deposition as well as associated cognitive deficits[123]. Histological analysis revealed co-localization of scavenger receptors and perivascular macrophages[123]. However, even though there was an increase in accumulation of PVM in the brain vessels, Aβ phagocytosis by macrophages was unchanged[123], suggesting that SR-B1 may be involved in migration but not phagocytic activity of macrophages. Similar observations were

shown in studies with TLRs, a family of pattern recognition receptors involved in innate immune function. Studies with AD mice with TLR-4 mutations showed increased Aβ accumulation and exacerbated cognitive deficits, suggesting a role for TLRs in Aβ uptake and clearance[124,125].

Interestingly, there is also evidence that PVM are detrimental in AD pathology; a recent study suggests that PVM may contribute the negative effects of Aβ on regulation of CBF[126]. More specifically, depletion of PVM abolished the vascular oxidative stress and neurovascular abnormalities with Aβ in AD mice[126].

1.6 Apolipoprotein E (ApoE)

Aside from the metabolic risk factors that contribute to the development of AD, genetic risk factors also play a role. Amongst them is apolipoprotein E (apoE), a class of apolipoproteins that is expressed highly in the liver. In the CNS, apoE is synthesized in the brain primarily by astrocytes[127]. ApoE is involved in lipid metabolism by transporting and delivering lipids through apoE receptors[128,129]. ApoE is present in both the plasma and in the brain, where it is synthesized primarily by hepatocytes and astrocytes, respectively, and the two apoE pools do not mix. In plasma, apoE is associated with many lipoproteins, whereas in the brain, apoE is on high density-like lipoprotein particles[130,131]. In contrast to plasma high-density lipoproteins (HDL), which has apoA-I as its primary apolipoprotein, brain "HDL" has apoE as its primary apolipoprotein[130].

In humans, apoE exists in 3 polymorphic allelelic states (ε2, ε3, and ε4) that subsequently encode different isoforms with varying structural and functional properties[129,132]. More specifically, the isoforms differ at amino acid residues 112 and 158: apoE3 has a cysteine at residue 112 and arginine residue at 158; apoE4 has arginine at both residues; and apoE2 has

cysteine at both sites[133]. The arginine residue at site 112 in the apoE4 isoform reduces the stability of the protein[134].

1.6.1 Apolipoprotein E (ApoE) as a risk factor for AD

The efforts of early researchers demonstrated that the apoE4 variant is a major risk variant for late-onset AD[135,136], with odds ratios increasing with more copies of the gene[137]. Indeed, accumulating evidence from early post-mortem studies to recent PET imaging studies find that AD patients with the *APOE4* allele, compared to patients with *APOE2* or *APOE3*, had increased vascular and amyloid plaque deposits[138–140], greater rates of volume decrease in the entorhinal cortex and hippocampus[57,141] and altered dendritic arborisation patterns[142]. The *APOE2* allele decreases AD[143] and slows the rate of cognitive decline[144] and hippocampal volume atrophy compared to carriers of the other *APOE* alleles[145]. More recently, a new study using PDAPP mice (a mouse model of amyloidosis) and APP.PS1/TRE4 mice (a mouse model that develops *APOE4*-dependent amyloid deposition), found that viral delivery of apoE2 reduced both soluble and insoluble Aβ[146]. Taken together, these genetic studies highlight the integral role for apoE in the onset and progression of AD, though the mechanisms through which these isoform-dependent effects occur are unclear.

1.6.2 ApoE and Aβ metabolism in AD pathology

ApoE is associated with Aβ in amyloid plaques, suggesting their interaction[147]. Subsequent *in vitro* studies find that synthetic Aβ peptides and apoE directly associate[148–150], although, in these older studies, both the structural integrity of both the Aβ and apoE components are suspect, and the ratios of their interactions vary widely and often bear no relationship to

physiological levels. Older studies that investigate isoform-dependent effects on plaque density are also conflicting, with some studies finding a positive association between APOE4 allele dose and increased plaque density[138,151], while other studies finding no association[152–154]. A more recent study with a larger sample size confirms a positive association between APOE4 allelic dose and increased neuritic plaques in AD[155]. Further support comes from a study in cognitively normal adults in which APOE4 allele dose is associated with lower Aβ42 in the cerebral spinal fluid (CSF)[156], which is an indicator of increased brain amyloid deposition[157]. A more recent study using Pittsburgh Compound B (PiB) PET imaging found an E4 allele dose-dependent increase in fibrillar Aβ burden in the brain in cognitively normal individuals[158]. Overall, this suggests that there may be an isoform-specific effect on Aβ deposition, and that apoE4 may be accelerating this deposition.

Early *in vitro* studies using unlipidated apoE particles demonstrated that apoE4 binds more rapidly to Aβ than apoE3[150,159], while later studies with lipidated apoE particles demonstrate that apoE2 and apoE3 formed more stable complexes with Aβ than apoE4[148,160,161]. A recent study comparing unlipidated versus lipidated apoE particles found that unlipidated apoE particles bind to Aβ with less affinity than lipidated apoE[162]. Moreover, lipidated binding of apoE to Aβ is highest for apoE2 > apoE3 >> apoE4[162].

ApoE may also be involved in Aβ fibrillization. One study found that apoE promoted Aβ fibrillization, in which the effect is strongest with the apoE4 isoform and the least with the apoE2 isoform[163]. Some studies find that apoE4 is more efficient than apoE3 at increasing Aβ40 aggregation[164,165], though later studies find that all isoforms are equipotent at inhibiting Aβ fibrillization *in vitro* through interfering with Aβ nucleation[166,167]. These conflicting results may

be due to differences in preparation of apoE (lipidated versus unlipidated) and Aβ (Aβ40 versus Aβ42), and differences in aggregation conditions (concentrations of apoE and Aβ)[127].

In terms of the effect of apoE on Aβ production, the studies have also been conflicting. Several studies suggest that apoE4 increases Aβ production through LRP1 and apoER2-mediated APP endocytosis[168,169] while other studies find no effect of apoE isoform on APP processing[170–172]. Studies also suggest that apoE may be involved in Aβ clearance. Various *in vitro* studies find that apoE aids in the binding and internalization of soluble Aβ by neurons, microglia and astrocytes[173–175], though overall no isoform-specific effect was found. ApoE has also been found to facilitate cellular degradation of Aβ[176,177].

Studies performed *in vivo* to address the effect on Aβ metabolism have been interesting, and suggests a more detrimental role of apoE on AD pathology. For example, early studies find that apoE deficiency leads to decrease in amyloid load[178–180]. Interestingly, the lack of apoE increases the levels of soluble Aβ prior to Aβ deposition[181]. A subsequent study using *in vivo* microdialysis to monitor Aβ levels in brain ISF confirms the role of apoE in Aβ metabolism prior to Aβ deposition[182].

There is also evidence that apoE is involved in Aβ clearance in an isoform-dependent manner. Accumulating evidence shows that, in mice, Aβ complexed with apoE is cleared from the brain at a slower rate than that of free Aβ[183–185]. While Aβ40 and Aβ42 was cleared rapidly from the brain through LRP1, Aβ binding to apoE4 redirected the clearance to VLDLR, which internalized the complex at a slower pace[185]. In contrast, Aβ binding to apoE2 or apoE3 was cleared by the brain by both LRP1 and VLDLR at a faster rate than Aβ-apoE4 complexes[185]. A more recent study using *in vivo* microdialysis showed that AD mice expressing human apoE4 cleared Aβ at a slower rate compared to AD mice expressing human apoE3[186]. A very recent

study using a novel *in vitro* model of human bioengineered blood vessels supports an isoform-dependent role of apoE in Aβ clearance across the vessel[187].

1.6.3 Other effects of apoE on AD pathology

Other than the role of apoE on Aβ metabolism, apoE may also be involved in additional processes that contribute to AD pathology. Synaptic dysfunction and loss are observed in AD patients[188,189]. In one study, AD mice expressing human apoE4 showed worsened synaptic and cholinergic deficits compared to those expressing apoE3 prior to amyloid plaque deposition[190]. A recent study found that apoE4 impairs synaptic plasticity such that long term potentiation (LTP), a neural correlate of learning and memory, is disrupted[191]. Other studies have found isoform-specific effects on dendritic spine density, which is also associated with learning and memory. In APOE targeted replacement (TR) mice, apoE4-TR mice have lower dendritic spine complexity compared to apoE3-TR mice[192,193].

AD is also characterized by neuroinflammation, which includes release of cytokines and chemokines, activation of the complement cascade, and reactive astrogliosis and microgliosis near amyloid plaques[194]. ApoE-deficient mice have an increased inflammatory response to Aβ, suggesting the role of apoE in neuroinflammatory regulation in AD[195,196]. Interestingly, apoE isoforms differentially regulate the inflammatory response[197]. In one study, apoE4-TR mice showed a greater inflammatory response to LPS stimulation in comparison to apoE3-TR mice[198]. Similarly in humans, apoE4 carriers showed increased inflammatory response that may relate to AD risk[199].

1.7 High Density Lipoprotein (HDL)

As lipids are water-insoluble, they must be transported with proteins. Lipoproteins play an important role in absorbing and transporting lipids in the small intestine, and transporting lipids between the liver and the peripheral tissues. Lipoproteins have a central hydrophobic core surrounded by a coat of phospholipids, free cholesterols and apolipoproteins. There are seven classes of lipoproteins sorted based on size, lipid composition and apolipoproteins: chylomicrons, chylomicron remnants, very low density lipoproteins (VLDL), intermediate density lipoproteins (IDL), low density lipoproteins (LDL), high density lipoproteins (HDL), and Lipoprotein (a)[200].

Characteristically, HDL is the smallest and densest of the circulating lipoproteins, owing to a proportionally higher protein:lipid ratio compared to other lipoprotein subclasses. One of the key proteins found in almost all HDL particles is apoA-I, which makes up 70% of protein content in HDL[201]. The lipid composition of HDL includes of a variety of lipids such as glycerophospholipids, cholesteryl esters, sphingomyelins, and triacylglycerols[202]. Overall, HDL particles consist of over 80 types of proteins and hundreds of types of lipids[203]. These constituents play important roles in the function of HDL such as lipid metabolism, anti-oxidation, and innate immune system[204].

1.7.1 HDL functions

HDL travels between organs, tissues and cells. One of its well-known functions is reverse cholesterol transport (RCT), a process by which cholesterol and phospholipids are collected from cells of peripheral tissues and transported to the liver for excretion[205]. This is an important function because most cells cannot catabolize cholesterol, and thus need a mechanism by which

to decrease their cholesterol content. ATP-binding cassette protein 1 (ABCA1) mediates the transport of lipids from the cells to lipid-poor HDL particles[206]. The cholesterol taken up by the HDL particle can be transferred to the liver in several ways. Cholesterol can be directly transferred to hepatic cells via HDL binding to the scavenger receptor class B, type I (SR-BI)[207,208]. Alternatively, cholesteryl ester transfer protein (CETP) can transfer the cholesterol from the HDL to apolipoprotein B-containing particles that are ultimately taken up by the liver[209].

HDL also demonstrates vasoprotective functions that are well-characterized in peripheral vessels. Studies find the HDL can inhibit VCAM-1 expression[210,211] as well as ICAM-1 and E-selectin in endothelial cells[212], suggesting its role in attenuating inflammation. HDL has also been shown to repair damaged endothelial cells and inhibit apoptosis[213], reduce lipid oxidation, and inhibit thrombotic activation[214].

1.7.2 HDL and the vasculature

The link between low HDL cholesterol (HDL-C) levels and cardiovascular disease was established in a seminal paper by Miller and Miller[215] and since then, a multitude of clinical and pre-clinical studies have confirmed this link[216,217]. Many animal studies support this association [218,219]. However, Mendelian randomization studies failed to show a causal relationship between HDL-C levels and cardiovascular disease[220–222]. Individuals with genetic deficiencies of *APOA-I* or *ABCA1* are shown to have low levels of HDL-C and an increased risk of coronary artery disease[223]. Interestingly, individuals with a specific mutation in the *APOA-I* gene which results in the apoA-I Milano version of the protein, have low levels of HDL-C but similar risks of atherosclerosis and coronary artery disease compared to individuals with normal HDL-C

levels[224]. Moreover, individuals with mutations in the gene encoding SR-BI (*SCARB1*) have high levels of HDL-C, but are at an increased risk for coronary heart disease[225]. Taken together, these studies suggest a more complex relationship between HDL-C and cardiovascular conditions.

1.7.3 Association between HDL and AD dementia

Various prospective studies were conducted to investigate whether there is an association between HDL levels and risk of dementia. For example, one study demonstrates a link between higher levels of HDL-C and lower risk of AD[226]. Similarly, decreased HDL-C and serum apoA-I concentrations are correlated with severity of AD, as measured by the Mini Mental State Examination (MMSE)[227]. A prospective cohort study consisting of Medicare recipients in the United States also found an association between higher levels of HDL-C and lower risk of AD[226].

However, some prospective studies fail to find this association. In one study that followed women in their 70's for 9 years, HDL-C was not found to be associated with dementia or AD[228]. Moreover, twin studies in which one twin developed dementia did not find a difference in HDL-C levels between the twin pairs[229]. Interestingly, a recent study found that in men without vascular pathologies, lower HDL-C levels were associated with dementia, while no association was found for women participants[230]. Several cross-sectional studies were conducted to address this possible link but were similarly met with conflicting results. One study found lower HDL-C levels in patients with AD compared to healthy controls[231], while another study only found this link specifically in late-onset AD patients[232]. Interestingly, one study found that AD patients with comorbid cardiovascular conditions and risk factors had lower HDL-C levels in comparison to healthy non-AD controls, but not AD patients without cardiovascular conditions

and risk factors[233]. However, other small-scale studies find no difference in HDL-C levels between AD participants and healthy controls[234–236].

Various factors could contribute to the controversy in these epidemiological studies[237]. First, there may be subjectivity and misclassification in the diagnosis of AD, which makes comparison between participants across the group difficult. Moreover, the different types of dementias may be co-morbid, and many dementias have mixed vascular and AD pathologies[238]. Participants of some of these studies are sampled from registered patients in health care systems, which may not be representative of the population of interest. More importantly, HDL-C levels may not be the correct clinical measure, as HDL-C quantifies only the average cholesterol content of circulating HDL particles, and this value may not necessarily correlate to HDL functions such as cholesterol efflux capacity[239–241]. Thus, HDL-C levels *per se* may not be as informative as evaluating HDL function in Aβ clearance.

1.8 TREM2

Triggering Receptor Expressed on Myeloid Cells 2 (TREM2) is a cell surface receptor on microglia and macrophages. The extracellular region of TREM2 consists of a single immunoglobulin superfamily domain that binds to various ligands polyanionic ligands[242,243]. After ligand binding, TREM2 signals through an adaptor protein DAP12, which recruits protein tyrosine kinase SYK through its immunoreceptor tyrosine-based activation motifs (ITAMs)[244] (Fig. 1.3). A cascade of signaling events occur such as protein tyrosine phosphorylation, phosphoinositide 3-kinase (PI3K) activation, calcium mobilization and mitogen-activated protein kinase (MAPK) activation[244]. TREM2 can also transmit intracellular signaling through another

adaptor protein DAP10, which recruits PI3K[245]. Altogether, TREM2 signaling is involved in promoting survival[246], proliferation[247], phagocytosis[248], and immune signaling[249].

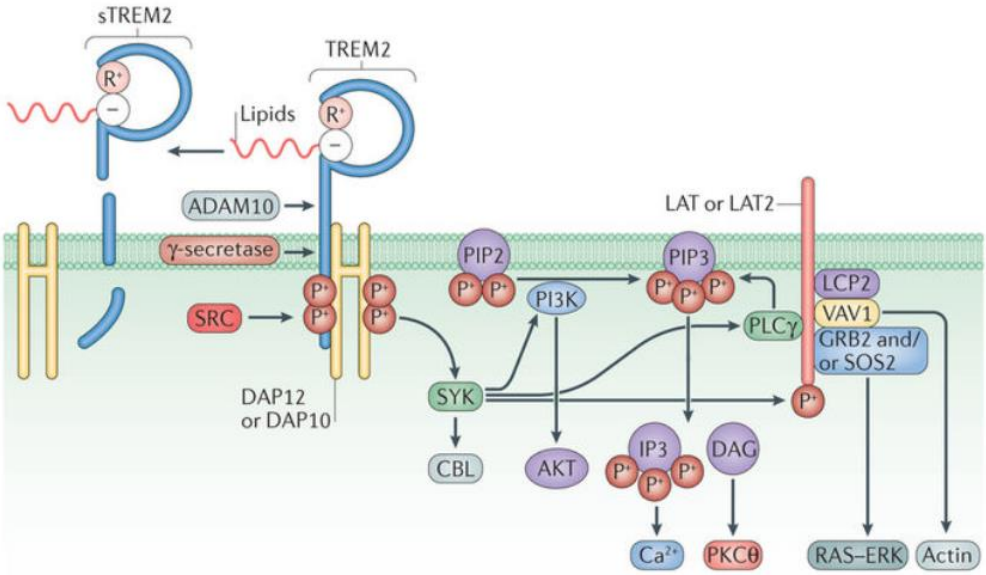

Figure 1.3 TREM2 signaling pathways

TREM2 signals through adaptor proteins DAP12 or DAP10, which initiates a cascade of signaling events. Reprinted with permission[244].

1.8.1 TREM2 and AD pathology

Recent whole-genome sequencing studies find variants in the TREM2 to be associated with AD risk[250,251]. The TREM2 R47H variant, which is an Arginine to Histidine change at amino acid 47, has an odds ratio of ~2.9 – 4.5 of developing AD, and seems to decrease age of AD onset by ~ 3 years[250,251]. Other variants such as R62H and D87N have also been found to be linked to AD[251].

Early studies to delineate the role of TREM2 in AD pathology found that knockdown of TREM2 in microglia inhibits phagocytosis of apoptotic neurons and increased the inflammatory

response, whereas overexpression of TREM2 increases phagocytosis and decreases the inflammatory response[248]. The idea that TREM2 is involved in phagocytosis is supported by another study in macrophages that finds that TREM2 is involved in phagocytosis of bacteria[252]. Other studies have found that overexpressing TREM2 in microglia also increases its capacity for Aβ phagocytosis[253,254]. TREM2 may be involved in regulating inflammation. Studies find that knocking down TREM2 results in an increased cytokine production in response to TLR ligands, suggesting that TREM2 may be mediating detrimental inflammation[248,255,256].

Studies investigating the role of TREM2 on Aβ accumulation show conflicting results. In one study using the 5XFAD mouse model of AD, TREM2-deficient microglia underwent apoptosis in response to Aβ deposition rather than becoming activated[246]. The study further demonstrated that TREM2 binds to various anionic and zwitterionic phospholipids, which are known to associate with fibrillar Aβ and be exposed on damaged neurons[246]. In contrast, microglia with the R47H variant of TREM2 have a reduced capacity to bind to phospholipids, suggesting that TREM2 may be detecting changes in the lipid microenvironment[246]. Another study knocking down and overexpressing TREM2 in APPswe/PS1de9 mouse model of AD suggests that TREM2 facilitates Aβ42 phagocytosis and inhibits Aβ42-triggered proinflammatory responses[254]. Moreover, overexpression of TREM2 ameliorated AD-related pathology including Aβ deposition, neuroinflammation, neuronal and synaptic loss, and cognitive deficits[254].

However, another study looking at TREM2 on infiltrating macrophages delineates the detrimental role of TREM2 in AD pathology. In the APPPS1-21 mouse model of AD, TREM2 is upregulated on myeloid cells surrounding amyloid plaques[257]. Moreover, lack of TREM2 resulted in reduced accumulation of Aβ and reduced inflammation[257], suggesting that TREM2

may be contributing to the inflammation. In a study using the same AD mouse model, loss of one copy of TREM2 did not affect Aβ pathology, but altered the morphology of plaque-associated microglia[258].

Taken together, these studies agree on the role of TREM2 in microgliosis, though its role in Aβ accumulation is controversial. Since the studies vary in its AD mouse model and age of the mice, it is possible that microglial response to Aβ occurs during a specific phase during the disease. Indeed, a recent study demonstrated that TREM2 deficiency ameliorates amyloid pathology during early stages of the disease, while later in the disease progression its deficiency exacerbates amyloid pathology[259]. More specifically, in the early disease progression, loss of TREM2 results in a decrease of number of amyloid plaques but an increase in plaque size later in pathology[259]. In late stages of the disease, TREM2 deficiency reduces inflammatory gene expression[259]. Interestingly, in all time points, TREM2 deficiency reduces myeloid cell internalization of amyloid[259]. The dynamic role of TREM2 during disease progression may be reflective of the dynamic role of myeloid cells in disease, such that they play a protective role early on with the clearance of Aβ, but become detrimental when they exacerbate inflammatory responses and lose their phagocytic abilities[260].

1.8.2 TREM2 and lipoproteins

Various studies suggest the interaction between TREM2 and lipoproteins. A study using TREM2-Fc fusion pull-down from cerebrospinal fluid (CSF) and serum identified apoE, apoA-I and apoA-II as TREM2 ligands[261]. Another study using a dot blot assay confirmed that apoE, apoA-I and apoB interact with TREM2. Interestingly, all isoforms of apoE, both unlipidated and lipidated, bind to TREM2 and this interaction is disrupted in the TREM2 R47H variant[261,262]. An

independent study found that TREM2 also interacts with lipoproteins such as HDL as well as LDL[263]. A study using bio-layer interferometry (BLI) found that TREM2 binds to apoE and Clusterin, as well as HDL, LDL, VLDL[264]. Moreover, they found that Aβ complexed with lipoprotein ligands of TREM2 such as Clusterin and LDL facilitated its uptake by microglia[264]. Another study also suggests the phagocytic role of lipoprotein binding to TREM2; apoptotic neurons that were covered in apoE increased microglial phagocytosis in a TREM2-dependent way[262]. Interestingly, a new study finds that the APOE-TREM2 pathway mediated the switch in microglia from a homeostatic phenotype to a neurodegenerative phenotype, suggesting that this pathway regulates microglial functional phenotype[265].

1.9 Objective

To investigate the beneficial effects of lipoproteins high density lipoprotein (HDL) and Apolipoprotein E (apoE) on perivascular cells in the cerebrovasculature.

1.10 Aims

Aim I: Investigate the effects of apoE-modulating compounds on pericyte migration

Aim II: Investigate the effects of high density lipoprotein (HDL) on apoE4-associated pericyte inflammation

Aim III: Investigate the effects of high density lipoprotein (HDL) on Aβ-induced inflammation and Aβ phagocytosis in macrophage

1.11 Hypothesis

I hypothesize that:

I: ApoE-modulating compounds decrease pericyte migration

II: HDL decreases apoE4-associated pericyte inflammation

III: HDL decreases Aβ-induced inflammation and increases Aβ phagocytosis in macrophages

Chapter 2: ApoE-modulating drugs on pericyte function

2.1 Introduction

Pericytes are cells that surround endothelial cells in the vasculature, and play an important role in maintaining blood brain barrier (BBB) integrity[99,266]. Pericyte death as well as pericyte migration away from the vasculature may cause the BBB to become leaky[267], which can leave the brain vulnerable to damage by serum-derived toxins. In AD brains, reduction of pericyte coverage of microvessels correlates with BBB disruption[49]. Pericyte-deficient mice show age-dependent BBB disruption and subsequent neurodegeneration[98]. Pericytes also contribute to clearance of Aβ, as pericyte-deficiency is associated with accelerated amyloid deposits in the brain and impaired amyloid clearance in AD mice[103]. Taken together, reduced pericyte coverage may further aggravate amyloid accumulation and contribute to AD pathology. Pericyte coverage is thus crucial to cerebrovascular health.

Pericytes produce apolipoprotein E (apoE), a major lipoprotein expressed in the brain that acts as a lipid carrier in the CNS. Humans have three APOE isoforms, which confer different risks for AD; the *APOE2* variant is associated with decreased AD risk[143], while the *APOE4* variant increases risk for late-onset AD, compared to the normal *APOE3* variant[135,136]. A recent study found that knocking down apoE in pericytes increases their migration, which can be rescued by addition of exogenous apoE3 but not apoE4[268]. This study found that apoE mediates pericyte migration through low-density lipoprotein receptor-related protein 1 (LRP1)[268], which is a major apoE receptor in the brain. Since pericytes are crucial to BBB integrity, which contributes to cerebrovascular health, increasing apoE levels may decrease pericyte migration, and ultimately protect the BBB.

Several compounds have been shown to increase apoE levels such as GW3965 and T0901317. These compounds have also been shown to increase ABCA1, which lipidates apoE. However, these compounds are agonists of liver-X-receptor (LXR), which promotes hepatotoxicity in humans and thus cannot be further developed as AD therapeutics.

We recently used high-throughput screens to identify several small molecule compounds that promote secretion of lipidated apoE from human astrocytoma cells. Importantly, the screen was designed with a counter-screen to exclude LXR agonists. The first class of compounds, which consists of P1 and P2, are antagonists of the P2X7 receptor. The P2X7 receptor is a purinergic receptor that is an ATP-gated cation-selective channel that plays a role in neurodegeneration and chronic inflammation[269]. P1 and P2 are structurally similar to AZD9056 (PubChem CID 10161381), which is a well-tolerated drug developed for rheumatoid arthritis, osteroarthritis, chronic obstructive pulmonary disease, and Crohn's Disease[270,271]. The second class of compounds is A1, which is an inhibitor of AXL receptor tyrosine kinase. Axl receptors have roles in the phagocytosis of apoptotic cells, as well as in inflammation[272,273]. Interestingly, there is evidence suggesting that Axl is involved in AD pathology. For example, microglia associated with AD plaques have upregulated AXL mRNA as well as other genes associated with immune response, overall contributing to neuroinflammation related to AD pathology[274]. Moreover, soluble Axl (sAXL) in plasma may be a potential biomarker of brain amyloid burden[275]. Increased baseline sAXL levels also correlate with Aβ42 in the CSF and potentially serve as a biomarker for brain amyloid pathology in AD[276].

The cerebrovasculature is important in AD. Firstly, most of the Aβ is cleared across the BBB[70–72]. Secondly, cerebrovascular damage such as BBB breakdown[47–50], loss of pericytes[46], reduced glucose utilization[52] and reduced CBF[54–56] are observed in AD patients. Lastly,

cardiovascular risk factors such as type 2 diabetes mellitus, hypertension and low physical activity increase the risk of AD, which is believed to be through damage to endothelial cells and the BBB[238,277,278]. Importantly, HDL, which is associated with improved endothelial cell function and reduced cardiovascular risk, cooperates with brain apoE to decrease Aβ-mediated endothelial cell inflammation and promote Aβ transport across a 3D human model of the cerebrovasculature[187,279]. Taken together, this suggests that the cerebrovasculature is an important target for repair in the development of AD therapeutics.

2.2 Methods

2.2.1 Cell culture

Primary fetal human brain vascular pericytes were commercially obtained from ScienCell. Three pericyte donors were available, with the following genotypes: Pericyte donor #1 (apoE4/E4); Pericyte donor #2 (apoE3/E3); and Pericyte donor #3 (apoE3/E4). Cells were maintained in Pericyte Media with 2% FBS and 1% penicillin and streptomycin. Media was changed every 2 – 3 days. Pericytes were utilized up to passage 9. Pericytes were subsequently sub-cultured when reaching 80 – 90% confluency.

2.2.2 Scratch-Wound Assay

A monolayer scratch assay was performed using the IncuCyte ZOOM live cell imaging system (Essen Biosciences). Pericytes were seeded at 10,000 cells/well on a gridded 96-well IncuCyte ImageLock Plate. Cells were treated with apoE-modulating compounds GW3965 (1uM), P1 (10uM), P2 (10uM), A1 (1uM) in Pericyte Media for 24 hours before the scratch-wound assay. Cells were treated with 10ug/mL mitomycin C (Sigma) in Pericyte Media for 2 hours before the

scratch-wound assay to prevent cell proliferation. Cells were scratched with a 96-pin mechanical wound-making device "WoundMaker" and washed with Pericyte Media once to remove cells from the scratch area. Media with drugs were added back into the wells after the wound was created. The plate was placed into the IncuCyte ZOOM device and images were collected every 2 hours for a total of 48 hours.

2.2.3 ApoE ELISA

Human apoE was quantified using commercial enzyme-linked immunosorbent assay (ELISA) kits (MABTech 3712-1H-6) following manufacturer's instructions.

2.2.4 Statistical Analysis

Data for pericyte migration were analyzed by Two Way analysis of variance (ANOVA) followed by a Sidak's multiple comparisons test. Data for apoE ELISA and Four Parameter Logistic curve fitting were analyzed by One Way ANOVA followed by a Tukey's multiple comparisons test for parametric data, and Kruskal-Wallis test followed by a Dunn's multiple comparisons test for nonparametric data. All statistical analyses were performed by GraphPad Prism v7. Data are displayed as mean ± standard error of mean (SEM). Since standard deviation (SD) is a measure of variability around mean of sample population, and SEM is a measure of precision of an estimated population mean, we chose to display the data with SEM because it represents how precisely we have determined the mean.

2.3 Results

2.3.1 Pericytes from all 3 donors produce apoE

The pericytes used in this study were obtained commercially (ScienCell) and derived from human fetal brain sources. The pericytes were from 3 separate donors, with varying *APOE* genotypes. Human pericyte donor #1 ("HP1") has an *APOE* genotype of E4/E4; human pericyte donor #2 ("HP2") has an *APOE* genotype of E3/E3; and human pericyte donor #3 ("HP3") has an *APOE* genotype of E3/E4. We confirmed that the pericytes used for this study secreted apoE after 24 hours in culture (**Fig. 2.1**). We find that all pericytes secrete apoE, and the levels of apoE secreted did not differ significantly between donors (**Fig. 2.1**).

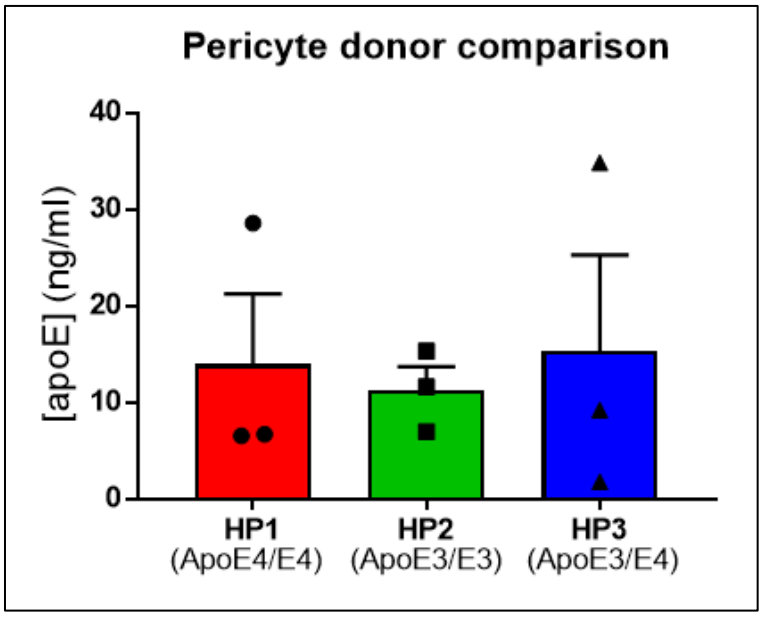

Figure 2.1 Pericytes from each pericyte donor produce apoE.

Media from each pericyte donor was measured for apoE levels with ELISA. No significant differences in secreted apoE levels are found when analyzed by Kruskal-Wallis test with Dunn's multiple comparisons test. Graph represents mean ± SEM, with N = 3 independent trials.

2.3.2 HP2 pericytes respond to apoE-modulating compounds

We tested whether the pericytes upregulate apoE secretion upon the treatment of apoE-modulating compounds P1, P2 and A1 for 72 hours. We used HP2 pericytes to test the drug effect because of its apoE3/E3 genotype. We found that HP2 pericytes respond to all apoE-modulating compounds with significant increase in apoE secretion (**Fig. 2.2**). We found that pericytes secreted 14.20 ± 7.33 ng/mL of apoE in the DMSO condition, 125.70 ± 37.48 ng/mL in the GW3965 condition, 210.2 ± 66.45 ng/mL in the P1 condition, 49.71 ± 19.02 ng/mL in the P2 condition, and 47.11 ± 11.74 ng/mL in the A1 condition. We also tested the drug effect on pericytes from different donors, and we see a trend towards increasing apoE secretion with drug treatment (**Fig. 2.3**).

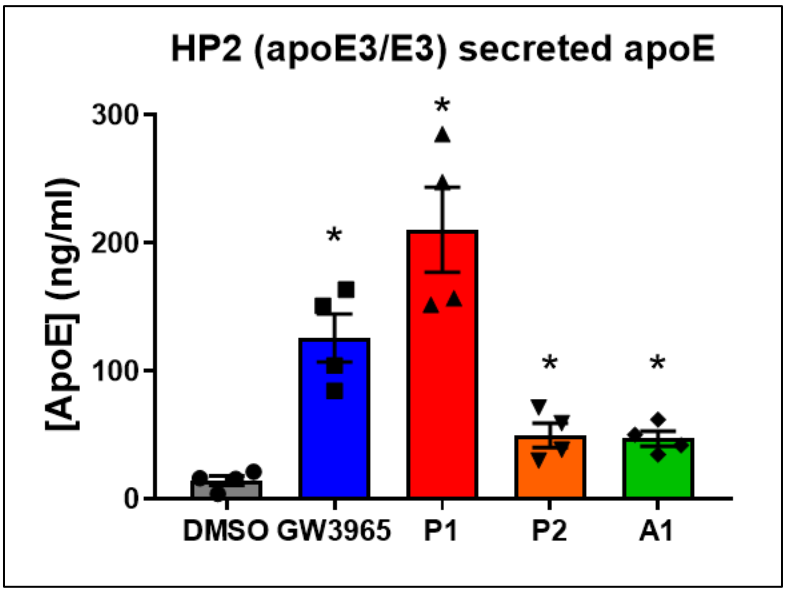

Figure 2.2 HP2 pericytes respond to apoE-modulating drugs

HP2 pericytes (apoE3/E3) were treated with apoE-modulating compounds GW3965, P1, P2 and A1 for 72 hours and measured for apoE secretion with ELISA. * $p < 0.05$ between DMSO and GW3965, P1, P2 and A1 as analyzed by blocked Two-Way ANOVA followed by Dunnett's multiple comparisons test. Graph represents mean ± SEM, with N = 4 independent trials.

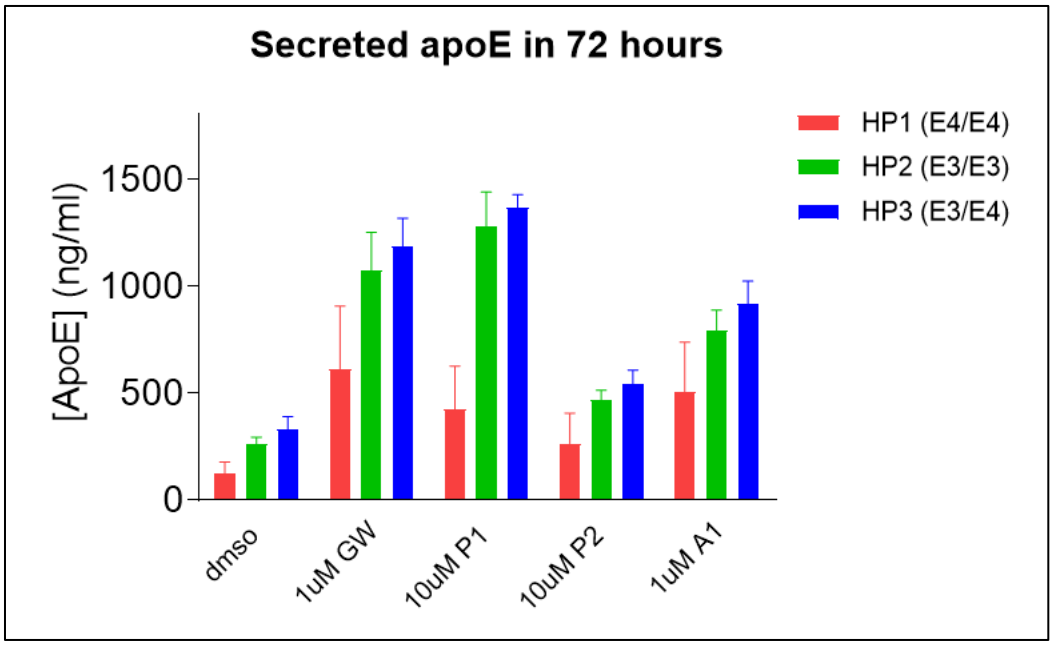

Figure 2.3 Pericytes from all donors show trend towards increasing apoE secretion with treatment with apoE-modulating compounds

Pericytes were measured for apoE secretion after treatment with apoE-modulating compounds for 72 hours. Pericytes from all donors showed an increase in apoE secretion with treatment with GW3965, P1, P2 and A1. Graph represents mean ± SEM, with data collected from N = 2 independent trials.

2.3.3 HP1 pericytes show slower migration with A1 treatment

Cell migration can be assessed with a scratch-wound assay, also known as the wound healing assay. The scratch-wound assay is an *in vitro* technique for measuring cell migration, in which the cells in a monolayer are scratched in a middle of the well and observed for migration across the wound over time. Historically, scratch-wound assays lacked standardization since there are variations in the wound, the imaging equipment and acquisition, and the analysis[280]. New platforms such as IncuCyte ZOOM™ real-time live imaging systems emerged to address the reproducibility of scratch-wound assays. Migration is quantified by "relative wound density", which is a ratio of the cell density in the wound area relative to the cell density outside the wound. Since the proportion of cells that migrate into the wound increases over time, so does the relative wound density.

In our study, we wanted to observe the effects of increasing apoE in pericytes on migration. Pericytes were treated with apoE-modulating compounds for 24 hours before they were imaged in the scratch-wound assay for 48 hours, which was the time required for the scratch-wound to close. Thus, the pericytes were treated with the apoE-modulating compounds for a total time of 72 hours, which was the time in which we observed an increase in apoE secretion (**Fig. 2.3**). Compared to addition of DMSO, the addition of GW3965, P1 or P2 did not affect pericyte migration, as measured by percentage relative wound density (**Fig. 2.4**). However, pericytes treated with Axl inhibitor A1 showed significantly slower migration in the 14 – 20 hour time point ($p < 0.05$) (**Fig. 2.4**).

We were also interested in extracting certain parameters from the curve for comparison. We used Four Parameter Logistic curve fitting in order to estimate the time to 50% relative wound density, as well as the maximum relative wound density, in all treatment groups. Four

Parameter Logistic curve fitting is a method commonly used as a curve fitting analysis for sigmoidal curves generated from assays such as ELISA and dose-response curves. For our purposes, we chose a functional form to yield meaningful and useful summaries for the curve. The four parameters that are extracted from this curve fitting method are: (1) minimum asymptote, which is typically the bottom of the curve; (2) maximum asymptote which is typically the top plateau of the curve; (3) the steepness of the curve, which is known as "Hill's Slope"; and (4) the inflection point, which is the point on the curve where the curvature changes direction or sign. In a dose-response curve, for example, the inflection point would be considered the "EC50", or the half maximal effective concentration. For our curve, the inflection point is the "time to 50% relative wound density" parameter.

Compared to HP1 pericytes treated with DMSO, in which the time to reach 50% relative wound density is 11.43 ± 2.39 hours, pericytes treated with A1 required 17.20 ± 4.21 hours to reach 50% relative wound density ($p < 0.05$) (**Fig. 2.5a**). However, pericytes in all treatment groups reach the same maximum relative wound density (**Fig. 2.5b**).

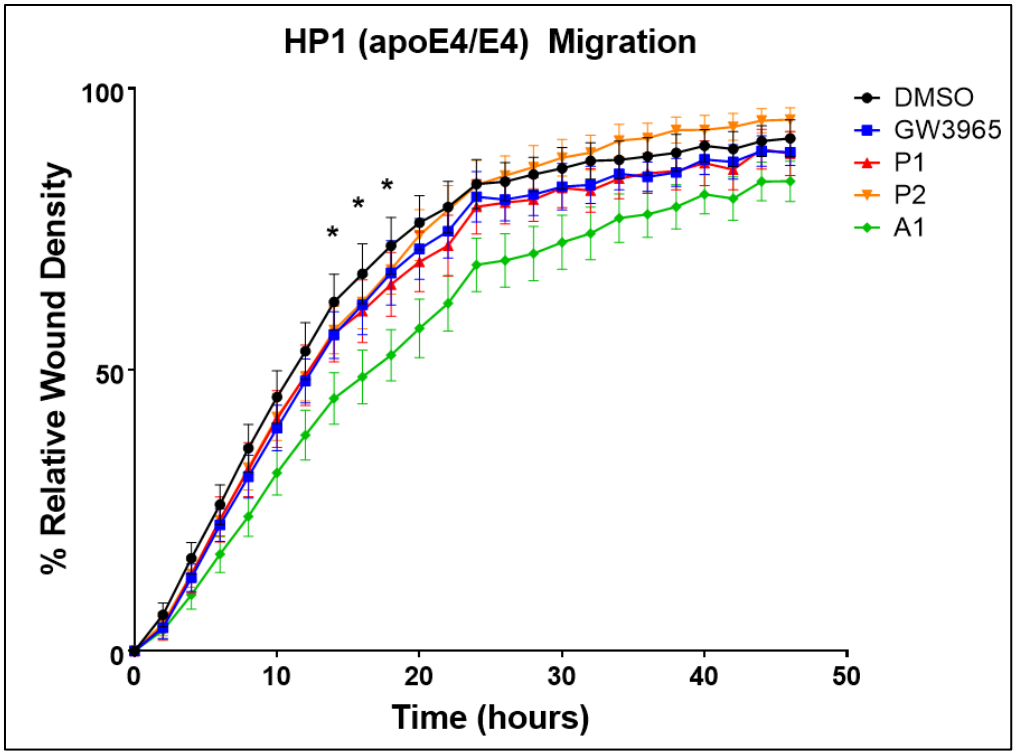

Figure 2.4 HP1 pericytes show slower migration with A1 treatment but not with GW3965, P1 or P2 treatment

HP1 (apoE4/E4) was treated with apoE-modulating compounds GW3965, P1, P2, and A1 for 24 hours before the scratch-wound assay as imaged and analyzed by IncuCyte. HP1 pericytes show a decrease in migration with A1 pre-treatment during hours 14 – 20 of the scratch-wound assay. * $p < 0.05$ between DMSO control and A1-treated pericytes as analyzed by Two-way ANOVA with Sidak's multiple comparisons test. Data are represented as mean ± SEM, with data collected from N = 7 independent trials.

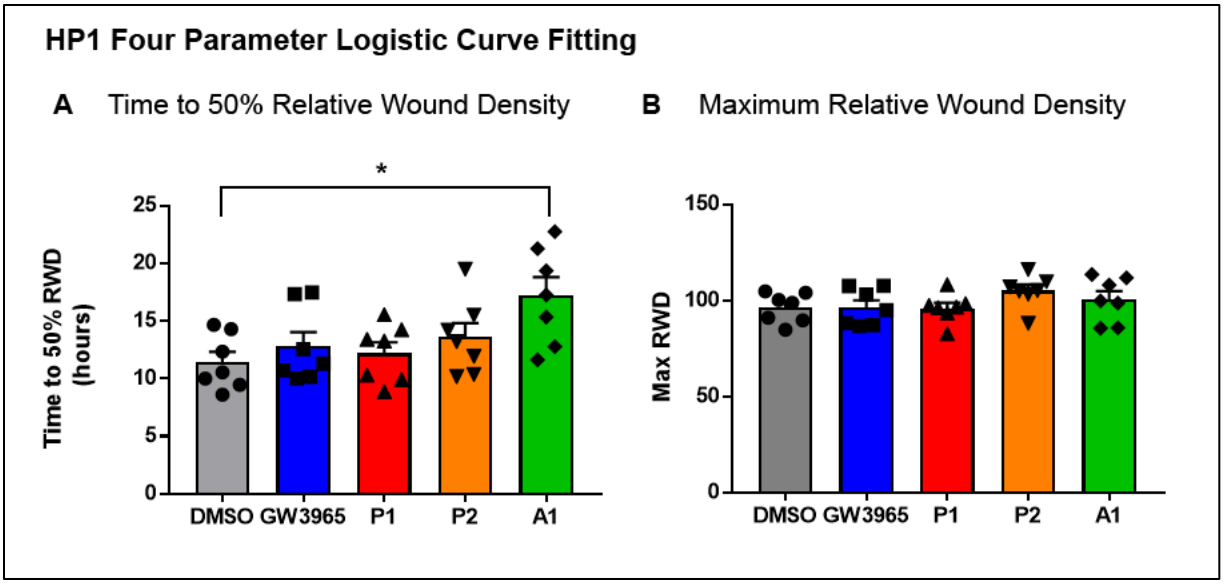

Figure 2.5 HP1 pericytes treated with A1 requires more time to reach 50% relative wound density

HP1 pericytes treated with A1, but not GW3965, P1, or P2, required more time needed to reach 50% relative wound density in a scratch-wound assay. HP1 pericytes treated with GW3965, P1, P2 and A1 did not show any differences in maximum relative wound density in a scratch-wound assay. *$p < 0.05$ as analyzed by Kruskal-Wallis with Dunn's multiple comparisons test. Data are represented as mean ± SEM, with data collected from N = 7 independent trials.

2.3.4 HP2 pericyte migration is not affected by GW3965, P1, P2 or A1 treatment

When HP2 pericytes (apoE3/E3) were treated with apoE-modulating drugs GW3965, P1, P2 or A1, pericyte migration did not change (**Fig 2.6**). Similarly, the time to reach 50% relative wound density as well as maximum relative wound density did not differ between treatment groups (**Fig 2.7**).

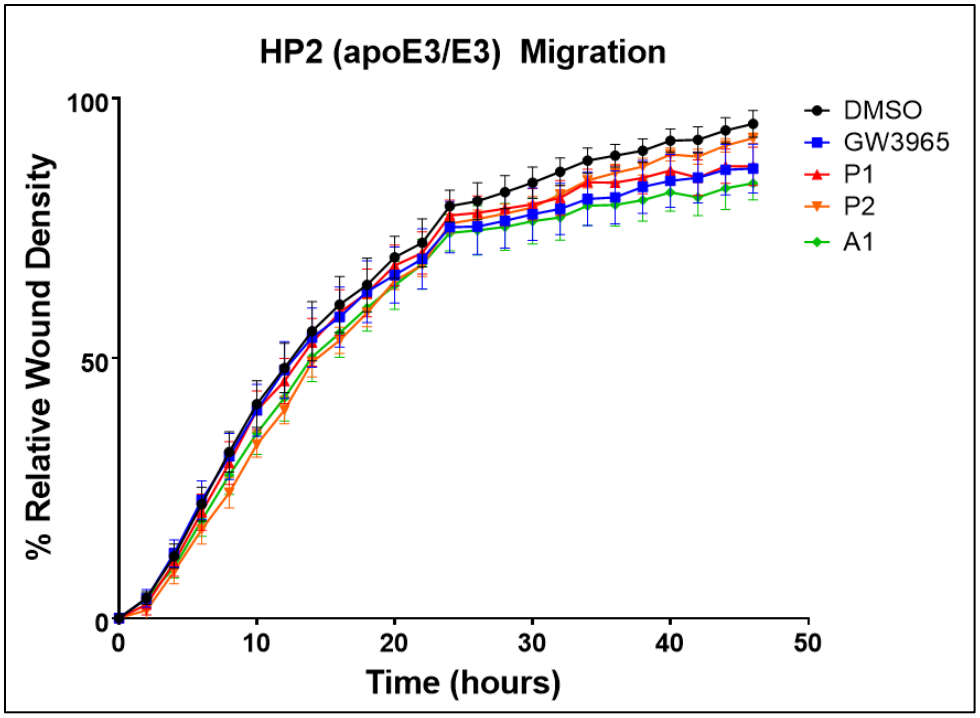

Fig 2.6. HP2 pericyte migration is not changed with GW3965, P1, P2 or A1 treatment

HP2 was treated with apoE-modulating compounds GW3965, P1, P2, and A1 for 24 hours before the scratch-wound assay as imaged and analyzed by IncuCyte. Data are not significant as analyzed by Kruskal-Wallis with Dunn's multiple comparisons test. Data are represented as mean ± SEM, with data collected from N = 7 independent trials.

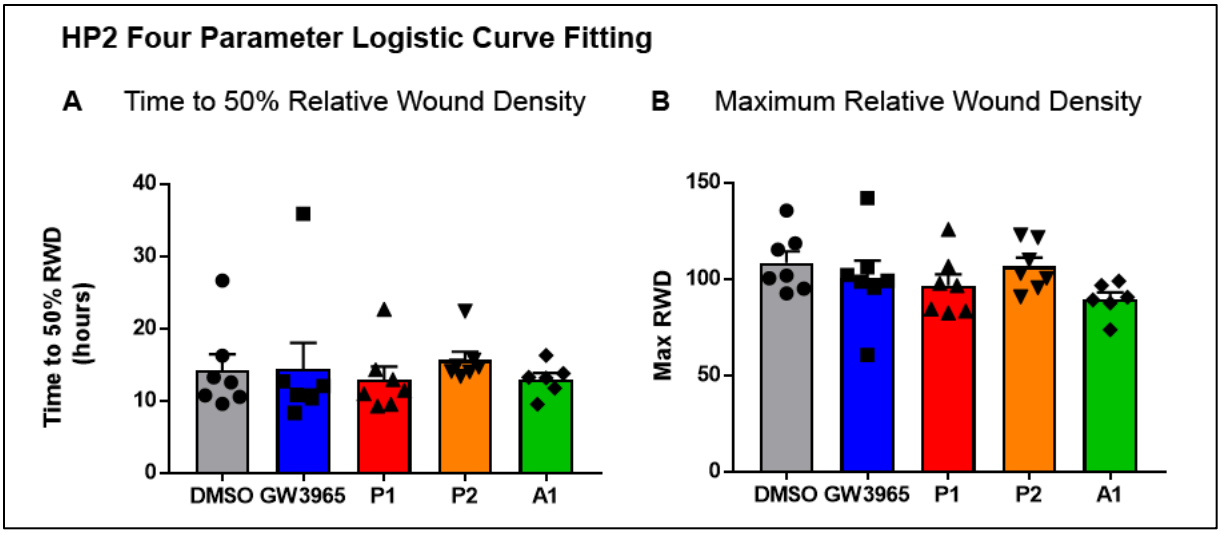

Fig 2.7 HP2 pericyte migration is similar between treatment groups in their time to reach 50% relative wound density and maximum relative wound density

HP2 pericytes treated with GW3965, P1, P2 and A1 show no difference in time to 50% relative wound density or maximum relative wound density in a scratch-wound assay as analyzed by Kruskal-Wallis with Dunn's multiple comparisons test. Data are represented as mean ± SEM, with data collected from N = 7 independent trials.

2.3.5 HP3 pericytes show slower migration with A1 treatment, but not with GW3965, P1, or P2 treatment.

HP3 pericytes (apoE3/E4) were treated with GW3965, P1, P2 or A1 for 24 hours before migration was observed in a scratch-wound assay. Pericytes treated with A1 showed slower migration ($p < 0.05$) at 8 to 22 hours into the assay, but not pericytes treated with P1 or P2 (**Fig. 2.8**). However, Four Parameter Logistic curve fitting showed no difference between treatment groups in their time to 50% relative wound density and maximum relative wound density (**Fig 2.9**).

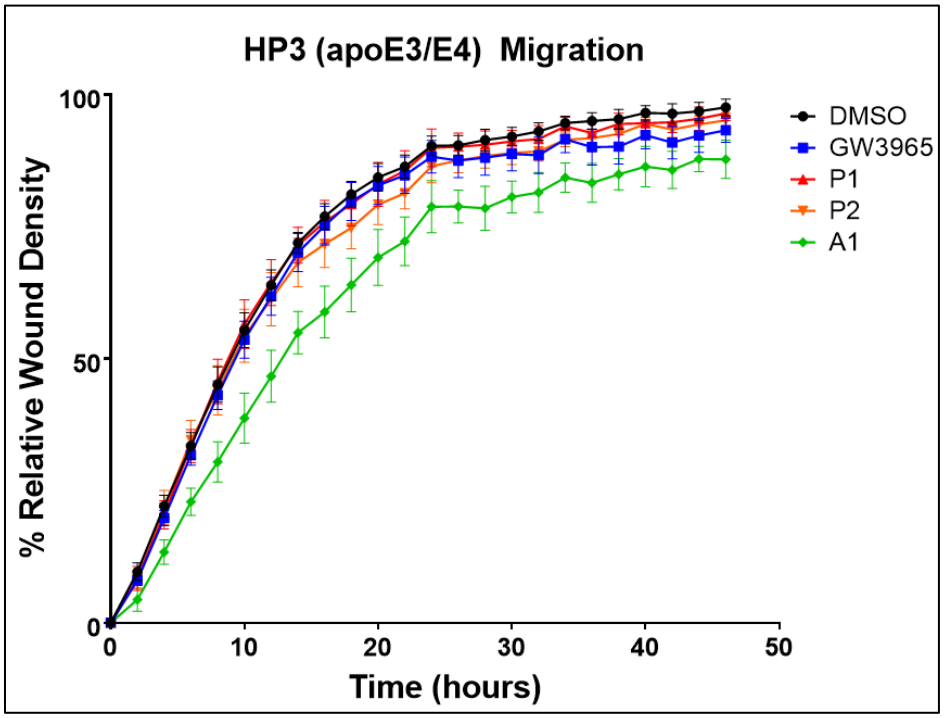

Figure 2.8 HP3 pericyte migration is not changed with treatment with apoE-modulating drugs

HP3 was treated with apoE-modulating compounds GW3965, P1, P2, and A1 for 24 hours before the scratch-wound assay as imaged and analyzed by IncuCyte. HP3 pericytes show a decrease in migration with A1 pretreatment during hours 8 – 22 of the scratch-wound assay. * $p < 0.05$ between DMSO control and A1-treated pericytes as analyzed by Two-way ANOVA with Sidak's multiple comparisons test. Data are represented as mean ± SEM, and collected from N = 7 independent trials.

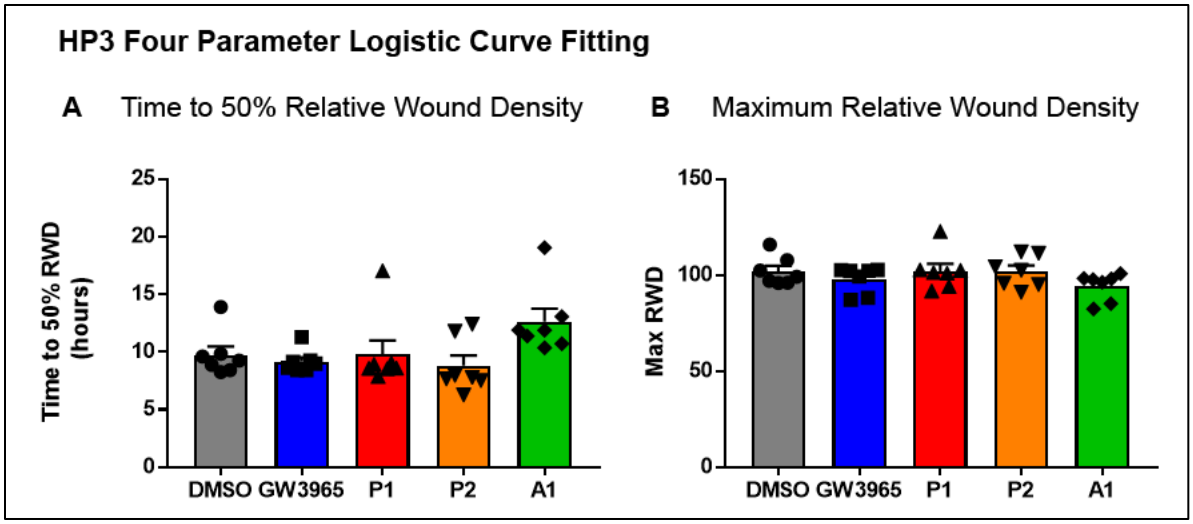

Figure 2.9 Four Parameter Logisitic Curve Fitting of HP3 pericyte migration curves

HP3 pericytes treated with GW3965, P1, P2 and A1 show no difference in time to reach 50% relative wound density or maximum relative wound density in a scratch-wound assay as analyzed by Kruskal-Wallis with Dunn's multiple comparisons test. Data are collected from N = 7 independent trials.

2.3.6 ApoE secretion is not increased in pericytes treated with A1 after scratch-wound assay

Media was collected from the wells after the scratch-wound assay in all treatment conditions to test for differences in secreted apoE with the addition of apoE-modulating drugs. However, only GW3965-treated pericytes in HP2 and HP3 showed increased apoE secretion after the scratch-wound assay (**Fig 2.10**). HP1 pericytes showed no changes in apoE secretion after the scratch-wound assay with any drug treatment (**Fig. 2.10a**). HP2 pericytes treated with GW3965 showed significantly increased apoE secretion of 177.8 ± 141.4 ng/mL compared to pericytes treated with DMSO, which had an apoE secretion of 50.42 ± 46.84 ng/mL ($p < 0.05$)

(**Fig 2.10b**). However, we observed no changes in apoE secretion in pericytes treated with A1 compared to the DMSO condition (**Fig. 2.10b**). Similarly, HP3 pericytes treated with GW3965 showed significantly increased apoE secretion of 122.2 ± 42.37 ng/mL compared to pericytes treated with DMSO, which had an apoE secretion of 23.46 ± 10.27 ng/mL ($p < 0.05$) (**Fig 2.10c**). No changes in apoE secretion were found in HP3 pericytes treated with A1 compared to the DMSO condition (**Fig. 2.10c**). Overall, this suggests that A1 is affecting pericyte migration in a way that does not depend on apoE levels.

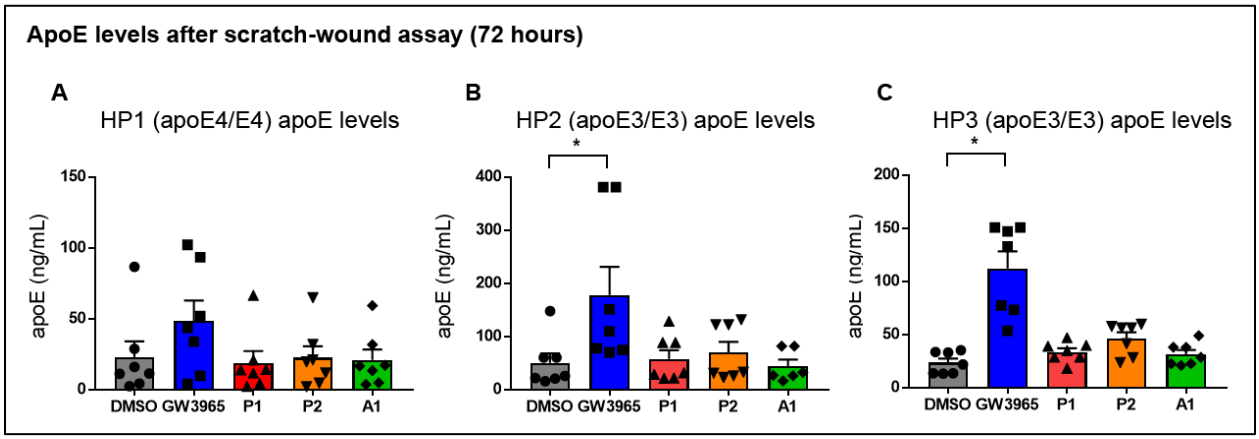

Figure 2.10 Pericytes treated with A1 do not show an increase in apoE secretion after scratch-wound assay

HP2 and HP3 but not HP1 showed increased apoE secretion with GW3965 treatment, while pericytes from all donors did not show a difference in apoE secretion with treatment with P1, P2 or A1 as collected from media after the scratch-wound assay. * $p < 0.05$ as analyzed by Kruskal-Wallis test with Dunn's multiple comparisons test. Data are represented as mean ± SEM, with data collected from N = 7 independent trials.

2.3.7 Pericytes treated with apoE-modulating compounds do not show increased apoE secretion at start of scratch-wound assay

As media for the apoE ELISA was collected from the wells after the scratch-wound assay, it is difficult to assess whether the increase in apoE levels in the GW3965 treatment condition was present before the scratch wound, and whether it would be affecting pericyte migration. Pericytes were thus seeded similarly on a 96-well plate and treated with apoE-modulating drugs for 24 hours, and media was collected to quantify apoE levels by ELISA. We found that apoE levels were not changed in any of the treatment groups, in any of the pericytes from different donors (**Fig. 2.11**).

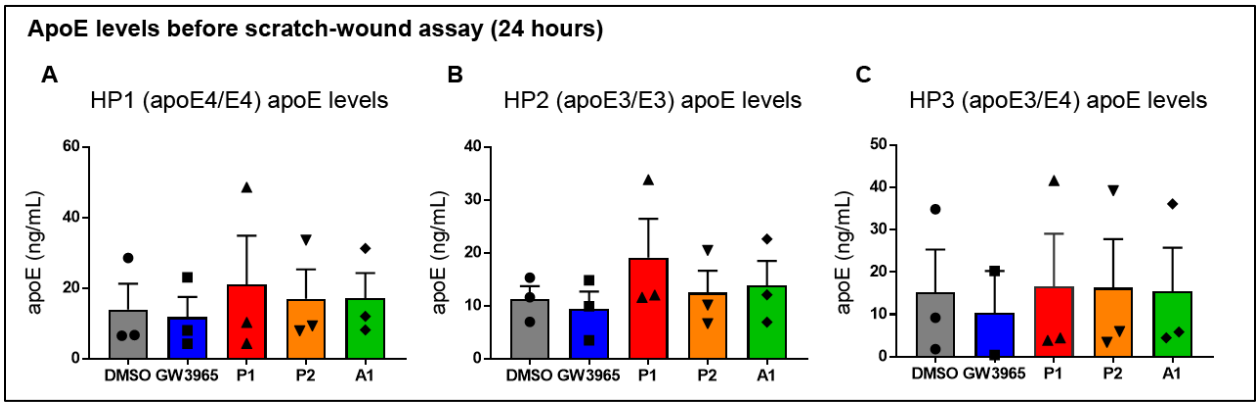

Figure 2.11 ApoE levels are not changed with GW3965, P1, P2 or A1 treatment

HP1, HP2 and HP3 pericytes showed no differences in apoE secretion with 24 hour treatment with apoE-modulating compounds before the scratch wound, as analyzed by Kruskal-Wallis test with Dunn's multiple comparisons test. Data are represented as mean ± SEM, with data collected from N = 3 independent trials.

2.3.8 Pericytes from the 3 different donors have different baseline migration

Thus far, we have observed differences in pericyte response to apoE-modulating drugs between donors in terms of their migration (**Fig. 2.4**, **Fig. 2.6**, **Fig. 2.8**). This could possibly be due to the inherent differences in migration between pericytes from different donors. Pericytes were thus analyzed on their baseline migration in the DMSO condition to compare migration rates for pericytes from all donors (**Fig 2.12a**). We found that there were no differences in migration at any time points when comparing HP1 (apoE4/E4) to HP2 (apoE3/E3) or HP3 (apoE3/E4) (**Fig. 2.12b, 2.12c**). However, when comparing HP2 (apoE3/E3) and HP3 (apoE3/E4), there were differences in migration in hours 8 to 24 ($p < 0.05$) (**Fig. 2.12d**). When Four Parameter Logistic curve fitting was performed, HP1 (apoE4/E4) vs. HP2 (apoE3/E3) and HP1 (apoE4/E4) vs. HP3 (apoE3/E4) did not differ in their time to reach 50% relative wound density (**Fig. 2.13a**). However, HP2 (apoE3/E3) required more time to reach 50% relative wound density (14.22 ± 5.91 hours) compared to HP3 (apoE3/E4) (9.74 ± 1.92 hours) ($p < 0.05$), suggesting a slower migration rate (**Fig. 2.13a**). Maximum relative wound density did not differ between any of the pericyte donors (**Fig 2.13b**), suggesting pericytes from all donors reach similar levels of cell density at the end of the scratch-wound assay.

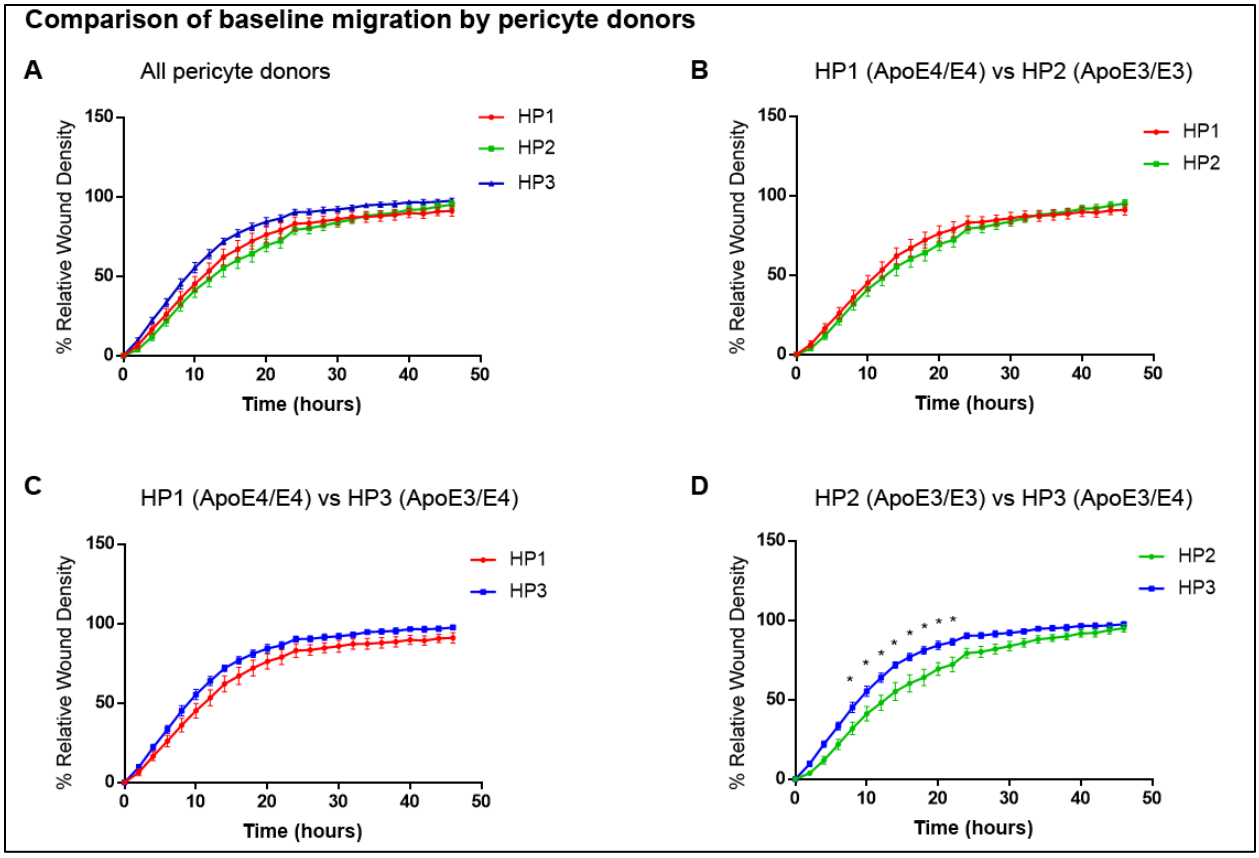

Figure 2.12 Baseline migration rates differ between HP2 and HP3

Pericytes were compared on their baseline migration in the DMSO condition. A) Differences in relative wound density over time in all pericyte donors. B) HP1 (apoE4/E4) and HP2 (apoE3/E3) did not show any differences in migration rates. C) HP1 (apoE4/E4) and HP3 (apoE3/E4) did not show any differences in migration rates. D) HP2 (apoE3/E3) and HP3 (apoE3/E4) differed in migration rates at time points 8 – 24 hours. * $p < 0.05$ as analyzed by Two-Way ANOVA with Sidak's multiple comparisons test. Data are represented as mean ± SEM, collected from N = 7 independent trials.

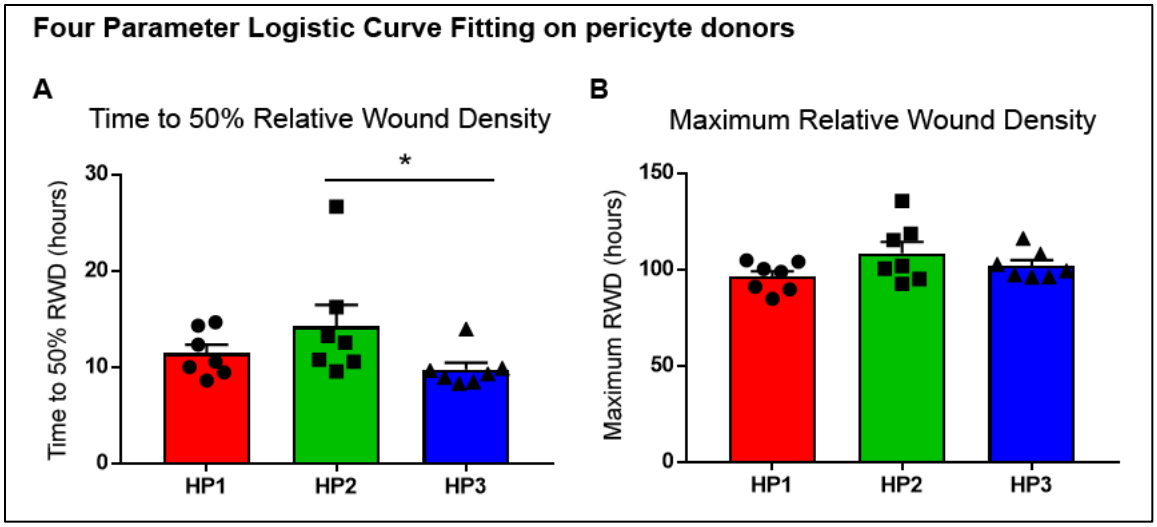

Figure 2.13 HP2 pericytes require more time to reach 50% relative wound density compared to HP3 pericytes

The time needed to reach 50% relative wound density was not different when comparing HP1 vs. HP2, or HP1 vs HP3. HP3 showed a slower time to 50% relative wound density compared to HP2 (* $p < 0.05$) as analyzed by Kruskal-Wallis test with Dunn's multiple comparisons test. Comparing all pericyte donors, there were no differences in Maximum RWD as analyzed by One-way ANOVA with Tukey's multiple comparisons test. Data are represented as mean ± SEM, with data collected from N = 7 independent trials.

2.4 Discussion

Here, we sought to increase apoE levels in pericytes with known apoE-modulating compounds. Previously, we tested these compounds on their apoE-modulating effects on pericytes in another experimental set-up in a 12-well plate (**Fig 2.3**), and we found increased apoE secretion after 72 hours. However, after the scratch-wound assay, we only observed an

increase in GW3965-treated HP2 and HP3 pericytes, but did not see an increase in GW3965-treated HP1 after 72 hours (**Fig. 2.10**). Moreover, we did not see increased apoE when the pericytes were treated with P1, P2 or A1 (**Fig. 2.10**). Though the pericytes in each of the different assays were treated similarly for 72 hours and with the same concentrations of apoE-modulating compounds, the differences in results we see may be due to the different conditions of the experimental set-up; the scratch-wound assay was conducted in a 96-well plate, whereas the previous experiment was conducted with a 12-well plate with a far higher number of cells per well. Moreover, the scratch-wound assay requires the removal of cells to make the wound, and this further reduction in the number of cells may be contributing to the lack of increase in apoE secretion. Importantly, the apoE secreted from these cells were not normalized to total protein, and thus were not standardized, making it difficult to compare between assays as well as between experimental conditions. For future experiments, protein levels in each well can be determined and subsequently the levels of apoE secreted from the pericytes can be expressed as a ratio of cell density.

Previous research found that knocking down apoE in pericytes increased their migration[268]. We thus hypothesized that increasing apoE levels may decrease their migration. We found that at the end of the scratch-wound assay, HP2 and HP3 pericytes treated with GW3965 had a significant increase in secreted apoE levels (**Fig 2.10**), but did not show a difference in migration despite the increase in apoE over the scratch-wound assay (**Fig 2.4**, **Fig 2.6**, **Fig 2.8**). This suggests that the increase in apoE levels over the 72 hours of the scratch-wound assay did not affect pericyte migration. Since the Casey et al. study showed that apoE mediates pericyte migration through receptor LRP1[268], it is possible that a ceiling effect was reached, and that the LRP1 receptors were already saturated with apoE. If this is true, pharmacologically increasing

apoE levels would not decrease pericyte migration. This can be verified by probing changes in LRP1 protein levels when the pericytes are treated with the different apoE-modulating compounds. If no changes in LRP1 levels are observed, this may explain the lack of differences seen in pericyte migration with the addition of GW3965, despite the overall increase in apoE levels over the assay. This can be also confirmed by increasing the number of LRP1 receptors, for example through virally overexpressing LRP1, and observing changes in migration.

However, since we collected the media from the pericytes after the scratch-wound assay, we cannot conclude that any changes in apoE levels we observed is directly affecting pericyte migration. To address this issue, we conducted another experiment in which we collected media from pericytes treated with the various apoE-modulating compounds for 24 hours (mimicking the time at which the pericytes would be scratched in a scratch-wound assay). We found that there was no increase in apoE levels in all conditions, with pericytes from all donors (**Fig. 2.11**). Since there is no change in apoE levels before the scratch-wound was implemented, we still cannot conclude that apoE levels observed during the course of the scratch-wound assay is necessarily affecting pericyte migration. Rather, if there is an increase in apoE levels right at the start of the scratch-wound, any changes in migration may be attributed to this change in apoE levels. Our experimental design thus did not adequately answer the question we proposed– whether an increase in apoE levels would affect pericyte migration – since we did not significantly increase the levels of apoE before the scratch wound was made. In retrospect, our experimental design should not have relied on the results of the apoE-modulating compounds in a 12-well assay, rather we should have tested these compounds and their effect on pericyte secretion of apoE in a 96-well, similar to a scratch-wound assay. Despite the same concentrations of the apoE-modulating compounds used in the different assays, it is possible that the different

cell densities and the different cell confluency between each experimental set-up are may affect apoE increase and secretion. Indeed, studies in macrophages find that apoE can be regulated in various ways; transcriptional regulation can be induced by exposure to cytokines, hormones and lipids in its microenvironment[281,282] while apoE secretion can also be stimulated by various apolipoprotein, and even apoE itself, which is observed to be isoform-dependent[283]. For future experiments, different concentrations of the apoE-modulating compounds can be added to the pericytes in a 96-well experimental set-up to validate the increase in apoE secretion by pericytes, such as a dose-response experiment. Subsequent testing of pericyte migration after treatment with these compounds with the determined conditions and concentrations can then be performed. If, from these experiments, we determine an effective dose of these compounds to observe an increase in apoE secretion by these pericytes, we can then use this to determine whether pericyte migration is affected in a scratch-wound assay. Further experiments can be done to test whether these compounds can be effective at decreasing permeability of the blood-brain barrier through an *in vitro* model with transwells.

Interestingly, we found that although A1 did not increase apoE levels, as measured at the end of the assay (**Fig 2.10**), A1 seemed to slow migration in HP1 and HP3 pericytes in the scratch-wound assay (**Fig. 2.4**, **Fig 2.8**). Whether this is an isoform-dependent effect is unclear, since there is only one sample for each apoE genotype. The decrease in pericyte migration seen with the addition of A1 without an increase in apoE levels suggests that A1 may be decreasing migration through an apoE-independent pathway. A1 is an inhibitor of Axl, which is an important receptor tyrosine kinase that plays a role in various cellular processes including proliferation, survival and migration[284]. The role of Axl in cellular migration has been delineated in many cancer studies, in which cell migration and metastasis occurs. In studies using colorectal

and cervical cancer cell lines, knockdown of Axl results in decreased migration[285,286]. Similarly, we see decreased migration in pericytes treated with our A1 compound (**Fig. 2.4**, **Fig 2.8**). Axl activity correlates with migratory phenotype in cells that includes increase in activated (GTP-bound) GTPase proteins Rho and Rac, decrease in epithelial mesenchymal transition (EMT)-associated transcription factors, and a decrease in matrix metalloproteinase MMP9 expression[287].

Studies in other cell types have shown that apoE mediates cell migration in both directions. For example, extensive studies have shown that apoE inhibits cell migration in smooth muscle cells through binding to LRP1 and subsequent induction of cyclic AMP and protein kinase A[288]. Interestingly, an isoform-dependent effect was seen as well, similar with apoE-mediated inhibition of pericyte migration, whereby apoE4 is less effective than apoE3 in inhibiting migration[288]. Another study showed that apoE is required for migration when stimulated with C5a and ATP, which was also found to be isoform-dependent whereby microglia expressing apoE4 or apoE2 were less effective in migrating to the stimuli[289]. ApoE has also been found to promote cell migration in lung carcinoma[290]. The curiously dual role for apoE in promoting and inhibiting migration may be due to the different cell types and possibly the different receptors to which it is binding.

In my study, I find that inhibition of the Axl receptor decreased pericyte migration in cells derived from 2 of 3 human donors (**Fig 2.4**, **Fig 2.8**). Whether apoE binds to Axl, and whether there is physiological significance to their binding, has not yet been studied. It is possible that apoE can be a ligand for Axl, since Axl plays a role in cell migration. A recent study that investigates the phenotypic switch from homeostatic microglia to neurodegenerative microglia finds a cluster of genes that are upregulated in neurodegenerative microglia, which includes *APOE* and *AXL* in the same cluster[265]. This could possibly hint at a transcriptional and

functional link, which may be salient in the dysfunctional microglia phenotype, since the TREM2-APOE pathway drives this phenotype[265]. Moreover, Axl is a receptor involved in phagocytosis[291], and apoE has been shown to also mediate phagocytosis[292], further supporting the plausible role for their interaction. Since the Axl receptor promotes migration, it is possible that apoE acts as a positive ligand for Axl, and thereby promotes migration as well. If this is true in pericytes, apoE may be mediating migration through the ratio of receptors that promote migration versus receptors that inhibit migration, such as through the ratio or balance of Axl receptors versus LRP1 receptors.

Many studies demonstrate the importance of pericytes at the vasculature[49,99,103,267]. Pericyte migration away from the vasculature can be thus detrimental, especially in cases in which vascular health is important, such as in AD. For example, in traumatic brain injury (TBI), pericytes detach from the basal lamina and migrate through the extracellular matrix to the site of injury[293]. Pericyte death after TBI has also been shown[294]. In diabetic retinopathy, pericyte migration and death leads to the loss of protection to the endothelial cells in the blood-retinal barrier[295], which leads to microaneurysms and changes in vascular permeability in the retina. However, pericyte migration is also beneficial in some cases. For example, pericyte migration is required for angiogenesis during development, specifically in early stages of capillary sprouting where they guide the outgrowth of endothelial cells[296]. Importantly, during injury, pericytes migrate to the site of the wound and contribute to the various stages of wound healing such as angiogenesis, inflammation, and tissue regeneration[297]. Thus, targeting pericyte migration specifically in the cerebrovasculature will be an important consideration for the therapeutic development of these compounds.

Chapter 3: ApoE4-associated inflammation in pericytes

3.1 Introduction

Pericytes contribute to the maintenance of blood brain barrier (BBB) integrity, which prevents toxins carried from the blood from entering the brain while still allowing the entry of nutrients and the exit of waste products. Rodent studies show that pericytes maintain a functional BBB[99,266]. In Alzheimer's Disease (AD), pericyte loss and degeneration are documented in post-mortem studies[46], and are accompanied by abnormal brain capillaries with tortuous vessels and fusiform dilations. Many studies demonstrate vascular changes such as a loss of cerebrovascular and BBB integrity in patients with AD[47,48,298], which is believed to lead to decreased capillary blood flow, neuronal injury, and impaired Aβ clearance[66]. According to the "Two Hit Hypothesis", when these vascular changes are combined with increased Aβ presence, subsequent neuronal injury, cognitive decline and neurodegeneration are observed, which ultimately presents as dementia[66].

The apoE4 genotype has been known to be associated with increased AD risk[135,136]. However, the specific mechanisms through which apoE4 contribute to the pathology of AD are unclear. Clinical studies suggest that the apoE4 genotype may accelerate vascular damage and BBB leakage in AD. In one study, advanced AD patients with apoE4 allele were more likely to have plasma proteins within microvessel walls, suggesting BBB leakage [47]. Similarly, a more recent study found that AD apoE4 carriers were more likely to show extravasation of immunoglobin G and fibrin, as well as pericyte degeneration[50]. Mice expressing human apoE4 show a leaky BBB compared to intact BBB seen in mice expressing human apoE3 or apoE2[266,299]. A model is suggested by which apoE4 is associated with upregulation of the CypA-NFκB-MMP9 inflammation pathway, leading to secretion of the gelatinase MMP9, and

ultimately enzymatic degradation of endothelial tight junctions of the BBB[266]. Interestingly, human apoE2 and apoE3 are not associated with upregulation of this pathway, pointing towards a genotype-specific inflammation in pericytes[266]. A recent study in humans lends support to this study, as cognitively normal apoE4 carriers were found to have increased evidence of BBB and increased levels of CypA and active MMP9 in the CSF[300]. However, further studies in human brain pericytes are necessary to understand the mechanisms by which apoE and other lipoproteins affect pericyte and cerebrovascular function.

We are particularly interested in the role of circulating high density lipoproteins (HDL) since HDL demonstrates vasoprotective functions that are well-characterized in peripheral vessels such as increasing NO production to dilate vessels, repairing damaged endothelial cells and inhibiting apoptosis[213], reducing lipid oxidation, and inhibiting thrombotic activation [214]. More relevantly, HDL has been shown to reduce inflammation in endothelial cells [210–212].

Whether HDL plays an anti-inflammatory role for brain vessels is unknown. Importantly, epidemiological studies correlate HDL levels with Mini Mental State Examination (MMSE) and Cognitive Ability Screening Instrument (CASI) scores[227,301], and high HDL levels are associated with reduced AD risk[226]. Importantly, these vasoprotective functions of HDL are compromised in metabolic conditions such as type II diabetes mellitus and coronary artery disease[213,302,303], which are also well-established risk factors for AD[304–306].

3.2 Methods

3.2.1 Cell Culture

Primary fetal human brain vascular pericytes were commercially obtained from ScienCell. Three pericyte donors were available, with the following genotypes: Pericyte donor #1 (apoE4/E4);

Pericyte donor #2 (apoE3/E3); and Pericyte donor #3 (apoE3/E4). Cells were maintained in Pericyte Media with 2% FBS and 1% penicillin and streptomycin. Media was changed every 2 – 3 days. Pericytes were utilized up to passage 9. Pericytes were subsequently sub-cultured when reaching 80 – 90% confluency. Pericytes were stimulated with human TNFα (Cedarlane, Cat. #300-01A) at concentrations of 10ng, 30ng and 100ng, as well as with human recombinant apoE4 (Abcam, ab50243) at concentrations of 1.6ug/mL and 8ug/mL for 24 hours in Basal Pericyte Media + 0.2% Bovine Serum Albumin (BSA).

3.2.2 Gelatin Zymography

Pericyte conditioned media was collected and separated on a 7.5% acrylamide gel containing 0.1% gelatin. Gels were washed twice for 30 minutes with washing buffer (2.5% Triton X-100, 50nM Tris HCl, 5mM CaCl2, 1uM ZnCl2) at room temperature with agitation. Gels were incubated with incubation buffer (1% Triton X-100, 50nM Tris HCl) for 48 hours at 37°C, stained with staining solution (40% methanol, 10% acetic acid, Coomassie Blue).

3.2.3 mRNA extraction and quantitative real-time polymerase chain reaction (qRT-PCR)

Cells were treated with Trizol (Invitrogen) to extract RNA. Nucleic acid levels were quantified using a nanodrop spectrometer at 260nm and nucleic acid purity was measured through absorbance at 230nm and 280nm. RNA was subsequently treated with DNAseI (Invitrogen). TaqMan Reverse Transcriptase Reagents Kit (Life Technologies) was used to reverse transcribe 1ug of RNA into complementary deoxyribonucleic acid (cDNA). Real-time quantitative PCR was performed with SYBR Green reagents (Applied Biosystems) with primers against MMP9

(Forward 5'-TTCAGGGAGACGCCCATTTC-3', Reverse: 5'-AACCGAGTTGGAACCACGAC-3'), CypA (Forward: 5'-GCCGAGGAAAACCGTGTACT-3', Reverse: 5'-GTCTGCAAACAGCTCAAAGGA-3'), and GAPDH (Forward: 5'-CCTGCACCACCAACTGCTTA-3', Reverse: 5'-CATGAGTCCTTCCACGATACCA-3'). Cycling conditions for MMP9 and CypA are: 95°C for 10 minutes, then 40 cycles at 95°C for 10 seconds, 60°C for 10 seconds and 72°C for 10 seconds, followed by melting at 95°C for 10 seconds, 65°C for 60 seconds, and 97°C for 1 second. Each sample was assayed in duplicate and normalized to GAPDH.

3.2.4 Protein extraction and Immunoblotting

Pericytes were lysed in RIPA buffer containing cOmplete protease inhibitor (Roche Applied Sciences) for 10 minutes and mechanically scraped. Lysed cells were spun at 12000 relative centrifugal force (RCF) for 10 minutes at 4°C. Protein concentrations were determined by a bicinchoninic acid (BCA) protein assay (Pierce). Cell lysates were subsequently heat-denatured at 95°C for 5 minutes. Equal amounts of total protein were electrophoresed through 10% sodium dodecyl sulfate (SDS)- polyacrylamide gel (PAGE) before being transferred to polyvinylidene difluoride (PVDF) membranes (Millipore). Membranes were blocked with 5% skim milk powder in PBST or 5% BSA in TBST for 1 hour and then incubated with antibodies VCAM-1 (EPR5047, ABCAM, 1:200) and ICAM-1 (EPY1442Y, ABCAM, 1:200) in blocking buffer at 4°C overnight. Membranes were washed with PBST or TBST and incubated with secondary antibody anti-rabbit or anti-mouse (1:10,000) for 1 hour. Membranes were washed and developed using enhanced chemiluminescence (ECL, Amersham). ChemiDoc MP Imager

(BioRad) was used to image the immunoblots. Densitometric images were captured with ImageJ and band densities were normalized to GAPDH as a loading control.

3.2.5 Immunocytochemistry

Coverslips were coated with poly-D-lysine (Sigma, Cat. #P6407-5MG). Primary human pericytes and primary human astrocytes were cultured on the coverslips for 2 days. Cells were fixed with 4% Paraformaldehyde (PFA) in PBS for 15 minutes and washed with 0.5M Tris HCl. Cells were washed 3 times with PBS before being permeabilized with 0.2% Triton X-100 in PBS for 7 minutes. Cells were washed 3 times in PBS and blocked with 5% BSA in PBS for 1 hour in room temperature. Cells were incubated with primary antibody PDGFRβ (Abcam, 1:100) in 1% BSA overnight at 4°C. Cells were washed 3 times in PBS and incubated with Alexa Fluor 594-conjugated goat anti-rabbit secondary (Molecular Probes, 1:600) in 1% BSA for 1 hour in room temperature. Slides were mounted using DAPI (Invitrogen) and sealed after 30 minutes. Coverslips were imaged with fluorescence microscopy (Zeiss).

3.2.6 Enzyme-linked Immunosorbent Assay (ELISA)

Human apoE was quantified using commercial enzyme-linked immunosorbent assay (ELISA) kits (MABTech, 3712-1H-6) following manufacturer's instructions.

3.2.7 Peripheral Blood Mononuclear Cells (PBMC) isolation

Peripheral Blood Mononuclear Cells (PBMC) was taken from healthy human donors. Blood was diluted in a 1:1 ratio with 1xPBS and added on top of Ficoll-Hypaque Lymphoprep (StemCell Technologies) in a SepMate Tube (StemCell Technologies). Diluted blood was spun at 1200g for

10 minutes at room temperature. After centrifugation, the bottom layer of erythrocytes and polymorophnuclear cells were discarded. The plasma layer, PBMC layer, and the lymophoprep solution were washed twice with 1xPBS and spun at 800g for 8 minutes. Supernatant was aspirated and the pellet as resuspended in 10mL of 1xPBS. Cells were counted with a hemocytometer and Trypan Blue was used as a viability stain. Cells were suspended in a concentration of 5 x 10^7 cells/mL in 1xPBS.

3.3 Results

3.3.1 MMP9 protein activity or mRNA were not detected in primary human pericytes

A previous study showed that mice expressing human apoE4 had a leaky BBB, which was due to the upregulation of the cyclophilin A (CypA) and increased MMP9 secretion from pericytes[266]. We thus sought to test the MMP9 upregulation and secretion from primary pericytes of different human donors and with different apoE genotypes. Stimulation of pericytes by TNFα was used as a positive control, since a previous study found that pericytes secrete MMP9 with TNFα stimulation[307]. Media from HP1 (apoE4/E4) was measured for MMP9 activity with TNFα and apoE4 stimulation in culture media by gelatin zymography (**Fig. 3.1**). However, MMP2 rather than MMP9 activity was observed (**Fig. 3.1**). Lysate from immortalized mouse microglia cell line (BV2 cells) were used as a positive control for MMP9 release as detected by gelatin zymography. MMP9 was also not detected in cells from HP2 (apoE3/E3) and HP3 (apoE3/E4) basally or stimulated with TNFα (10ng, 100ng) as well as apoE4 (**Fig. 3.2**).

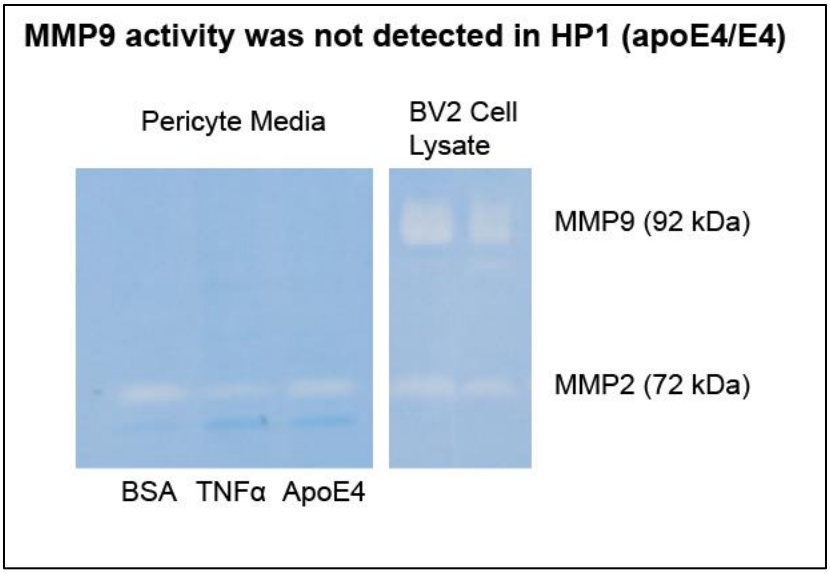

Figure 3.1 MMP9 activity was not detected in HP1 pericytes with TNFα or apoE4 stimulation

HP1 (apoE4/apoE4) did not basally secrete MMP9 as measured by gelatin zymography. Stimulation with TNFα or apoE4 did not induce MMP9 release. BV2 cell lysate was used as positive control to show MMP2 and MMP9 expression. Data are representative gelatin zymograms from N = 3 independent trials.

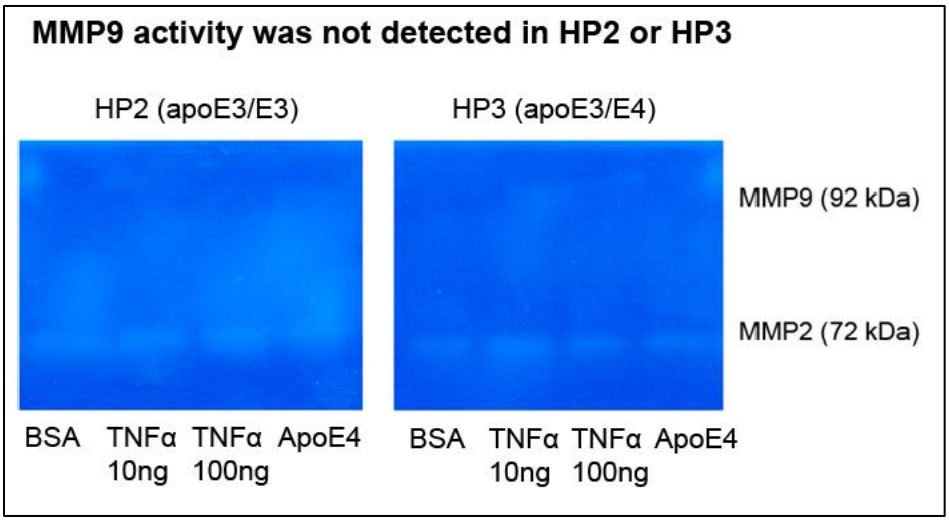

Figure 3.2 MMP9 activity was not detected in HP2 or HP3 pericytes with TNFα or apoE4 stimulation.

HP2 (apoE3/apoE3) and HP3 (apoE3/apoE4) did not secrete MMP9 with TNFα (10ng/mL, 100ng/mL) stimulation or apoE4 (1.6ug/mL) stimulation as measured by gelatin zymography. Data are representative gel zymogram shown N = 3 independent trials.

MMP9 mRNA levels were probed with qRT-PCR. Pericyte MMP9 mRNA levels are near the limit of detection, as seen by high Cq value (>30). Addition of TNFα in various concentrations (10ng/mL, 30ng/mL, 100ng/mL) or apoE4 in various concentrations (1.6ug/mL, 8ug/mL) did not change MMP9 expression in all pericytes (**Fig. 3.3**)

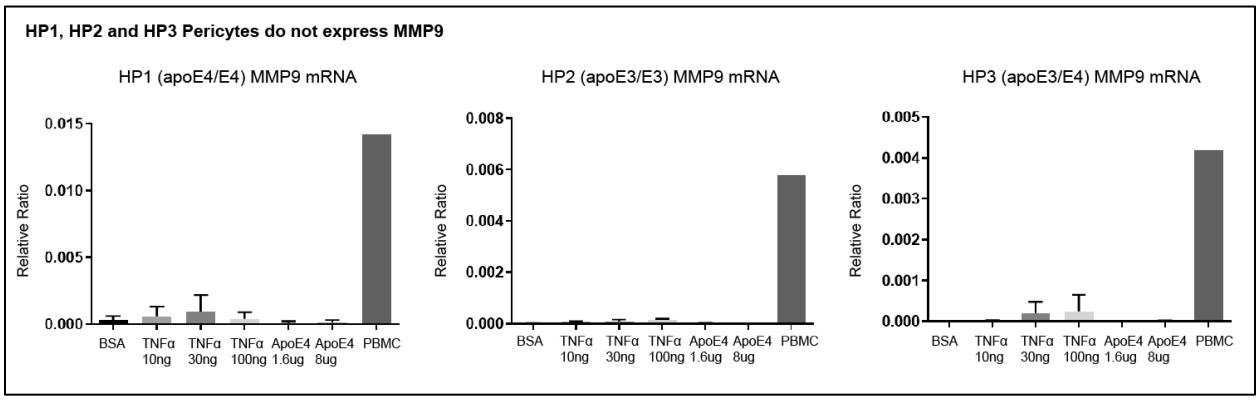

Figure 3.3 Pericytes from all donors do not express MMP9 mRNA

Addition of TNFα (10ng/mL, 30ng/mL, 100ng/mL) or apoE4 (1.6ug/mL, 8ug/mL) did not change MMP9 expression in all pericytes as measured by qPCR. Cq values for MMP9 are all pericyte conditions are >30. Peripheral blood mononuclear cells (PBMC) were used as a positive control for MMP9 signal. Data are represented in mean ± SD, with data from N = 2 independent trials.

3.3.2 Cyclophilin A (CypA) mRNA levels are not changed with TNFα stimulation

In the pericyte inflammation pathway delineated by Bell et al., mice expressing apoE4 had higher levels of pro-inflammatory cytokine cyclophilin A (CypA), which activates the NF-kB-MMP-9 pathway and causes BBB breakdown[266]. Since we previously did not see MMP9 expression or release in pericytes from all donors, we wanted to see whether CypA mRNA was upregulated in these pericytes. We wanted to trigger the inflammatory process in pericytes with TNFα to see whether CypA mRNA could be upregulated. We found that addition of TNFα or apoE4 in various concentrations did not change CypA mRNA levels in pericytes from all donors (**Fig. 3.4**).

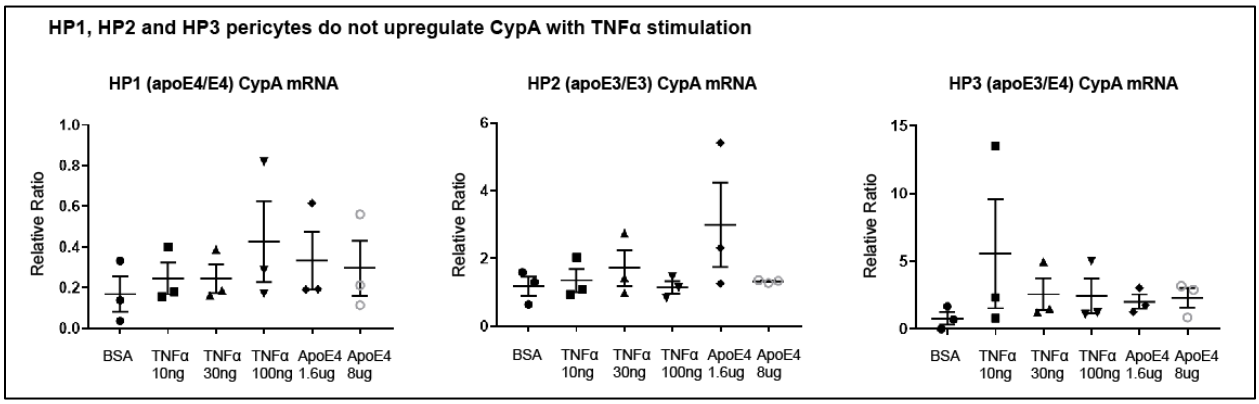

Figure 3.4 Pericytes from all donors do not upregulate CypA levels with TNFα stimulation

Addition of TNFα (10ng/mL, 30ng/mL, 100ng/mL) or apoE4 (1.6ug/mL, 8ug/mL) did not change CypA expression in HP1, HP2 or HP3 as measured by qPCR. Data are not significant. Mean ± SEM are shown, with data combined over N = 3 trials.

3.3.3 HP1 shows upregulation of inflammatory markers ICAM and VCAM-1 with TNFα stimulation

Since the pericytes did not upregulate CypA or MMP9 mRNA in response to TNFα stimulation, we then tested whether these pericytes are valid models of inflammation. We stimulated HP1 pericytes with TNFα and probed cellular levels of vascular cellular adhesion molecule 1 (VCAM-1) and intracellular cellular adhesion molecule 1 (ICAM-1). Both VCAM-1 and ICAM-1 are adhesion molecules that aid in the trafficking of immune cells across the vessel walls to allow the inflammation cascade to occur. Pericytes are known to express both VCAM-1 and ICAM-1[93]. We found that both VCAM-1 and ICAM-1 were upregulated with TNFα stimulation, as shown by immunoblotting (**Fig. 3.5**). This suggests that these pericytes still respond to inflammatory stimuli.

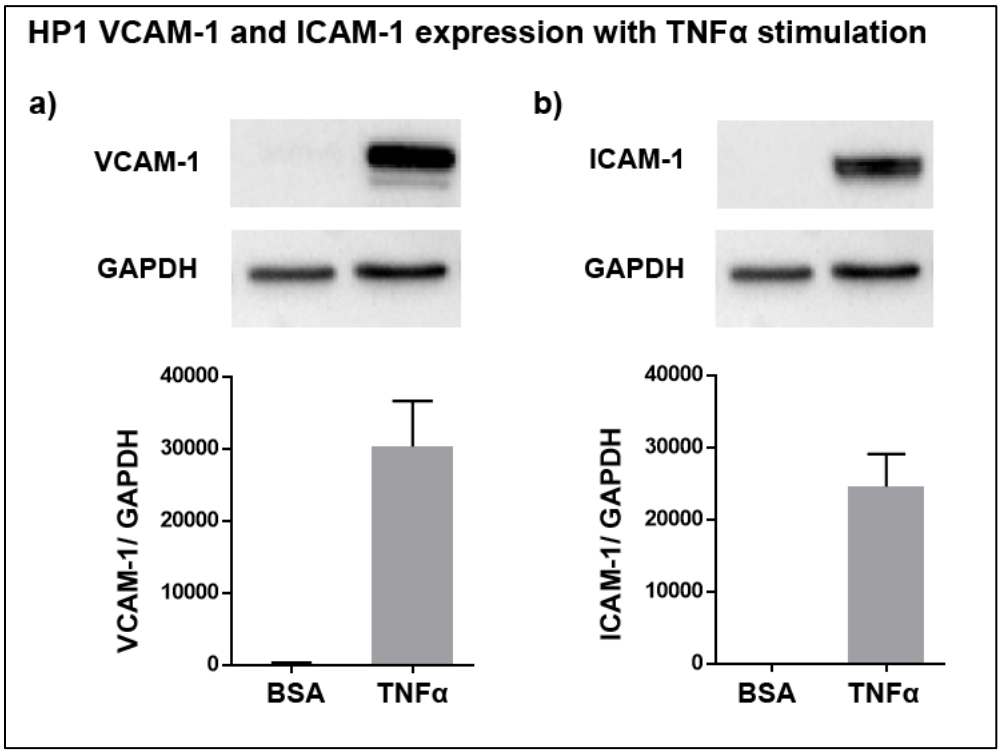

Figure 3.5 HP1 pericytes show upregulation of VCAM-1 and ICAM-1 with TNFα stimulation

Vascular cell adhesion molecule 1 (VCAM-1) and intercellular adhesion molecule 1 (ICAM-1) were upregulated with TNFα stimulation in HP1 pericytes as shown by Western Blot. Data are shown mean ± SEM, and with representative immunoblots from N = 3 independent trials.

3.3.4 Pericytes were validated for pericyte marker PDGFRB

We lastly wanted to confirm that these commercial pericytes were pericytes, since they did not express CypA or MMP9. We probed their expression of pericyte marker Platelet Derived Growth Factor Receptor Beta (PDGFRβ) through immunocytochemistry. We found that pericytes from all three donors express PDGFRβ (**Fig. 3.6**).

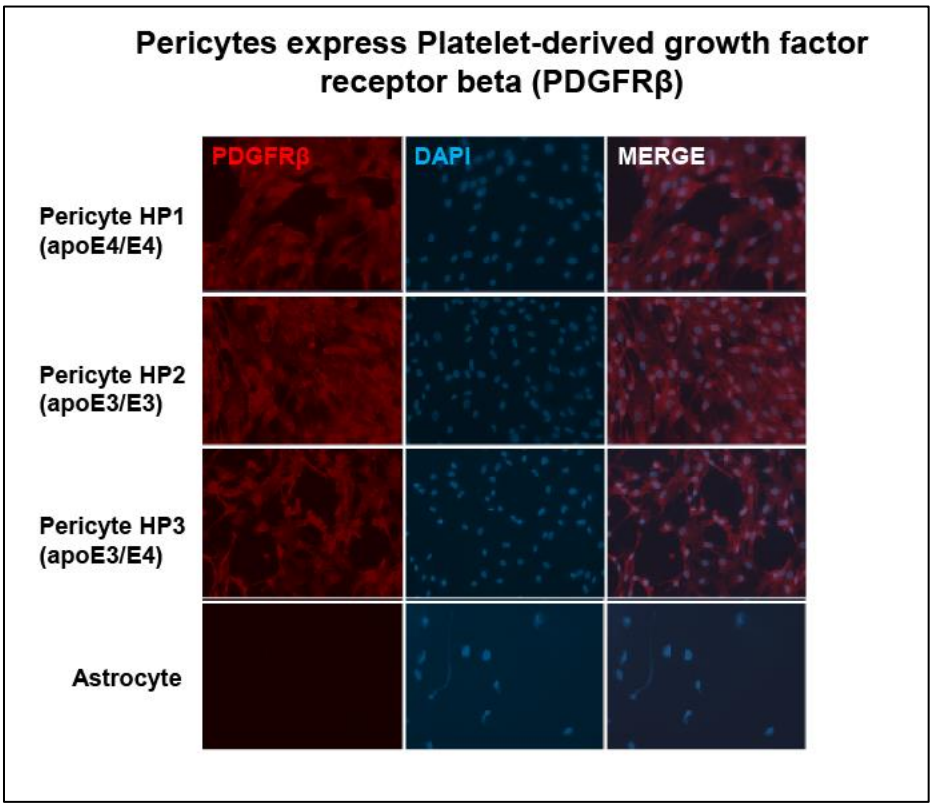

Figure 3.6 Pericytes from all 3 donors express PDGFRβ

Pericytes from all donors were probed for expression of pericyte marker PDGFRβ through immunocytochemistry. Coverslips were counterstained with DAPI. Astrocytes were used as a negative control.

3.4 Discussion

In this study, we aimed to test the anti-inflammatory effects of HDL on apoE4-associated inflammation in human pericytes. However, despite evidence showing that pericyte inflammation occurs in mouse pericytes[266], we did not reproduce these results using commercially obtained human primary pericytes. Specifically, we did not observe MMP-9 production (**Fig. 3.1**, **Fig. 3.2**) as well as mRNA expression (**Fig. 3.3**) in our primary human pericytes. Further, human pericytes also did not upregulate CypA with inflammatory stimuli, suggesting that the CypA-MMP9 inflammatory pathway may be unique to murine pericytes. As the quality control measures by which commercially-obtained pericytes are evaluated are not explicitly stated, we independently validated that these cells are indeed pericytes by confirming their PDGFRβ expression, and validated that they could indeed respond to inflammatory stimuli by confirming that TNF-α induced upregulation of ICAM-1 and VCAM-1 protein levels.

Several reasons could contribute to the lack of reproducibility of the CypA-MMP9 pathway in our hands. First, and most likely, this could be a difference in species that is contributing to the difference in activation of this inflammation pathway. For example, human and mouse brain pericytes differ in their cytokine and chemokine response to LPS stimulation[93]. Important species differences exist in vascular cell immunological functions, in terms of their major histocompatibility complex (MHC) molecules, their co-stimulators, antigen presentation

and T-cell recruitment[308]. Studies are also conflicting whether gene-expression patterns of mouse models are similar to human inflammatory conditions[309,310]. It is possible that human pericytes do not activate the CypA-NFκB-MMP9 pathway that is observed in mouse pericytes. Thus, a species differences may explain why our primary human pericytes do not recapitulate what others have observed in mouse pericytes. Species differences could be tested by comparing human and murine pericytes in head-to-head assays.

Our *in vitro* culture conditions may also be contributing to the lack of MMP-9 expression and release in our pericytes. For example, there may be important factors with which to culture and grow pericytes in order to induce expression of certain proteins. Many studies using cancer cells find that MMP-9 expression can be upregulated in various cells when co-cultured with tumor and stromal cells[311–314]. MMP9 release from human pericytes may require additional factors in its environment that allows it to be released, which may not found in our *in vitro* culture conditions, where our pericytes are grown in isolation and on plastic cell culture dishes. Indeed, cells grown in plastic or glass culture can exhibit altered cell proliferation, differentiation, morphology, and lifespan[315]. In order to mimic *in vivo* conditions, cells can also be grown with extracellular matrix (ECM) components with which they are usually in contact, and this has been shown to improve culture conditions, with cells surviving longer in culture and more differentiated phenotype observed[315]. The components of ECM include proteoglycan and fibrous proteins such as collagen, fibronectin and laminin, which structurally support the cells and facilitates cell-to-cell communication[316]. Moreover, transmembrane proteins that link cells to the ECM such as integrins, are involved in signalling pathways that regulate a variety of cell phenotypes such as proliferation, morphology, survival, adhesion and differentiation[317]. In future studies, pericytes can be grown in culture with ECM components, such as with the use of

Matrigel, which is a gelatinous mixture of proteins derived from mouse tumor cells that is commonly used as a basement membrane matrix[318]. The use of matrix membranes in cell culture has been demonstrated to be conducive to *in vivo* cellular behaviour that is otherwise not observed in traditional cell culture; for example, endothelial cells will form capillary-like structures *in vitro* when grown on top of reconstituted basement membrane extracellular matrix such as Matrigel[319]. It is possible that our pericytes may express the CypA-NFκB-MMP9 inflammation pathway if they were cultured in a more contextual manner with ECM components.

MMPs are also regulated by tissue inhibitors of metalloproteinases (TIMPs), which are endogenous inhibitors of MMPs. MMP-9 secretion is tightly regulated this way, such that its endogenous inhibitor TIMP-1 is bound to and inhibits latent MMP-9 before secretion, thereby preventing its function[320]. It is possible that our culture conditions allow MMP9 inhibitors to inhibit MMP9 release and thus may not be conducive to its release. Interestingly, though, we did observe the release of MMP-2 in pericytes from all donors (**Fig. 3.1**, **Fig 3.2**, **Fig. 3.3**). Future experiments to test this hypothesis could be to probe the presence of TIMP-1 and other TIMPS, as well as testing their activity.

Mice expressing apoE4 were found to have higher levels of cytokine CypA[266]. In our study, we asked whether CypA would be upregulated with pro-inflammatory stimuli, but we did not compare CypA levels across pericytes of different genotypes. Future experiments should test whether pericytes with apoE4 (HP1, HP3) show CypA upregulation compared to non-apoE4 pericytes. Moreover, in the model of apoE4-associated pericyte inflammation, NF-kB is upregulated with apoE4 pericytes. We can thus probe whether NF-kB is also upregulated in apoE4 pericytes.

Our failure to observe the upregulation of this pathway led us to hypothesize that the cells we have at hand may not have been pericytes. However, we found that these pericytes can be activated by classic pro-inflammatory stimuli (**Fig. 3.5**), and express the pericyte marker PDGFRβ (**Fig 3.6**). It is possible that although these pericytes express classic pericytes markers, they do not express the specific inflammatory pathway we are interested in studying. They are thus unable to address the question we initially posed, and thereby also raise questions about the potential of the murine CypA-NFκB-MMP9 pathway to be translated to humans.

Chapter 4: The role of HDL in TREM2-mediated functions in macrophages

4.1 Introduction

TREM2 is a cell surface receptor on myeloid cells such as microglia and macrophages. Recent whole-genome sequencing studies find variants in the TREM2 receptor to be associated with AD risk[250,251], such as the TREM2 R47H variant, which has an odds ratio of ~2.9 – 4.5 of developing AD, and decreases age of AD onset by ~ 3 years[250,251]. Animal studies show that TREM2 seems to be important in AD pathology. In one study, TREM2-deficient 5XFAD mice model of AD had increased hippocampal Aβ burden and increased cortical neuron loss[246]. Meanwhile, TREM2-overexpressing APPswe/PS1de9 mouse model of AD had decreased Aβ deposition, neuroinflammation and neuronal and synaptic loss, which was accompanied by improved cognitive function[254]. However, it is important to note that other studies suggest a detrimental role of TREM2 in AD pathology. For example, in one study using APPPS1-21 mouse model of AD, loss of TREM2 resulted in reduced accumulation of Aβ and reduced inflammation[257]. A newer study tried to address this controversial role of TREM2 by looking at the effect of TREM2 deficiency in different stages of disease. The results of the study suggest that TREM2 may be detrimental to amyloid pathology in early stages of the disease while being beneficial in later stages[259].

The specific mechanisms through which TREM2 lends its beneficial effects on AD pathology are unclear. Studies suggest that TREM2 may be involved in phagocytosis. One study finds that TREM2 facilitates Aβ42 phagocytosis in microglia[254], while other studies suggest its role in clearance of apoptotic neurons[248,321]. TREM2 has also been found to be important for regulating inflammation; loss of TREM2 increased microglia production of pro-inflammatory

cytokines by LPS stimulation[248,321]. Moreover, TREM2 has also been found to inhibit Aβ42-triggered pro-inflammatory responses in microglia[254]. However, much is still unknown about how TREM2 is involved in regulating inflammation and phagocytosis, and which ligands govern these functions.

Various studies suggest that lipoproteins bind to TREM2. Early studies find that apoE, apoA-I, and apoB are TREM2 ligands[261,262]. Lipoproteins such as HDL and LDL have also been found to interact with TREM2[263]. The functional significance of these interactions are unclear, though a recent study found that lipoprotein binding to TREM2 facilitates Aβ uptake by microglia[264]. Moreover, apoE seemed to facilitate phagocytosis; apoptotic neurons covered in apoE increased microglial phagocytosis in a TREM2-dependent way[262]. A new study also finds that the APOE-TREM2 pathway mediates the switch in microglia from a homeostatic phenotype to a neurodegenerative phenotype, suggesting that this pathway regulates microglial functional phenotype[265].

Though most studies investigating the role of TREM2 on AD use microglia as a model, we hope to use perivascular macrophages (PVM), which are macrophages located in the perivascular space along the blood vessels. We are using monocyte-derived macrophages to model PVM since they are similar to blood-derived macrophages in terms of their immunophenotype and morphology[109]. We are particularly interested in PVM because of their proximity to the vasculature and their ability to promote capillary stability and contribute to BBB integrity[115,118]. Importantly, PVM are found to remove Aβ aggregates in the brain through phagocytosis, and aid in the clearance of Aβ effluxed from the brain parenchyma into the circulation[119,120].

4.2 Methods

4.2.1 Peripheral Blood Mononuclear Cell (PBMC) Isolation

Peripheral Blood Mononuclear Cells (PBMC) were taken from 6 healthy human donors (range 25 – 39, mean 30.67 ± 5.31, 2 females and 4 males). Blood was diluted in a 1:1 ratio with 1xPBS and added on top of Ficoll-Hypaque Lymphoprep (StemCell Technologies) in a SepMate Tube (StemCell Technologies). Diluted blood was spun at 1200g for 10 minutes at room temperature. After centrifugation, the bottom layer of erythrocytes and polymorophnuclear cells were discarded. The plasma layer, PBMC layer, and the lymophoprep solution were washed twice with 1xPBS and spun at 800g for 8 minutes. Supernatant was aspirated and the pellet as resuspended in 10mL of 1xPBS. Cells were counted with a hemocytometer and Trypan Blue was used as a viability stain. Cells were suspended in a concentration of 5×10^7 cells/mL in 1xPBS.

4.2.2 Monocyte enrichment and macrophage differentiation

The EasySep Human Monocyte Enrichment Kit (Cat# 19059, StemCell Technologies) was used to isolate monocytes from PBMCs following manufacturer's instructions. Monocytes were plated at a density of 1×10^6 cells/mL in a 12-well dish with RPMI 1640 supplemented with 10% FBS, 1% streptomycin and penicillin, and addition of 50ng/mL M-CSF (STEMCELL TECHNOLOGIES). Cells were fed on day 3 and day 6 with M-CSF (50ng/mL) and were used in experiments on day 7.

4.2.3 Phagocytosis assay and flow cytometry

Macrophages were pre-treated with 50ug HDL (Lee biosolution, Cat # 361-10) for 2 hours and then incubated with FITC-Aβ42 (Bachem, Cat # M-2585.0500) for 3 hours at 37C in the dark.

Macrophages are harvested by mechanical dissociation as well as dissociation with enzyme-free, PBS-based cell dissociation buffer (Gibco, Cat# 13151014). Cells are suspended in ice-cold FACS buffer (1xPBS, 2% fetal bovine serum (FBS)) in polystyrene round bottom tubes. Cells are washed twice with FACS buffer and spun at 400g for 5 minutes. Cells are stained with primary-conjugated antibodies (anti-human CD14 PECy7, 1:400 dilution, clone 61D3 eBiosciences, Cat 25-0149-42; PE Rat anti-mouse CD11b, 1:200 dilution, clone M1/70, BD Biosciences, Cat #557397; anti-human/mouse TREM2 Alexa Fluor 647 conjugated, 1:100 dilution, clone 237920, R&D systems, Cat # FAB17291R) as well as a Fixable Viability Dye eFluor 506 (1:1000, eBiosciences, Cat 65-0866-14). Tubes are mixed gently and briefly vortexed before incubating for 20 minutes at 4C in the dark. After primary antibody staining, cells are washed twice with FACS buffer and mean fluorescence was analyzed with flow cytometry.

4.2.4 Immunocytochemistry (ICC)

Monocytes were cultured on the coverslips for 7 days with MCSF added on Day 3 and Day 6. On Day 7 when monocytes have differentiated into macrophages, cells incubated with 0.1uM FITC-Aβ42 for 3 hours. Cells were then fixed with 4% paraformaldehyde (PFA) in PBS for 15 minutes and washed with 0.5M Tris HCl. Cells were washed 3 times with PBS then blocked with 5% BSA in PBS for 1 hour in room temperature. Cells were incubated with primary antibody Wheat Germ Agglutinin (WGA), Alexa Fluor 488 Conjugate (1:1000, Invitrogen Cat #W11261) in 1% BSA overnight for 10 mins at room temperature. Cells were washed 3 times then mounted on slides with DAPI (Invitrogen) and sealed after 30 minutes. Coverslips were imaged with confocal microscopy (Leica).

4.2.5 Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Cells were treated with Trizol (Invitrogen) to extract RNA. Nucleic acid levels were quantified using a nanodrop spectrometer at 260nm and nucleic acid purity was measured through absorbance at 230nm and 280nm. RNA was subsequently treated with DNAseI (Invitrogen). TaqMan Reverse Transcriptase Reagents Kit (Life Technologies) was used to reverse transcribe 1ug of RNA into complementary deoxyribonucleic acid (cDNA). Real-time quantitative PCR was performed with SYBR Green reagents (Applied Biosystems) with primers against IL-1β (Forward: 5'-AGAAGTACCTGAGCTCGCCA-3', Reverse: 5'-CTGGAAGGAGCACTTCATCTGT-3'), TNFα (Forward: 5'-TGCACTTTGGAGTGATCGGC-3', Reverse: 5'-GCTTGAGGGTTTGCTACAACA-3'), and GAPDH (Forward: 5'-CCTGCACCACCAACTGCTTA-3', Reverse: 5'-CATGAGTCCTTCCACGATACCA-3'). Cycling conditions for IL-1β and TNFα are: 95°C for 10 minutes, then 40 cycles at 95°C for 10 seconds, 57°C for 10 seconds and 72°C for 10 seconds, followed by melting at 95°C for 10 seconds, 65°C for 60 seconds, and 97°C for 1 second. Each sample was assayed in duplicate and normalized to GAPDH.

4.3 Results

4.3.1 Monocyte-derived macrophages are characteristic of macrophages

We first wanted to characterize the macrophages we differentiated from monocytes isolated from peripheral blood mononuclear cells (PBMCs) from healthy human donors. Monocytes were isolated from PBMCs with a monocyte enrichment kit and subsequently cultured for 7 days in macrophage colony stimulating factor (M-CSF) to allow differentiation into macrophages[322]. We used M-CSF as a differentiation factor to avoid differentiating

monocytes into activated macrophages. We observed that the monocytes increased in size and increased adherence to the culture plates over the 7 days (**Fig. 4.1**), which is morphologically characteristic of macrophages. We also used flow cytometry to probe macrophage markers, and observed the presence of CD11b and CD14 in these monocyte-derived macrophages (**Fig. 4.2**). Moreover, we confirmed the presence of the TREM2 receptor in these macrophages (**Fig. 4.2**).

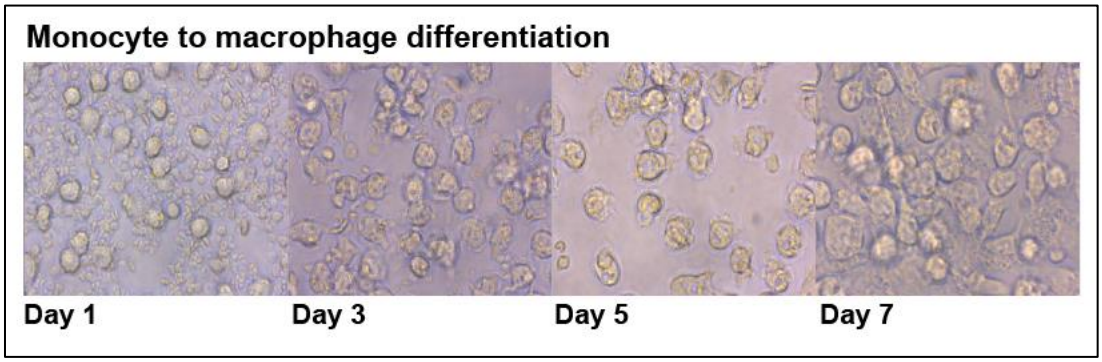

Figure 4.1 Monocyte-derived macrophages were morphologically characteristic of macrophages

Monocytes isolated from PBMCs were cultured with M-CSF for 7 days. Cells increased in size and increased adherence to the culture plates within the 7 days. Cells were imaged with light microscope at Day 1, Day 3, Day 5 and Day 7.

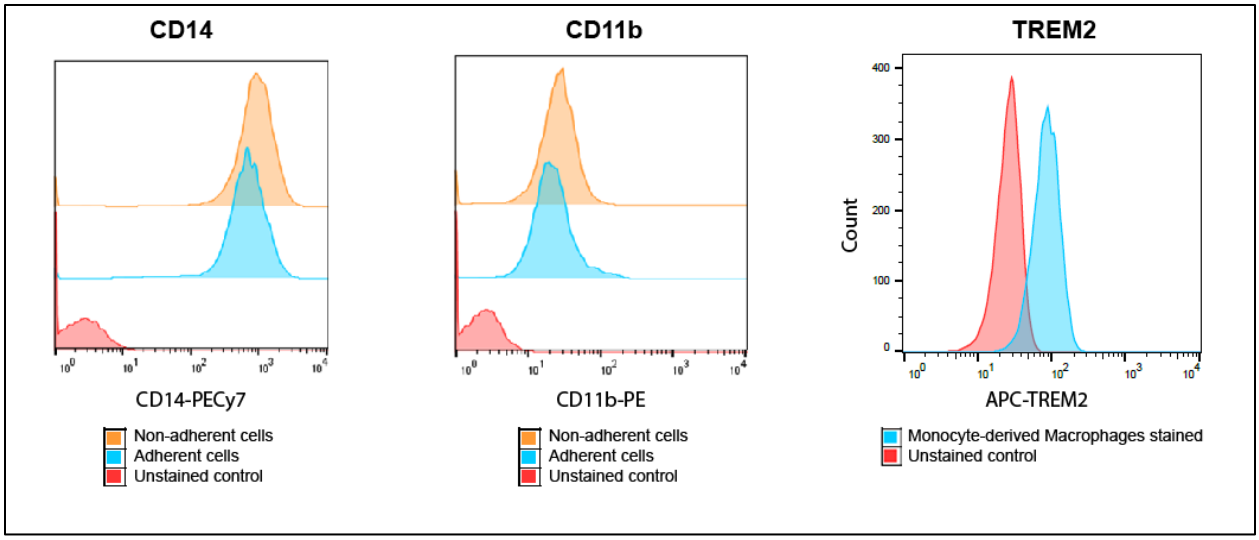

Figure 4.2 Monocyte-derived macrophages stain positive for macrophage markers CD14, CD11b and TREM2

Monocyte-derived macrophages were stained for CD14, CD11b, and TREM2 through flow cytometry. Data are represented as histograms of cell surface molecule expression, while expression levels are shown on log scale. Data are collected from N = 1 independent experiment.

4.3.2 Monocyte-derived macrophages take up FITC-Aβ42

Next, we wanted to test whether these monocyte-derived macrophages take up FITC-Aβ42. We incubated the macrophages with different doses of FITC-Aβ42 and observed a dose-dependent increase in mean fluorescence with increasing dose of FITC-Aβ42, up until 1uM FITC-Aβ42 (**Fig. 4.3**). Interestingly, we observed that macrophages take up 5uM FITC-Aβ42 less than 1uM FITC-Aβ42, possibly due to the increased fibrils that may be forming at a higher concentration of FITC-Aβ42 (**Fig. 4.3**). We further confirmed that the FITC-Aβ42 is being taken up by the cell by confocal microscopy. Macrophages were cultured on coverslips and stained

with Wheat Germ Agglutinin (WGA), which marks the cell membrane, allowing us to visualize the internalization of the FITC-Aβ42. We found that the FITC-Aβ42 taken up by the macrophages were localized inside the cell membrane, suggesting that the FITC-Aβ42 were not simply adhering to the outside of the cells, but were actually internalized (**Fig. 4.4**).

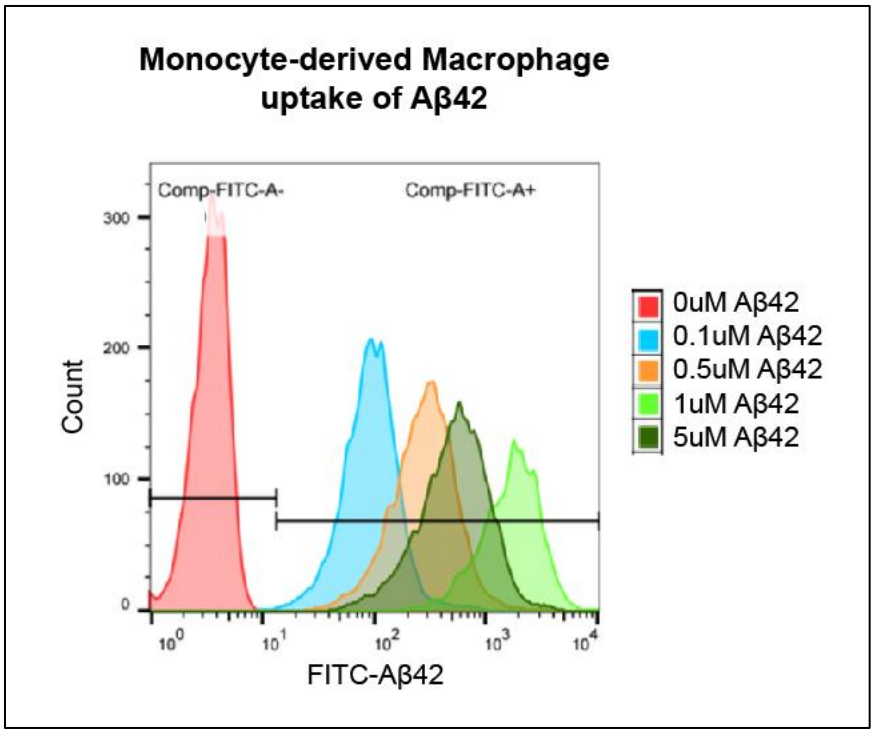

Figure 4.3 Monocyte-derived macrophages uptake FITC-Aβ42

Monocyte-derived macrophages were incubated for 3 hours with 0uM, 0.1uM, 0.5uM, 1uM and 5uM FITC-Aβ42. Fluorescence of cells was quantified with flow cytometry. Data are represented as histograms of fluorescence, while fluorescence levels are shown on log scale.

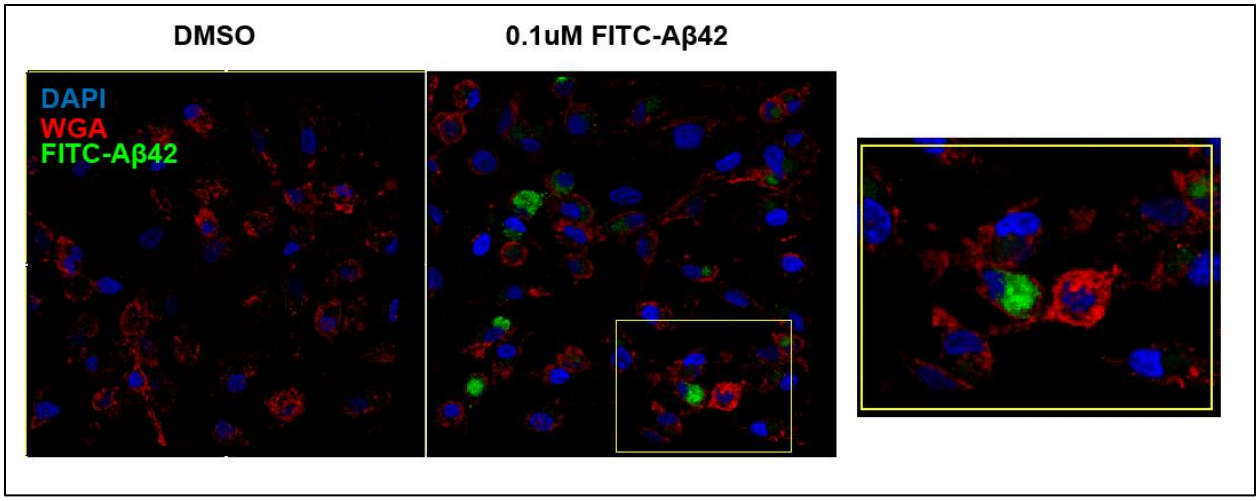

Figure 4.4 FITC-Aβ42 taken up by monocyte-derived macrophages are localized within cells

Monocyte-derived macrophages were incubated with 0.1uM FITC-Aβ42 for 3 hours. Coverslips were subsequently stained with Wheat Germ Agglutinin (red) and counterstained with DAPI (blue). Data are collected from N = 1 independent experiment.

4.3.3 HDL has no effect on monocyte-derived macrophage uptake of FITC-Aβ42

A previous study showed that TREM2 facilitates Aβ phagocytosis in microglia[254]. We hypothesized that addition of TREM2 ligands would increase its phagocytic capacity, and chose to use HDL because it is a lipidated particle found in tissue. Since 0.1uM FITC-Aβ42 was sufficient to shift the mean fluorescence of the monocyte-derived macrophages (**Fig. 4.3**), we used this concentration for subsequent experiments. We found that compared to macrophages in the DMSO condition, macrophages incubated with FITC-Aβ had a higher mean fluorescence ($p < 0.05$) as measured by flow cytometry, suggesting uptake of FITC-Aβ42 by the cells (**Fig. 4.5**). However, HDL pre-treatment did not affect macrophage uptake of FITC-Aβ42 (**Fig. 4.5**).

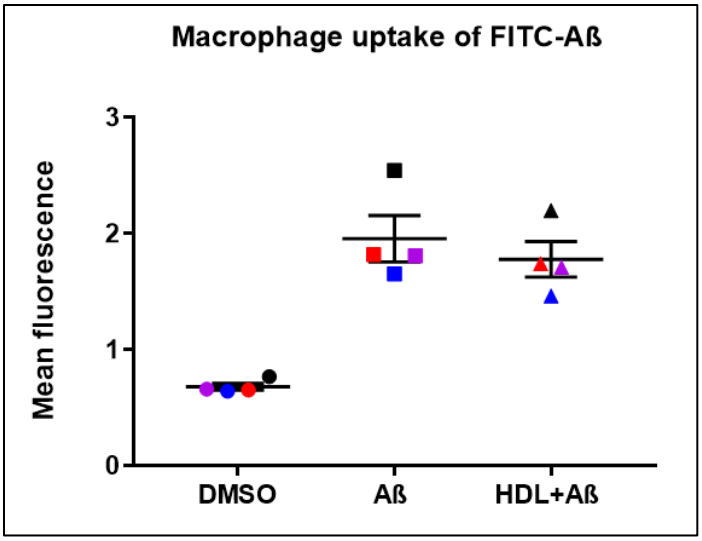

Figure 4.5 HDL pre-treatment of monocyte-derived macrophages has no effect on uptake of FITC-Aβ42

Monocyte-derived macrophages were pre-treated with HDL for 2 hours before incubation with 0.1uM FITC-Aβ42 monomers for 3 hours. Log transformed data of mean fluorescence as measured by flow cytometry. $p < 0.05$ as analyzed by Kruskal-Wallis test with Dunn's multiple comparisons test. Data are represented as mean ± SEM, collected from N = 3 independent healthy human donors in N = 4 independent trials. Sex of the donors are: black (M), red (M), blue (F) and purple (F).

4.3.4 Aβ-induced inflammation varies between donors

Since the Jiang et al. study also showed that TREM2 mediated Aβ-induced inflammation[254], we also wanted to test whether HDL pre-treatment would decrease this inflammation in macrophages. We find that different donors respond to Aβ stimulation as well as HDL pre-treatment differently, in terms of their IL-1β and TNFα expression (**Fig. 4.6**).

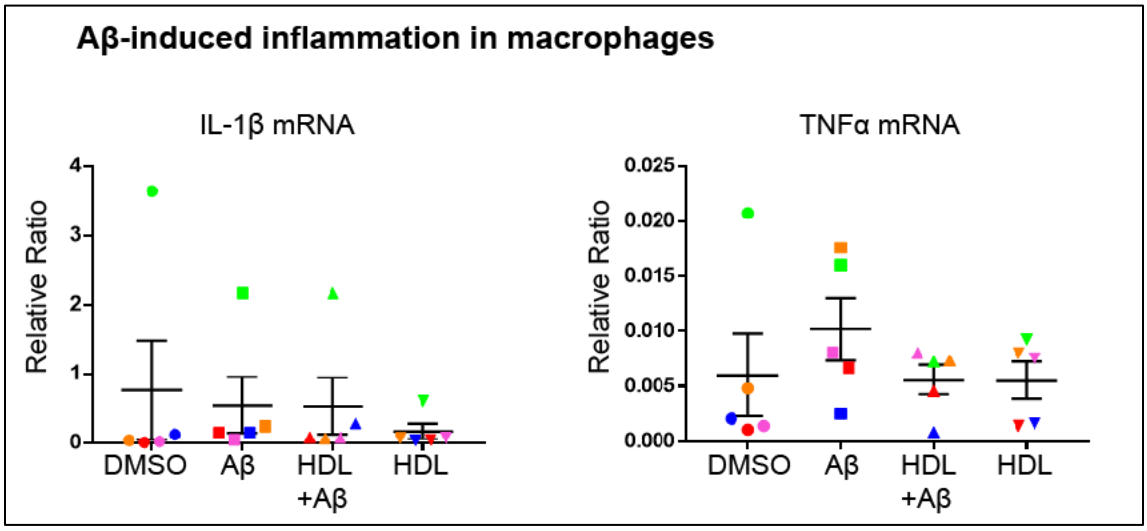

Figure 4.6 Both HDL treatment and Aβ42 stimulation of monocyte-derived macrophages showed inconsistent effects amongst donors

Monocyte-derived macrophages were pre-treated with HDL for 2 hours before 1uM monomeric Aβ42 stimulation. Macrophages from different healthy human donors do not consistently respond to Aβ42 stimulation or HDL pre-treatment in their IL-1β and TNFα cytokine expression as measured by qRT-PCR. Data are represented as mean ± SEM, collected from N = 5 independent healthy human donors. Sex of each donor are: red (M), orange (F), pink (F), green (M), and blue (M).

4.4 Discussion

In this study, we wanted to test the effects of lipoproteins on TREM2-mediated functions. A previous study showed that TREM2 is involved in Aβ-mediated phagocytosis and Aβ-induced inflammation in microglia[254]. Since studies find that HDL and apoA-I bind to TREM2[262–264], we hypothesized that they may be possible ligands for TREM2 and mediate TREM2 functions.

Here, we find that monocyte-derived macrophages are able to take up FITC-Aβ42 (**Fig. 4.3**, **Fig. 4.4**), suggesting that they may be able to clear Aβ in the system. However, we found that HDL pre-treatment of these macrophages does not affect its uptake of FITC-Aβ42 (**Fig. 4.5**). Several factors could be contributing to this lack of effect. It is possible that despite evidence showing that HDL binds to TREM2, this binding does not have biological significance. Thus, in our experiments, we see no effect of HDL pre-treatment because there may not be any downstream signalling occurring. Another reason that HDL pre-treatment does not affect TREM2-mediated uptake of Aβ42 could be that the TREM2 receptors may be fully occupied by its ligands already (such as apoE), and that addition of HDL would not affect TREM2-mediated functions. Future experiments to test this hypothesis would be increasing the dose of HDL (in a dose-response experiment) to see a possible effect, assuming the high concentrations of HDL can displace the occupying ligand on the TREM2 receptor. Moreover, these experiments only addressed whether Aβ42 is taken up in the macrophages, and not whether degradation of Aβ42 occurs, which is important in the context of AD. Further experiments can be conducted to test whether degradation occurs, which can be performed by testing the gradual loss of Aβ42 protein, as well as probing the activity of degradation enzymes.

We also find that individual human donors vary in their response to HDL pre-treatment as well as their Aβ-induced inflammation (**Fig. 4.6**). Variability exists between individuals in terms of their monocytes, which may be affecting their characteristics when we differentiate the monocytes into macrophages. For example, individual differences are found in human monocyte activation, whereby monocytes from some individuals are more inflammatory than others[323,324]. Interestingly, the inflammatory profile of human monocytes was found to be related to altered lipid levels, such that monocytes had an increased inflammatory state for individuals with an

increased cardiovascular risk[324]. More specifically, levels of HDL-C are negatively correlated with the production of IL-1β,[324]. Similarly, in our experiments, there were individual differences in how the monocyte-derived macrophages responded to Aβ42 stimulation. Future experiments to test this idea could be to probe whether HDL-C levels in individuals were related to whether their macrophages had a higher inflammatory response to Aβ stimulation.

In our experiments, we used HDL because of its biological relevance; HDL is lipidated and can be found in tissues. Future experiments can be performed to probe whether other TREM2 ligands such as apoE can affect Aβ phagocytosis and Aβ-induced inflammation in these monocyte-derived macrophages. Indeed, studies have shown that apoE may also be involved in mediating inflammation in macrophages; macrophages deficient in apoE have increased inflammatory response compared to normal macrophages in mice[325,326]. More recently, a study showed that in a mouse macrophage cell line, apoE inhibits LPS-stimulated activation of macrophage, by decreasing release of cytokines TNFα, IL-1β and IL-6[327]. Addition of apoE may thus be able to ameliorate Aβ-induced inflammation in macrophages.

Lastly, it is possible that the results we see are due to the method by which we decided to differentiate our monocytes into macrophages. Both granulocyte-macrophage colony-stimulating factor (GM-CSF) and macrophage colony-stimulating factor (M-CSF) are strong hematopoetic growth factors for differentiating monocytes to an uncommitted macrophage phenotype. There is debate in the literature of whether using GM-CSF or M-CSF would influence their ability to polarize human monocytes into different macrophage subtypes (M1 versus M2 phenotype). For example, many studies on macrophage polarization has been investigated in mice, wherein mouse GM-CSF pre-orients the macrophage into an M1 phenotype while M-CSF pre-orients the macrophage into an M2 phenotype[328,329]. This is especially an important consideration because

the polarization of the macrophages affects its phagocytic capacity and inflammatory profile. Classically activated (M1) macrophages are characterized by their higher levels of pro-inflammatory cytokines while alternatively activated (M2) macrophages have more efficient phagocytic activity[117]. In our studies, we justified our use of M-CSF to differentiate our monocytes to macrophages in order to have a "non-activated" macrophage phenotype, especially because we stimulated the macrophages with Aβ afterwards. If we used GM-CSF to differentiate our monocytes into macrophages, we may have an already activated macrophage and thus subsequent inflammatory stimuli may not further activate the macrophages. However, a recent study investigating human monocyte to macrophage differentiation find that both GM-CSF and M-CSF yielded similar functional phenotypes after M1 and M2 polarization, suggesting the similarity in these two growth factors in differentiating monocytes into uncommitted macrophages[330]. In follow-up experiments, we can validate whether our macrophages were more polarized (if at all) towards a more M1 or M2 phenotype by looking at specific markers and also testing their cytokine and chemokine release. It may also be possible that there are individual differences in how monocytes respond to these growth factors, which may affect their ability to differentiate into macrophages as well as their function as mature macrophages. In our experiments, we could have added an additional step of checking macrophage markers for every experiment in which we differentiate macrophages to ensure that the similarity of the macrophages between different donors.

Chapter 5: Conclusion

In this thesis, we aimed to test the beneficial effects of lipoproteins (HDL, apoE) on perivascular cells (pericytes, perivascular macrophages) in the brain. In Chapter 2, we tested whether pharmacologically increasing apoE in pericytes can decrease their migration, thereby protecting the BBB. We found that increasing apoE levels with GW3965 throughout the scratch-wound assay was not associated with changes in pericyte migration. Interestingly, we also found that Axl inhibitor A1 slowed pericyte migration in an apoE-independent way. In Chapter 3, we tested whether HDL can attenuate the CypA-NFkB-MMP9 inflammatory pathway associated with apoE4 pericytes. Unfortunately and unexpectedly, we found no evidence that this pathway exists in primary human pericytes and thus could not test our hypothesis. Lastly, in Chapter 4, we tested whether HDL can affect TREM2-mediated functions such as Aβ phagocytosis and Aβ-induced inflammation in macrophages. We found that HDL does not affect Aβ phagocytosis in macrophages. Moreover, we found that there were individual differences in how macrophages from different donors respond to Aβ. Taken together, we failed to show a beneficial effect of lipoproteins on perivascular cell function in the context of AD.

One of the hypotheses in this thesis is that increasing apoE levels would be beneficial in AD. Whether increasing apoE levels or decreasing apoE levels would be beneficial as a therapeutic approach for AD has been robustly debated for the last decade. An early study found that deletion of mouse *APOE* gene dramatically reduced the deposition of fibrillar Aβ in amyloid model mice[178]. Moreover, high concentrations of apoE were found to form co-aggregates with Aβ[331]. Isoform-specific effects were found, in which apoE4 promoted Aβ aggregation more than apoE3[164]. ApoE was also found to increase the level of Aβ oligomers, with apoE4 > apoE3> apoE2[332]. However, it is important to note that there are studies suggesting that apoE inhibits

rather than facilitates Aβ aggregation[167,333], which is believed to be because apoE associates with the Aβ nucleus and prevents the Aβ seeding. AD mice expressing only one copy of the *APOE* gene had significantly less Aβ accumulation, plaque deposition, and microgliosis compared to mice with two copies of the same apoE isoforms (both *APOE3* and *APOE4*)[334]. Moreover, apoE-knockout mice clear Aβ from the brain more efficiently than control mice[182]. ApoE thus plays an undeniable role in Aβ accumulation and deposition, though recent studies demonstrate that this may depend on the stage of the disease; it was shown that reducing apoE expression in an AD mouse model prior to plaque deposition decreased Aβ accumulation[335]. Moreover, expression of apoE4 during the seeding stage of amyloid development resulted in decreased Aβ clearance and increased Aβ aggregation[336].

Conversely, many studies demonstrate the beneficial effects of increasing apoE. For example, Aβ degradation may be facilitated by apoE, as observed in astrocytes[176] and microglia[174]. Moreover, stimulating the LXR pathway[337,338] and the retinoid X receptor (RXR) pathway[339] helps Aβ clearance and restores memory in AD mice. This effect may be due to both increasing apoE levels as well as the lipidation of apoE. Indeed, reduction of amyloid burden by LXR agonist GW3965 depends on the expression of ABCA1 in AD mice[340]. Increasing lipidated apoE may be a viable therapeutic option for AD. In Chapter 2, we find that an increase in apoE levels did not necessarily affect pericyte migration. It is possible that increasing apoE only in specific cell types yield beneficial results. Moreover, we did not confirm the upregulation of ABCA1 and lipidation of apoE particles by the apoE-modulating drugs in our study – which should be tested in future experiments.

How the different apoE isoforms come into play is unclear; for example, would simply increasing lipidation of apoE particle be beneficial – regardless of the isoform? Would increasing

the lipidation of apoE4 rescue its detrimental effects? Are the lipidated apoE particles beneficial or are the unlipidated apoE particles detrimental? These are questions that would have been interesting to answer with our experimental paradigm in Chapter 2 if we had more donors of each genotype. Importantly, addressing these questions can potentially aid in the development of therapeutics for AD patients of various apoE genotypes. A new study by the Holtzman group found that removing unlipidated, aggregated apoE with antibodies in AD mice expressing human apoE4 reduced Aβ accumulation[341]. These anti-apoE antibodies may offer important therapeutics to AD patients expressing apoE4.

HDL has many protective effects in the vasculature. Aside from its classical function of collecting lipids from various cells in the body and transporting them to the liver for excretion (reverse cholesterol transport), HDL also demonstrates anti-inflammatory effects for endothelial cells[210–212]. Recently, it was shown that the anti-inflammatory effects of HDL in the peripheral blood vessels also apply to the endothelial cells in the brain. In this study, HDL pre-treatment of brain endothelial cells suppress Aβ-induced PBMC adhesion[279]. HDL can also repair damaged endothelial cells, reduce lipid oxidation and inhibit thrombic activation[214].

Some clinical studies demonstrate the link between HDL levels and risk of dementia[226,227], suggesting that dysfunctional HDL may be contributing to AD, though many studies report no association[228,229,234–236]. The protective role of HDL in AD has been highlighted in animal studies. For example, the genetic overexpression of human apoA-I and thereby increase of functional HDL prevented the development of cognitive deficits in a mouse model of AD[342] while apoA-I deficiency exacerbated cognitive deficits in AD mice[343]. However, it is important to note that physiological difference between mice and humans must be taken account when evaluating the translational value of these studies – especially with regards to lipid

metabolism. Importantly, mice lack cholesteryl ester transfer protein (CETP)[344]. This protein, which is expressed in humans, shuttles cholesteryl ester from HDL to apoB-containing lipoproteins such as LDL and VLDL[345]. For this reason, the majority of cholesterol is carried by HDL in mice[346]. This difference in lipid metabolism between humans and mice may explain why mice are resistant to atherosclerosis[347]. Because of the pitfalls of using animal models to study vascular diseases, and by extension AD, a disease in which there are important cardiovascular contributions, the use of human cells to study AD offer results that hold more translational value. Recently, it was shown that HDL and apoE facilitates Aβ clearance across a bioengineered vessel composed entirely of human cells[187], suggesting a beneficial role of HDL in AD pathology, lending further support to HDL-directed therapies in clinical trials for AD.

Studies on the effects of HDL on macrophages are few, and conflicting. One study found that HDL exerts anti-inflammatory effects by down-regulating expression of TLR-induced cytokines[348], while another study found that HDL exerted pro-inflammatory effects by enhancing TLR-induced signaling and activating the PKC-NF-kB/STAT1-IRF1 inflammatory pathway and increased cytokine expression[349]. In macrophage-derived foam cells, HDL exerted anti-inflammatory effects[350]. The difference in HDL preparations (commercial HDL, reconstituted HDL, and native HDL from healthy donors) may be the reason for the different effects. In terms of the effect of HDL on macrophage phagocytosis, one study found that HDL acts as an opsonin to increase bacteria phagocytosis by a monocyte cell line[351] while another study found that apolipoproteins of HDL (apoHDL) but not HDL increased macrophage phagocytosis of zymosan particles[352]. In these studies, the notion follows that HDL or apoHDL binds to the bacteria or zymosan and this facilitates uptake by the monocytes and macrophages. In Chapter 4, we found that HDL did not change macrophage uptake of Aβ42. Many studies report the interactions

between HDL and Aβ42. Soluble Aβ42 is shown to interact with HDL[353], as well as HDL components apoA-I, apoA-II, apoE and apoJ[354]. The interaction between purified apoA-I and Aβ peptide has been shown to inhibit fibril formation[355]. Recently, it was shown that HDL delays Aβ fibrillization[279], which may suggest a role for HDL in mediating the balance between different forms of Aβ. Thus, in our study, it is possible that Aβ is binding to HDL, but this binding is not facilitating Aβ uptake in the macrophages, suggesting that these macrophages do not prefer taking up HDL-bound Aβ compared to Aβ itself. Further experiments should be conducted to confirm the interaction of these two species in our experiments, similar to previous studies in which immunoprecipitation and biotinylated Aβ were used to detect an interaction.

Altogether, we have designed these experiments to explore the role of lipoproteins in AD – to test how apoE contributes to the mechanisms that underlie AD pathogenesis and test the idea that apoE and HDL are beneficial. With our use of human cells, we aimed to bridge the gap between work in animal models and human clinical studies. Ultimately, we wanted to narrow down the specific role of the vasculature in AD, and put forth the notion that apoE and HDL can be potential targets for therapeutics in AD.

Despite mounting pre-clinical evidence that enhancing lipoprotein function is a means for AD therapeutics, the clinical work in this avenue has been sparse and met with difficulties. Much work has been done on studying the therapeutic effects of LXR and RXR agonists. For example, LXR agonist T0901317 and GW3965 improves cognitive functions in mice[177,340]. RXR agonist bexarotene showed amyloid clearance as well as reversal of cognitive deficits in AD mice[339], though several labs have difficulties replicating the results[356–359]. However, despite its tolerance in mice, these compounds present challenges for human use because of the negative side effects. LXR agonists induce the transcription of fatty acid synthase (FAS) and sterol regulatory

element-binding protein 1-c (SREBP-1) gene, leading to hypertriglyceremia and hepatic steatosis[360,361] while RXR agonist bexarotene causes increased CETP activity and subsequently dyslipidemia[362], and hypothyroidism[363]. Development of non-LXR and RXR agonists that modulate apoE levels may offer therapeutic value without the detrimental side effects. Recently, several compounds have been identified to increase apoE secretion and ABCA1 levels in CNS cells, and do not directly activate the LXR pathway[364], which have been used in our studies in Chapter 2. These compounds may offer benefits of increased apoE functionality without the negative side effects of LXR agonists. Further work in developing apoE-modulating compounds should be done in mice models, in which tolerance can be tested.

Other HDL-directed therapies include niacin, which was found to be effective in raising HDL-C levels[365], but has no effect on reducing cardiovascular events[366]. Two CETP inhibitors, torcetrapib and dalcetrapib, were also shown to elevate HDL-C levels but also failed to reduce cardiovascular events[367,368]. Recently, evacetrapib was evaluated to have no effect on the rates of cardiovascular events[369]. However, another CETP inhibitor anacetrapib showed more promise, as its use resulted in a lower incidence of coronary events[370]. Currently, the effects of CETP inhibitors on the brain are unknown, and further work needs to be done to evaluate its effect on modulating AD pathology. Some work has also been done on developing reconstituted or recombinant HDL that can be infused into the circulation to directly elevate HDL levels. An early study on infusing apoA-I Milano, a genetic variant of apoA-I that is associated with reduced cardiovascular disease, showed regression of atherosclerotic plaques[371]. Purified human apoA-I that is reconstituted with soy phosphotidylcholine has also been tested in patients with acute coronary syndrome. This form of reconstituted HDL was found to improve plaque characterization but did not change atheroma volume, which is the proportion of total vessel wall

occupied by atherosclerotic plaque[372]. Studies in AD mice showed that administration of oral apoA-I mimetic peptide improves cognitive function and reduces amyloid burden[373]. It was also recently demonstrated that reconstituted HDL decreases soluble brain Aβ in APP/PS1 mice[374].

Moving forward, the role of lipoproteins in preserving cerebrovascular integrity should be further explored in the context of AD. Importantly, further work should be done on the beneficial effects of lipoproteins HDL and apoE on pericytes and perivascular macrophages, since these perivascular cells are important to the integrity and function of the cerebrovasculature. For example, it has been shown that Aβ42 induced human pericyte death in culture[375]. A follow-up study by the same group found that Aβ-induced degeneration of human brain pericytes depends on the apoE genotype, in which cell death was highest in pericytes with apoE4/E4 genotype, and lowest in pericytes with apoE2/apoE3 genotype[376]. The addition of apoE was found to protect against the toxicity of Aβ in a dose-dependent way[377]. Future experiments can test whether administration of apoE-modulating drugs can rescue the toxic effects of Aβ on pericytes in culture. Moreover, since Aβ has been known as an inflammatory agent in various CNS cells[279,378], it would be worthwhile to test whether Aβ can induce an inflammatory response in pericytes, and whether HDL can attenuate this inflammation. Further testing can be done on effects of lipoproteins on other TREM2-mediated functions in macrophages. For example, apoA-I and apoE are found to bind to TREM2 in several *in vitro* studies[248,262,264]. Further testing as to whether these lipoproteins play a role in mediating Aβ phagocytosis and Aβ-induced inflammation in macrophages can be done. Such work can improve upon our understanding of how lipoproteins can influence cerebrovascular health and ultimately pave way for new therapeutics for AD.

5.1 Limitations

One of the limitations of this study is the small sample size. For the pericyte work in Chapters 2 and 3, we had 3 donors of separate genotypes. In Chapter 4, healthy human volunteers are required for blood donation to obtain the monocytes, so donor availability was a limiting factor. This lack of donor availability made it difficult to do technical replicates, since a larger volume of blood donation would be needed. Moreover, the same donors could not donate blood too frequently. Another limitation that comes along with using human donors is both variability between each pericyte donor, as well as between each monocyte donor. This also introduces variability in the results obtained. There are many ways in which the variability of results can be reduced. For the pericyte experiments, for example, pericytes and monocytes were seeded at the same density and grown for the same number of days. Overall, more steps could have been taken to improve the rigor of the experiments and their analysis. Specifically, more work could have been done to decrease variability in the macrophage experiments. For example, checking the purity of the monocyte isolation could have been performed, such that the population of monocytes isolated from PBMCs. Moreover, one well of the plate could have been dedicated to checking the quality of the differentiation from monocytes to macrophages, by probing the appropriate markers for macrophages.

For the limited sample size of pericyte and monocyte donors, it is difficult to draw firm conclusions about the results, and generalize these conclusions to what is actually seen in the population. Moreover, for the macrophage experiments, it is also difficult to draw firm conclusions about results from individual donors, since there is also variability within an individual, depending on the different conditions when comparing experiments. In comparison, the pericyte work yields more consistency in results within a donor, and the data is more robust –

especially the pericyte migration experiments, in which an N = 7 replicates were performed, as determined by power calculations. Moreover, the use of the IncuCyte live imaging system reduced variability in results, as the scratch-wounds were performed by a mechanical pin device (as opposed to with a pipette tip) and the analysis of the migration is performed by their software – overall reducing human error.

The macrophage experiments, in contrast, had more variability which was more difficult to control, because of the variable nature of monocytes within an individual at different times and conditions, as well as the variability of monocytes between individuals. Moreover, the monocytes had to undergo differentiation over 7 days into macrophages, which also introduced variability in results. Thus, the experiments with monocyte-derived macrophages yielded the least robust results. For example, when the macrophages were stimulated with Aβ, we found that they responded differently depending on the donor. Moreover, HDL pre-treatment response also varied between donors.

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