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Ameliorating Cholesterol Homeostasis in Activated Microglia as an Approach for Developing Novel Therapeutics for Alzheimer's Disease

By

OSCAR MANUEL MUÑOZ HERRERA

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

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Approved:

Angela M. Zivkovic, Chair

Heike Wulff

Izumi Maezawa

Committee in Charge

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ABSTRACT

Establishing mitochondrial cholesterol enrichment as a fundamental causal mechanism underlying the dysfunctional clearance of neurotoxic substances by microglia would help pave the way toward the discovery of potential new therapeutics for Alzheimer's disease (AD). The dissertation is divided into four chapters with the corresponding objectives:

1. The first chapter is a literature review describing the mechanisms by which cholesterol has been implicated in influencing microglia phenotype and function, and the implications for a role in contributing to the pathophysiology of AD. We describe both what is known and the research gaps that exist and should be explored for the discovery of novel therapies for AD
2. The second chapter is an investigation on the effects of a 36h fast on HDL particle size distribution by transmission electron microscopy (TEM), and ability to efflux cholesterol from activated microglia. Importantly, HDL from 36h fasted individuals effluxed cholesteryl esters from immortalized human microglia (HMC3) loaded with cholesterol + amyloid beta oligomers (AβO) 10-fold more effectively than HDL from postprandial individuals, and microglia treated with cholesterol+AβO had reduced apolipoprotein E (ApoE) secretion, which was attenuated more effectively by HDL from 36h fasted individuals than HDL from postprandial individuals.
3. In the third chapter we use a multi-stimulant approach to test the usefulness of the HMC3 microglia cell line in duplicating critical aspects of the dysfunctional microglia phenotype and to better mimic the multi-factorial nature of AD, which is associated with multiple comorbidities, including diabetes and cardiovascular disease. HMC3 microglia were treated with cholesterol (Chol), AβO, lipopolysaccharide (LPS), and fructose individually

and in combination. HMC3 microglia demonstrated changes in morphology consistent with activation when treated with the combination of Chol+AβO+fructose+LPS. Multiple treatments increased the cellular content of Chol and cholesteryl esters (CE), but only the combination treatment of Chol+AβO+ fructose+LPS increased mitochondrial Chol content. Microglia treated with combinations containing cholesterol+AβO had lower apolipoprotein E (ApoE) secretion, with the combination of Chol+AβO+fructose+LPS having the strongest effect. Combination treatment with Chol+AβO+fructose+LPS also induced APOE and TNF-α expression, reduced ATP production, increased reactive oxygen species (ROS) concentration, and reduced phagocytosis events.

INTRODUCTION

Since the conception of Alzheimer's disease (AD) it has been known that an aberrant accumulation of lipid is characteristic of the brain in AD. Further understanding has elucidated that cholesterol enrichment in the plasma membranes of neurons is causally linked to excessive production of the cytotoxic amyloid beta (Aβ) peptide through a lipid raft-dependent mechanism.

Cholesterol is an essential lipid component for various cellular structures and organelles, and its trafficking occurs via various distinct pathways. Disruption in cholesterol transport and trafficking can lead to aberrant cellular function. Multi-omic analysis of postmortem brain samples from AD patients revealed that the concentrations of free cholesterol were elevated in microglial endocytic vesicles suggesting that microglial cholesterol handling may be directly involved in AD pathology. Importantly, it has been shown that the ability of microglia to degrade Aβ is dependent on their ability to efflux cholesterol. Homeostatic microglia are protective, participating in the clearance of amyloid beta (Aβ) and other cellular debris; on the other hand, activated or disease-associated microglia (DAM) drive neuroinflammation and neurodegenerative processes in the AD brain. However, it is not clear how or even whether aberrant cholesterol metabolism in microglia contributes to the pathology of AD, and what kinds of treatment approaches might be useful in ameliorating cholesterol-mediated microglial dysfunction. In this dissertation, the evidence for the role of aberrant cholesterol handling in microglia in the pathophysiology of AD is reviewed in Chapter 1.

High-density lipoproteins (HDL) play a critical role in reverse cholesterol transport (RCT) and have recently been shown to perform an array of other essential functions, including regulation of immune function and inflammation. HDL amount has been linked to better cardiovascular, renal, and cognitive health. However, simply raising HDL concentrations is not a

safe strategy for improving such health outcomes. Instead, research has turned to searching for other HDL-related therapeutic targets. One focus is HDL functionality and structure which have both been found to be reliable predictors of cardiovascular outcomes. Evidence is emerging that HDL particles from different physiological states have differential functional effects. In Chapter 2 of this dissertation, we show that HDL particles from individuals who were fasted for 36 hours are functionally superior that HDL particles from individuals in the postprandial state. Specifically, we show that HDL from fasted individuals are significantly better at effluxing cholesteryl esters from activated microglia, providing preliminary evidence that certain types of HDL might be therapeutically beneficial for patients at risk for or suffering from AD.

It is more and more clear that AD is a multifactorial disease. For example, AD is associated with multiple comorbidities, including diabetes and cardiovascular disease, with as many as 80% of AD patients developing diabetes. Although hyperglycemia is by definition the primary metabolic dysregulation in diabetes, high fructose concentrations are a key driver of aberrant lipid accumulation in the liver, and high fructose intake has been found to be a causal factor in the development of insulin resistance and metabolic dyslipidemia. Other factors have also been found to be potentially causal in the progression of AD pathophysiology, including bacterially derived factors such as LPS. In order to better recapitulate this multifactorial setting in AD, in Chapter 3 of this dissertation we developed an in vitro approach using a human microglia cell line stimulated with not just amyloid beta peptides, but also cholesterol, fructose and LPS, to generate an activated microglia phenotype. The goal is to further develop and validate this as a high-throughput screening tool that could be useful to test molecules with a potential to ameliorate the dysfunctional microglia phenotype.

Chapter 1: Microglia and Cholesterol Handling: Implications for Alzheimer's Disease

Oscar M. Muñoz Herrera[1] and Angela M. Zivkovic[1]

[1]Department of Nutrition, University of California – Davis, Davis, CA, 95616

ABSTRACT

Cholesterol is essential for brain function and structure, however altered cholesterol metabolism and transport are hallmarks of multiple neurodegenerative conditions, including Alzheimer's disease (AD). The well-established link between apolipoprotein E (APOE) genotype and increased AD risk highlights the importance of cholesterol and lipid transport in AD etiology. Whereas more is known about the regulation and dysregulation of cholesterol metabolism and transport in neurons and astrocytes, less is known about how microglia, the immune cells of the brain, handle cholesterol, and the subsequent implications for the ability of microglia to perform their essential functions. Evidence is emerging that a high-cholesterol environment, particularly in the context of defects in the ability to transport cholesterol (e.g., expression of the high-risk APOE4 isoform), can lead to chronic activation, increased inflammatory signaling, and reduced phagocytic capacity, which have been associated with AD pathology. In this narrative review we describe how cholesterol regulates microglia phenotype and function, and discuss what is known about the effects of statins on microglia, as well as highlighting areas of future research to advance knowledge that can lead to the development of novel therapies for the prevention and treatment of AD.

Keywords: microglia; cholesterol; Alzheimer's disease

INTRODUCTION

Since Alzheimer's disease (AD) was first described by Alois Alzheimer over a century ago [1], it has been known that an aberrant accumulation of lipid "saccules" as they were called then, essentially an enrichment in intracellular lipids, is characteristic of the brain in AD. Since then, we have gained a much more sophisticated understanding of how lipid accumulation and aberrant lipid metabolism contribute to the etiology of AD. It is now clear, for example, that cholesterol enrichment in the plasma membranes of neurons is causally linked to production of the cytotoxic amyloid beta (Aβ) peptide [2] through a lipid raft-dependent mechanism. Cholesterol is an essential lipid component for various cellular structures and organelles, and its trafficking occurs via various distinct pathways which include endocytosis/phagocytosis, transport to the plasma membrane and repurposing of cholesterol by removal from the plasma membrane [3–17]. Disruption in cholesterol transport and trafficking can lead to aberrant cellular function. For example, in Niemann-Pick Type C disease, which involves an autosomal recessive mutation causing neurodegeneration, aberrant cholesterol metabolism is linked to Aβ deposition similar to the pathology occurring in AD [18]. In mouse models hypercholesterolemia results in glial cell hyperactivation, accelerating amyloid pathology in the brain [19]. In zebrafish exposure to high cholesterol (4% weight/weight cholesterol) for 19 days resulted in higher brain mRNA expression of proinflammatory markers and elevated brain mRNA of cluster of differentiation molecule 11B, a microglia marker, in a type 2 diabetes model [20].

However, it is not yet clear whether unregulated cholesterol drives pathology or if cholesterol is simply worsening an already pathological process. Much more is known about the cholesterol-mediated regulation of cellular function in neurons and astrocytes in the context of AD [21–23]; however not as much is known about how microglia, the immune cells of the brain,

respond to high cholesterol environments. Microglia are quickly emerging as key players in the pathophysiology of AD [24–29]. Genome-wide association studies have consistently found that genes expressed predominantly or exclusively in microglia in the brain, such as triggering receptor expressed on myeloid cells 2 and myeloid cell surface antigen CD33, are associated with AD risk [30–34]. Disease-associated microglia (DAMs), or activated microglia found to be enriched in plaque areas of the AD brain [25,35–39], are directly linked to neuroinflammation, which has been shown to be a critical driver of pathology in AD [40]. Although a vast literature from the cardiovascular field points to cholesterol accumulation in macrophages, the peripheral cousins of microglia, in causing the formation of "foam cells" which are in turn causally linked to the process of atherosclerosis [41–43], not much is known about how microglia respond to excessive cholesterol accumulation.

Multi-omic analysis of postmortem brain samples from AD patients revealed that the concentrations of free cholesterol were elevated in microglial endocytic vesicles [44] suggesting that microglial cholesterol handling may be directly involved in AD pathology. Importantly, it has been shown that the ability of microglia to degrade Aβ is dependent on their ability to efflux cholesterol [45]. Thus, there is already compelling evidence that cholesterol-mediated regulation of microglial function may be an important contributing factor in AD. Here, we review what is known about cholesterol-mediated regulation of microglia phenotype and function, discuss the impacts of statins, which inhibit the synthesis of cholesterol, on microglia function, and highlight areas of future research in the context of AD.

2. Cholesterol-Mediated Regulation of Microglia Phenotype

Microglia are classically characterized as immune cells predominantly found in a homeostatic state, only moving outside of that resting or homeostatic phenotype to respond

acutely to the infiltration of foreign and harmful substances [46,47]. However, recent studies have revealed that microglia are in fact very dynamic, occupying a diverse set of states, with different functional phenotypes [48,49]. Microglia perform different roles in different environments, falling into four broad categories: injury-response microglia, proliferative-region-associated microglia (PAM), disease-associated microglia (DAM), and lipid droplet-accumulating microglia. Injury-response microglia can be induced by lysolecithin injection in mice [50], which leads to the upregulation of lipoprotein lipase and apolipoprotein E (APOE) among other effects [50]. Microglia that surround oligodendrocytes during the first week after birth are highly phagocytic, identified as PAM, and share a very similar profile at the transcriptional level with DAM, such as the increased expression of lipid metabolic genes [51]. Researchers have identified the transition into the DAM state by the downregulation of typical microglia markers (e.g., C-X3-C motif chemokine receptor 1 and adenosine diphosphate receptor P2Y12) and the activation of phagocytic and lipid metabolic genes; as well as having overlapping signatures with injury-response microglia and PAM [50,51]. Elements of the DAM profile are highly conserved from PAM to DAM in mouse models [36,51], behaving with similar phenotypic states as PAM in the zebrafish model [52], indicating that both processes are highly regulated. Even though the murine DAM gene profile from an AD-inducible model is detected in human DAM, the majority of those genes are dispersed across multiple subgroups of microglia instead of being specific to DAM in humans, highlighting species-specific differences in microglia phenotype [53].

Lipids are essential to the brain for both structure and function [54]; however, an overload of lipids can lead to catastrophic damage. When lipid molecules in a given area surpass a critical concentration they begin to aggregate, form micelles, and even form crystals (e.g., cholesterol

crystals), which then act as detergents and structures that lyse and otherwise damage cells and their components [55]. Thus, lipid storage and disposal mechanisms have emerged to protect cells from these extreme events. Cells usually respond to excess cholesterol by inducing the cholesterol efflux machinery, including APOE [56]. Although beyond the scope of this review article, cholesterol metabolism and regulation have been described thoroughly elsewhere [57]. Briefly, the sterol regulatory element binding protein family is involved in regulating genes involved in cholesterol synthesis, transport, and efflux. Induction of the sterol regulatory element binding protein transcriptional program in response to low or high concentrations of cholesterol detected in the endoplasmic reticulum membrane activates and deactivates, respectively, the transcription of genes involved in lipid biosynthesis and import vs. storage and efflux [58,59], including the expression of low-density lipoprotein receptor [58,60] for cholesterol import, and the adenosine triphosphate-binding cassette (ABC) family, in particular ABCA1, for cholesterol efflux [61–63]. However, when the capacity to utilize or efflux excess lipids has been surpassed all cells have the ability to form lipid droplets as a way to temporarily store the excess [64]. When the storage of lipids in lipid droplets becomes chronic and/or the number of lipid droplets starts to exceed the normal threshold, there can be effects on the ability of cells to perform their normal functions [65].

In the case of microglia, lipid droplet-accumulating microglia, have reduced phagocytosis capacity[66]. They show an enhanced phagocytic uptake of lipid, which exacerbates the lipid droplet accumulation burden, and promotes chronic and self-sustained microglial activation [67]. Sustained inflammation further pushes microglia into an hyperactivated state, which exacerbates neuroinflammation and damages blood–brain barrier integrity [68]. Whereas cholesterol excess leads to lipid droplet accumulation and chronic microglial activation, dysregulated cholesterol

concentration in the opposite direction can also be problematic. For example, in mouse models with an interleukin-10 receptor knockout (specifically in astrocytes) there is prolonged neuroinflammatory response to peripheral lipopolysaccharide (LPS), with interleukin-10 receptor signaling deficits and a lack of cholesterol biosynthesis both leading to the inability to resolve microglial activation [69]. It is still unclear in which context injury-response microglia, PAM and DAM become aggressors vs. protectors [70], but there is ongoing research to understand the dynamics of change in microglia phenotype so that we can better understand how to intervene to modulate phenotype to prevent or decrease microglia-driven neurodegeneration [71].

3. Cholesterol-Mediated Regulation of Microglia Function

One of the main functions of microglia is to remove debris and other cytotoxic molecules in a constant effort to maintain a homeostatic environment [48,72]. When microglia fail to perform this essential function a number of downstream effects can occur and lead to disease development. For example, the failure to keep up with the clearance of Aβ monomers contributes to the formation of Aβ oligomers and eventually plaques, which are a hallmark of AD pathophysiology [73–75].

One of the metabolic pathways, in addition to cholesterol efflux and storage, that can be activated in the presence of cholesterol is the generation of a variety of cholesterol metabolites, which in turn can either act as regulators of downstream pathways or have direct deleterious effects. Oxysterols are generated in animals, including humans, by enzymatic means as well as non-enzymatic means. Cholesterol can be oxidized to 25-hydroxycholesterol by cholesterol 25-hydroxylase, and to 27-hydroxycholesterol by sterol 27-hydroxylase [76]. While 25-hydroxycholesterol can also be generated by non-enzymatic oxidation by reactive oxygen species (ROS), 7-ketocholesterol is generated exclusively through non-enzymatic means, for example via

oxidation by ROS [76]. Oxidized cholesterol is fundamental for generating a pro-inflammatory environment for microglia [77]. In rodent microglia, cholesterol oxides confer cytotoxic effects by potentiating the effects of LPS and nitric oxide production, promoting programmed cell death [78]. Specifically, 25-hydroxycholesterol was observed to induce the highest mRNA levels of nitric oxide synthase in combination with LPS in these cells [78]. Similarly, 27-hydroxycholesterol in vitro treatment of rodent microglia cell lines induced accumulation of ROS and the subsequent activation of the pro-inflammatory interleukin-6/signal transducer and activator of transcription 3 signaling pathway [79]. In turn, in the presence of increased ROS the proportion of sterols of non-enzymatic origin increases, and promotes a chronic DAM state [80]. Studies have also shown that 25-hydroxycholesterol can increase the area of the lipid bilayer as well as affecting the orientation of lipids within the membrane [81], increasing membrane permeability [82–84], which has a direct influence on cell death activation [85]. It has also been reported that 27-hydroxycholesterol can induce cellular senescence in microglia through oxidative damage [79,86,87], and that 7-ketocholesterol promotes cellular death by altering biogenesis and peroxisomal activity through oxidative stress [88,89]. Investigators report 7-ketocholesterol released during chronic inflammation indirectly induces neuronal damage mediated by activated microglial cells [90]. It is not yet clear how the relative and absolute concentrations of all of these cholesterol species impact overall microglial function and phenotype.

There is evidence that microglia that are unable to maintain proper cholesterol metabolism, leading to lipid droplet accumulation, have a pro-inflammatory lipidomic profile [91]. A number of approaches to reduce this cholesterol-induced microglia dysfunction have been investigated. The liver X receptor, which is induced by oxysterols, agonistically promotes an

anti-inflammatory environment in the central nervous system (CNS) of rodent models and their primary microglia [92]. Liver X receptor-mediated suppression of inflammation and lipid recycling has also been shown to mitigate disease severity at the microglial level in rodent models [93]. These reports in rodent models suggest that reducing cholesterol via liver X receptor activation could be an approach for clearing the burden from microglia and restoring their functionality.

An additional way in which cholesterol can negatively impact microglia function is through mechanisms involving membrane proteins [94]. The enrichment of cholesterol in plasma membranes potentiates the formation of lipid rafts, which increases the physical proximity of raft-associated proteins. An example of how this can be detrimental when excessive is the case of overactivation via LPS. In a high-cholesterol membrane environment, monomers of Toll-like receptors are in close proximity to each other, enabling the formation of Toll-like receptor dimers, which in turn leads to pro-inflammatory signaling in response to activation by LPS [95]. In murine models chronic LPS activation leads to increased Aβ deposition [96,97].

A high cholesterol diet (3% cholesterol) has been shown to induce a pro-inflammatory profile in rodent microglia models by activating the inflammasome [98]. Furthermore, the literature indicates that cholesterol load causes chronic inflammation in microglia [99]. A high fat, high cholesterol diet (21% fat), administered for 18 weeks increased the presence of interleukin-6 in the microglia and plasma of wild-type and APOE-/-mice [100]. The APOE4 isoform of APOE has been consistently associated with an increased risk for AD in genome-wide association studies [101]. The ApoE4 protein encoded by the APOE4 gene has been shown to have a significantly reduced capacity to induce cholesterol efflux from a variety of cell types compared to APOE3 [102]. In a human microglia cell line expressing APOE4 it was observed

that excess cholesterol leads to higher levels of inflammation [103], highlighting that a reduced capacity to efflux cholesterol, particularly in an environment of excess cholesterol, is associated with microglial activation. Together, these findings suggest that a high cholesterol environment, particularly in genetically susceptible individuals with a reduced capacity to transport and efflux cholesterol (e.g., APOE4 carriers) leads to chronic microglia inflammation and activation, reducing the ability of microglia to respond to additional stressors.

In the CNS, increasing cholesterol leads to reduced phagocytosis by phagocytes [104], and conversely, depleting cholesterol with methyl-β-cyclodextrin increases phagocytosis. Depleting cholesterol using methyl-β-cyclodextrin enhanced phagocytic activity in primary rat microglia when treated with cholesterol and LPS [105]. Alternatively, one group reported that the accumulation of esterified cholesterol in microglia as a result of the dysfunction of the transmembrane structure triggering receptor expressed on myeloid cells 2 (trem2), a receptor for lipidated ApoE and other lipids [106,107], did not evoke changes in their phagocytic capacities [108]; suggesting that the concern should not lie solely on the amount of cholesterol microglia are exposed to, but their capacity to traffic cholesterol. Moreover, microglia cultured from mice lacking ABCA1 exhibit augmented LPS-induced secretion of tumor necrosis factor α (TNF-α) and decreased phagocytic activity hand in hand with decreased ABCA1/APOE expression, which are involved in cellular cholesterol efflux [56,109,110]. Loss-of-function of ABCA7, which also impairs the ability of microglia to efflux cholesterol, accelerated enzymatic activity on the amyloid precursor protein, impaired microglial Aβ clearance and impaired the ability of microglia to perform phagocytosis, contributing to the development of AD [111,112]. In a mouse AD model using a knockout of the protein translocator protein 18 kDa, a molecular sensor specific to glial cells in the brain, it was shown that there is increased Aβ deposition in the brain

and a decreased number of microglia undergoing phagocytosis compared to control mice [113], highlighting the importance of effective microglial phagocytosis in the prevention of AD.

Accumulating evidence demonstrates that defects in cholesterol metabolism, particularly in the context of a variety of high cholesterol environments, are deleterious to microglia function. However, it is not yet clear how to reverse this defective phenotype and correct the underlying cholesterol transport defect in order to improve microglia function. It is also not yet clear how the DAM phenotype can be reversed.

4. Effects of Statins on Microglia

Statins are among the most highly prescribed drugs for the management of hypercholesterolemia (plasma total cholesterol > 200 mg/dL) and cardiovascular disease risk. As a result, millions of patients have been prescribed this class of drugs, which inhibit cholesterol biosynthesis. Clinical studies have shown that patients treated with statins, or a combination of statins plus medications to control blood pressure, have a reduced risk for developing AD and dementia [114,115]. Statins have been found to have generally anti-inflammatory properties [116-118], which may be part of their mechanism of action in the context of AD. It has been established that statins have the ability to cross the blood–brain barrier [119]. Thus, it is plausible that this class of drugs could have direct effects on a variety of cells within the CNS, including microglia. In experimental AD rodent models, the statin atorvastatin had a positive impact on cognitive performance and dampened the activated state of microglia, downregulating inflammatory signaling at the mRNA level [120], suggesting that the inhibition of cholesterol synthesis in the context of AD may have beneficial effects via the reduction in neuroinflammation associated with microglia. It has been observed that in microglia statins can

lead to a number of effects, including effects on phagocytic activity, on cell activation, and inflammatory signaling.

Firstly, statins have been found to improve phagocytic activity in human microglia and comparable cell models; however, with some unresolved questions about the underlying mechanisms involved [121–124]. Statins increase phagocytic activity in microglia and glia-like cells in part by inhibiting the secretion of pro-inflammatory signals, including TNF-α [125,126]. However, some studies found that changes in phagocytic activity may be linked to both cholesterol-dependent and cholesterol-independent mechanisms, with both increases and decreases in pro-inflammatory cytokine expression reported [105,127]. Further research is needed to understand both the cholesterol-dependent and cholesterol-independent effects of different statin formulations on microglia phagocytic activity.

Chronic microglia activation and concurrent pro-inflammatory signaling can result in damage to neighboring cells [90] and in turn diminish the capacity of microglia to perform normal function [96,97]. The impact of statins on inflammatory signaling has been explored in rat cellular models, in which the LPS induced inflammatory response in microglia was dampened by reducing the expression of pro-inflammatory cytokines that include TNF-α [128]. Statins are directly involved in attenuating the effects of TNF-α, the release of associated factors, and the modulation of receptors in this signaling cascade [120,129]. Furthermore, statins can also have an inhibitory effect on the production of superoxide free radical [123,130], protecting cells from oxidative damage, inducing the production of ionized calcium-binding adaptor molecule 1, regulating lipid metabolism and phagocytosis [131–133].

Statins can reduce microglial activation and as a result reduce the propensity for neurodegenerative disease [124,134]. Statins can inhibit microglial activation by blocking the

activation of pro-inflammatory cascade pathways [25,130,135] and by promoting the enzymatic degradation of Aβ [136]. Statins have also been found to attenuate the expression of matrixins in microglia, reducing pro-inflammatory signaling [130,137,138], inhibiting the expression of prostaglandin-endoperoxide synthase 2, and redirecting microglia away form an activated state and toward a homeostatic state [130,139]. Studies in human cell lines and in rat neonatal cortical microglia found that atorvastatin reduced the secretion of pro-inflammatory cytokine interleukin-6 [125]. Simvastatin was not able to reduce pro-inflammatory cytokine interleukin-6 secretion and hindered cell viability in both human microglia cell lines and rat neonatal cortical microglia [125]. However, since Simvastatin is a prodrug in which the lactone ring must be opened to be activated, and since this activation step was not undertaken in this study prior to treatment, it is not clear whether Simvastatin would also have the expected anti-inflammatory effects in microglia. Future studies are needed to further confirm the effects of different statin drugs on microglia.

An increase in APOE expression has been demonstrated to be a defining factor in the conversion of homeostatic microglia to DAM [36]. Several lines of evidence have now shown that statins may be important in regulating microglia function in part through their effects on APOE. Administration of statins reduced brain APOE mRNA levels, ApoE protein level and ApoE secretion, attenuating the effects of the high-cholesterol diet [140]. In vitro experiments have also shown that APOE expression can be increased in the CNS in response to cellular stress, and that simvastatin was able to reduce that expression [141]. In mice, inhibition of TNF-α signaling increased APOE mRNA and protein levels, whereas inflammatory signaling otherwise dampens APOE expression in microglia [142]. It is still unclear how these various anti-inflammatory mechanisms are activated by the different statin compounds, and the different

hypotheses from different reports dependent on the model under observation [143]. However, the evidence thus far, as shown in Table 1, suggests that statins could be a viable approach, whether directly through cholesterol-mediated or indirectly through cholesterol-independent mechanisms, to modulate microglial activation and functionality.

Table 1. Summary of studies highlighting the effects of statins on microglia.

Study	Study Type	Model	Statin (Dose)	Outcome Measure	Results
Wang, 2018 [120]	In vivo	Sprague Dawley male rats (age 7 to 8 weeks), 250–300 g	Atorvastatin 5- and 10-mg/kg (chronic)	Number of Iba-1-positive microglia	Reduced number of Iba-1 positive microglia.
Ewen, 2013 [122]	In vivo	Sprague–Dawley male rats (12 week of age)	Atorvastatin 2, 5, and 10 mg/kg	TNF-α and IL-10 levels, and infiltration at site of injury	Atorvastatin decreased TNF-α and increased in IL-10 levels, and number of activated microglia.
Lindberg, 2005 [125]	In vitro and in vivo	CHME-3 human cell line; primary rat microglia	Atorvastatin 0.1, 1, 5, and 20 mM or Simvastatin 0.1, 1, 5, and 10 mM; Atorvastatin 1, 5, and 20 μM	Microglial Secretion of IL-6	Atorvastatin reduce IL-6 secretion of stimulated human and rat microglia.
Townsend, 2004 [127]	In vitro and in vivo	BALB/c mice microglia	Lovastatin 10 μM	IL-6, TNF-α and IL-β1 concentrations and phagocytosis activity	Lovastatin reduced IL-6, TNF-α and IL-β1 concentrations and attenuated impaired phagocytosis in primary mouse microglia.
Pahan, 1997 [128]	In vitro	Isolated primary rat microglia from mixed cultures	Lovastatin 10 μM	Nitiric Oxide, TNF-a, IL-1b, and IL-6 concentrations	In LPS stimulated primary rat microglia, lovastatin reduced nitric oxide, TNF- α, IL-1β, and IL-6 in supernatant.
Yongjun, 2013 [130]	In vitro	Primary human microglia	Atorvastatin 0.1 mM	MT1-MMP expression	Reduced microglia expression of MT1-MMP.
Kata, 2016 [131]	In vitro	Primary rat microglia	Rosuvastatin 1 μM	Iba-1 immunoreactivity; phagocytosis activity; IL-10, IL-1b and TNF-α production.	In microglia challenged with LPS, rosuvastatin reduced IL-1 β, TNF-α production and phagocytosis, IL-10 and Iba1 immunoreactivity was increased.

Chu, 2015 [135]	In vivo	Sprague–Dawley male rats	Atorvastatin 10 mg/kg/day	pNFκB immunostaining	Proinflammatory pNFκB proteins were decreased by atorvastatin in microglia, following surgery.
Tamboli, 2010 [136]	In vivo	BV-2 mouse microglia	Lovastatin 5 μM	Aβ degration, Westen blot	Lovastatin enhanced the degradation of extracellular Aβ by microglial cells.
Petanceska, 2003 [140]	In vivo	C57/BL6 mice and BV-2 cell line	Lovastatin 5 μM and atorvastatin 5 μM	ApoE Western blot	In mice, lovastatin reduced ApoE secretion. Atorvastatin reduced the levels of both cellular and secreted ApoE.

5. Conclusions

Exposure to excess cholesterol has been shown to drive several aspects of pathological microglia states, including increased inflammatory signaling and decreased phagocytic capacity, both of which have been implicated in AD pathophysiology. The overall picture that is emerging, as depicted in Figure 1, is that in a high cholesterol environment, high cholesterol concentrations in neuronal plasma membranes lead to higher production of Aβ due to colocalization of amyloid precursor protein and γ secretase, which in turn leads to a higher burden of Aβ that needs to be cleared by microglia. At the same time, high cholesterol concentrations impair the ability of microglia to clear Aβ, and increase microglial inflammatory signaling and ROS production. All of this further drives the accumulation of Aβ oligomers and eventually plaque formation, as well as creating a pro-inflammatory environment that contributes to neurodegeneration. The highly prescribed, generally safe, statin drugs may be a viable approach for regulating cholesterol and reducing neuroinflammation but further research on their specific effects on microglia are needed. Further research is also needed to understand how defective or suboptimal cholesterol

metabolism impacts microglia phenotype and function so that novel targeted therapies to restore microglia to their functional, homeostatic state can be developed.

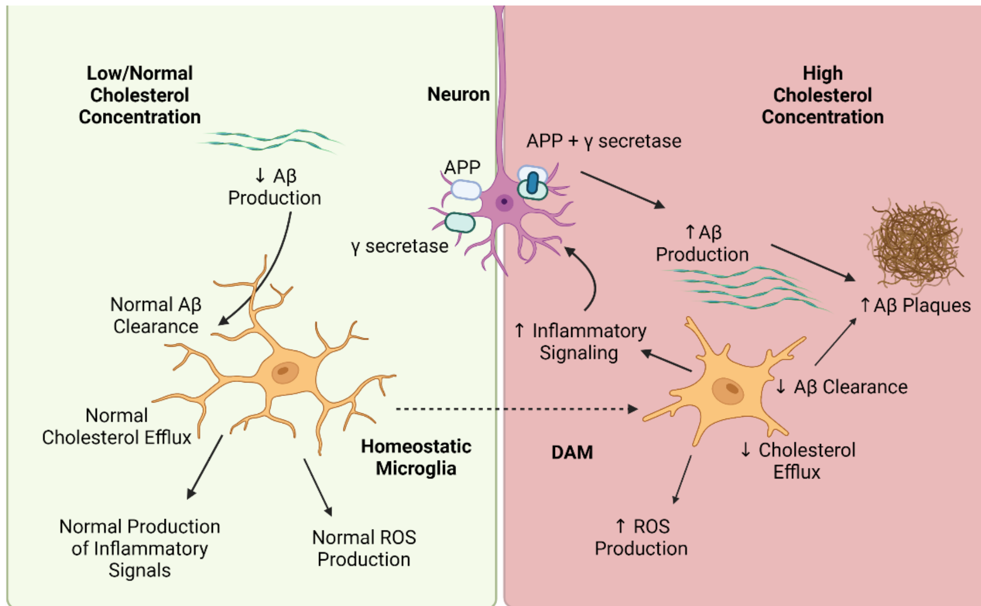

Figure 1. High cholesterol concentrations and disease-associated microglia (DAM). When cholesterol concentrations are low or normal the amount of amyloid-β (Aβ) production is low, coupled with normal microglia Aβ clearance, and a homeostatic microglia phenotype with normal levels of cholesterol efflux, normal production of inflammatory signals and reactive oxygen species (ROS). Conversely, exposure to high cholesterol concentrations leads to increased production of Aβ by neurons due to colocalization of amyloid precursor protein (APP) and gamma secretase in the plasma membrane. Concurrently, high cholesterol concentrations lead to induction of the DAM phenotype, in which increased inflammatory signaling, increased production of ROS, and decreased cholesterol efflux hinder the ability of microglia to clear Aβ, further increasing the concentration of Aβ oligomers and driving plaque formation. Created with BioRender.com.

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Chapter 2: HDL from 36h Fasted Participants Potently Promote Efflux of Cholesteryl Ester from Activated Microglia

Joanne K. Agus [1] and Oscar M. Muñoz Herrera [1], Christopher Rhodes [1], Jack Jingyuan Zheng [1], Chenghao Zhu [1], Maurice Wong [3], Xinyu Tang [1], Izumi Maezawa [2], Lee-Way Jin [2], Carlito B. Lebrilla [3], Danielle J. Harvey [4], Angela M. Zivkovic [1]

[1]Department of Nutrition, University of California – Davis, Davis, CA, 95616

[2]Department of Pathology and Laboratory Medicine, University of California – Davis, Davis, CA, 95616

[3]Department of Biochemistry and Molecular Medicine, University of California – Davis, Davis, CA, 95616

[4]Department of Public Health Sciences, University of California – Davis, Davis, CA, 95616

ABSTRACT

Little is known about how prolonged fasting affects HDL particles. Here, we investigated the effects of a 36h fast on HDL particle size by transmission electron microscopy (TEM), and their ability to efflux cholesterol from activated microglia. Plasma from individuals participating in a study investigating the effects of a 36h fast were analyzed. HDL particles were isolated by ultracentrifugation followed by size exclusion chromatography. Importantly, HDL from 36h fasted individuals effluxed cholesteryl esters from microglia loaded with cholesterol + amyloid beta oligomers (AβO) 10-fold more effectively than postprandial HDL, and microglia treated with cholesterol+AβO had reduced apolipoprotein E secretion, which was attenuated more effectively by HDL from 36h fasted individuals than HDL from postprandial individuals. These findings suggest that prolonged fasting alters the structure, composition and function of HDL particles.

INTRODUCTION

High-density lipoproteins (HDL) play a critical role in reverse cholesterol transport (RCT) and have recently been shown to perform an array of other essential functions, including regulation of immune function [1–4] and inflammation [4–6]. HDL particles exert their immuno-regulatory and anti-inflammatory effects in part by modulating cholesterol content in plasma membranes [2,3,7], as well as lipid droplets and cellular organelles, in a complex process involving multiple pathways [7].

HDL amount (often measured as HDL-cholesterol (HDL-C) has been linked to better cardiovascular [8], renal [8,9], and cognitive health [2,10–15] and is predictive of longevity [16]. However, HDL-C concentrations have recently been found to have a U-shaped curve with respect to mortality outcomes, with both low (<30 mg/dL) and high (>100 mg/dL) HDL-C being associated with increased risk of mortality [17]. Thus, simply raising HDL-C is not a safe strategy for improving outcomes related to HDL. Instead, research has turned to searching for other HDL-related therapeutic targets. For example, HDL functionality (i.e. cholesterol efflux capacity) and structure (i.e. particle count and size distribution) have both been found to be more predictive of cardiovascular outcomes than HDL-C [18–20]. Evidence shows that both the functionality and structure of HDL particles is affected by multiple factors, including overall metabolic state [21,22]. While it is clear that somehow improving HDL is an important therapeutic strategy for decreasing the risk of chronic diseases such as cardiovascular disease and Alzheimer's disease (AD), it is not clear exactly what aspect of HDL should be altered to improve outcomes, and how.

Interest in HDL particles in the context of AD has been growing given recent findings linking HDL with protection from AD in humans, and with reversal of neurodegeneration in

animal models. High plasma HDL-C is associated with a strong reduction in AD risk (HR 0.4) [2], conversely, AD patients have low concentrations of ApoA-I, the defining HDL protein [10]. Mice injected with reconstituted HDL peripherally experienced reductions in brain soluble amyloid beta (Aβ) peptides [11], and overexpression of peripheral ApoA-I reduced brain Aβ burden, reduced neuroinflammation, and preserved cognitive function [12]. Furthermore, AD patients were found to have reduced HDL functionality, i.e. lower cholesterol efflux capacity (CEC) [28].

In addition to the well-known Aβ- and tau-related pathological hallmarks of AD [29], intracellular lipid deposition is characteristic of AD. Excessive storage of cholesteryl esters (CE) has been found in the AD brain [30]. In neurodegenerative conditions, microglia accumulate cholesterol and lipid-rich debris [31]. Microglia, specialized phagocytic cells of the brain, play a critical role in the clearance of Aβ, which is involved in the pathogenesis of AD [32,33]. The ability of microglia to remove Aβ is influenced by their cellular cholesterol clearance capacity [34]. It is not known to what extent Aβ impairs microglial cholesterol efflux capacity to cholesterol acceptors (i.e. HDL), especially in the context of excess cholesterol. Intracellular Aβ degradation is mediated by the cholesterol efflux function via ApoE [35], which is induced by HDL particles.

We and others have previously shown that the HDL proteome, lipidome, and functional capacity can be remodeled through diet [36–38], and that these alterations can be achieved within a relatively short period of time [5,39,40]. However, comparatively little is known about the effects of prolonged fasting on HDL. Several studies have shown that different forms of decreasing meal frequency, from intermittent fasting to time-restricted eating patterns, can lead to increases in HDL-C, improvements in CEC [41], and alterations in HDL particle size

distribution. Additionally, a recent study showed that fasting-mimicking diets can reduce neuroinflammation in mice [42].

In this study, we sought to determine whether prolonged fasting, exerts beneficial effects in the context of AD via its effects on HDL particle functionality, specifically the ability to efflux cholesterol and CE from microglia.

2. Materials and Methods

Study Design

Plasma samples were obtained from a previously conducted study of a single bout of prolonged fasting in healthy human volunteers [43]. Full clinical study protocols and inclusion and exclusion criteria can be found at clinicaltrials.gov; NCT03487679. Briefly, the study involved 20 participants, 10 males and 10 females, aged 20-40 with no reported health conditions or extreme dietary and exercise patterns. Study participants first came in on Day 1 after an overnight fast (12 hours) at around 8am (Base-line: 12h fasted, Timepoint A) for a baseline blood draw, and two hours after their last meal at around 8pm for their baseline postprandial blood draw (Fed: 2h postprandial prior to 36h of fasting, Timepoint B). Participants tracked every food and beverage consumed over the entire course of Day 1. After the evening meal on Day 1 they began their 36-hour water-only fast until the morning of Day 3, when they came in for a blood draw (Fasted: 36h fasted, Timepoint C) at around 8AM. After this blood draw they were instructed to consume the exact same foods and beverages as those they consumed on Day 1, and then came in for a final blood draw two hours after their last meal on Day 3 (Refed: 2h postprandial after 36h of fasting, Timepoint D), again at around 8PM. Participants were monitored for fasting

compliance with glucose monitoring every 2 hours during the study, and ketone body concentrations were later also confirmed.

HDL Isolation

HDL particles were isolated from plasma using a validated, optimized method (Zheng, 2021). Briefly, 500 µL starting plasma was thawed and placed under 4.1 ml density solution of 1.006 g/ml in 4.7 ml OptiSeal tube (Beckman-Coulter, IN, USA). Then, samples were ultracentrifuged for 30 min at 110,000 RPM using a fixed angle rotor (TLA-110, k factor=13, Beckman-Coulter). The top 4ml were removed from the OptiSeal and the remaining 700 µl of sample was mixed with 1.1 ml 1.34 g/ml KBr density solution to create 1.8 ml of a 1.21 g/ml KBr-Lipoprotein and plasma protein fraction. This fraction was pipetted under 2.8 ml of 1.21 g/ml KBr density solution in the OptiSeal tube, and topped off with 100 µl of 1.21 density solution for a second ultracentrifuge spin of 3 hours and 30 minutes at 110,000 RPM. After the second spin, the first 2 ml of the tube was isolated and filtered through 50 KDa Amicon Ultra-4 for 8 minutes at 4500 RPM to 250 µl, of which 200 µl was injected into an FPLC (AKTA P-920) with a size exclusion chromatography (SEC) column (Superdex 200 GI 10/300), and the system was run at a flow rate of 0.5 ml/min. The HDL fraction was selected based on elution time collecting between the troughs of the LDL-HDL peak and the HDL-albumin peak. The 4 mL of total eluted volume representing the HDL fraction was concentrated using Amicon 50 KDa filters, 2% sucrose was added as a cryoprotectant [44] and samples were aliquoted into 20 µL fractions before storage at -80 °C.

Microglia Experiments

HMC3 microglia were cultured using EMEM (ATCC 30-2003), penicillin-streptomycin (10,000 U/mL, Thermo Fisher, 15140122) and 10% fetal bovine serum (FBS, ATCC 30-2020). HMC3 were seeded on 96-well microplates (Corning; Costar; 3916) 40,000 cells per 200 µL, per well. Seeded cells were allowed to adhere for at least 7 hours. Following adherence, media was replaced with EMEM without phenol red (Thermo Scientific, C837K00) to reduce background signal, penicillin-streptomycin (10,000 U/mL, Thermo Fisher, 15140122) and 10% fetal bovine serum (FBS, ATCC 30-2020), along with the corresponding treatment.

To determine the appropriate doses of cholesterol and AβO for the experiments, microglia were loaded with water soluble cholesterol (Sigma-Aldrich, C4951, 40mg cholesterol per gram and 960mg methyl-β-cyclodextrin per gram) at various concentrations (5, 10, 20, 30, 40, 50 µg/mL), for 6 hours, and cytotoxicity was measured using a commercially available kit (Promega CellTox™ Green Cytotoxicity Assay (Cat.# G8741) following the manufacturer's protocol. Microglia were then treated with water soluble cholesterol at 10 µg/mL or 20 µg/mL concentration +/- amyloid β oligomer (AβO) (1-42) (HFIP-treated) (BACHEM, H-7442) at various concentrations (0.1, 0.25, 0.5 µM) for 24 hours and cytotoxicity was measured as above.

Microglia were treated with AβO at 2 µM for 24 hours. At the 18h time-point, the HMC3 were loaded with water soluble cholesterol at 20 µg/mL for 6 hours. At the 21h time-point, the cells were supplemented with HDL at 0.1 mg/mL for 3 hours. At 24h, the supernatant in all wells was removed and stored at -80°C. HDL from 18 out of 20 of the individuals who participated in the study were included due to lack of sufficient material from 2 of the participants. HDL pools from the baseline 2h postprandial state (time point B) and the 36h fasted state (time point C) were prepared to generate adequate HDL for the in vitro experiments. HDL samples from each time point were thawed on ice and 10µL of each isolate from each participant were taken and

combined to generate a pooled HDL sample from each time point with protein concentration determined by microBCA assay. HMC3 cells were lysed using chloroform (Sig-ma-Aldrich, C2432), isopropyl alcohol (Fisher Scientific, 67-63-0) and Np40/ Igepal Ca 630 (Sigma-Aldrich, 9002-93-1), at 7:11:0.1, respectively. The cellular content of unesterified and esterified cholesterol (CE) was measured using the Total Cholesterol Assay (Cell Biolabs, Inc, STA-390) kit following the manufacturer’s instructions. A portion of the culture supernatant was probed for ApoE content using the Human ApoE ELISA (Cell Biolabs, Inc, STA-367) following the manufacturer's instructions. The remaining portion of the supernatant was used for analysis of the lipoprotein content, size and structure via electron microscopy.

Electron Microscopy and particle analysis

Transmission electron microscopy (TEM) analysis was performed as reported [6,40] with minor modifications. Briefly, 4 microliters of sample at 0.1 mg/ml were loaded on a glow-discharged 200-mesh carbon-coated TEM grid (TedPella Inc., CA, USA) for 1 minute. The grid was then blot-dried with filter paper and loaded with 2% w/v uranyl formate negative stain solution (pH 4.6), which was quickly blot-dried with a filter paper. The loading and drying of the negative stain solution was repeated 4 more times. After the last negative stain solution was removed and the grid was completely dried, it was stored in a dark environment until sample imaging. Sample grids were imaged using TEM (Talos L120C, Thermo Fisher) combined with a bottom-mounted CCD camera (Ceta, Thermo Fisher) at 36,000x magnification and 80 kV electron beam voltage.

Micrographs were processed and particle diameters were obtained using FIJI [15]. Images were processed with the bandpass filter function to filter structures down to 40 pixels and up to 10 pixels. The threshold of the images was then set by the “Moments” mode. The Analyze

Particles function was used to obtain particle size in area (nm^2) and shape information (e.g. circularity, aspect ratio, roundness), using the following parameters: Area: 19.625 - 706.5 nm^2; Circularity = 0.15 – 1.00; Exclude on edges; Include holes. Selected particle data were then filtered using additional geometrical parameters (aspect ratio < 1.5, roundness > 0.5) to remove non-spherical particles. For data analysis, particle size for each selected particle was reported as nm in diameter.

Statistical Analysis

Two-way ANOVA tests were applied to determine the changes in cholesterol concentration and ApoE secretion according to the treatments and HDL conditions. A one-way ANOVA test was applied to detect differences in particle sizes in treatments across different HDL conditions. P-values of host hoc pairwise comparisons were adjusted using the Tukey method.

3. Results

All of the participants were determined to be compliant for fasting throughout the 36 hours, as monitored by blood glucose readings and ketone body concentrations [43]. Participants also consumed the same foods and beverages on day 1 and day 3 of the study, as per the study design [43].

3.1 Effects of HDL From Fasted Individuals vs. HDL From Postprandial Individuals on Microglia Cholesterol Content and ApoE Secretion

We challenged HMC3 microglia with cholesterol (Chol), Aβ oligomers (AβO), or cholesterol + AβO (Chol+AβO) to investigate how microglia Chol handling is affected by these treatments, simulating the environment in the AD brain. We also investigated the effects of adding HDL

particles from individuals in the postprandial state (Timepoint B) and after a 36h water-only fast (Timepoint C). Cell death increased in response to increasing doses of Chol (Supplemental Fig. 1). A final dose of 20 µg/mL for 6 hours of Chol and 2 µM for 24 hours of AβO were selected based on the fact that the doses of Chol were well tolerated by the HMC3 microglia under 30 µg/mL for no longer than 6 hours, and it was clear that AβO was not considerably contributing to cell-death at lower µM concentrations for 24 hours. The average particle size of the pooled HDL from fasted and postprandial individuals determined by TEM showed that the mean diameter of the fasted state HDL particles was significantly increased compared to the post-prandial state HDL (Supplemental Fig. 2).

Chol and Chol+AβO treatment but not AβO alone increased total cellular Chol concentrations, and in all three treatments as well as in the control, untreated cells, adding HDL from 36h fasted individuals significantly attenuated the Chol increase, whereas HDL from postprandial individuals only decreased cellular Chol in Chol and Chol+AβO treated cells (Fig. 1A). Strikingly, only HDL from 36h fasted individuals but not HDL from postprandial individuals was capable of reducing the cellular content of CE in AβO and Chol+AβO treated cells, with the strongest effect observed in the Chol+AβO cells (Fig. 1B). The amount of ApoE secreted into the supernatant was significantly reduced in AβO and Chol+AβO cells (Fig. 1C), suggesting that at least part of the observed accumulation of cellular Chol was due to decreased efflux via ApoE. Again, HDL from 36h fasted individuals were significantly better at stimulating ApoE secretion than HDL from postprandial individuals, and this effect was strongest in AβO treated cells (Fig. 1D). The diameter of secreted ApoE particles in the supernatant was significantly reduced in Chol+AβO treated cells (Fig. 1E), suggesting that when microglia are challenged with

Chol+AβO their Chol efflux is impaired not only because they secrete less ApoE but also because they secrete smaller particles, which carry less Chol per particle.

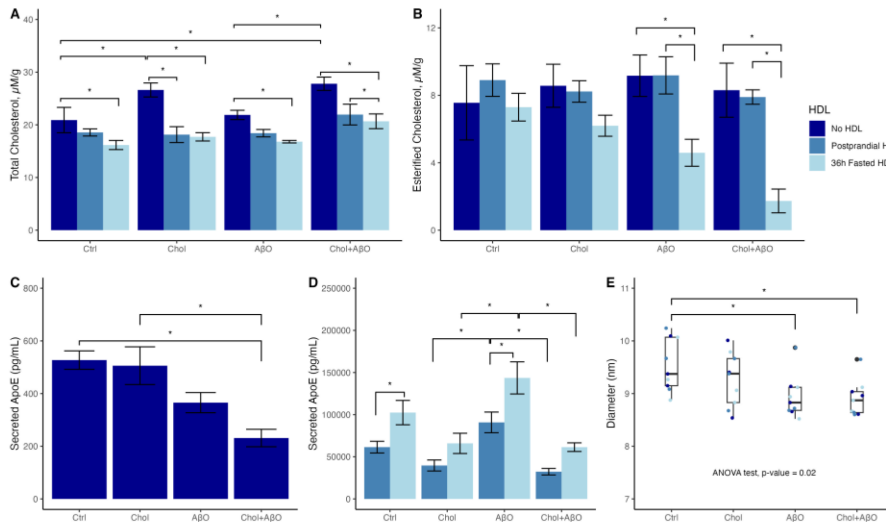

Figure 1. The effects of cholesterol (Chol), amyloid beta oligomers (AβO) or Chol+AβO vs. control, and high density lipoproteins (HDL) from postprandial individuals or HDL from 36h fasted individuals vs. no HDL on HMC3 microglia: A) Total cellular cholesterol, and B) esterified cholesterol content. Effects of Chol, AβO or Chol+AβO on: C) Apolipoprotein E (ApoE) secreted into the supernatant with no HDL incubation, D) with HDL from postprandial individuals vs. HDL from 36h fasted individuals incubation. E) Diameter of particles secreted into the supernatant. Shown are means ± SE (n=3 independent cultures).

4. Discussion

Higher plasma HDL and cerebrospinal fluid HDL concentrations are associated with better cognitive function and a lower risk of dementia and AD [61–65]. However, it is not clear how to intervene to improve HDL as an approach for AD prevention and management. In our study we found that 36 hours of fasting improved the ability of HDL particles to efflux CE from microglia activated with Chol+AβO. In this study we found that the average particle size of HDL from individuals in the fasted state was significantly larger than HDL particles from those same

individuals in the postprandial state. Future studies are needed to determine whether the HDL particles from 36h fasted individuals were more effective at inducing cholesterol efflux from activated microglia due to their size difference alone, or also by additional differences in composition and structure. For example, it is entirely possible that small HDL in one physiological state are more functional and less deleterious than small HDL in another condition. With quadrillions of HDL particles circulating per mL of plasma, it is easy to imagine that even more refined subclass categorization beyond what has been achieved thus far (based on size alone, apoprotein content alone, charge and size, or density +/- size) will be required for a complete understanding of HDL biology and to translate these findings to therapeutic solutions for AD.

Even though cellular levels of CE were not significantly increased in HMC3 microglia by treatment with Chol, AβO or Chol+AβO, the HDL from 36h fasted individuals were able to efflux CE, which is only found in lipid droplets intracellularly, from AβO and Chol+AβO treated microglia to concentrations well below all control conditions. This is of importance because one group has reported that peripheral ApoA-I HDL can penetrate the blood–brain barrier (BBB) through the scavenger receptor class B type I (SR-BI)-mediated transcytosis system [71], which suggests the possibility that peripheral HDL may interact with brain cells such as microglia to efflux excess intracellular cholesterol, affecting their function and phenotype. Cholesterol handling in microglia is essential because high cholesterol concentrations impair the ability of microglia to clear Aβ, and increase microglial inflammatory signaling and ROS production [72]. All of this further drives the accumulation of Aβ oligomers and eventually plaque formation, as well as creating a pro-inflammatory environment that contributes to neurodegeneration [72–75]. HDL may be protective against the progression of AD in the brain by modulating Aβ synthesis

by regulating cholesterol concentrations in the cell, which in turn, may enhance Aβ clearance and degradation [76,77].

In this study we also found that both Chol alone and Chol+AβO but not AβO alone increased cellular cholesterol content in HMC3 microglia. Both HDL from fasted and post-prandial state individuals attenuated the cholesterol increase; however, in Chol+AβO treated cells neither type of HDL was able to reduce cellular cholesterol to control levels. Furthermore, ApoE secretion was reduced in the presence of AβO and even further in Chol+AβO treated cells but not cells treated with Chol alone. HDL from fasted individuals stimulated better ApoE secretion than HDL from postprandial individuals, and this effect was strongest in AβO treated cells. The diameter of these secreted ApoE particles in the supernatant was significantly reduced in Chol+AβO treated cells. Together these findings suggest that this "double hit" of Chol+AβO impairs the ability of microglia to efflux cholesterol both in the presence and absence of cholesterol acceptor (i.e. HDL).

Our study revealed that 36 hours of fasting is a physiologically potent intervention to alter HDL function and profile when compared to 12 hours baseline fasted and post-prandial time points. However, at this time it is not clear how to implement fasting into a preventative nutritional strategy for AD because it is not yet clear how to appropriately dose the fasting regimen to improve health outcomes. Future studies are needed to better understand the specific compositional and structural changes in HDL particles in the 36h fasted state, and other potential mechanisms by which prolonged fasting may be a beneficial intervention as a treatment approach in AD.

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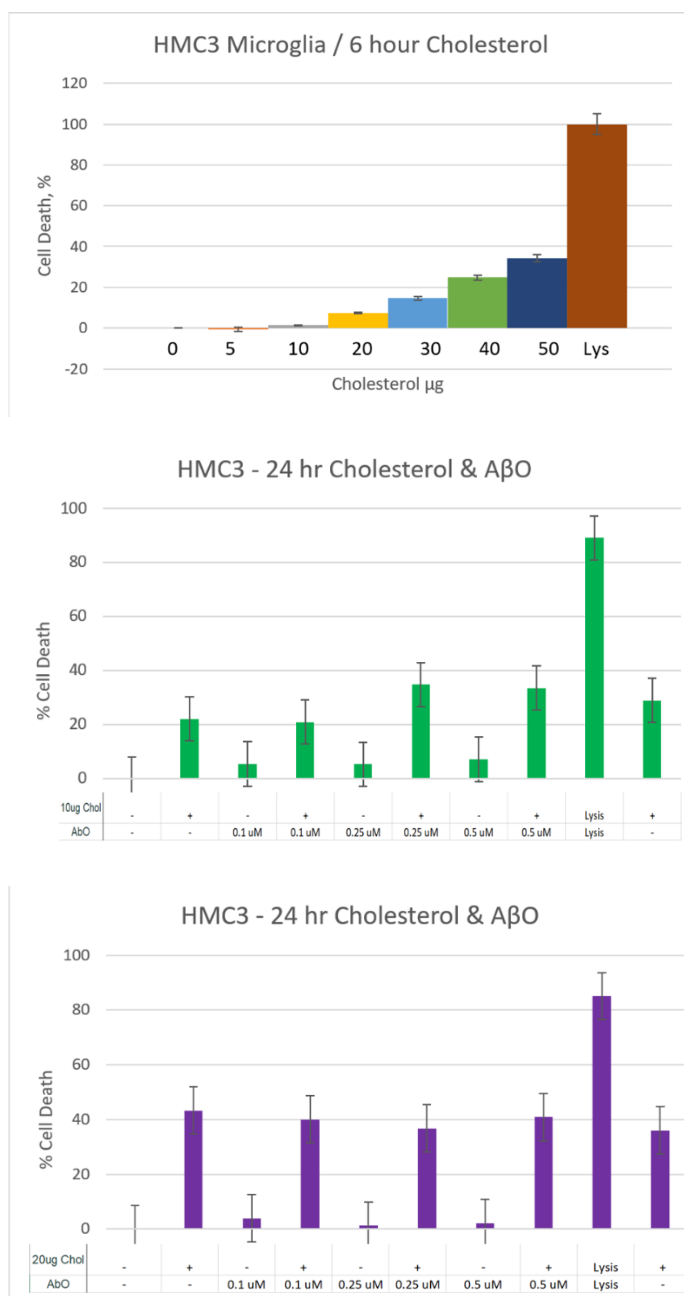
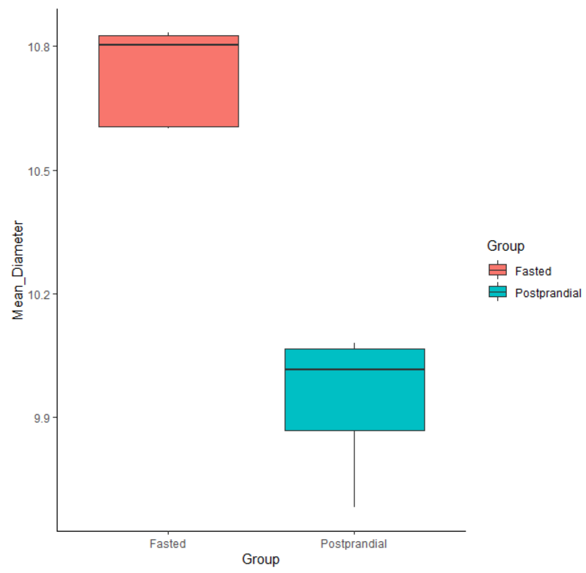
Supplemental materials for Chapter 2: HDL from 36h Fasted Participants Potently Promote Efflux of Cholesteryl Ester from Activated Microglia



Supplemental Figure 1. HMC3 Cytotoxicity Assay. A) Cholesterol loaded HMC3 microglia (varying doses), for 6 hours. B) Cholesterol (10µg/mL) loaded and AβO treated HMC3 microglia, for 24 hours. C) Cholesterol (20µg/mL) loaded and AβO treated HMC3 microglia, for 24 hours.



Supplemental Figure 2. The mean diameter of fasted HDL is larger than the mean diameter of postprandial HDL.

Chapter 3: A Promising High-Throughput Screening Tool of Activated Microglia for Alzheimer's Therapeutic Discovery Using the HMC3 Human Microglia Cell Line

Oscar M. Muñoz Herrera[1], Brian Hong[1], Ulises Ruiz Mendiola[2], Izumi Maezawa[2], Lee-Way Jin[2], Carlito B. Lebrilla[3], Danielle J. Harvey[4], Angela M. Zivkovic[1*]

[1]Department of Nutrition, UC Davis; [2]Department of Pathology and Laboratory Medicine, UC Davis Medical Center; [3]Department of Chemistry, University of California; [4]Department of Public Health Sciences, UC Davis; Davis, California 95616 United States

ABSTRACT

Research has found that genes specific to microglia are among the strongest risk factors for Alzheimer's disease (AD), and that microglia are critically involved in the etiology of AD. Thus, microglia are an important therapeutic target for novel approaches for the treatment of AD. High-throughput in vitro models to screen molecules for their effectiveness in reversing the pathogenic, pro-inflammatory microglia phenotype are needed. In this study, we used a multi-stimulant approach to test the usefulness of the human microglia cell 3 (HMC3) cell line, immortalized from a human fetal brain-derived primary microglia culture, in modeling critical aspects of the dysfunctional microglia phenotype. HMC3 microglia were treated with cholesterol (Chol), amyloid beta oligomers (AβO), lipopolysaccharide (LPS), and fructose individually and in combination HMC3 microglia demonstrated changes in morphology consistent with activation when treated with the combination of Chol+AβO+fructose+LPS. Multiple treatments increased the cellular content of Chol and cholesteryl esters (CE), but only the combination treatment of Chol+AβO+ fructose+LPS increased mitochondrial Chol content. Microglia treated with combinations containing cholesterol+AβO had lower apolipoprotein E (ApoE) secretion, with the combination of Chol+AβO+fructose+LPS having the strongest effect. Combination treatment with Chol+AβO+fructose+LPS also induced APOE and TNF-α expression, reduced ATP production, increased reactive oxygen species (ROS) concentration, and reduced phagocytosis events. These findings suggest that HMC3 microglia treated with the combination of Chol+AβO+fructose+LPS may be a useful high-throughput screening model amenable to testing on 96-well plates to test potential therapeutics to improve microglial function in the context of AD.

INTRODUCTION

Microglia are known to be critically involved in the etiology of Alzheimer's disease (AD) [1,2]. Homeostatic microglia are protective, participating in the clearance of amyloid beta (Aβ) and other cellular debris; on the other hand, activated or disease-associated microglia (DAM) drive neuroinflammation and neurodegeneration processes in the AD brain [3]. Due to this critical role of microglia in AD, and the failure of Aβ-based therapies to improve AD outcomes, microglia have emerged as an important therapeutic target to prevent and treat AD. Low cost, high throughput screening in vitro models are needed to test a wide variety of molecules for their potential to reverse this deleterious DAM phenotype. It has been established that induced pluripotent stem cell (iPSC)-derived microglia are superior to cell lines and animal model-derived cells in replicating the complex, multi-faceted phenotypes of microglia in human brains, and continue to be necessary to fully understand the mechanisms and underlying biology of microglia in the context of AD [4–13]. However, neither iPSC-derived microglia nor microglia isolated from animals are well-suited for high-throughput screening applications because they are expensive, difficult to grow and/or difficult to scale up. In this study the objective was to determine whether the human microglia cell 3 (HMC3) cell line, immortalized from a human fetal brain-derived primary microglia culture, could act as a suitable model of DAM phenotype, which could be used to screen large numbers of molecules for their ability to reverse specific dysfunctional aspects associated with activated microglia. Several different treatments alone and in combination with each other were assessed for their ability to generate critical aspects of the DAM phenotype, including alterations in apolipoprotein E (ApoE) expression and secretion, cytokine expression, mitochondrial dysfunction, and capacity to perform phagocytosis. The

treatments included cholesterol (Chol), lipopolysaccharide (LPS), Aβ, and fructose. These treatments were chosen on the basis of the following observations.

Chol is critically involved in the pathophysiology of AD, from neurons, which are known to overproduce pathogenic Aβ peptides in the context of high plasma membrane Chol concentrations [14–19], to microglia, which are known to accumulate Chol and lipid-rich debris in multiple neurodegenerative conditions [20]. Genome-wide association studies point to Chol and microglia as key players in AD, with APOE, the main Chol transporter in the CNS, and TREM2, a monocyte-specific receptor, being among the strongest genetic risk factors for AD across populations [21]. When cells are faced with excess Chol they can esterify it and store the resulting cholesteryl esters (CE) in lipid droplets. Lipid droplet accumulation is a hallmark of the AD brain [22]. The ability of microglia to remove Aβ is influenced by their cellular Chol clearance capacity [23]. Intracellular Aβ degradation is mediated by the Chol efflux function of ApoE [24]. DAM show reduced expression of homeostatic genes with an increase in lipid metabolism and phagocytosis genes [25]. In microglia, high Chol concentrations lead to induction of the DAM phenotype, in which increased inflammatory signaling, increased production of reactive oxygen species (ROS), and decreased Chol efflux hinder the ability of microglia to clear Aβ, further increasing the concentration of Aβ oligomers and driving plaque formation [24,26].

Although the function of the blood-brain-barrier (BBB) is to keep deleterious molecules from entering the brain, BBB function may be impaired in AD [27–30], and deleterious, pro-inflammatory molecules such as LPS have been found to cross the BBB [31,32]. High LPS concentrations induce cognitive impairment and neuroinflammation in the mouse brain [33]. In

humans, a two-to-three-fold increase in LPS has been detected in the AD brain [34–36]. LPS is a potent pro-inflammatory activator of monocytic cells, including microglia.

AD is associated with multiple comorbidities, including diabetes and cardiovascular disease [37], with as many as 80% of AD patients developing diabetes [38]. Although hyperglycemia is by definition the primary metabolic dysregulation in diabetes, high fructose concentrations are a key driver of aberrant lipid accumulation in the liver [39,40], and high fructose intake has been found to be a causal factor in the development of insulin resistance and metabolic dyslipidemia [41]. Recently, short-term fructose intake was shown to impact hippocampal plasticity even in the absence of overt peripheral insulin resistance [42], and a new hypothesis linking brain fructose metabolism in the etiology of AD is emerging [43].

2. Materials and Methods

Study Design

Two sets of experiments were conducted using the HMC3 human microglia cell line (ATCC, CRL-3304) to evaluate the effects of treatment with Aβ oligomer (AβO), fructose, Chol and LPS, individually and in combination with each other. One set of experiments was performed on 6-well plates and the second set on 96-well plates. Both approaches, prior to analysis, differ only by the number of cells seeded per well, where microglia for the 6-well plates (GenClone, 25-105MP) were seeded at 400,000 cells per 2 mL, per well; and the microglia on 96-well microplates (Corning; Costar; 3916) were seeded at 40,000 cells per 200 μL, per well. In both sets of experiments HMC3 microglia were cultured using EMEM (ATCC 30-2003), penicillin-streptomycin (10,000 U/mL, Thermo Fisher, 15140122) and 10% fetal bovine serum (FBS, ATCC 30-2020). Seeded cells were allowed to adhere for at least 7 hours. Following adherence,

media was replaced with EMEM without phenol red (Thermo Scientific, C837K00) to reduce background signal, penicillin-streptomycin (10,000 U/mL, Thermo Fisher, 15140122) and 10% fetal bovine serum (FBS, ATCC 30-2020), along with the corresponding treatment, where applicable. Microglia were treated with amyloid β-Protein oligomer (AβO) (1-42) (HFIP-treated) (BACHEM, H-7442) at 2 μM for 24 hours, or fructose (Millipore Sigma, Fructose F0550000 100MG) at 50 mM for 24 hours [44], or the combination of both. At the 18h time-point, the HMC3 were loaded with water soluble Chol (Sigma-Aldrich, C4951) at 20 μg/mL, for 6 hours. At the 21h time-point, the cells were treated with LPS (Millipore Sigma, L4005-100MG) at 100 ng/mL for 3 hours. At 24 hours, the supernatant in all wells was removed, and stored at -80°C for analysis. Additionally, in all instances of Chol quantification, lysing was performed using a solvent prepared with chloroform (Sigma-Aldrich, C2432), isopropyl alcohol (Fisher Scientific, 67-63-0) and Np40/ Igepal Ca 630 (Sigma-Aldrich, 9002-93-1), at 7:11:0.1, respectively.

The first set of experiments performed with 2 technical and 2 biological replicates required the use of 6-well plates to generate sufficient material for the analysis of gene expression by qPCR, cellular and mitochondrial Chol concentrations, and ApoE content in the supernatant. Immediately following the treatments, the supernatant was removed and stored at -80°C. The microglia were harvested for RNA extraction and qPCR analysis and for mitochondrial isolation. The supernatant was probed for ApoE content. Chol measurements were performed on lysed samples of whole-cell microglia and lysed isolated mitochondria.

For qPCR analysis, cells were harvested using Cell scraper, 2-position blade, size: M (SARSTEDT, 83.3951). Total RNA from cultured cells was extracted using RNeasy Plus Mini Kit (QIAGEN, 74134). cDNA was synthesized using 100 ng RNA and iScript Reverse Transcription Supermix (BioRad). qPCR was performed using SsoFast™ EvaGreen Supermix

and CFX96 qPCR system (BioRad). The forward/reverse primer sequences used are listed in Table 1. Gene expression was normalized to an endogenous gene, β-actin. Relative cDNA levels for the target genes were analyzed by the $2^{-\Delta\Delta Ct}$ method.

Gene	Primer Sequence (Invitrogen)
IL-1β (Human)	FW: CCACAGACCTTCCAGGAGAATG RV: GTGCAGTTCAGTGATCGTACAGG
IL-6 (Human)	FW: CCAGCTATGAACTCCTTCTC RV: GCTTGTTCCTCACATCTCTC
TNF-α (Human)	FW: CTCTTCTGCCTGCTGCACTTTG RV: ATGGGCTACAGGCTTGTCACTC
β-Actin (Human)	FW: TCAAGATCATTGCTCCTCCTGAG RV: ACATCTGCTGGAAGGTGGACA

Gene	Primer Sequence (Bio-Rad)
ApoE (Human)	qHsaCED0044297

Table 1. Primers and their sequences used for qPCR analysis. Interleukin 1β (IL-1β), interleukin 6 (IL-6), tumor necrosis factor alpha (TNF-α), apolipoprotein E (ApoE).

Mitochondria were isolated using a commercially available kit (with Dounce Homogenizer) (abcam, ab110171) following the manufacturer’s instructions. The cellular content of unesterified and esterified Chol was measured using the Total Cholesterol Assay (Cell Biolabs, Inc, STA-390) following the manufacturer’s instructions. A portion of the culture supernatant was probed for ApoE content using Human ApoE ELISA (Cell Biolabs, Inc, STA-367) following the manufacturer's instructions.

The second set of experiments was conducted in 96-well plates with 3 technical and 3 biological replicates to measure the mitochondrial function and phagocytosis capacity. Immediately following the treatments, the supernatant was removed in order to peform ATP quantification (abcam, ab83355), ROS measurement (abcam, ab113851 DCFDA / H2DCFDA) and Phagocytosis activity (Molecular Probes, Vybrant, V-6694) according to manufacturer’s instructions for each kit.

To assess cellular morphology pictures were taken using the Summit SK2-14X 14.0MP PC/MAC Digital Microscope Camera (OptixCam, TMS-SK2-14X).

Statistical Analysis

Data were analyzed on R version 4.1.1 (R Foundation for Statistical Computing, Vienna, Austria). We inspected for normality using Shapiro-Wilk test. Data that were non-normally

distributed were log2 transformed. For qPCR analysis, the $2^{-\Delta\Delta CT}$ method was used to analyze the relative changes in gene expression. For multiple comparison analysis, an ANOVA post-hoc Dunnett's test was performed to compare treatments to the control. Results were considered statistically significant at $P < 0.05$.

3. Results

Effects of treatments on expression of pro-inflammatory cytokine genes and APOE in HMC3 microglia

As shown in **Figure 1** treatments containing LPS generally resulted in an induction of tumor necrosis factor alpha (TNF-α) and interleukin 6 (IL-6) expression. LPS and fructose+LPS also induced IL-1β. AβO-containing treatments and fructose alone but not AβO alone induced ApoE expression. The combination treatment Chol+AβO+Fru+LPS induced ApoE and TNF-α expression the most, whereas Chol alone decreased ApoE expression.

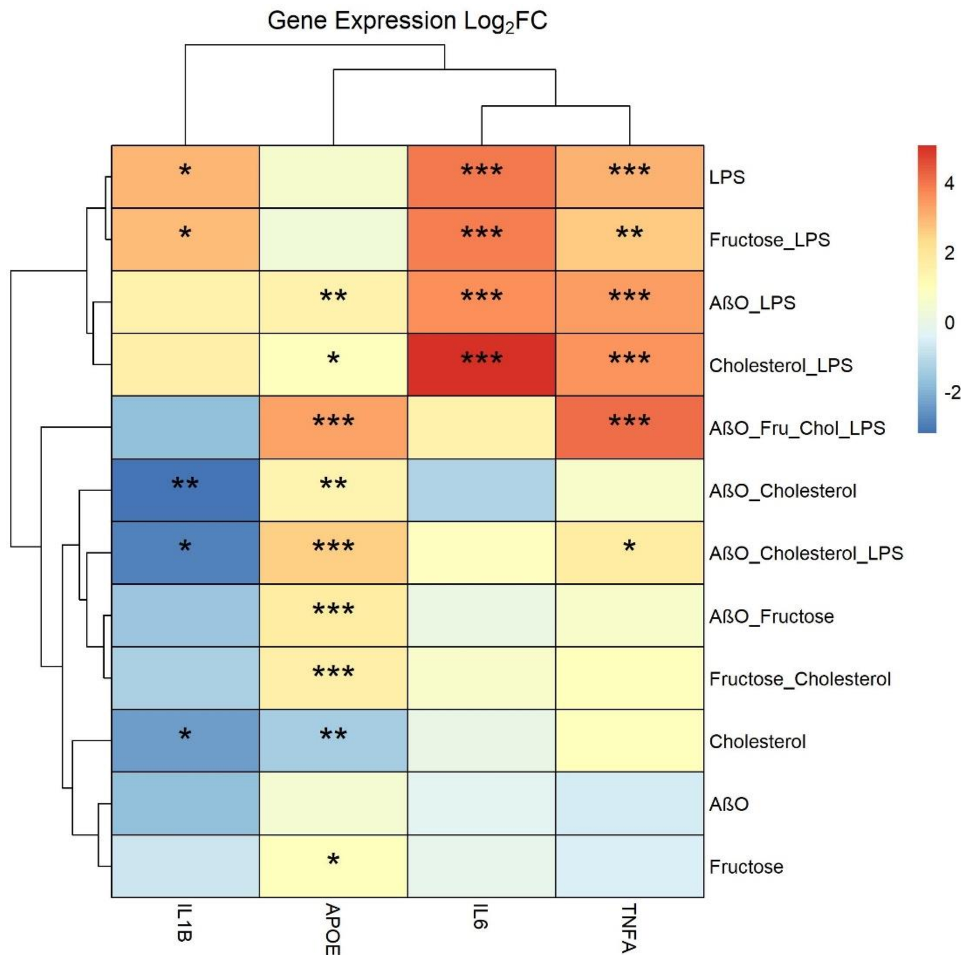


Figure 1. Effects of treatments on expression of pro-inflammatory cytokine genes and APOE in HMC3 microglia. Heat map from qPCR analysis from treated HMC3 microglia on 6-well plate. Terms: Lipopolysaccharide (LPS), amyloid beta oligomer (AβO), cholesterol (Chol), fructose (Fru), tumor necrosis factor alpha (TNFA), interleukin 6 (IL6), interleukin 1β (IL1B), apolipoprotein E (APOE) expression. n=2 independent cultures. Data presented as fold change, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Effects of treatments on whole-cell total and esterified cholesterol concentrations, mitochondrial total cholesterol concentrations and ApoE secretion in HMC3 microglia

It was observed that whole-cell Chol content was increased by all AβO and Chol-containing treatments but not AβO or Chol alone (**Figure 2A**). Fructose alone and in combination increased cellular cholesterol the most (**Figure 2A**). Esterified cholesterol concentrations were increased by AβO alone and fructose alone, as well as by all combination treatments, except

AβO+Chol, with the AβO+Fru+Chol+LPS having the strongest effect (**Figure 2B**). Mitochondrial total Chol content was significantly increased only by the combination of AβO+Fru+Chol+LPS (**Figure 2C**). In contrast to the observation of increased APOE gene expression (**Figure 1**), ApoE secretion was decreased in AβO-containing combination treatments, by the combination of Fructose+Chol and especially by AβO alone (**Figure 2D**).

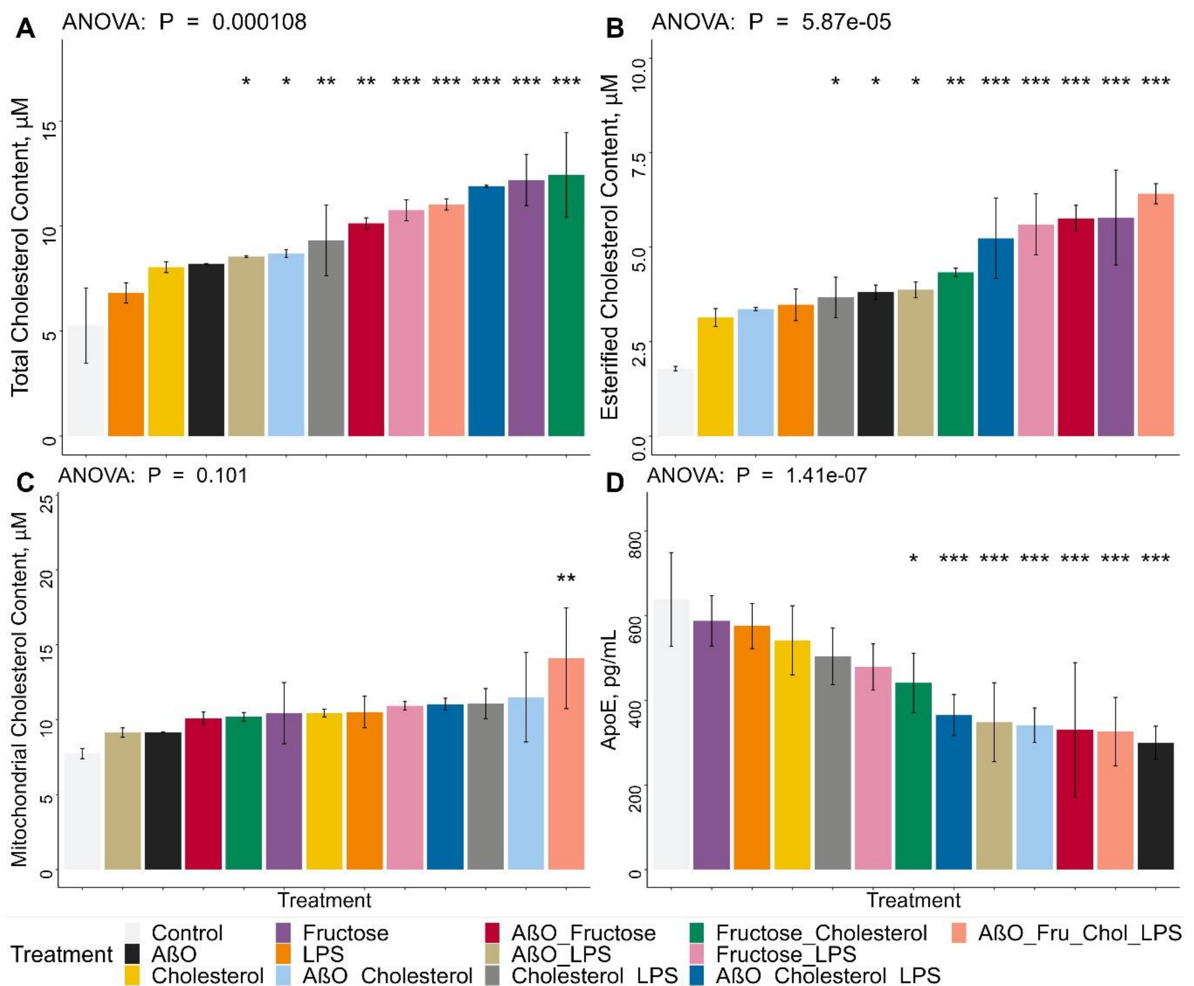


Figure 2. Effects of treatments on whole-cell total and esterified cholesterol concentrations, mitochondrial total cholesterol concentrations and ApoE secretion in HMC3 microglia. A) Total cholesterol concentration measurement performed on lysed whole-HMC3 microglia following treatments on 6-well plates. Fluorescence readings were compared to the cholesterol standard curve. n=2, two biological replicates. B) Esterified cholesterol concentration calculated from the fluorescent reading of the reaction without the esterase enzymatic component; performed on lysed whole-HMC3 microglia following treatments on 6-

well plates. Fluorescence readings were compared to the cholesterol standard curve. n=2, two biological replicates. C) Mitochondria total cholesterol concentration measurement performed on lysed isolated HMC3 microglial-mitochondria following treatments on 6-well plates. Fluorescence readings were compared to the cholesterol standard curve. n=2, two biological replicates. D) ApoE ELISA quantification performed on the supernatant from treated HMC3 microglia on 6-well plate. Fluorescence readings were compared to the human ApoE standard curve. n=2, two biological replicates. Lipopolysaccharide (LPS), amyloid beta oligomer (AβO), cholesterol (Chol), fructose (Fru), apolipoprotein E (ApoE) protein. Data presented mean ± SD unpaired two-tailed t test, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Effects of treatments on HMC3 microglia ROS and ATP concentrations and phagocytic activity

ATP production was significantly reduced by AβO+Chol, AβO+Chol+LPS, and especially by the combination of AβO+Fru+Chol+LPS which reduced ATP production the most (**Figure 3A**). Whole-cell reactive oxygen species (ROS) production was significantly increased by AβO alone and the combination of AβO+Fru+Chol+LPS (**Figure 3B**). Phagocytosis events were significantly lower in cells treated with AβO alone and AβO in combination with fructose, LPS, and the combination of Fru+Chol+LPS (**Figure 3C**). LPS alone and fructose+LPS also reduced phagocytosis (**Figure 3C**).

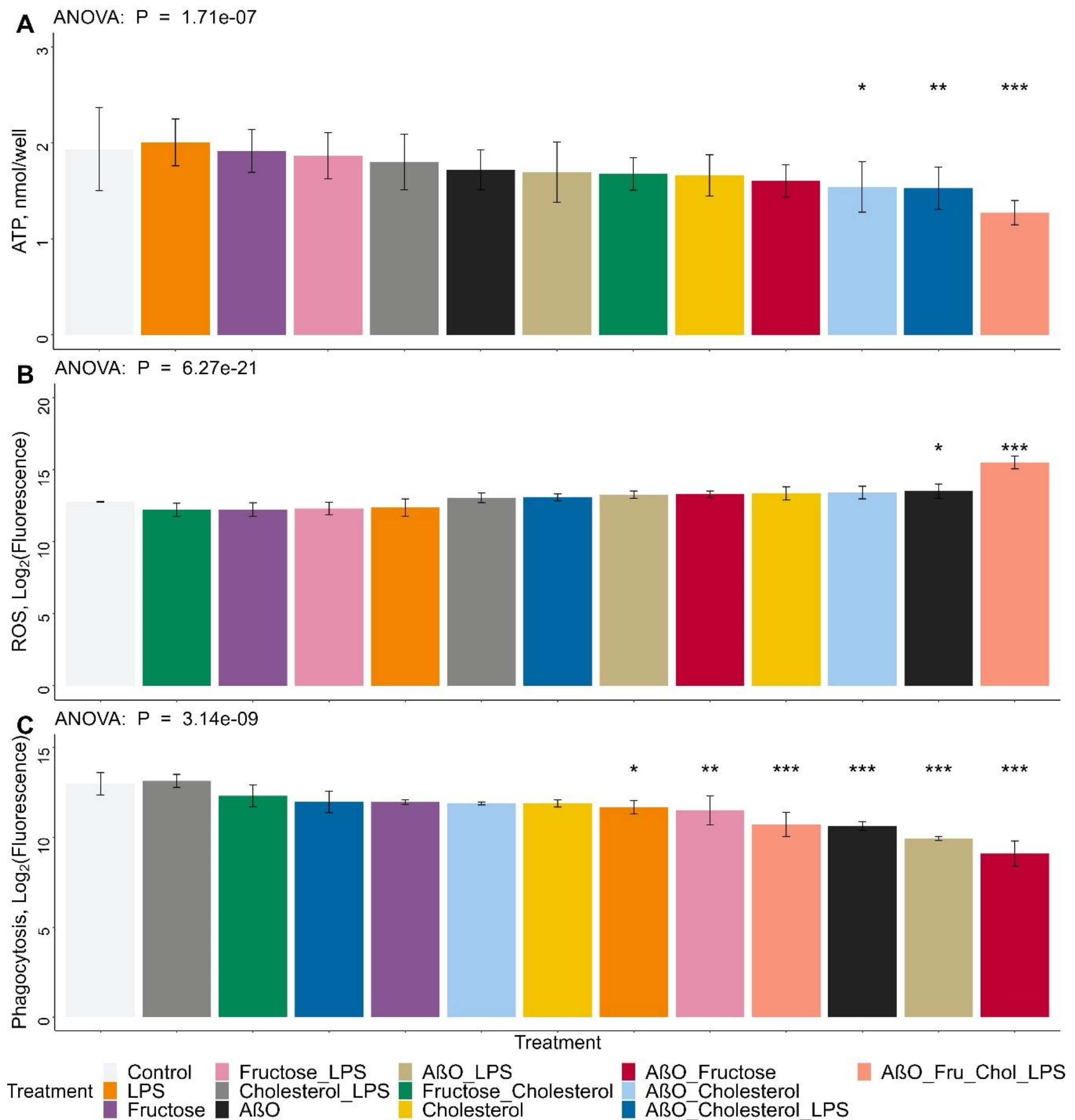
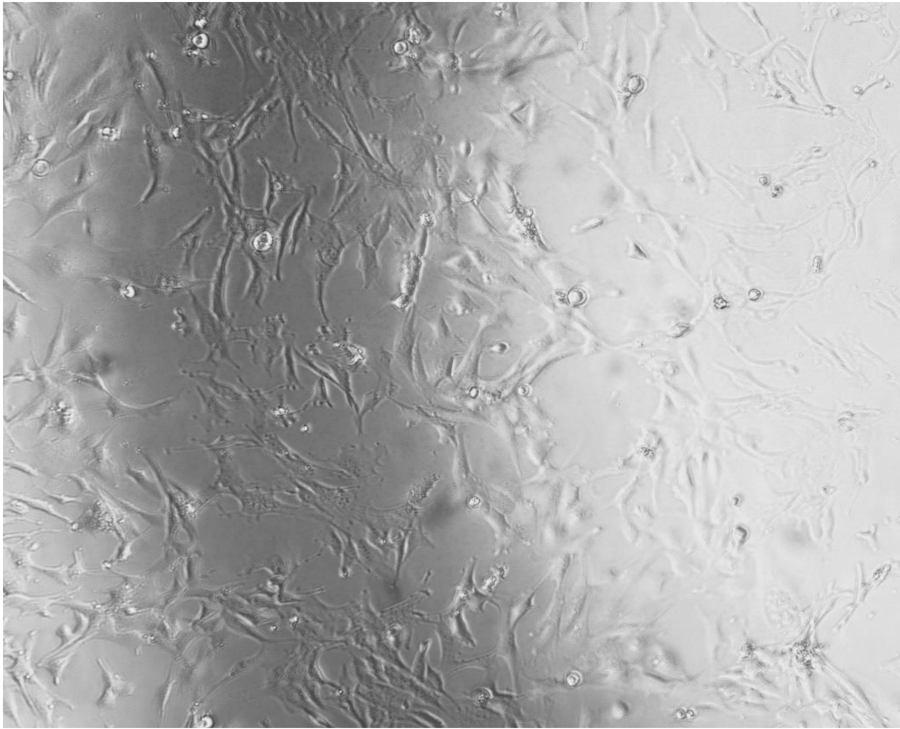


Figure 3. Effects of treatments on HMC3 microglia ROS and ATP concentrations and phagocytic activity A) Whole-cell adenosine triphosphate (ATP) fluorescence measurements on 24-hour period treated HMC3 microglia on 96-well plates. Fluorescence readings were compared to the ATP standard curve. n=3, three biological replicates B) Whole-cell reactive oxygen species (ROS) fluorescence measurements by reaction with DCFDA / H2DCFDA, on 24-hour period treated HMC3 microglia on 96-well plates. n=3, three biological replicates. C) Phagocytosis activity, quantified by fluorescein-labeled Escherichia coli (K-12 strain) readings on 24-hour period treated HMC3 microglia on 96-well plates. n=3, three biological replicates. Terms:

Lipopolysaccharide (LPS), amyloid beta oligomer (AβO), cholesterol (Chol), fructose (Fru). Data presented mean ± SD unpaired two-tailed t test, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

Effects of treatments on HMC3 microglia morphology

Untreated HMC3 microglia at timepoint 0 hours (**Figure 4A**) can be described as elongated and spindly. HMC3 microglia following a 24-hour period of treatment with the combination of AβO+Fru+Chol+LPS, on the other hand, can be described as contracted, semi-circular shapes (**Figure 4B**). HMC3 microglia at the 24h time-point, following AβO treatment appear to roughly keep the elongated and spindly morphology (Supplemental Figure 1A). Similarly, HMC3 microglia at the 24h time-point following Chol) AβO+Chol, fructose+Chol and Chol+LPS treatment (Supplemental Figure 1C, 1F, 1H, and 1J, respectively), also keep the elongated and spindly morphology. Following AβO+Fru, AβO+LPS and AβO+Chol+LPS treatment (Supplemental Figure 1E, 1G, and 1K, respectively) HMC3 microglia have a mixed morphology, with roughly 30% to 50% of the cell population abandoning the elongated and spindly morphology and adopting a contracted, semi-circular shape. Following fructose, LPS and Fru+LPS treatment (Supplemental Figure 1B, 1D, and 1I, respectively) HMC3 microglia have the contracted, semi-circular shape roughly 60% to 80% of the time, while the spindly and elongated morphology makes up the minority of the microglial population.



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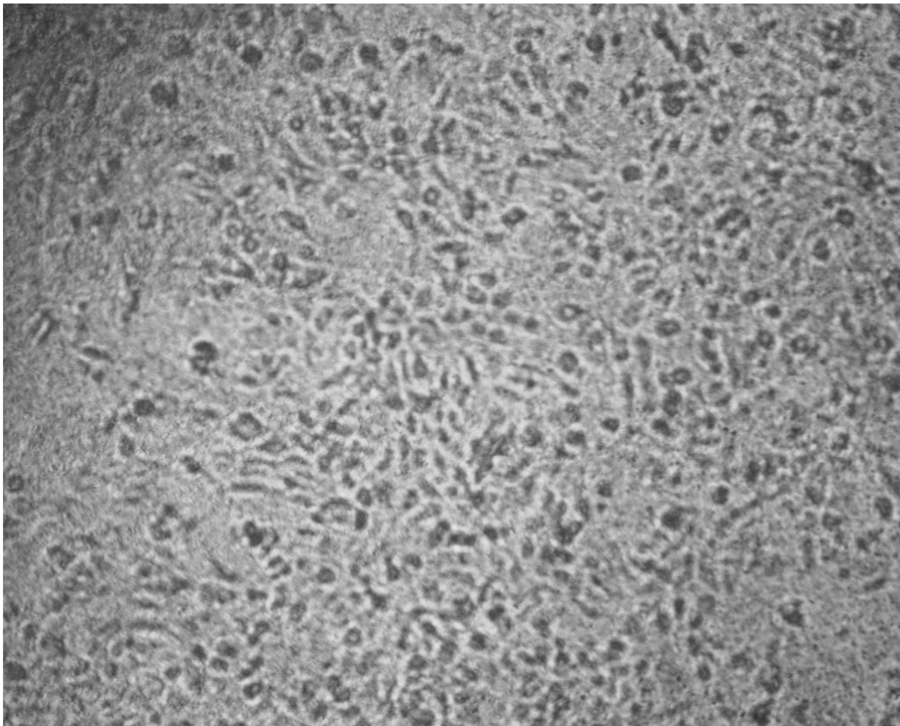

B

Figure 4. Effects of treatments on HMC3 microglia morphology A) Untreated HMC3 microglia at timepoint 0 hours, on 96-well plates. n=3, three biological replicates. Digital

Microscope Camera was used for the picture. B) HMC3 microglia following the 24-hour period treatment with AβO+Fru+Chol+LPS. n=3, three biological replicates. Digital Microscope Camera was used for the picture. Terms: Lipopolysaccharide (LPS), amyloid beta oligomer (AβO), cholesterol (Chol), fructose (Fru).

4. Discussion

AD is defined by the accumulation of Aβ [45] and DAM were defined by their colocalization with Aβ plaques [46], thus Aβ is the most obvious mediator of the DAM phenotype. Aβ deposition induces the expression of neurotoxic pathways, including pro-inflammatory cytokines [47]. In our study, AβO alone reduced the concentration of ApoE secretion to one of the lowest concentrations and increased whole-cell esterified Chol concentrations, in addition to increasing ROS concentrations and reducing phagocytic activity. AβO-containing treatments (AβO+LPS, AβO+Chol, AβO+fructose and AβO+Chol+LPS) induced APOE expression, but reduced ApoE secretion, while also increasing whole-cell total Chol and CE. AβO+fructose and AβO+LPS additionally reduced phagocytosis.

However, there are additional factors that are known to drive pathophysiology in AD, and in fact the amyloid hypothesis has recently come into question in the AD field, due to emerging evidence that some individuals can have no neurological symptoms even in the presence of very high Aβ plaque burden [48], and due to recent failures and controversies with regard to the effectiveness of drugs targeting Aβ [49]. AD is likely a multi-factorial disease with poor management of concentrations of additional factors such as fructose, LPS, and Chol also playing roles in the development of microglia dysfunction [37–43]. In fact, in our experiments, the biggest changes were induced by the combination treatment containing all 4 stimulants (AβO+Fru+Chol+LPS), which increased cytokine expression, increased APOE expression but resulted in one of the lowest levels of ApoE secretion and some of the highest concentrations of

whole-cell total Chol, CE, and mitochondrial Chol concentrations, as well as increasing ROS concentrations to the highest level, reducing ATP concentrations to the lowest level, and reducing phagocytic activity.

APOE is highly expressed in microglia, and APOE4 is shown to promote the neurodegeneration-associated inflammatory phenotype of mouse microglia [50] and alter functions of human microglia-like cells (iMGLs) [51]. It has been suggested that APOE4 confers a proinflammatory DAM phenotype [51,52]. ApoE4 carriers have lower cholesterol efflux capacity and lower mitochondrial function [53]. APOE4 iMGLs are fundamentally unable to mount normal microglial functionality when compared to APOE3, and APOE4 genotype impaired phagocytosis and migration, and aggravated inflammatory responses of iMGLs [54]. Future studies are needed to determine the APOE genotype of the HMC3 microglia cell line.

In previous work it was shown that primary microglia from mice fed a diet constituting 29% fat, 34% sucrose, and 1.25% cholesterol (w/w) plus 42 g/L glucose and fructose (55%/45%, w/w) in drinking water had increased expression of IL-6 and TNF-α [55]. Fructose alone induced APOE expression, while Chol alone dampened APOE and IL-1β expression. Additionally, fructose alone increased whole-cell total Chol and CE content. The combination of Chol+fructose induced APOE expression; however, this combination reduced ApoE secretion to similar concentrations as AβO+Fru+Chol+LPS. Chol+fructose also increased whole-cell total and esterified Chol to even greater concentrations than their individual effects. But it was only the AβO+Fru+Chol+LPS treatment that increased mitochondrial total Chol concentrations. ATP concentration was only significantly reduced by the combined treatment with Chol+fructose, and not by either individually; and phagocytosis was only significantly reduced with AβO+Fru+Chol+LPS. When the expression of rate-limiting enzymes in glucose metabolism are

inhibited in primary microglia and in the BV2 microglia cell line, fructose-6-phosphate inhibits ATP production and phagocytosis [56]. However, in this study we did not find a significant reduction in ATP concentration or phagocytosis when HMC3 were treated with fructose alone, however, the combination treatment including Chol+AbO+Fructose+LPS did decrease both ATP production and phagocytosis events.

In our study, LPS-containing treatments induced the expression of pro-inflammatory cytokines. iMGLs robustly responded to LPS with significant induction in all measured cytokines (IL-6, TNFα, among others) [54], similarly observed in HMC3 microglia [57]. TNF-α, IL-6, IL-1β are also expressed in primary human microglia when treated with LPS and when treated with Aβ 1-42 during a 6 hour period [58]. However, in our experiments, we did not see the expression of these genes with AβO alone. LPS-treated HMC3 cells were previously shown to exhibit an appearance characteristic of activated microglia that generally had larger cell bodies, fewer branches, and an amoeboid phenotype [57]. In our experiments, AβO+LPS and AβO+Chol+LPS treatments shift a portion of the microglia to that amoeboid phenotype. An even greater percentage of the microglia experience that same morphological shift with the LPS treatment, in agreement with Baek *et al*, and the Fru+LPS treatment. However, the potency of the morphological shift away from the elongated and spindly, to the semi-circular, fried-egg morphology was better achieved with the combination treatment of Chol+AbO+Fructose+LPS. LPS stimulation in the BV2 microglia cell line did not result in an increase of total and esterified Chol levels [59], which is in agreement with our observations in HMC3 microglia that LPS alone did not influence cellular Chol content. .

We demonstrate in this paper that HMC3 microglia, a verified human cell line [4], treated with a combination of Chol+AbO+Fructose+LPS replicate multiple critical aspects of the DAM

phenotype, including increased expression of pro-inflammatory cytokines, increased intracellular Chol accumulation, increased mitochondrial Chol accumulation, decreased ApoE secretion, decreased mitochondrial function, and decreased phagocytosis activity. Thus, HMC3 microglia treated with Chol+AbO+Fructose+LPS is a promising tool that could be used in high-throughput screening platforms to test potential therapeutics compatible with this 24h treatment window [60,61]. The advantage of this tool for high-throughput screening is several-fold: 1) the HMC3 microglia cell line is easy and affordable to grow and maintain, 2) treatment with the combination of Chol+AbO+Fructose+LPS appears to adequately reproduce critical aspects of the DAM phenotype observed in AD, 3) and the readouts (total cellular Chol, ATP, and ROS content, ApoE content in the supernatant, phagocytic activity and cell morphology) are also easy and affordable to measure. Future studies can now test this new screening tool to discover candidate molecules capable of reversing the induced cellular dysfunction, which can then be further tested for their effectiveness with more sophisticated methodologies including iPSC cells and animal models.

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