THE EFFECTS OF DIETARY FATTY ACIDS ON MICROGLIAL IMMUNE RESPONSES

by

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Abstract

The increasing number of Canadians with dementia is projected to reach one million by 2033. The most common cause of dementia is Alzheimer's disease (AD). Modifiable risk factors for AD, including dietary factors, should be investigated to inform recommendations for disease prevention. Dietary fatty acids, including polyunsaturated fatty acids (PUFAs), monounsaturated fatty acids (MUFAs) and saturated fatty acids (SFAs), are modifiable dietary components. PUFAs affect immune functions in the periphery and the central nervous system (CNS). Microglia are CNS immune cells that release inflammatory mediators including chemokines, cytokines, reactive nitrogen species (RNS) and reactive oxygen species (ROS). In AD, microglia are chronically activated by abnormal proteins, which results in excessive production of inflammatory mediators, promoting neuron death.

The fatty acids studied in this thesis included the n-3 and n-6 PUFAs α-linolenic acid (ALA) and linoleic acid (LA), the MUFA oleic acid (OA), and the SFA stearic acid (SA). While this thesis presents experiments containing two glucose conditions, effects of fatty acids on the microglial release of RNS, ROS, monocyte chemoattractant protein (MCP)-1 and neurotoxins were examined using high glucose conditions (17.0-17.3 mM). Gas chromatography assessed fatty acid content of cells exposed to fatty acids in the presence of 5.6 mM and 17.0 mM glucose. The Griess assay assessed RNS, followed by immunoblotting for inducible nitric oxide synthase (iNOS). Chemiluminescence was used to measure ROS, and enzyme-linked immunosorbent assays were used to detect MCP-1. Neuronal SH-SY5Y cells were incubated with supernatants from microglia exposed to fatty acid to assess impact of released neurotoxins.

In activated BV-2 microglia cultured in high glucose media (17.0 mM), fatty acids ALA and LA reduced microglial RNS release, and LA reduced the expression of iNOS. In microglia cultured in high glucose media (17.0-17.3 mM), no effects of fatty acids on phagocytosis, ROS release and MCP-1 release were identified. Except for SA, each fatty acid studied did not affect neurotoxic microglial secretions. My thesis demonstrates that dietary fatty acids inhibit select immune responses of microglia cultured in high glucose (17.0 mM). This data extends our knowledge basis needed for evidence-based dietary recommendations to prevent CNS inflammation.

Lay Summary

Each year, there are 10 million new global cases of dementia, and as the population ages, this number will increase. The most common cause of dementia is Alzheimer's disease (AD), which is associated with the death of neurons. Diet may be important in the development of this disease. To test whether dietary fats play a preventative role in AD, my thesis research investigated how four different dietary fats altered the responses of brain cells known as microglia. Microglia are immune cells required to maintain the healthy environment of the brain. In AD, microglia excessively release factors that cause inflammation, including reactive nitrogen species (RNS). This results in an environment that is toxic to neurons. After microglia were cultured in high glucose and treated with each fat, I observed decreased RNS levels. Thus, these fats should be further investigated for their protective roles in altering microglial responses involved in AD.

Preface

I am responsible for identification of the knowledge gap, as well as experimental work, with the exception of measuring monocyte-chemoattractant protein 1 and microglia-mediated neurotoxicity experiments. My contribution to these experiments was a supervisory role, with the experimental work completed by Carolyn Lee. Additionally, primary mouse microglia phagocytosis experiments were conducted by myself, but several replicates were analysed in a blinded fashion by Hannah Young. I was responsible for all other aspects of the described research, experimental procedures, and data analysis.

During my M.Sc. studies I authored the following publication:

Lowry, J.R., and Klegeris, A. (2018) Emerging Roles of Microglial Cathepsins in Neurodegenerative Disease. Brain Research Bulletin, 139:144-156. doi: 10.1016/j.brainresbull.2018.02.014

I also co-authored the following publications:

McKenzie, J.A., Spielman, L.J., Pointer, C.B., **Lowry, J.R.**, Bajwa, E., Lee, C.W., Klegeris, A. (2017) Neuroinflammation as a Common Mechanism Associated with the Modifiable Risk Factors for Alzheimer's and Parkinson's Diseases. Current Aging Science, 10:158-176. doi: 10.2174/1874609810666170315113244

Pointer, C.B., Slattery, W.T., Spielman, L.J., McKenzie, J., **Lowry, J.R.**, Lee, C., Klegeris, A. (2016). Neuroimmune interactions of astrocytes with other central nervous system cell types. In *New Developments in Astrocytes Research* [in press]. New York, NY: Nova Science Publishers, Inc.

Schindler, S., Spielman, L., Pointer, C., Slattery, W., Bajwa, E., McKenzie, J., **Lowry, J.**, Klegeris, A. (2015). An Interplay between Neuroinflammation and Modifiable Risk Factors of Alzheimer's Disease, Parkinson's Disease and Amyotrophic Lateral Sclerosis. In R. Dawson (Ed.), *Neuroinflammation in Disease: Risk Factors, Management and Outcomes* (pp. 1-55). New York, NY: Nova Science Publishers, Inc.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

Aβ	Amyloid-β Peptide
AA	Arachidonic Acid
AD	Alzheimer's Disease
ALA	α-Linolenic Acid
ANOVA	Analysis of Variance
APP	Amyloid Precursor Protein
APS	Ammonium Persulfate
BBB	Blood-Brain Barrier
BCA	Bicinchoninic Acid
BNAD	β-Nicotinamide Adenine Dinucleotide
BSA	Bovine Serum Albumin
CBS	Calf Bovine Serum
CHL	Chemiluminescence
CNS	Central Nervous System
COX	Cyclooxygenase
CTCF	Corrected Total Cell Fluorescence
DAPI	4'6-Diamidino-2-Phenylindole
dH_2O	Deionized H_2O
DHA	Docosahexaenoic Acid
DMEM	Dulbecco's Modified Eagle Medium: F12/Ham
DMF	Dimethylformamide

DMSO	Dimethyl Sulfoxide
DPH	Diaphorase
ECL	Enhanced Chemiluminescence
eNOS	Endothelial Nitric Oxide Synthase
FMLP	N-Formylmethionine-Leucyl-Phenylalanine
GC-FID	Gas Chromatography with a Flame Ionization Detector
GPR	G Protein-Coupled Receptor
HBSS	Hanks' Balanced Salt Solution
HDL	High-Density Lipoprotein
HETE	Hydroxyeicosatetraenoic Acid
HODE	Hydroxyoctadecadienoic Acid
HRP	Horseradish Peroxidase
IFN	Interferon
IL	Interleukin
iNOS	Inducible Nitric Oxide Synthase
INT	Iodonitrotetrazolium Chloride
JAK/STAT	Janus Kinase/Signal Transducers and Activators of Transcription
LA	Linoleic Acid
LDH	Lactate Dehydrogenase
LDL	Low-Density Lipoprotein
LOD	Limit of Detection
LOX	Lipoxygenase
LPL	Lipoprotein Lipase

LPS	Lipopolysaccharide
MCP	Monocyte Chemoattractant Protein
MeDi	Mediterranean Diets
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide
MUFA	Monounsaturated Fatty Acid
NADH	Nicotinamide Adenine Dinucleotide
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NF-κB	Nuclear Factor κ-Light-Chain-Enhancer of Activated B Cells
NFT	Neurofibrillary Tangle
NO	Nitric Oxide
OA	Oleic Acid
OD	Optical Density
PBS	1 X Phosphate-Buffered Saline
PBS-T	1 X Phosphate-Buffered Saline-Tween
PFA	Paraformaldehyde
PUFA	Polyunsaturated Fatty Acid
RIPA	Radioimmunoprecipitation Assay
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
SA	Stearic Acid
SDS	Sodium Dodecyl Sulfate
SFA	Saturated Fatty Acid
SIP	Stock Isotonic Percoll

SMP	Skim Milk Protein
TBI	Traumatic Brain Injury
TBS-T	Tris-Buffered Saline-Tween
TEMED	Tetramethylethylenediamine
TLR	Toll-like Receptor
TNF	Tumor Necrosis Factor

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I would like to express my deepest gratitude towards all of the incredible people who have supported me during my studies at the University of British Columbia Okanagan campus. First, I wish to express my sincere appreciation to Dr. Andis Klegeris for advising me throughout the research process, as well as helping me develop and hone my professional abilities. I wish to also extend a special thank you to my committee members, Dr. Sanjoy Ghosh and Dr. Frederic Menard, whose thoughts and advice have proven invaluable during this process. Thank you to past and current members of Dr. Ghosh's lab for your assistance with collecting and analysing gas chromatography data. My appreciation also goes to members of Dr. Barker's lab, for their fluorescence microscopy expertise and assistance with data collection using microscopy and immunoblotting.

I would like to acknowledge with a special thank you to past and current members of the Laboratory for Cellular and Molecular Pharmacology, for their help troubleshooting, editing, and laughing with me in times of stress. To Nijiati Abulizi and Nishat Tasnim, thank you for the insightful discussions, and support throughout each step of my thesis.

It would be impossible to acknowledge everyone who has contributed to my success during my graduate degree. I wish to thank all of my friends and family who have supported me during this process. Thank you for supporting me throughout my university journey, listening to my struggles, and offering words of encouragement. To my parents: your unwavering support is how I have come this far. Thank you for everything. Finally, I wish to thank my partner for their encouragement, emotional support, and patience during hours upon hours of "science talk".

For my parents

Chapter 1: Introduction

1.1 Cells of the Central Nervous System (CNS)

The central nervous system (CNS) is comprised of neuronal and non-neuronal cells, which have unique physiological functions. Neurons are the main excitable cells of the brain, whereas glia, the immune and helper cells of the brain, support the functions of neurons. The main types of glia include astrocytes, microglia and oligodendrocytes, and they offer both trophic and neuroimmune support to the CNS (Hanisch and Kettenmann, 2007; Lee and MacLean, 2015; Ransohoff, 2016). The above types of glia and their critical functions in CNS homeostasis are described in Figure 1.

Astrocytes primarily function as support cells of the brain. Astrocytes associate with endothelial cells and pericytes in the blood brain barrier (BBB), and regulate the passage of molecules, including nutrients and oxygen required for neuron metabolism (Lecrux and Hamel, 2011). Astrocytes support neurons through their responses to neuronal signals, such as release of the signaling molecule nitric oxide (NO), in addition to regulating neurotransmitter abundance in the CNS and participating in the turnover of neuronal synapses by phagocytosis (Ben Haim et al., 2015; Chung et al., 2013). Furthermore, astrocytes become activated to release pro-inflammatory signals in response to disruptions in CNS homeostasis, including an altered environment of "healthy" neuronal signaling molecules, or an accumulation of abnormal proteins (Ben Haim et al., 2015).

Another type of glia, oligodendrocytes, provide physical support to neurons in the form of the myelin sheath (Jessen, 2004), which is an insulating physical barrier for neurons. The myelin sheath also participates in neuronal metabolism, providing a supporting role in ion and energy regulation (Saab and Nave, 2017).

Microglia are the resident immune cells of the brain that constantly monitor and maintain the microenvironment of the CNS. Microglia promote CNS homeostasis through immune responses, phagocytosis of cellular debris and dysfunctional neuronal synapses, regulation of neuronal synapse activity and neurotransmitter recycling (Brown and Neher, 2014; Clark et al., 2015; Hanisch and Kettenmann, 2007). Microglia can detect a wide range of endogenous and exogenous stimuli that promote their activation, resulting in the release of signaling molecules, which can be anti-inflammatory or pro-inflammatory depending on the activating stimulus. For example, stimuli including endogenous cytokines such as tumor necrosis factor (TNF)-α or interferon (IFN)-γ, and exogenous mediators such as bacterial lipopolysaccharide (LPS) promote a pro-inflammatory phenotype of microglia (Brown and Vilalta, 2015; Hanisch and Kettenmann, 2007; Lee and MacLean, 2015). Upon activation, microglia release pro-inflammatory mediators including reactive oxygen species (ROS) and reactive nitrogen species (RNS), as well as cytokines and chemokines, to facilitate removal of the activating stimulus (Lee and MacLean, 2015). Microglial activation and their secretion of pro-inflammatory mediators is a significant feature of Alzheimer's disease (AD) and other neurodegenerative diseases (Brown and Vilalta, 2015).

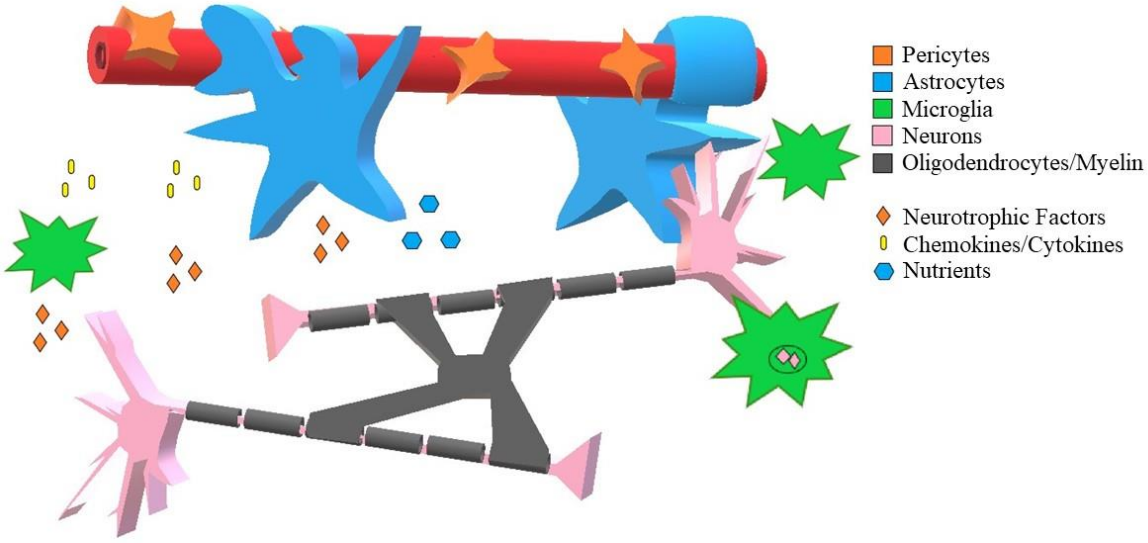

Figure 1. Glia of the central nervous system (CNS). Glia regulate CNS functions by the release of neurotrophic factors, pro-inflammatory and pro-resolving chemokines and cytokines, and nutrients (Brown and Vilalta, 2015; Hanisch and Kettenmann, 2007; Lee and MacLean, 2015). The end feet of astrocytes associate with pericytes and endothelial cells of the blood brain barrier (BBB) to regulate the flow of nutrients (Lee and MacLean, 2015), whereas microglia are the primary immune cells of the CNS. Astrocytes and microglia can phagocytose neuronal synapses to eliminate weak connections as well as regulate synapse activity (Chung et al., 2013; Clark et al., 2015; Hanisch and Kettenmann, 2007). Oligodendrocytes provide metabolic and axonal support, including development of the myelin sheath (Jessen, 2004; Saab and Nave, 2017).

1.2 CNS Inflammation and Alzheimer's Disease (AD)

Abnormal protein aggregation in the CNS is an important feature of several neurodegenerative diseases. The hyperphosphorylation of tau protein and aggregation of toxic amyloid-β (Aβ) species are the two hallmarks of AD. Tau protein is an abundant intra-neuronal cytoskeletal protein, and tau hyperphosphorylation causes accumulation of neurofibrillary tangles (NFTs), which can lead to necroptotic neuron death (Caccamo et al., 2017; Kametani and Hasegawa, 2018). The second pathological hallmark of AD, which is the aggregation of Aβ into soluble Aβ oligomers and insoluble plaques, is due to excessive cleavage of the amyloid precursor protein (APP) (Ferreira and Klein, 2011). The presence of abnormal protein in the AD

brain causes adverse microglial activation (Bronzuoli et al., 2016). Moreover, microglial activation in turn promotes a chronic state of neuroinflammation, which propagates further activation of surrounding microglia. Due to the non-specific nature of change in the glial secretome upon activation, a positive feedback loop is generated, wherein microglial activation leads to neuroinflammation and excessive neuron death (Spielman et al., 2014). Some experts believe that this neuroinflammatory cycle is largely responsible for the disease progression and cognitive decline seen in AD (Whittington et al., 2017). Additionally, several other recently described modifiable risk factors may affect the chronic neuroinflammation observed in AD (Freeman et al., 2014; McKenzie et al., 2017).

1.3 Modifiable Risk Factors for AD Development

Modifiable risk factors for AD include certain cardiovascular conditions and type 2 diabetes mellitus, whose pathologies share common features of systemic and CNS inflammation (McKenzie et al., 2017). Dietary factors may also be important AD risk factors, and the types of fats consumed in the diet may be particularly significant (Freeman et al., 2014). Past studies have often focused on comparisons of high-fat (over 40-45% energy from fat) and low-fat (<10-30% energy from fat) diets, as the high caloric intake in high-fat diets is relevant to the development of obesity (Schrauwen and Westerterp, 2000; Schwingshackl and Hoffmann, 2013). Obesity is characterized by systemic and CNS inflammation and increased body mass (Guillemot-Legris and Muccioli, 2017). Systematic review comparing low-fat to high-fat diets found that both diets can be associated with certain beneficial aspects related to cardiovascular disease prevention (Schwingshackl and Hoffmann, 2013). Low-fat and high-fat diets may show these complex effects due to compositional changes of individual fatty acids between these diets, which are

difficult to control in human studies. It remains important to elucidate the cellular roles of such a significant part of human diet, namely dietary fatty acids, on human immune status.

An extensive understanding of the dietary composition of fatty acids and their physiological effects are required to provide evidence-based dietary recommendations. These recommendations may be beneficial for the prevention of type 2 diabetes mellitus and potential comorbid conditions including AD. Dietary patterns prevalent in North America and Northern Europe, often recognized as following a "western diet" pattern, are characterized by moderate fat intake (35-40% energy) and low intake of n-3 polyunsaturated fatty acids (PUFAs) compared to n-6 PUFAs (Feart et al., 2012; Simopoulos, 2016, 2011). As PUFAs play key roles regulating fatty acid oxidation, triglyceride content, and body weight in animals, the low intake ratio of n-3 to n-6 PUFAs in the western diet may contribute to the development of obesity (Hennig and Watkins, 1989; Massiera et al., 2010; Simopoulos, 2016, 2011; Ukropec et al., 2003). Further, many dietary fatty acids have systemic and CNS immunomodulatory activities, which highlight the possible relevance of these dietary factors in the development of AD (Layé et al., 2018; McKenzie et al., 2017).

1.4 Dietary Factors

Dietary factors may protect from age-related cognitive decline and the development of AD; however, meta-analyses have failed to find a significant positive impact of altered dietary fatty acids including n-3 PUFAs on cognition (Cooper et al., 2015; Lehert et al., 2015; Xu et al., 2015). Many of these studies focused on Mediterranean diets (MeDi), which have low caloric content and high levels of monounsaturated fatty acids (MUFAs) with an increased ratio of n-3 to n-6 PUFAs (Lehert et al., 2015; Nowson et al., 2018; Van De Rest et al., 2015; Xu et al.,

2015). Studies have confirmed the association between MeDi and reduced risk of age-related cognitive decline or dementia (Van De Rest et al., 2015). These results suggest that diet impacts cognition and dementia, and dietary fat may be a contributor (Martínez-Lapiscina et al., 2013; Nowson et al., 2018; Zazpe et al., 2008). As it is difficult to isolate dietary variables in human studies, data from *in vitro* experiments will be useful in determining the effects of fatty acids prominent in the MeDi on cells of the CNS. There is some i*n vitro* data to suggest an anti-inflammatory contribution of high content of a MUFA, oleic acid (OA), and an n-3 PUFA, docosahexaenoic acid (DHA), which are prominent in the MeDi (Debbabi et al., 2017). In this study, OA and DHA both reduced ROS production and apoptosis of microglial BV-2 cells following exposure to a bioactive sterol, 7-ketocholesterol. These types of studies may provide insight into particular types of fatty acids in the MeDi that may contribute to the beneficial outcomes of these diets on human health.

The potential beneficial effects of the MeDi on cognition contrast those of the western diet, which is generally characterized by high caloric content from highly processed foods, high saturated fatty acid (SFA) content, and low n-3 PUFA to n-6 PUFA ratio (Cordain et al., 2005; Simopoulos, 2016). For instance, an estimated ratio of n-3 to n-6 PUFAs from dietary intake within the United States in 2016 was approximately 17:1 (Simopoulos, 2016). Recently, it was shown that mice reared on a mock western diet, which contained 16.4% total fat compared to 4.6% in the control diet, including 8% saturated fat compared to 1% in the control diet, displayed increased activation of both microglia and astrocytes (Graham et al., 2016). Limited evidence suggests that saturated fats may be a risk factor for cognitive decline in humans, further highlighting the negative impacts of the western diet, in comparison to the protective benefits of the MeDi (Eskelinen et al., 2008; Lee et al., 2010). These studies have been confirmed, by

Baierle et al. (2014), who found a positive correlation between human serum DHA and increased cognition, alongside a correlation of serum SFAs with reduced cognitive function (Baierle et al., 2014).

Thus, individuals who consume western diets with high levels of SFAs and low levels of n-3 PUFAs compared to n-6 PUFAs may have an increased risk of cognitive decline, compared to individuals who do not regularly consume high levels of SFAs and n-6 PUFAs. Understanding how fatty acids from diet affect the brain and impact neuroinflammation is important to help explain the mechanisms of the potential link between dietary fatty acid and cognition, which is still poorly understood.

1.5 Dietary Fats and the CNS

1.5.1 Ingestion of Dietary Fats

Dietary fats in the form of free (unesterified) fatty acids are absorbed in the small intestine (Wang et al., 2013). Triglycerides and phospholipids are two common esterified fatty acids in diet that are cleaved by lipases into smaller fatty acid chains; these free fatty acids are repackaged into triglycerides, which are esterified into lipoproteins for transport through the lymphatic system into circulation, or they are transported directly by the portal venous system to the liver (Sigalet and Martin, 1999; Wang et al., 2013).

The circulatory system transports free and esterified fatty acids to the BBB, before entry into the CNS (Banks et al., 2018; Bazinet and Laye, 2014). Upon entering circulation, lipoproteins, including chylomicrons, low-density and high-density lipoproteins (LDLs and HDLs) are carried to the tissue, where they can bind to lipoprotein lipase (LPL) present in endothelium. LPL releases triglycerides from HDLs and LDLs, some of which remain in

circulation. In circulation, triglycerides undergo recycling reactions to free fatty acids. When in circulation, free fatty acids bind to serum albumin (in μM concentrations, in a ratio of 0.5 to 2 free fatty acids per each albumin protein), with a much lower level (nM concentrations) of serum fatty acids not bound to albumin (Richieri and Kleinfeld, 1995; Spector, 1975). The fatty acid carboxyl group binds to the cationic sites of serum albumin, with additional hydrophobic interactions between the fatty acid chain and albumin side chains (Spector et al., 1969).

Both free fatty acids and esterified fatty acids in lipoproteins are thought to be able to enter the CNS by passive diffusion and by active transport across the BBB (Banks et al., 2018; Bazinet and Laye, 2014; Layé et al., 2018). Within the CNS, fatty acids and their metabolites play several regulatory roles. Fatty acids and their metabolites are important in cell membrane organization (Shaikh and Teague, 2012), inter- and intra-cellular signaling (Bazinet and Laye, 2014), β-oxidation of fatty acids to generate energy (primarily in astrocytes) (Auestad et al., 1991), and intra-cellular storage in lipid droplets (Daemen et al., 2016). Fatty acids affect cellular receptors by interacting directly with receptors, or by producing fatty acid metabolites that interact with receptors. Within cells, including neurons, fatty acids and their metabolites can be re-formed from membrane phospholipids when they are recycled through reactions known as the Land's cycle (Bazinet and Laye, 2014; Tabe et al., 2016).

1.5.2. Fatty Acid Composition of CNS Glia

Some fatty acids can be formed in the CNS, including the MUFA OA, and the SFA stearic acid (SA). In the CNS, fatty acids are preferentially formed through the elongation pathway of fatty acid synthesis from malonyl-CoA compared to *de novo* fatty acid synthesis (Aeberhard and Menkes, 1968). For instance, SA is produced by the elongation of palmitic acid.

The MUFA OA can be produced from SA, which has been demonstrated in astrocytes of the developing rat brain, but this has yet to be described for the adult CNS (Tabernero et al., 2002). However, there is a variant of the enzyme that converts SA to OA, stearoyl-coA desaturase-5, which is expressed in the adult human brain (Wang et al., 2005).

Though MUFAs and SFAs can be produced in the CNS, the 18-carbon PUFAs, α-linolenic acid (ALA) and linoleic acid (LA), are essential in the diet. Once ingested, they can be converted in the liver to their metabolites DHA and the n-6 PUFA arachidonic acid (AA), respectively (Layé et al., 2018). Structures of the four 18-carbon fatty acids, ALA, LA, OA and SA are shown in Figure 2.

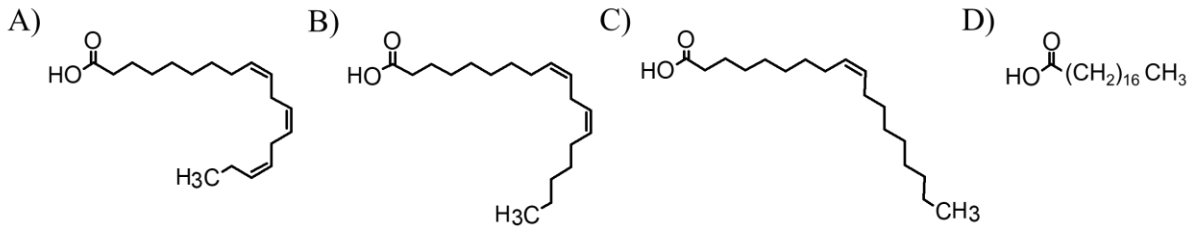

Figure 2. Structures of the 18 carbon fatty acids assessed in this study: (A) α-linolenic acid (ALA), (B) linoleic acid (LA), (C) oleic acid (OA), and (D) stearic acid (SA). This figure was created using ACD/ChemSketch, version 2017.2.1, Advanced Chemistry Development, Inc., Toronto, ON, Canada, www.acdlabs.com, 2017.

Fatty acid metabolism in microglial cells is currently not well understood. BV-2 mouse microglia are composed of higher proportions of both SFAs and MUFAs, compared to PUFAs (Button et al., 2014). These microglia have been shown to express mRNA of stearoyl-coA desaturase-2, which allows for conversion of SA to OA. Microglial membrane fatty acid composition was altered upon pro-inflammatory stimulation by LPS, which was associated with reduced activity of stearoyl-coA desaturase-2 (Button et al., 2014). Fatty acid treatment may also affect the organization of cellular membranes; for instance, DHA can affect a number of cell

membrane functions, including the ability to remodel microglial organelles and lipid rafts (Hellwing et al., 2018; Tremblay et al., 2016). Studies have shown *in vivo* altered PUFA composition in astrocytes and oligodendrocytes when rats were fed diets with different fatty acid compositions (Bourre et al., 1984). *In vitro*, the addition of LA also increased n-6 to n-3 PUFA phospholipid ratios of neonatal rat astrocytes (Murphy, 1995). In comparison to astrocytes, the fatty acid composition of microglial cells is largely unknown.

Intracellular fatty acid content, particularly of membrane phospholipids, may be an important component of the phagocyte response. Peripheral mouse macrophages treated with unsaturated fatty acids displayed an increased rate of phagocytosis of both zymosan particles and viable bacteria, when compared to the phagocytic activity of macrophages without fatty acid treatment (Adolph et al., 2012; Calder et al., 1990). In macrophages, the addition of unsaturated fatty acids including ALA or LA has been shown to double total unsaturated phospholipid content (Calder et al., 1990). This increased phagocytic effect in macrophages treated with unsaturated fatty acids may be due to either altered membrane fluidity upon fatty acid exposure, or changes in the reactive states of the macrophages (reviewed by Schumann, 2016). The hypothesis that increased membrane fluidity promotes phagocytosis is supported by literature, as compounds that increase fluidity, such as the sterol intermediate lanosterol, also enhance phagocytosis (Adolph et al., 2012; Araldi et al., 2017). Since microglia are phagocytes, their responses may also be affected by compositional membrane changes upon fatty acid treatment, but this has not been established for microglia.

1.6 Dietary Fats and Alzheimer's Disease

Dietary factors may impact cognition and AD pathogenesis; however, research has been unable to identify a fatty acid treatment that specifically lowers risk of AD development. Some research indicates a role of n-3 PUFA phospholipids in reducing cognitive decline (Phillips et al., 2012); yet, systematic reviews have failed to validate these findings (Burckhardt et al., 2016; Sydenham et al., 2012). These systematic reviews found no association between dietary n-3 PUFA intake and cognitive decline in humans with dementia as well as non-demented adults. Studies have indicated that DHA levels are reduced in the cerebrospinal fluid and brains of AD patients (Fonteh et al., 2014; Martín et al., 2010); however, conflicting reports indicate that DHA levels did not change (Fraser et al., 2010), or even increased in AD brains (Snowden et al., 2017).

Some metabolites of LA are found at increased levels in the plasma of AD patients compared to non-demented individuals, including 13-hydroxyoctadecadienoic acid (HODE), an important regulator of inflammation (Vangaveti et al., 2016; Yoshida et al., 2009). Limited evidence suggests that metabolism of the n-6 PUFA AA into its metabolites may be increased in the brains of individuals with AD (Esposito et al., 2008); however, other studies found that levels of AA were reduced in AD brains when compared to brains from non-demented individuals (Snowden et al., 2017). The inconsistencies in these research findings indicate a clear gap in understanding the link between fatty acid profiles and neurodegenerative disease pathology and thus merit further study.

MUFAs are a key component of the MeDi, such as oleamide, a metabolite of the MUFA OA found in fermented dairy products. In the 5x familial AD transgenic mouse model, which overexpresses human APP, dietary supplementation with fermented dairy products reduced

hippocampal $A\beta_{1-42}$ (Ano et al., 2015). Oral administration of oleamide in mice also increased *ex vivo* phagocytosis of $A\beta_{1-42}$ when its production was induced in primary microglia, demonstrating the potential neuroprotective effects of this fatty acid. Thus, while levels of various types of fatty acids may be dysregulated in AD brains, little is known about this dietary factor in the development of AD.

1.7 Immune Responses Induced by Fatty Acids

Whether the PUFAs ALA and LA have direct cell-modifying effects is a current source of controversy. Bioactive metabolites of these fatty acids are responsible for immunomodulatory effects and cell signaling activity, and thus these metabolites have been a current focus in research on inflammation (Bazinet and Laye, 2014; Vangaveti et al., 2016; Weylandt et al., 2012). From microarray analysis, high plasma ratios of LA to ALA in humans were found to be associated with increased expression of inflammatory markers, such as toll-like receptor (TLR)-8 (Olsen et al., 2013). A study involving Wistar rats showed that a diet rich in ALA promoted macrophage immune effects that were comparable to a diet rich in DHA (fish oil), which included increased phagocytic activity of neutrophils (Schiessel et al., 2016). However, due to various reactions that convert ALA to DHA and other metabolites, it is unknown which factors may be responsible for the altered immune responses. *In vivo* research has largely failed to elucidate direct inflammatory effects specific to the PUFAs ALA and LA alone.

Intake of the n-3 PUFA ALA in diet may induce protective functions of glial cells in the CNS. For example, following ischemia, rats given a diet enriched in ALA displayed reduced microglial activation (Liu et al., 2014). Similar results were reported in a study on mice with traumatic brain injury (TBI), where reduced astrocyte activation was observed for mice reared on

a diet that was supplemented with ALA (Desai et al., 2016). These outcomes may be neuroprotective, as ischemia and TBI are accompanied by persistent glial activation (Guruswamy and ElAli, 2017; Li et al., 2018; Ramlackhansingh et al., 2011). Neurodegeneration may follow either ischemia or TBI, and abnormal proteins may accumulate in TBI, linking these conditions to neurodegenerative disease (Johnson et al., 2010; Xiong et al., 2013; Zlokovic, 2011). Cerebroventricular injection of ALA in rats, followed by ischemic damage, reduced the presence of reactive microglia that was indicated by the microglial marker Iba-1 by almost 50% compared to rats that did not receive ALA (Liu et al., 2014). ALA may have a role in reducing the activation of microglia and altering their neuroimmune responses.

In the periphery, LA has demonstrated immunomodulatory functions. In primary human Muller cells, a type of retinal glia, treatment with LA induced the release of cytokines that promote inflammation, such as interleukin (IL)-6 (Capozzi et al., 2016). Other studies have found an anti-inflammatory role of LA treatment, which reduced TNF-α levels in mouse macrophages stimulated with LPS (Martins De Lima-Salgado et al., 2011). In activated mouse peripheral macrophages, the addition of either ALA or LA reduced both nitrite levels and ROS release (Ambrozova et al., 2010). This thesis is focused on exploring the effects of the fatty acids ALA and LA on microglial immune function. Research to explain which immune functions are specifically modulated by ALA and LA is currently lacking, whereas the primary metabolites of these PUFAs including DHA and AA have been extensively studied.

DHA is an important n-3 PUFA and a significant component of phospholipid membranes in brain tissue (Salem et al., 2001). This BBB-permeable fatty acid is acquired through diet or metabolized from ALA in the liver (Bazinet and Laye, 2014; Nguyen et al., 2014). DHA may play an important role in converting microglia from a pro-inflammatory activated state to an anti-

inflammatory and pro-resolving state, which is necessary for brain tissue repair following injury and inflammation (Harvey et al., 2015; Heras-Sandoval et al., 2016). DHA activates pro-resolving and anti-inflammatory pathways through the action of its bioactive metabolites, resolvins and neuroprotectins (Weylandt et al., 2012). These metabolites reduce the neuroinflammatory response and promote homeostasis. For instance, neuroprotectin D1 has been shown to reduce expression of cyclooxygenase (COX)-2 and TNF-α in a primary human neuronal-glial co-culture model (Zhao et al., 2011). Similarly, the family of resolvins can decrease mouse microglial immune responses, as measured by reductions in Iba-1 and TNF-α (Xu et al., 2013). Thus, the action of DHA on microglial immune responses has been extensively studied.

n-6 PUFAs, including AA and its derivatives, have been well-known for their pro-inflammatory actions. LA is the precursor to AA, and these fatty acids can be metabolized enzymatically or through oxidation reactions with ROS to produce a variety of metabolites (Niki, 2009; Vangaveti et al., 2016; Yoshida et al., 2015). Enzymatically, LA and AA are metabolized by lipoxygenase (LOX) and COX enzymes to generate immunomodulatory mediators, including 13-HODE, which is a regulator of inflammation in peripheral cells (Czapski et al., 2016; Vangaveti et al., 2016). AA-derived eicosanoids, including thromboxanes, prostaglandins and leukotrienes, are generally pro-inflammatory with some immunomodulatory functions (Alashmali et al., 2016; Levi et al., 1998). AA is converted to prostaglandins by COX enzymes, which are active in the brain (Eskilsson et al., 2017; Janssen et al., 2018; Vane et al., 1998). LOX enzymes metabolize AA to produce the eicosanoid molecules hydroxyeicosatetraenoic acids (HETEs) and leukotrienes (Czapski et al., 2016); this metabolism was shown to occur in microglia (Matsuo et al., 1995; Rademacher et al., 2004; Slepko et al., 1997). A positive

correlation between the level of brain AA phospholipids and activation of both microglia and astrocytes has been demonstrated (Xu et al., 2016). The release of AA from microglial cells has also been shown to promote microglial survival (Palomba et al., 2009), which may promote prolonged neuroinflammatory outcomes. Thus, metabolites of LA, including AA, may promote increased severity and duration of microglial pro-inflammatory responses.

Bioactive metabolites produced from AA and DHA in microglia may affect the immune status of the CNS in non-demented and AD brains. Fatty acids and their metabolites in the CNS can have a variety of pro-resolving and anti-inflammatory actions, as well as pro-inflammatory actions. Investigating the specific effects of fatty acids, including ALA and LA, on neuroinflammation will help to clarify the role of PUFAs in the progression of AD.

Measuring the effects of OA, a MUFA, has shown conflicting results related to neuroimmune function. For instance, OA increased the BV-2 mouse microglial secretion of matrix metalloproteinase-9, which is a pro-inflammatory mediator (Button et al., 2014). However, anti-inflammatory effects of OA have also been reported, such as the reduction of inducible nitric oxide synthase (iNOS) after LPS-stimulation in BV-2 cells and primary rat microglia (Oh et al., 2009). In addition, OA increased superoxide anion release by human neutrophils, but decreased superoxide anion release by BV-2 mouse microglia (Hatanaka et al., 2006; Oh et al., 2009). These data may be explained by differences in cell types and experimental methods used for superoxide detection. OA also improved neuronal viability after toxicity was induced by the exposure to an SFA (Kwon et al., 2014). Based on these results, further studies are required to elucidate the effects OA has on microglial function.

An amide metabolite of OA, oleamide, has anti-inflammatory potential. Oleamide and OA treatment reduced TNF-α levels in primary mouse microglia (Ano et al., 2015). Similarly,

OA and oleamide reduced levels of iNOS and prostaglandin E2 levels in primary rat microglia, and reduced ROS levels in BV-2 microglia, demonstrating the anti-inflammatory potential of these fatty acids (Oh et al., 2009; Oh et al., 2010b). Thus, OA and its metabolites may exert anti-inflammatory activity; however, there is some controversy in literature regarding this role.

The SFA SA can interact with multiple receptors and pathways to elicit pro-inflammatory and apoptotic effects. Both SA and its metabolites, which include the elongated SFAs arachidic acid and behenic acid, upregulated protein levels of pro-inflammatory cytokines including TNF-α and IL-1β in rat brain (Milanski et al., 2009). SA increased the microglial release of matrix metalloproteinase-9 from BV-2 microglia (Button et al., 2014). SFAs are known to stimulate the release of pro-inflammatory mediators through activation of TLR-4 pathways in the periphery (Lee et al., 2001). However, apart from the action on cell surface receptors, SFAs including SA can also induce inflammation upon accumulation in the cell. SA was shown to promote the activation of endoplasmic reticulum stress pathways in macrophages including c-Jun N-terminal kinase (Anderson et al., 2012), independent of any TLR-2 or TLR-4 receptor activity. Thus, SFAs have multiple mechanisms through which they may promote inflammation. In BV-2 microglia, SA treatment increased the production of iNOS, TNF-α, IL-1β and IL-6 when compared to untreated cells (Wang et al., 2012). The SFA palmitic acid was also shown to elicit pro-inflammatory responses of BV-2 microglia through the TLR-4 pathway. As the known pro-inflammatory effects of SA have been found in very few studies specific to microglia, it is necessary to further ascertain microglial responses following SA exposure.

1.8 Model Cell Lines

In this thesis, BV-2 cells, THP-1 cells and HL-60 cells were used to model microglia, and SH-SY5Y cells were used to model neurons. BV-2 cells were used as a microglia model for gas chromatography analysis of cellular fatty acid content, nitrite measurement, and iNOS detection by western blotting. The utility of BV-2 cells, which is an immortalized mouse microglial cell line, has recently been refuted as there are some proteins expressed in primary microglia that are not expressed in BV-2 cells upon exposure to LPS (Das et al., 2016). However, a similar pattern of expression of monocyte chemoattractant protein (MCP)-1, iNOS and many other chemokines, cytokines and other inflammatory mediators was observed when comparing BV-2 microglia and primary mouse microglia, when they were exposed to LPS (Das et al., 2016; Henn et al., 2009). Therefore, BV-2 cells are an established microglial model with cellular activity comparable to that of primary microglia, particularly for the experiments performed herein.

THP-1 cells are human monocytic cells, which were used as a model of microglia. THP-1 cells show gene expression patterns that are similar to macrophages. For example, the TNF-α gene expression response to different LPS exposure times showed the same fold-change patterns when THP-1 cells and peripheral blood mononuclear cell-derived macrophages were compared (Sharif et al., 2007). THP-1 cells have been shown to respond in a similar neurotoxic manner as microglia upon various methods of stimulation (Klegeris et al., 2005; Little et al., 2014). THP-1 cells were used to study secretion of MCP-1 and neurotoxins. SH-SY5Y cells are human neuroblastoma cells that are commonly used to model neurons; they express neuronal markers and secrete the neurotransmitter dopamine (Korecka et al., 2013; Kovalevich and Langford, 2013; Ross et al., 1981). Neurotoxicity is an established effect of activated THP-1 cells on SH-SY5Y neurons (Gouveia et al., 2017; Klegeris et al., 2005; Klegeris and McGeer, 2003).

HL-60 cells were used to model the phagocyte respiratory burst. HL-60 cells are human promyelocytic cells that acquire a neutrophil-like phenotype upon treatment with dimethyl sulfoxide (DMSO) and are ideal for stimulating respiratory bursts to release ROS (Levy et al., 1990). Neutrophils and macrophages both use the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase-mediated respiratory burst response to produce ROS (Benakis et al., 2015; Thomas, 2017).

1.9 Research Questions and Hypotheses

The **central hypothesis** of my research is that the 18-carbon fatty acids ALA, LA, OA and SA regulate select microglial immune responses. I hypothesized that ALA (the n-3 PUFA) and OA (the MUFA) would inhibit the immune responses of microglia, whereas LA (the n-6 PUFA) and SA (the SFA) would enhance the microglial immune responses.

The **specific research hypotheses** tested through the experimental work described in this thesis included:

1. **The fatty acid composition of BV-2 microglia cultured in high glucose media (17.0 mM) is altered by fatty acid treatment.**

   We hypothesized that treatment with each of the fatty acids, ALA, LA, OA, and SA, would result in increased levels of n-3 PUFA, n-6 PUFA, MUFA, and SFA, respectively, due to incorporation of these fatty acids into microglial membrane phospholipids.

2. **Fatty acids affect the phagocytic activity of primary microglia cultured in high glucose media (17.0 mM).**

   We hypothesized that the unsaturated fatty acids ALA, LA and OA increase phagocytic responses of microglia, which can be beneficial to maintain the immune status of the

CNS. Alternatively, we hypothesized that SA reduces phagocytic activity, which could be detrimental for CNS homeostasis.

3. **Fatty acids regulate select microglial responses.**

    i. Fatty acids affect levels of mediators released by microglia, including RNS, iNOS, ROS, and MCP-1, when cells were cultured in high glucose media (17.0 to 17.3 mM).

    We hypothesized that ALA and OA reduce levels of RNS, ROS, and MCP-1 as well as iNOS expression, associated with adverse microglial immune responses. We hypothesized that LA and SA increase the levels of RNS, ROS, and MCP-1 in addition to upregulating iNOS expression in microglia.

    ii. Fatty acids alter microglia-mediated neurotoxicity, after culturing microglial and neuronal cells in high glucose media (17.0 mM).

    We hypothesized that ALA and OA reduce microglial secretion of neurotoxins. Conversely, LA and SA were hypothesized to increase microglial neurotoxicity.

Chapter 2: Materials and Methods

2.1 Chemicals and Reagents

The following reagents were purchased from Sigma Aldrich (Oakville, ON, Canada): ammonium persulfate (APS), β-nicotinamide adenine dinucleotide (BNAD), fatty acid-free bovine serum albumin (BSA), 4',6-diamidino-2-phenylindole (DAPI) with Fluoroshield™, diaphorase (DPH), DMSO, DNase, extravidin alkaline phosphatase, N-formylmethionine-leucyl-phenylalanine (FMLP), glucose, iodonitrotetrazolium chloride (INT), linoleate sodium salt, LPS, luminol sodium salt, mini cOmplete™ protease inhibitor cocktail, paraformaldehyde (PFA), phosphoric acid, N-(1-naphthyl)ethylenediamine dihydrochloride, sodium lactate, sodium oleate, sodium stearate, sulfanilamide, tetramethylethylenediamine (TEMED), D-(+)-trehalose dihydrate, and Triton® X-100 detergent.

ThermoFisher Scientific (Ottawa, ON, Canada) supplied cell culture reagents, which included: antibiotic solution containing penicillin G (10000 U/mL), streptomycin (10000 μg/mL) and amphotericin B (25 μg/mL), calf bovine serum (CBS), Dulbecco's Modified Eagle Medium: F12/Ham (DMEM-F12) containing 17.5 mM glucose, ethanol, Hanks' balanced salt solution (HBSS), papain, percoll, 0.05% trypsin, and 0.25% trypsin. Materials for various assays were also purchased from ThermoFisher Scientific including: 40% 29:1 acrylamide/bis-acrylamide mixture, diethanolamine, dimethylformamide (DMF), hydrochloric acid, phosphatase tablets, sodium bicarbonate, sodium chloride, sodium dodecyl sulfate (SDS), sodium nitrite, Supersignal West Pico PLUS enhanced chemiluminescence (ECL) substrate, and Tris base.

ALA, DHA, and AH-7614 were purchased from Cayman Chemical (Ann Arbor, MI, USA). The MCP-1 enzyme-linked immunosorbent assay (ELISA) kit and mouse IFN-γ were purchased from Peprotech (Montreal, QC, Canada). Bromophenol blue, methanol, nitrocellulose,

1 X phosphate-buffered saline (PBS) tablets, Ponceau S stain, and Tween-20 were purchased from Van Waters and Rogers International (Mississauga, ON, Canada).

The anti-actin antibody (1-19; catalogue #SC-1616-R) was purchased from SantaCruz Biotechnology (Mississauga, ON, Canada). Anti-iNOS polyclonal antibody (catalogue #160862) was purchased from CedarLane Laboratories (Burlington, ON, Canada). Anti-biotin horseradish peroxidase (HRP)-linked antibody (catalogue #7075) and anti-rabbit IgG HRP-linked antibody (catalogue #7074S) were purchased from New England Biolabs, Ltd. (Whitby, ON, Canada).

2.2 Equipment and Supplies

A class two, type IIA biological safety cabinet was used to maintain sterility during seeding and treatment of cells, including supernatant and protein collection. All cell lines were cultured in T75 flasks (Sarstedt, Montreal, QC, Canada) in a Steri-Cycle High-Efficiency Particulate Air Class 100 CO_2 incubator (Model #370, ThermoFisher Scientific) with a humidified atmosphere of 5% CO_2 and 95% air. A hemocytometer (ChangBioscience, Fremont, CA, USA) was used to manually count cells before seeding. A Sorvall RT1 centrifuge (ThermoFisher Scientific) was used to collect cell pellets prior to seeding cells and during extractions of primary mouse microglia. An Accuspin^{TM} 400 centrifuge (ThermoFisher Scientific) was used for fatty acid extraction prior to the gas chromatography analysis, and a Sorvall Legend Micro 17 centrifuge (ThermoFisher Scientific) was used for processing protein samples prior to western blotting. A FLUOstar Omega microplate reader (BMG LabTech, Guelph, ON, Canada) was used for all assays requiring absorbance measurements in 96-well microplates, including Griess, ELISA, 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) and lactate dehydrogenase (LDH) assays. For western blotting, a Mini-

PROTEAN electrophoresis cell (Bio-Rad, Mississauga, ON, Canada) was used with a BioRad Transblot electrophoresis transfer cell. Plastic 24-well plates, 96-well plates, 10 cm and six cm tissue culture dishes were used (Corning, NY, USA). Petri dishes and eight-well chamber slides were purchased from ThermoFisher Scientific.

2.3 Cell Lines

BV-2 cells were generously donated by Dr. G. Garden (University of Washington, Seattle, WA, USA). Human THP-1 cells and HL-60 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA), and the human SH-SY5Y cells were gifted by Dr. R. Ross (Fordham University, NY, USA).

Antibiotic solution that was used throughout all experiments contained penicillin G (10000 U/mL), streptomycin (10000 μg/mL) and amphotericin B (25 μg/mL). In all experimental media, antibiotic solution was diluted 100-fold. F0 media refers to DMEM-F12 containing antibiotic solution alone. F2 media refers to DMEM-F12 (without phenol red) containing antibiotic solution as well as two percent CBS, F5 media refers to DMEM-F12 containing antibiotic solution as well as five percent CBS, and F10 media refers to DMEM-F12 containing antibiotic solution as well as 10% CBS (Table 1). Low glucose media contained 5.6 mM glucose, while all other media used for maintaining and seeding cells contained 16.5 mM to 17.5 mM glucose. Trypsin was used to detach adherent cells from the flask bottom, with 0.05% trypsin used for SH-SY5Y cells, and 0.25% trypsin used for BV-2 cells.

Table 1. The media preparations used throughout all experiments. Antibiotic solution containing penicillin G (10000 U/mL), streptomycin (10000 µg/mL) and amphotericin B (25 µg/mL) was diluted 100-fold into all media.

Medium Abbreviation	CBS (%)	Presence of Phenol Red Marker	Glucose Level (mM)
F0	-	Yes	17.5
F0 (clear)	-	No	17.5
F2 (clear)	2	No	17.3
F5	5	Yes	17.0
F10	10	Yes	16.5
Low Glucose F0	-	Yes	5.6
Low Glucose F5	5	Yes	5.6
Low Glucose F10	10	Yes	5.6

2.4 Preparation of Fatty Acid Stocks and Conjugation with Bovine Serum Albumin

The fatty acids ALA, LA, OA, and SA were dissolved (100 mM) in 70% ethanol (in deionized H_2O (dH_2O)) by heating for approximately 10 min (37ºC for ALA, LA, and OA; 70ºC for SA). Each 100 mM fatty acid stock was diluted 40-fold with 6% (w/v) BSA in F0 (clear) media. Fatty acid was conjugated to BSA by incubating at 37ºC for one hour. The resulting fatty acid/BSA solutions had a final concentration of 2.5 mM fatty acid/6% BSA, and were sterile filtered (0.2 µM filter) prior to use in the cell culture experiments. Vehicle solution was prepared through a 40-fold dilution of 70% ethanol with 6% (w/v) BSA solution in F0 (clear) media. This vehicle solution (final concentration of 1.8% ethanol, 6% BSA) was similarly incubated at 37ºC for one hour, and sterile filtered (0.2 µM filter).

2.5 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) Assay

MTT is commonly used to determine viability of cultured cells. The MTT assay determines viability by measuring metabolic activity of electron transport system components in mitochondria, including succinate dehydrogenase and nicotinamide adenine dinucleotide (NADH)-linked substrates, which reduce tetrazolium dye to formazan crystals (Liu et al., 1997). Importantly, MTT is a sensitive metabolic measure that was shown to correlate with cell viability (Fotakis and Timbrell, 2006; Hansen et al., 1989; Mosmann, 1983). As the MTT reaction to formazan product increases linearly with increasing cell density, an important caveat is that MTT can also measure cellular proliferation (Hansen et al., 1989; Hussain et al., 1993). Cell culture experiments commonly include two different assays (e.g. MTT and LDH) in parallel to provide a more robust analysis of cell viability and death rate. The tetrazolium dye MTT was dissolved in sterile PBS (5 mg/mL) and was added to cell culture medium at the final concentration of 0.5 mg/mL. After one hour incubation, the formation of purple formazan dye could be observed in live cell cultures, and a solution of 20% (w/v) of SDS in 50% DMF (diluted in dH_2O) equal to the total well volume was added to dissolve this formazan dye. The MTT and SDS-DMF mixture was incubated for three to 12 h, followed by agitation. Absorbance of the dissolved formazan dye solution (100 μL in 96-well plates) was measured at 570 nm by the FLUOstar Omega microplate reader.

Analysis of the MTT assay consisted of subtracting the lowest value of raw absorbance of either the media or lysis sample from the raw absorbance values of each sample, followed by normalization as a percentage of the control cells, as shown by the following formula.

$$((Absorbance_{Sample} - Absorbance_{Media\ or\ Lysis}) / Absorbance_{Control}) \times 100\% \quad (1)$$

Where Media = F5 Media Alone; Lysis = BV-2 cells + F5 Media + 1% Triton X-100; Control = BV-2 Cells + F5 Media. Cells in the lysis sample were lysed immediately prior to the MTT assay.

2.6 Lactate Dehydrogenase (LDH) Assay

The LDH cell death assay is often paired with a viability assay such as MTT as part of best practice cell culturing experiments (Fotakis and Timbrell, 2006). The LDH assay measures cell death by the detection of an intracellular enzyme, LDH, which is released from the cytoplasm upon membrane damage (Chan et al., 2013; Decker and Lohmann-Matthes, 1988; Fotakis and Timbrell, 2006). INT (20 µL of two mg/mL stock in 10% DMSO) was added to cell supernatant (100 µL) to a final INT concentration of 0.3 mg/mL. Absorbance at 492 nm was measured immediately ($OD_{Initial}$). DPH, BNAD, and lactate were diluted in PBS and added to each well (30 µL total), for final concentrations of 0.03 mg/mL, 0.06 mg/mL, and 0.72 mg/mL, respectively. After 30 min incubation in a 37°C incubator, absorbance was measured at 492 nm (OD_{Final}). This reaction detects the conversion of lactate to pyruvate, through a series of redox reactions that produces formazan (Burd and Usategui-Gomez, 1973; Decker and Lohmann-Matthes, 1988). Optical density (OD) and cell death were calculated by the following formulae.

$$OD_{Sample} = OD_{Final} - OD_{Initial} \quad (2)$$

$$\text{Cell Death (\%)} = (OD_{Sample} - OD_{Media}) / (OD_{Lysis} - OD_{Media}) \times 100\ \% \quad (3)$$

Where OD_{Media} = absorbance of F5 media alone; OD_{Lysis} = absorbance of BV-2 cells + F5 Media + 1% Triton X-100. Cells from the lysis sample were lysed immediately prior to the LDH assay reactions.

2.7 Gas Chromatography Measurement of Microglial Fatty Acid Content

2.7.1 BV-2 Seeding and Fatty Acid Treatment

BV-2 cells (0.1 million cells/mL) were seeded at a well volume of 0.5 mL (using 24-well plates) in F5 media (containing either 5.6 mM or 17.0 mM glucose). Cell cultures were incubated at 37ºC in a CO_2 incubator with 5% CO_2 overnight (16 to18 h) to allow the BV-2 cells to adhere to the plates. A description of all controls is provided in Table 2. Media were removed from all wells and fresh F5 media were added 30 min prior to the addition of fatty acid/BSA solutions for each of ALA, LA, OA, and SA. The fatty acid/BSA solutions (2.5 mM fatty acid/6% BSA; see 2.4) were first diluted 10-fold in F0 media followed by 10-fold dilution into cell cultures to achieve final well concentrations of 25 μM for each fatty acid. Vehicle control was obtained by a 10-fold dilution of the vehicle solution (the 1.8% ethanol and 6% BSA solution; see 2.4) in F0 media followed by 10-fold dilution into cell cultures achieving final well concentrations of 0.02% ethanol, and 0.06% BSA. Following the addition of the fatty acid/BSA and vehicle solutions to BV-2 cell cultures, these culture plates were incubated at 37ºC in a CO_2 incubator with 5% CO_2 for 24 h.

Table 2. A description of control wells used in the gas chromatography preparation of BV-2 cells cultured in media containing low and high glucose (5.6 mM and 17.0 mM), and exposed to fatty acids.

Control Label	Description
Control	BV-2 cells cultured in F5 media* and no fatty acid treatment
Vehicle	BV-2 cells cultured in F5 media*, with a final concentration of 0.06% BSA and 0.02% ethanol

* = DMEM-F12 (5.6 mM or 17.0 mM glucose) with antibiotic solution and 5% CBS

2.7.2 BV-2 Cell Lysis and Collection

After the 24 h incubation, adherent BV-2 cells were washed three times with one mL PBS, which was then aspirated from the cells. Lysis buffer (100 µL; 150 mM sodium chloride, 1% Triton® X-100, and 50 mM Tris buffer, pH 8) was added to all samples (fatty acid-treated cells, BV-2 control cells and vehicle cells), which were frozen at -20 ºC.

2.7.3 Fatty Acid Extraction and Gas Chromatography

The lipid extraction protocol was modified from the simplified method by Kang and Wang (2005). Each cell lysate was incubated with either low glucose (5.6 mM) or high glucose (17.0 mM; 100 µL cell lysate diluted in 100 µL lysis buffer; 200 µL total), mixed with 1.2 mL hexane (EMD Millipore, Etobicoke, ON, Canada) and 1.2 mL boron trifluoride (14% in methanol; Alfa Aesar, Tewksbury, MA, USA), and heated (one hour at 100 ºC) with periodic mixing. The samples were added to two mL of double distilled H_2O and centrifuged at 1400 rpm (500 x g) for two min. Centrifugation was followed by collection of the upper hexane phase, containing the extracted fatty acids. Extracted fatty acid samples were analysed using gas chromatography with a flame ionization detector (GC-FID; Thermo Scientific^{TM} Trace 1300 gas

chromatograph). Data analysis was performed by manually comparing the chromatogram peaks to a panel of standard peaks using Thermo Scientific™ Dionex™ Chromeleon software. This software was used to visualize the fatty acid peaks, and output the integrated areas of individual peaks as a percentage (%) of the total area obtained by integrating all peaks.

2.7.4 BV-2 Cell Viability in Fatty Acid Measurement Experiments

BV-2 cell viability was determined after BV-2 cells were cultured in media containing low or high glucose concentrations (5.6 mM and 17.0 mM, respectively), and treated with the different fatty acids at 25 μM for 24 h. The fatty acid/BSA stock solution (2.5 mM of fatty acid/6% BSA) was diluted in F0 to the fatty acid concentrations of 0.1 mM, 0.25 mM. 0.5 mM, and 1 mM. These were further diluted to the final concentrations of 10 μM, 25 μM, 50 μM, and 100 μM. The vehicle control wells contained BV-2 cells with a final BSA concentration of 0.2% and a final ethanol concentration of 0.07%, which corresponded to BSA and ethanol concentrations of the highest concentration of fatty acid treatment, 100 μM.

2.7.5 Fatty Acid Content Measured in Microglia Cultured in Low Glucose Medium (5.6 mM)

Experimental protocols in 2.7.1. to 2.7.4 were repeated using all low glucose media (F0, F5, and F10 described in Table 1, containing 5.6 mM of glucose) and 25 μM fatty acid treatments only.

2.8 Measurement of Nitrite Concentration in BV-2 Cell Supernatant

2.8.1 BV-2 Cell Seeding and Fatty Acid Treatment in High Glucose Medium (17.0 mM)

BV-2 cells (0.1 million cells/mL) were seeded into 24-well plates at a well volume of 0.5 mL in F5 media (high glucose media containing 17.0 mM glucose). Cells were allowed to adhere in a CO_2 incubator for 16 to 18 h. Media were removed from all wells and fresh F5 media were added 30 min prior to the addition of fatty acid/BSA solutions. Fatty acid solutions were prepared from the fatty acid/BSA stock solutions (2.5 mM fatty acid/6% BSA; see 2.4) and were diluted in F0 to the concentrations of 0.01 mM, 0.1 mM, 0.25 mM, and 1 mM. Following a 30 min incubation period, the different concentrations of fatty acids were added (ALA, LA, OA or SA) to their respective wells. For BV-2 cells that were later stimulated with LPS, final fatty acid concentrations were 1 μM, 10 μM, 100 μM, and 250 μM; for BV-2 cells that were later stimulated with IFN-γ, final fatty acid concentrations were 1 μM, 10 μM, 25 μM, 100 μM, and 250 μM. Control and vehicle wells are described in Table 3. After 24 h fatty acid treatment in a CO_2 incubator at 37 °C with 5% CO_2, LPS (50 μg/mL) or IFN-γ (5000 U/mL) diluted in PBS was added for 24 h. Final concentrations of LPS or IFN-γ diluted in cell culture media were 0.5 μg/mL and 50 U/mL, respectively. PBS was the vehicle solution for both LPS and IFN-γ, and was added at this time to any unstimulated wells. After the final incubation, supernatants were collected (50 μL) to be used in the Griess assay to measure nitrite. Cell viability was determined by the MTT assay using the protocol described in 2.5.

Table 3. A description of control wells used in experiments measuring nitrite or iNOS with BV-2 cells exposed to fatty acids, after BV-2 cells were cultured in high glucose media (17.0 mM).

Control Labels	LPS or IFN-γ Stimulation	Description
Unstimulated Control	-	BV-2 cells cultured in F5 media*, and no fatty acid treatment
Stimulated Control	LPS	BV-2 cells cultured in F5 media*, no fatty acid treatment, and 0.5 μg/mL LPS
Vehicle	-	BV-2 cells cultured in F5 media*, with a final concentration of 0.6% BSA, 0.18% ethanol and 100-fold PBS dilution (LPS-stimulation vehicle)
Stimulated Vehicle	LPS	BV-2 cells cultured in F5 media*, with a final concentration of 0.6% BSA, 0.18% ethanol and 0.5 μg/mL LPS
Unstimulated Control	-	BV-2 cells cultured in F5 media* and no fatty acid treatment
Stimulated Control	IFN-γ	BV-2 cells cultured in F5 media*, no fatty acid treatment, and 50 U/mL IFN-γ
Vehicle	-	BV-2 cells cultured in F5 media*, with a final concentration of 0.6% BSA, 0.18% ethanol and 100-fold PBS dilution (IFN-γ-stimulation vehicle)
Stimulated Vehicle	IFN-γ	BV-2 cells cultured in F5 media*, with a final concentration of 0.6% BSA, 0.18% ethanol and 50 U/mL IFN-γ

* = DMEM-F12 (17.0 mM glucose) with antibiotic solution and 5% CBS

2.8.2 Griess Assay for Nitrite Measurement

NO in blood is quickly converted to nitrite and nitrate (Preik-Steinhoff and Kelm, 1996). The Griess assay detects nitrite in cell culture media and other solutions (Bryan and Grisham, 2007). In cell-free conditions with media exposed to air atmosphere, NO was shown to degrade fully into nitrite within three min, while nitrate was below the detection limit (Ridnour et al., 2000). Therefore, nitrite alone is widely used as an indirect measure of NO in cell culture media (Bryan and Grisham, 2007). In this assay, sulfanilamide binds to nitrite, in acidic conditions, to

form a diazonium ion. This ion binds N-(1-naphthyl)ethylenediamine, forming a product that absorbs maximally at 550 nm. NO_2 standard solutions (0.01 μM to 40 μM) in F5 media were prepared for a standard curve of different nitrite concentrations. Solution C was prepared by mixing solution A (2% sulfanilamide; 5% phosphoric acid in dH_2O) and solution B (0.2% N-(1-naphthyl)ethylenediamine dihydrochloride in dH_2O) in a one to one ratio immediately prior to use. One volume of solution C was added to one volume of BV-2 supernatant samples or NO_2 standard, followed by absorbance measurement at 550 nm using the FLUOstar Omega microplate reader. The following formulae were used to calculate nitrite concentrations in experimental samples.

$$\text{Corrected } OD_{Sample} = OD_{Sample} - OD_{Media} \quad (6)$$

$$\text{Nitrite } (\mu M) \text{ in Supernatant} = \text{Corrected } OD_{Sample} / m \quad (7)$$

Where OD_{Sample} = absorbance of BV-2 supernatant sample; OD_{Media} = absorbance of F5 media; *m* = slope of the standard nitrite curve.

The limit of detection (LOD) was calculated by the following formulae.

$$LOD_{Absorbance} = \text{Media Average} + 2 \text{ x (Standard Deviation of } OD_{Media}) \quad (8)$$

$$LOD\ (\mu M) = (LOD_{Absorbance} - b) / m \quad (9)$$

Where Media = F5 Media; *b* = the intercept of the standard curve; *m* = slope of the standard curve.

2.8.3 Griess Assay in Experiments Using G Protein-Coupled Receptor (GPR)-120 Antagonist

BV-2 cells (0.1 million cells/mL) were seeded into 24-well plates at a well volume of 0.5 mL in F5 media (high glucose media containing 17.0 mM glucose). Cells were allowed to adhere in a CO_2 incubator for 16 to 18 h. Media were removed from all wells and fresh F5 media were added 30 min prior to treatment with the G protein-coupled receptor (GPR)-120 antagonist AH-7614. Vehicle controls were prepared as described in Table 4. AH-7614 (10 mM) was prepared

in 100% DMSO, which was further diluted 10-fold with 50% (v/v) DMSO in PBS. After 30 min incubation at 37 °C, AH-7614 was added to the cell culture medium to a final concentration of one μM. Incubation at 37°C was repeated for an additional 30 min prior to the addition of fatty acid/BSA solutions. ALA and LA solutions were prepared from the fatty acid/BSA stock solutions (2.5 mM fatty acid/6% BSA; see 2.4) and were diluted in F0 to one mM, followed by dilution into cell culture medium to a final concentration of 100 μM. After 24 h, LPS (50 μg/mL in PBS) was added for an additional 24 h incubation to achieve a final concentration of 0.5 μg/mL LPS. After the final incubation, supernatants were collected (50 μL) to be used in the Griess assay to measure nitrite (see 2.8.2). Cell viability was determined by the MTT assay using the protocol described in 2.5.

This experiment was repeated using DHA as a positive control. DHA in 100% ethanol was diluted to 0.5 g/L in PBS, and then diluted in F0 to 0.25 mM. DHA was added at a final concentration of 25 μM to BV-2 cells cultured as previously described in high glucose media (17.0 mM). Vehicle controls were as described in Table 4, with modifications including 0.01% ethanol and no BSA.

Table 4. A description of control wells used in a cell culture experiment measuring nitrite with BV-2 cells cultured in high glucose media (17.0 mM) that were exposed to fatty acids and the GPR-120 antagonist AH-7614.

Control Labels	LPS or IFN-γ Stimulation	Description
Unstimulated Control	-	BV-2 cells cultured in F5 media*, and no fatty acid treatment
Stimulated Control	LPS	BV-2 cells cultured in F5 media*, no fatty acid treatment, and 0.5 μg/mL LPS
Vehicle	-	BV-2 cells cultured in F5 media*, with a final concentration of 0.2% BSA, 0.07% ethanol, 100-fold PBS dilution (LPS-stimulation vehicle) and 0.05% DMSO (AH-7614 vehicle)
Stimulated Vehicle	LPS	BV-2 cells cultured in F5 media*, with a final concentration of 0.2% BSA, 0.07% ethanol, 0.5 μg/mL LPS and 0.05% DMSO (AH-7614 vehicle)
Non-Inhibited Vehicle With Fatty Acid	LPS	BV-2 cells cultured in F5 media*, with a final concentration of 100 μM fatty acid, and 0.05% DMSO (AH-7614 vehicle)

* = DMEM-F12 (17.0 mM glucose) with antibiotic solution and 5% CBS

2.9 Detection of Inducible Nitric Oxide Synthase (iNOS) by Western Blotting

2.9.1 Fatty Acid Treatment and Stimulation

BV-2 cells (0.2 million cells/mL) were seeded onto six cm tissue culture dishes in two mL of F5 media (high glucose media containing 17.0 mM glucose). Cells were allowed to adhere for 16 to 18 h, fresh media was added for 30 min at 37°C with a 5% CO_2 and 95% air atmosphere. Fatty acid/6% BSA stock solutions (see 2.4) were prepared as described in 2.8.1. Cells were treated with different concentrations of fatty acids (final concentrations: 100 μM and 250 μM for experiments using LPS as stimulus; 25 μM, 100 μM and 250 μM for experiments using IFN-γ as stimulus) or vehicle solution for 24 h. Refer to Table 3 for a summary of prepared

controls. After treatment and incubation at 37°C with a 5% CO_2 and 95% air atmosphere for 24 h, cells were stimulated as previously described with LPS (final concentration of 0.5 μg/mL) or IFN-γ (final concentration of 50 U/mL).

2.9.2 Protein Collection

After fatty acid treatment and stimulation, cells were washed once with one mL PBS, followed by lysing of cells with radioimmunoprecipitation assay (RIPA) buffer (refer to Appendix A) containing 1 X mini cOmplete™ protease inhibitor cocktail. Following incubation for five min, cells were gently scraped off the plates using a rubber policeman and centrifuged at 10 000 x g for 20 min. Supernatants from lysed cells were collected for western blotting, and cell debris pellets were discarded. Cell viability was determined by MTT assay using the previously described protocol in 2.5, and following identical seeding, treatment and stimulation parameters as cells prepared for western blotting analysis.

2.9.3 Bicinchoninic Acid (BCA) Protein Assay

The Pierce™ Bicinchoninic Acid (BCA) Protein Assay Kit (Thermo Scientific) was used to normalize protein as per the manufacturer's protocol. Eight provided BSA standards were diluted in RIPA buffer (25 to 2000 μg/mL standard BSA concentrations). Extracted proteins from lysed BV-2 cells were diluted four-fold in RIPA buffer. Working stock was prepared by diluting 50 volumes of BCA kit Reagent A for every volume of BCA kit Reagent B, and 200 μL of this working stock was added to 25 μL of each sample/standard in duplicate using a 96-well plate. This mixture was agitated for 30 s, followed by 30 min incubation in 37°C and measurement of absorbance at 570 nm. A standard curve was used to interpolate the protein

concentration of each sample for western blot analysis. LPS-stimulated BV-2 samples were diluted to 1.5 mg/mL of protein, and IFN-γ-stimulated BV-2 cells were diluted to 1 mg/mL of protein.

2.9.4 Polyacrylamide Gel Preparation

A polyacrylamide resolving gel was prepared by mixing 1.3 mL of 40% 29:1 acrylamide/bis-acrylamide mixture, 1.3 mL of 1.5 M Tris at pH 8.8, 0.1 mL of 20% SDS, 50 µL of 10% APS, and 2.3 mL dH_2O to produce the following final concentrations of the reagents: 10% 29:1 acrylamide/bis-acrylamide mixture, 0.4 M Tris pH 8.8, 0.4% SDS, 0.1% APS. Five µL of TEMED (0.1%) was added, and the gel was allowed to set between one mm glass plates. One mL 70% ethanol was poured over the gel to ensure that bubbles did not disrupt the gel. After 20 min, polymerization was complete, ethanol was removed, and dH_2O was used to rinse any unpolymerized product from the gel. The stacking gel was prepared by combining 0.9 mL of 40% 29:1 acrylamide/bis-acrylamide mixture, 0.6 mL 1.0 M Tris at pH 6.8, 0.2 mL 20% SDS, 50 µL of APS and 3.3 mL dH_2O to produce the final concentrations of the reagents: 7.1% acrylamide, 0.1 M Tris pH 6.8, 0.8% SDS, 0.1% APS. 10 µL of TEMED (0.2% TEMED) was added to the stacking gel mixture, which was immediately poured over the lower gel. The stacking gel was allowed to polymerize for 10 to 15 min. A combination of equal volumes of the visual ladder OptiLadder (Applied Biological Materials, Inc., Vancouver, BC, Canada) and biotinylated protein ladder (New England Biolabs) was heated to 100°C for two min. Protein samples (1 to 1.5 mg/mL in 1 X Laemmli sample buffer; refer to Appendix A) were denatured at 90°C for 4 min prior to loading 7.5 µg of protein sample on the 10% SDS polyacrylamide gel using specialized loading tips. 20µL of ladder mixture was loaded in the same manner.

Preliminary data showed detectable iNOS protein in a 10% SDS polyacrylamide gel after loading 7.5 µg of protein from LPS-stimulated BV-2 cell lysate. Therefore, for this experiment, 7.5 µg protein was loaded in all 10% SDS polyacrylamide gels. Gels were run at 160 V for 60 to 75 min in 1 X Laemmli running buffer. Proteins from each gel were transferred onto a nitrocellulose membrane for 75 min at a current of 0.3 A using a BioRad Transblot Electrophoresis Transfer Cell (Bio-Rad) in ice-cold 1 X Towbin transfer buffer (refer to Appendix A). Nitrocellulose membranes were then incubated for 10 to 15 min in Ponceau stain (0.02% Ponceau S, 0.3% trichloroacetic acid, 0.3% sulfosalicylic acid in dH_2O) followed by one wash with tris-buffered saline-tween (TBS-T) buffer (refer to Appendix A) to remove any background staining. Membranes were imaged with the Fluorchem® FC2 image system using the AlphaView Q 3.2.2.0 gel acquisition software (Cell Biosciences, Inc.) and the gel analysis function of ImageJ software.

2.9.5 Immunoblotting

Several five min washes with TBS-T at room temperature were used to remove the Ponceau stain. When the membrane was clear of all Ponceau stain, it was blocked for 60 min in 5% skim milk protein (SMP) dissolved in TBS-T. The gel was cut between the 50 kDa and 60 kDa markers, using the visual ladder as a guide; one half of the membrane was incubated with the anti-iNOS antibody and the other with the anti-actin antibody. Blocked membranes were incubated in primary antibody (1:200 anti-iNOS antibody diluted with 5% SMP in TBS-T, or 1:1000 anti-actin antibody diluted with 5% SMP in TBS-T) at 4°C overnight, followed by five 10 min TBS-T washes. Both membrane halves were incubated in secondary antibody containing anti-rabbit secondary antibody to bind the primary antibody, and anti-biotin antibody to bind the

biotinylated protein ladder. Secondary antibody incubation (1:1000 anti-rabbit IgG secondary antibody diluted with 5% SMP in TBS-T and 1:2000 anti-biotin antibody diluted with 5% SMP in TBS-T) occurred for one hour at room temperature, followed by five 10 min TBS-T washes. Membranes were incubated in ECL reagent (SuperSignal West Pico PLUS) in the dark for five min. Membranes were imaged with the Fluorchem® FC2 image system using the AlphaView Q 3.2.2.0 gel acquisition software and analysed with the gel analysis function of ImageJ software. Western blotting data were displayed as a ratio of the signal from the iNOS-containing protein band divided by the total protein signal obtained by Ponceau staining.

2.10 Reactive Oxygen Species (ROS) Detection

The HL-60 cell line was used to detect ROS released by respiratory burst. Differentiated HL-60 cells can be primed by LPS to undergo enhanced respiratory burst upon exposure to the bacterial peptide FMLP. First, HL-60 cells were plated at 0.2 million cells/mL in petri dishes containing 12 mL of F10 media with 1.3% DMSO. These HL-60 cells were left to differentiate to their neutrophil-like phenotype during five-day incubation at 37°C with an atmosphere of 5% CO_2 and 95% air.

After five days, differentiated HL-60 cells were seeded into 96-well plates. Cells primed with LPS were seeded at one million cells/mL, while unprimed cells were seeded at two million cells/mL, each in 250 µL of F2 (clear) media (containing 17.3 mM glucose). Fatty acid solutions (ALA, LA, OA, and SA) were prepared from the fatty acid/6% BSA stock solutions (see 2.4). Fatty acid solutions were diluted in F0 (clear) media to achieve concentrations of 0.01 mM, 0.1 mM and 1 mM. Vehicle stock (see 2.4) was diluted in F0 (clear) media 2.5-fold. After 30 min incubation, this vehicle solution was diluted in HL-60 cell culture media for a final concentration

of 0.6% BSA and 0.18% ethanol. Fatty acid solutions (10 μL) were diluted in the cell culture media to final concentrations of 1, 10, and 100 μM. Table 5 summarizes the control samples used in this experiment.

Table 5. A description of control samples used in experiments studying the effects of fatty acids on LPS-primed HL-60 cell respiratory burst when cells were cultured in high glucose media (17.3 mM).

Control Labels	LPS Priming	Description
Unprimed Control	-	HL-60 cells cultured in F2 media*, and no fatty acid treatment
Unprimed Vehicle	-	HL-60 cells cultured in F2 media*, with a final concentration of 0.6% BSA, 0.18% ethanol and 100-fold PBS dilution (LPS-stimulation vehicle)
Primed Vehicle**	0.5 μg/mL	HL-60 cells cultured in F2 media*, with a final concentration of 0.6% BSA, 0.18% ethanol and 0.5 μg/mL LPS

* = DMEM-F12 (17.3 mM glucose) with antibiotic solution and 2% CBS

** = Control not used for experiments with unprimed HL-60 cells

After 24 h treatment with fatty acid solutions, LPS stock was prepared (50 μg/mL in PBS), and this stock was diluted into the cell culture media to a final concentration of 0.5 μg/mL. This LPS treatment primed the differentiated HL-60 cells, which was shown to intensify the neutrophil respiratory burst response (Forehand et al., 1989). Unprimed HL-60 cells did not

receive this LPS treatment. The following day, a sufficient volume of media was removed to leave 85 μL in each well. Luminol (10 mg/mL dissolved in PBS) and FMLP (1 mM in DMSO) stock solutions were prepared. FMLP was further diluted in PBS to a 20 μM solution. The FLUOstar OMEGA plate reader was used, and the different injectors of the plate reader were used to add luminol and FMLP solutions. Light intensity measurements were recorded every 21 s (one cycle) for 60 cycles. Luminol (13 μL) was injected after five cycles of measurements, followed by FMLP (8 μL) at 15 cycles of measurements to induce the respiratory burst response. For light intensity analysis, background levels were measured during cycles 0 to 14, and the respiratory burst intensity measurements were taken during cycles 15 to 39. Cell viability was determined by MTT assay using the previously described protocol in 2.5; however, luminol and FMLP were not injected to avoid interference between MTT and luminol. The following formulae were used to calculate chemiluminescence.

$$\text{Corrected Chemiluminescence (CHL)} = \text{Sample CHL} - \text{Background CHL} \quad (13)$$

$$\text{CHL (\%)} = (\text{Corrected CHL} / \text{Unprimed Vehicle CHL}) \times 100\% \quad (14)$$

Where the Sample CHL and Background CHL are measured from 15 to 39 cycles, and 0 to 14 cycles, respectively. Each value is a measure of the area under the curve of the respiratory burst, which were analysed using the MARS data analysis software (BMG LabTech). This software was used to subtract Sample CHL and Background CHL, and then this chemiluminescence value

was normalized to the CHL intensity measured from unprimed, but FMLP-stimulated HL-60 cells.

2.11 Measurement of Monocyte Chemoattractant Protein (MCP)-1 Concentration

THP-1 cells were first seeded into 24-well plates at one million cells/mL in one mL of F5 media (high glucose media containing 17.0 mM glucose). Fatty acid solutions were prepared from the fatty acid/6% BSA stock solutions (see 2.4). Fatty acid solutions were diluted in F0 media to achieve concentrations of 0.01 mM, 0.1 mM and 1 mM. After 30 min, 100 μL of each fatty acid solution (ALA, LA, OA, and SA) were added to THP-1 cells to final concentrations of 0 μM, 1 μM, 10 μM, 100 μM, and 250 μM. Vehicle solution was added to THP-1 cells to the final concentration of 0.18% (v/v) ethanol and 0.6% (w/v) BSA. Following 24 h incubation, cells were stimulated with LPS (final concentration 0.5 ng/mL) and IFN-γ (final concentration 150 U/mL) for 48 h. After incubation, THP-1 supernatants were collected and stored in -20°C for the ELISA.

ELISAs were performed following the manufacturer's protocol for the detection of human MCP-1 (Peprotech). Primary antibody at a 1:400 dilution in coating solution (50 μL; refer to Appendix A) was added to each well of a 96-well plate and left to adhere to the plastic overnight at 4°C. After incubation, coating solution was removed and blocking solution (180 μL; 1% SMP and 1% heat-shock treated BSA, ThermoFisher Scientific) was then added to each well for one h at 37°C. The plate was washed twice with PBS-Tween (PBS-T) (refer to Appendix A). During the second wash, PBS-T was left in each well, followed by aspiration from individual wells and the addition of 100 μL of THP-1 supernatant samples or MCP-1 standards into their respective wells. To prepare the standards, MCP-1 protein was diluted in F5 media (six

concentrations between 0.0032-10 ng/mL). Standards and supernatants were left in the plate overnight at 4°C.

After the overnight incubation, plates were washed three times with PBS-T. 100 µL of secondary antibody diluted 1:200 in blocking solution were added to each well, and incubated for one hour at 37°C. Each well was washed four times with PBS-T, and 100 µL of extravidin alkaline phosphatase (prepared 1:10 000 in blocking solution) were added to each well for 45 min at 37°C. Each well was then washed five times with PBS-T. One tablet of phosphatase substrate was dissolved in five mL of diethanolamine, and 100 µL of this phosphatase substrate solution (final concentration one mg/mL) were added to each well. Immediately after phosphatase substrate solution was added, the FLUOstar OMEGA plate reader was used to measure absorbance at 405 nm ($OD_{Initial}$). Absorbance was measured again four h after incubation at 37°C (OD_{Final}).

A standard curve was used to determine the MCP-1 concentrations in all samples. The formulae used were as follows.

$$OD_{Sample} = OD_{Final} - OD_{Initial} \quad (10)$$

$$\text{MCP-1 concentration (ng/mL)} = (OD_{Sample} - OD_{Media}) / m \quad (11)$$

Where OD_{Media} = absorbance of F5 media, and m is the slope of the MCP-1 linear standard curve.

The LOD was calculated by the following formula.

$$LOD = (2 \times \text{Standard Deviation of } OD_{Media}) / m \quad (12)$$

Where OD_{Media} = absorbance of F5 media, and *m* is the slope of the standard curve.

2.12 Microglial-Mediated Neurotoxicity

2.12.1 Fatty Acid Treatment of THP-1 Cells

THP-1 cells were used to model microglia-mediated neurotoxicity, where THP-1 supernatants were transferred onto SH-SY5Y neurons. THP-1 cells were first seeded into 24-well plates at one million cells/mL in one mL of F5 media (high glucose media containing 17.0 mM glucose). Fatty acid solutions from the fatty acid/6% BSA stock solutions were prepared as described (see 2.4). Fatty acid solutions were diluted in F0 media to achieve concentrations of 0.01 mM, 0.1 mM and 1 mM. After 30 min, 100 µL of each fatty acid solution (ALA, LA, OA, and SA) were added to THP-1 cells to final concentrations of 0, 1, 10, 100, and 250 µM. Vehicle solution was added to THP-1 cells to the final concentration of 0.18% (v/v) ethanol and 0.6% (w/v) BSA. Following 24 h incubation, cells were stimulated with LPS (final concentration 0.5 ng/mL) and IFN-γ (final concentration 150 U/mL) for 48 h.

2.12.2 THP-1 Supernatant Transfer on SH-SY5Y Neurons

SH-SY5Y neurons were seeded into 24-well plates at a density of 0.4 million cells/mL in 400 µL of F5 media (high glucose media containing 17.0 mM glucose). After 24 h, SH-SY5Y media were aspirated, and supernatants (400 µL) from THP-1 cells that were stimulated with

LPS and IFN-γ for 24 h were transferred onto SH-SY5Y neurons. On the same day, 100 µL of THP-1 supernatants were used in the LDH assay to measure cell death, and the remaining 300 µL of THP-1 cells were used in the MTT assay to measure cell viability in 2.5. After 72 h incubation of the SH-SY5Y cells at 37°C in 5% CO_2 and 95% air atmosphere, 100 µL of SH-SY5Y supernatants were used in the LDH assay, and the remaining 300 µL of SH-SY5Y cells were used in the MTT assay described in 2.5.

2.13 Statistical Analysis

Student's paired t-test was used to assess the significance of the effects of fatty acids on the viability of BV-2 cells (3.1.1). Several analyses were performed using the two-way randomized block analysis of variance (ANOVA). These included assessing the effects of extracellularly added fatty acids on the microglial fatty acid content (3.1.2), as well as on MCP-1 release by THP-1 cells (3.3.3), and on cell viability and death in the microglia-mediated neurotoxicity assays (3.4). One-way randomized block ANOVAs followed by Dunnett's multiple comparison procedure were used for all other statistical tests. These tests assessed the effects of fatty acids on the microglial release of RNS, expression of iNOS, and release of ROS (3.3.1 to 3.3.2).

Chapter 3: Results

3.1 The Effects of Exogenous Fatty Acids on Microglial Fatty Acid Content Measured by Gas Chromatography

3.1.1 BV-2 Cell Viability and Death

The MTT and LDH assays were used to determine the effects of extracellularly-added fatty acids on viability and death of BV-2 cells, respectively (described in 2.7.4 and 2.7.5). Culturing BV-2 cells for the MTT assay, the LDH assay, and the measurement of microglial fatty acid content was performed in parallel. Cells were cultured in both high glucose (17.0 mM) and low glucose (5.6 mM) media. Different concentrations (10 μM, 25 μM, 50 μM, or 100 μM) of each fatty acid (ALA, LA, OA and SA) or its vehicle solution (0 μM) were added to BV-2 cells for 24 h. After incubation, the production of formazan dye was quantified by the MTT assay, and the release of LDH was measured. Results were normalized as a percentage of the signal obtained from untreated BV-2 cells that were also cultured in either low or high glucose media (5.6 mM or 17.0 mM, respectively).

For comparison with the gas chromatography data, in which 25 μM of fatty acids were added to BV-2 cell cultures, viability data for only the 25 μM concentration of each fatty acid are presented (Figure 3). BV-2 cells cultured in high glucose media (17.0 mM) and treated with ALA, LA, and OA did not display significant differences in viability or death compared to BV-2 cells treated with vehicle (0 μM) solution (Figure 3; A-C, E-G). In high glucose media (17.0 mM), SA treatment alone significantly decreased cell viability according to the MTT assay when compared to cells treated with vehicle solution (Figure 3; D), but SA treatment did not affect cell death as assessed by the LDH assay (Figure 3; H).

For cells cultured in low glucose (5.6 mM) media, ALA and OA did not affect viability of BV-2 cells determined by the MTT assay, when compared to cells treated with vehicle (0 μM) solution (Figure 4; A, C), but ALA and OA treatments significantly increased cell death as shown by the LDH assay (Figure 4; E, G). LA and SA treatments significantly decreased BV-2 cell viability (Figure 4; B, D). LA, but not SA, also significantly increased BV-2 cell death, when compared to the death of cells treated with its vehicle solution (Figure 4; F, H).

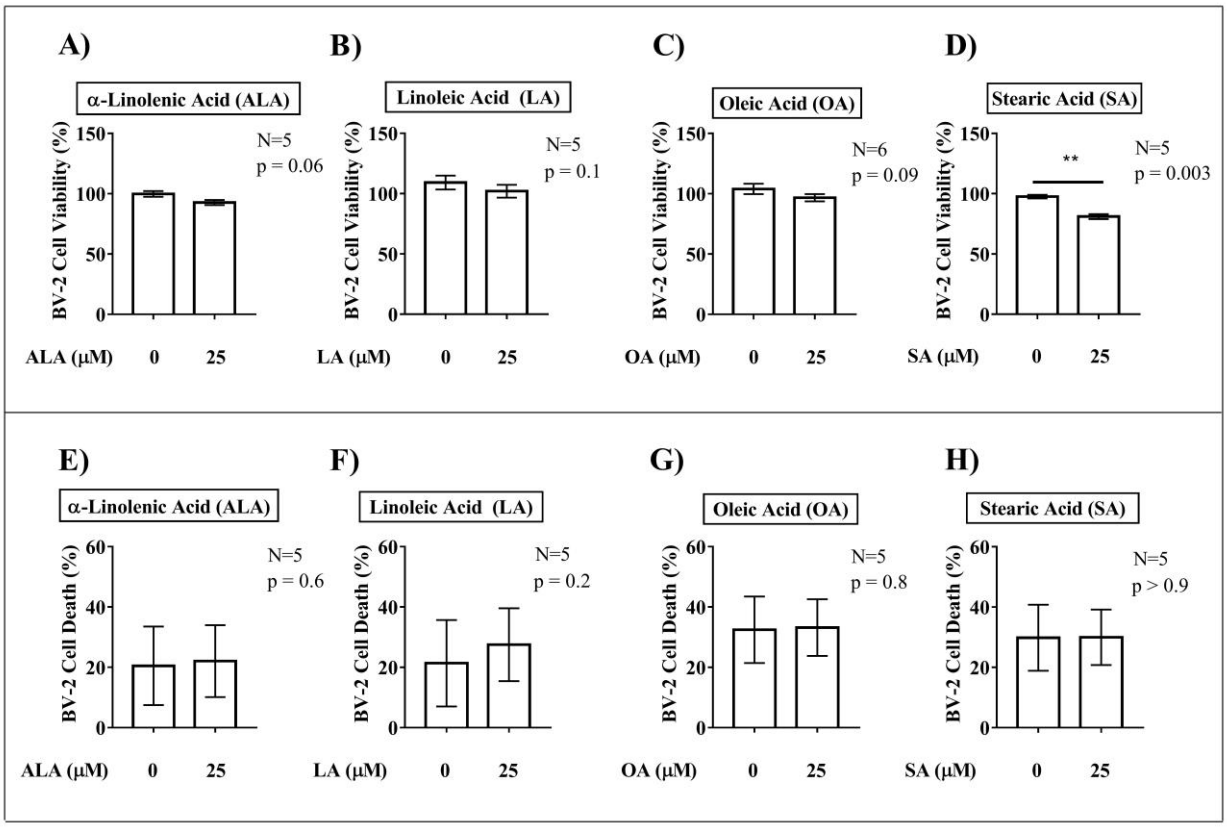

Figure 3. The effects of α-linolenic acid (ALA), linoleic acid (LA), oleic acid (OA), and stearic acid (SA) on viability and death of unstimulated BV-2 mouse microglia cultured in high glucose medium (17.0 mM). BV-2 cells were treated for 24 h with ALA, LA, OA, SA or their vehicle solution (0 μM fatty acid, 0.07% v/v ethanol and 0.2% w/v BSA). Concentrations of fatty acids (μM) are shown on the abscissa. After incubation, viability of ALA-treated (A), LA-treated (B), OA-treated (C), and SA-treated (D) BV-2 cells was assessed by the MTT assay. Cell death of ALA-treated (E), LA-treated (F), OA-treated (G), and SA-treated (H) BV-2 cells was assessed by the LDH assay. Data (means ± S.E.M.) are expressed as a percentage of the viability of untreated BV-2 cells (A-D) or LDH released by lysed BV-2 cells (E-H) without fatty acid or vehicle treatment, from four independent experiments. ** $p < 0.01$, different from cells in the absence of fatty acids (0 μM), according to Student's paired t-test (p values are indicated on the figure).

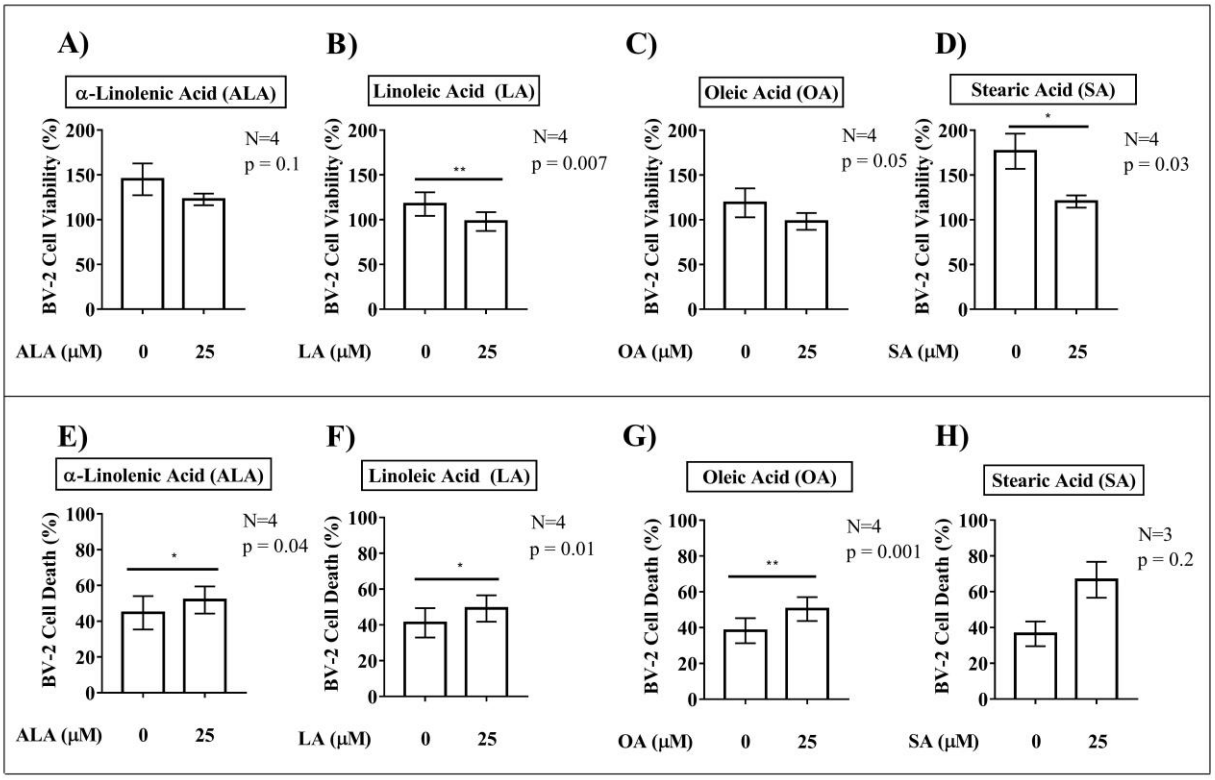

Figure 4. The effects of α-linolenic acid (ALA), linoleic acid (LA), oleic acid (OA), and stearic acid (SA) on viability and cell death of unstimulated BV-2 mouse microglia cultured in low glucose medium (5.6 mM). BV-2 cells were treated for 24 h with ALA, LA, OA, SA or their vehicle solution (0 μM fatty acid, 0.07% v/v ethanol and 0.2% w/v BSA). Concentrations of fatty acids (μM) are shown on the abscissa. After incubation, viability of BV-2 cells treated with (A) ALA, (B) LA, (C) OA, and (D) SA was assessed by the MTT assay. Cell death of BV-2 cells treated with (E) ALA, (F) LA, (G) OA, and (H) SA was assessed by the LDH assay. Data (means ± S.E.M.) are expressed as a percentage of the viability of untreated BV-2 cells (A-D) or the cell death of untreated and lysed BV-2 cells (E-H), from four independent experiments. * $p < 0.05$, ** $p < 0.01$, different from cells in the absence of fatty acids (0 μM), according to Student's paired t-test (p values are indicated on the figure).

3.1.2 Microglial Fatty Acid Content

Experiments were performed as described in 2.7.1 to 2.7.3. BV-2 cells were treated with 25 μM of ALA, LA, OA, and SA for 24 h. After incubation, BV-2 cells were washed three times with PBS, cells were lysed, and fatty acids were extracted. GC-FID was used to measure the fatty acid content in microglial extracts. Data are presented as the area of each peak relative to

the total area of peaks on the corresponding chromatogram obtained from either vehicle solution (0 μM) or fatty acid treated cells (25 μM).

Four types of fatty acids were measured: SFAs, MUFAs, n-6 PUFAs, and n-3 PUFAs. For BV-2 cells cultured in high glucose, SFA showed the highest relative content, corresponding to approximately 60% of total fatty acids, followed in decreasing order by MUFAs, n-6 PUFAs and n-3 PUFAs (Figure 5). The fatty acid content of cells treated with 25 μM of ALA, LA, OA, or SA were compared to the fatty acid content of cells cultured in the absence of fatty acids (0 μM) by two-way ANOVA; no statistically significant effect of fatty acid treatement was observed (Figure 5; A-D). For BV-2 cells cultured in low glucose, the highest relative fatty acid content was for SFAs, which corresponded to 50 to 60% of total fatty acids (Figure 6; A-D). Following SFAs, in decreasing order, were MUFAs, n-6 PUFAs and n-3 PUFAs. The fatty acid content of cells treated with 25 μM of ALA, LA, OA, or SA was compared to the fatty acid content of cells cultured in the absence of fatty acids (0 μM) by two-way ANOVA; no statistically significant effect of fatty acid treatment was observed.

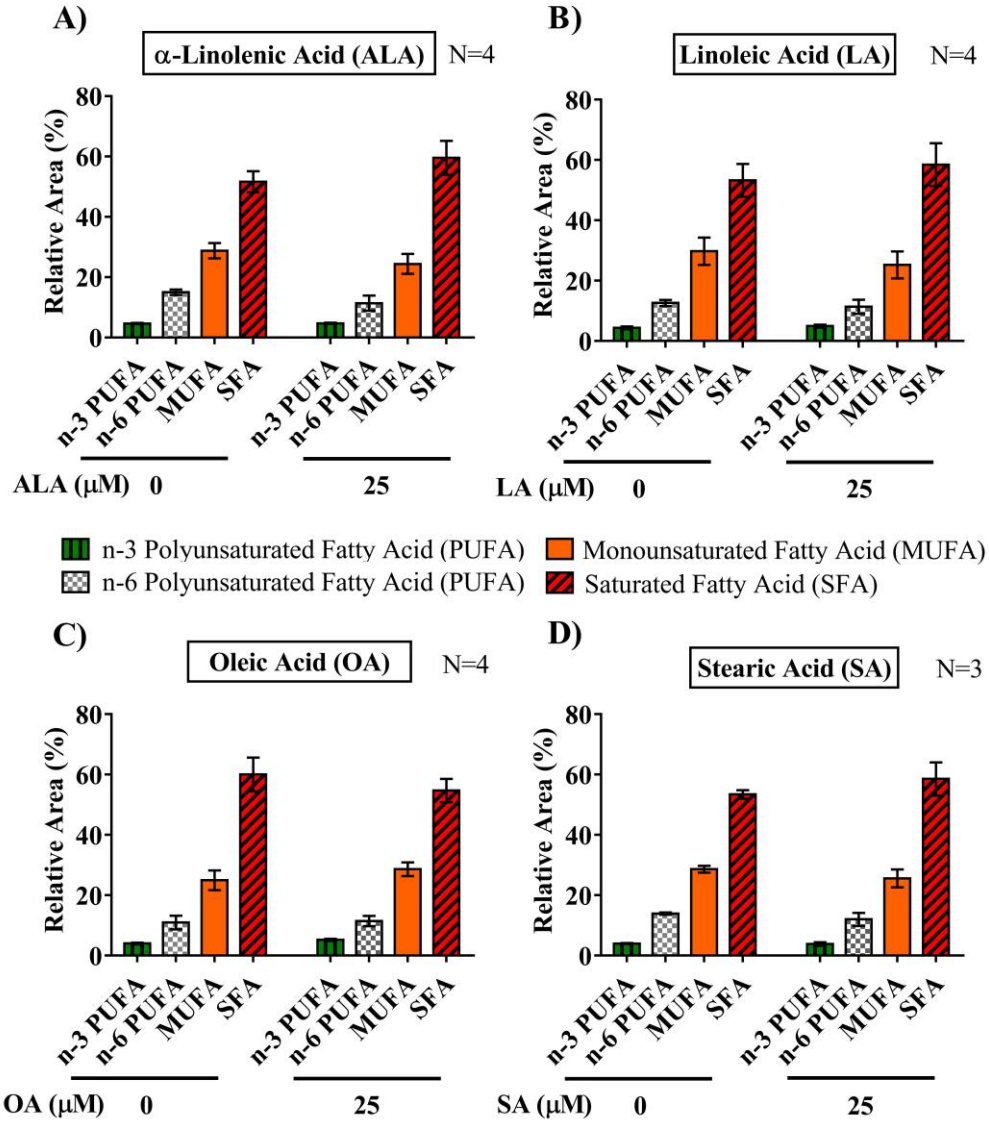

Figure 5. The fatty acid profile of BV-2 mouse microglia that were exposed to α-linolenic acid (ALA), linoleic acid (LA), oleic acid (OA), and stearic acid (SA) and cultured in high glucose medium (17.0 mM). BV-2 cells were treated for 24 h with 25 μM of (A) ALA, (B) LA, (C) OA, (D) SA or their vehicle solution (0 μM fatty acid, 0.02% v/v ethanol and 0.06% w/v BSA). The concentrations of fatty acids (0 μM or 25 μM) and fatty acid types are shown on the abscissa. Extracted fatty acids were analysed using gas chromatography with a flame ionization detector (GC-FID). Data (means ± S.E.M.) from three or four independent experiments are presented as the peak area corresponding to four different types of fatty acids (%), relative to the total chromatogram peak area determined by manual peak labelling and calculated by Thermo Scientific™ Dionex™ Chromeleon software. No statistically significant differences were observed between fatty acid-treated and vehicle-treated cells by two-way ANOVA (p values > 0.9).

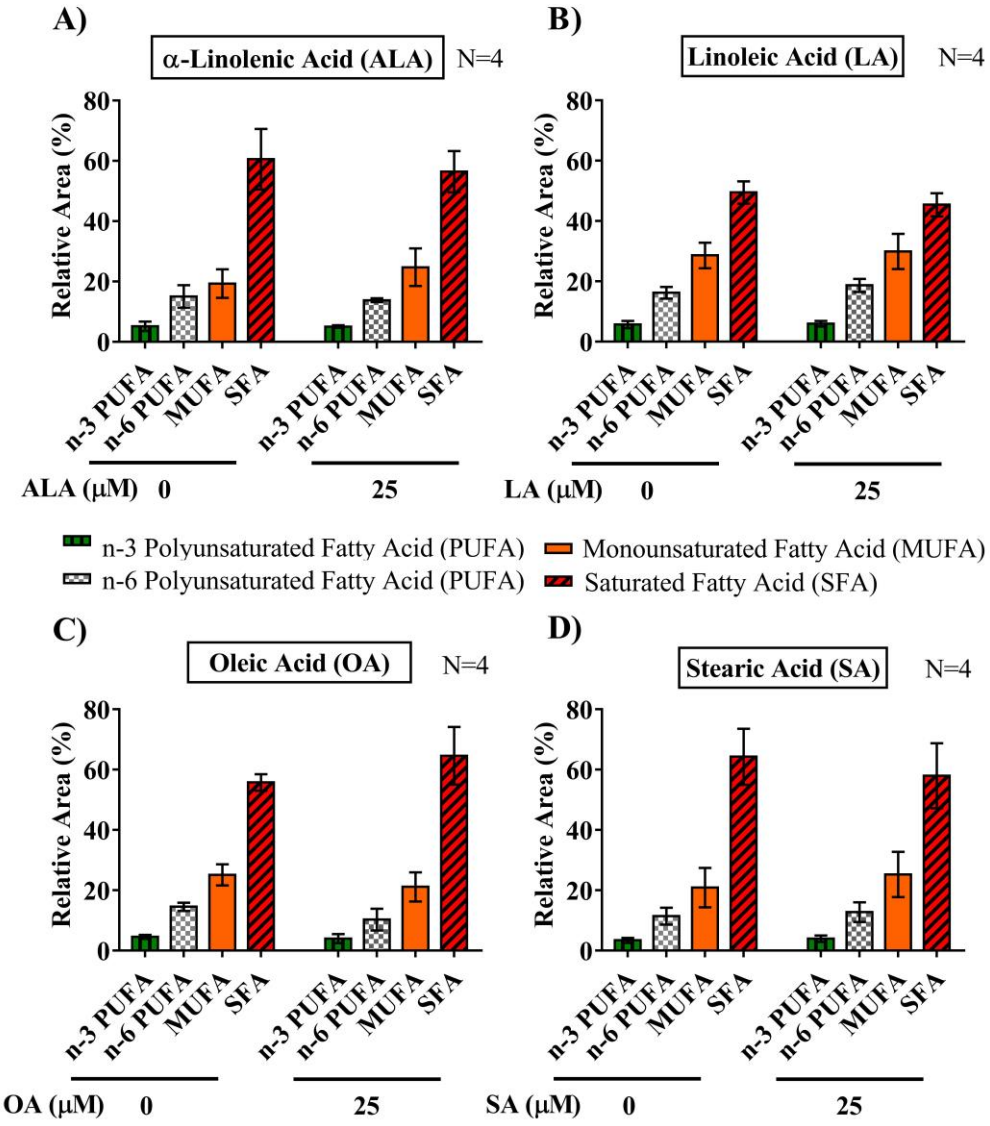

Figure 6. The fatty acid profile of BV-2 mouse microglia that were exposed to α-linolenic acid (ALA), linoleic acid (LA), oleic acid (OA), and stearic acid (SA) and cultured in low glucose medium (5.6 mM). BV-2 cells were treated for 24 h with 25 μM of (A) ALA, (B) LA, (C) OA, (D) SA or their vehicle solution (0 μM fatty acid, 0.02% v/v ethanol and 0.06% w/v BSA). The concentrations of fatty acids (0 μM or 25 μM) and fatty acid types are shown on the abscissa. Extracted fatty acids were analysed using gas chromatography with a flame ionization detector (GC-FID). Data (means ± S.E.M.) from four independent experiments are presented as the peak area corresponding to four different types of fatty acids (%), relative to the total chromatogram peak area determined by manual peak labelling and calculated by Thermo Scientific™ Dionex™ Chromeleon software. No statistically significant differences were observed between fatty acid-treated and vehicle-treated cells by two-way ANOVA (p values > 0.9).

3.2 The Effects of Exogenous Fatty Acids on Primary Microglial Phagocytosis

Primary microglia were extracted, as described in Appendix B.1. Preliminary experiments indicated that treatment of primary mouse microglia with 25 μM of ALA, LA, OA or SA did not affect their phagocytosis of latex beads (see Appendix B.2).

3.3 The Effects of Exogenous Fatty Acids on the Microglial Immune Response

3.3.1 The Effects of Fatty Acids on Nitrite Levels and iNOS Expression

3.3.1.1 LPS-Stimulated BV-2 Cell Viability and Cell Death

Experiments were performed as described in 2.8.1. Different concentrations (1 μM, 10 μM, 100 μM, or 250 μM) of each fatty acid (ALA, LA, OA and SA), or their vehicle solution (0 μM), were added for 24 h to BV-2 cells cultured in high glucose media (17.0 mM). After incubation, cells were left unstimulated or were stimulated with LPS (0.5 μg/mL) for 24 h, and the MTT and LDH assays were used to measure viability and death of BV-2 cells, respectively.

Results were compared to the data obtained from cells stimulated in the absence of fatty acids (0 μM). For ALA-, and LA-treated and LPS-stimulated BV-2 cells cultured in high glucose media (17.0 mM), no significant differences in viability were observed across all fatty acid concentrations (Figure 7; A, B). The only significant difference in cell viability observed with OA-treated cells was an increase in viability seen at the 250 μM concentration (Figure 7; C). SA treatment at 100 μM and 250 μM significantly decreased viability of LPS-stimulated BV-2 cells cultured in high glucose media (17.0 mM; Figure 7; D).

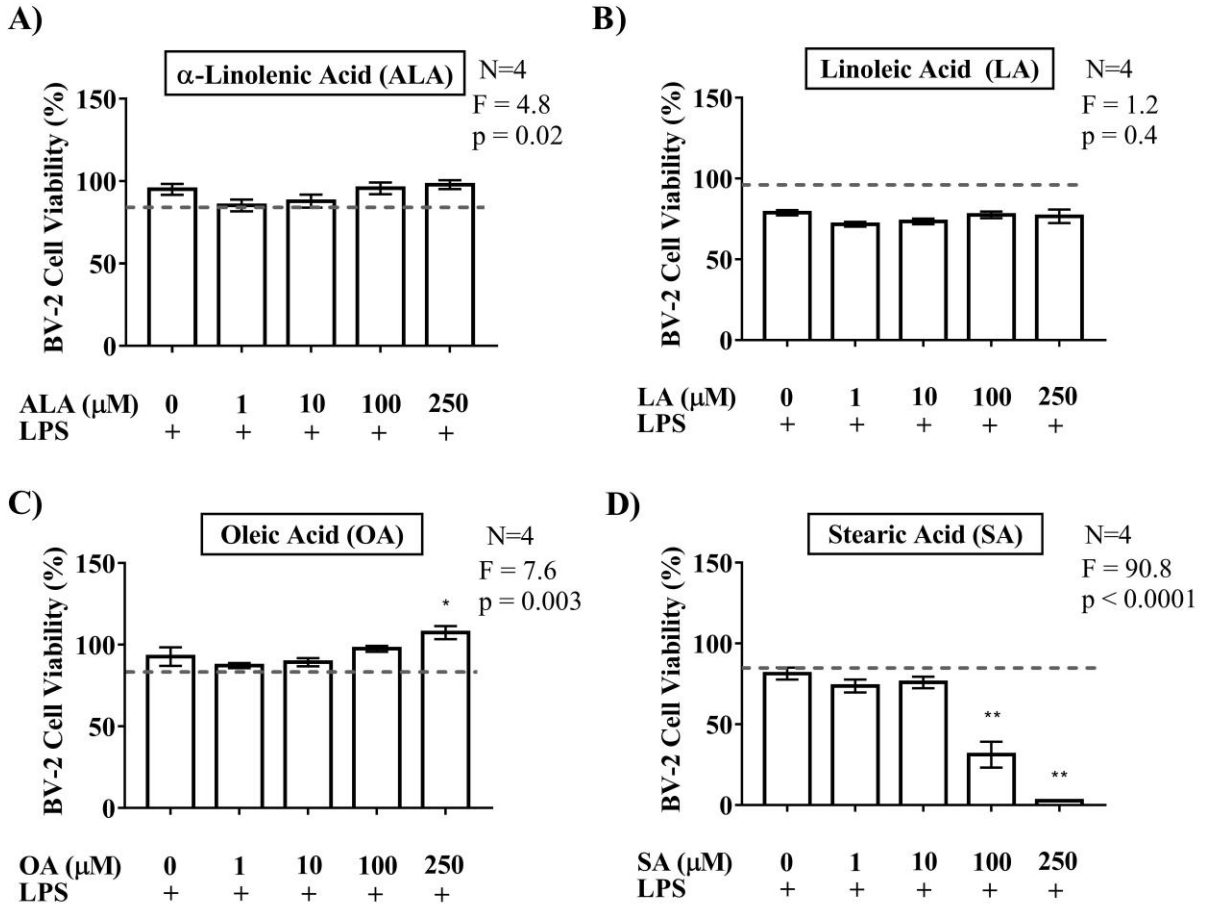

Figure 7. The effects of α-linolenic acid (ALA), linoleic acid (LA), oleic acid (OA), and stearic acid (SA) on the cell viability of LPS-stimulated BV-2 mouse microglia cultured in high glucose medium (17.0 mM). BV-2 cells were treated for 24 h with ALA, LA, OA, SA or their vehicle solution (0 μM fatty acid, 0.18% v/v ethanol and 0.6% w/v BSA). After incubation, cells were left unstimulated or were stimulated with LPS (0.5 μg/mL) for 24 h. Concentrations of fatty acids (μM) are shown on the abscissa. The MTT assay assessed viability of (A) ALA-treated, (B) LA-treated, (C) OA-treated, and (D) SA-treated BV-2 cells after LPS stimulation. Data are expressed as a percentage of the viability of untreated and unstimulated BV-2 cells. Data (means ± S.E.M.) from four independent experiments are presented. The dashed line represents viability of unstimulated BV-2 cells treated with both fatty acid vehicle and LPS vehicle solutions. * $p < 0.05$, ** $p < 0.01$, different from cells stimulated in the absence of fatty acids (0 μM), according to randomized block design one-way ANOVA (F and p values indicated), followed by Dunnett's multiple comparison procedure.

Cell death was assessed by the LDH assay. Results were compared to the data obtained from cells stimulated in the absence of fatty acid (0 μM). ALA, LA or OA treatments did not affect death of stimulated BV-2 cells assessed by the LDH assay (Figure 8; A - C). 100 μM and 250 μM of SA significantly increased cell death of LPS-stimulated BV-2 cells (Figure 8; D).

3.3.1.2 Nitrite Concentration in Supernatants of BV-2 Cells Stimulated by LPS

Nitrite concentration (μM) was assessed by the Griess assay. Experiments were performed as described in 2.8.1 to 2.8.2. Different concentrations (1 μM, 10 μM, 100 μM, or 250 μM) of each fatty acid (ALA, LA, OA, and SA), or their vehicle solution (0 μM), were added for 24 h to BV-2 cells cultured in high glucose media (17.0 mM). After incubation, BV-2 cells were left unstimulated or were stimulated with LPS (0.5 μg/mL) for 24 h, followed by measuring nitrite in their supernatants.

Nitrite was undetected in the supernatants of BV-2 cells that were not stimulated with LPS (data not shown; nitrite detection limit = 1.2 ± 1.1 μM). Results were compared to data obtained from the supernatants of BV-2 cells stimulated in the absence of fatty acid (0 μM). BV-2 cells that were cultured in high glucose media (17.0 mM), treated with ALA, LA, and SA (100 μM and 250 μM), and stimulated with LPS, displayed reduced supernatant nitrite concentrations (Figure 9; A, B, D). OA had no significant effect on the nitrite concentration in supernatants from LPS-stimulated BV-2 cells (Figure 9; C).

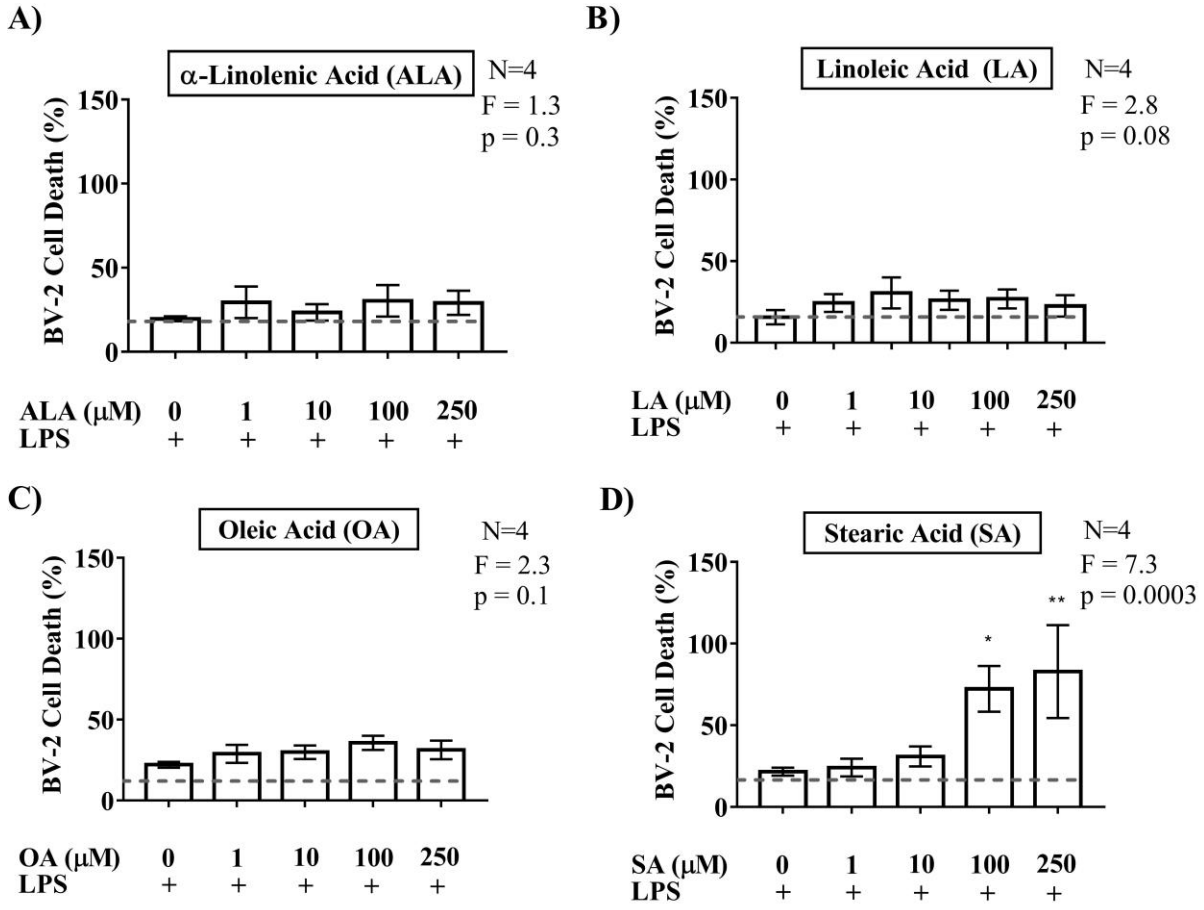

Figure 8. The effects of α-linolenic acid (ALA), linoleic acid (LA), oleic acid (OA), and stearic acid (SA) on the cell death of LPS-stimulated BV-2 mouse microglia cultured in high glucose medium (17.0 mM). BV-2 cells were treated for 24 h with ALA, LA, OA, SA or their vehicle solution (0 μM fatty acid, 0.18% v/v ethanol and 0.6% w/v BSA). After incubation, cells were left unstimulated or were stimulated with LPS (0.5 μg/mL) for 24 h. Concentrations of fatty acids (μM) are shown on the abscissa. LDH activity was measured in supernatants from BV-2 cells treated with (A) ALA, (B) LA, (C) OA, and (D) SA, followed by LPS stimulation. Data are expressed as a percentage of LDH released by untreated and lysed BV-2 cells. Data (means ± S.E.M.) from four independent experiments are presented. The dashed line represents cell death of unstimulated BV-2 cells treated with both fatty acid vehicle and LPS vehicle solutions. * $p < 0.05$, ** $p < 0.01$, different from cells stimulated in the absence of fatty acids (0 μM), according to randomized block design one-way ANOVA (F and p values indicated), followed by Dunnett's multiple comparison procedure.

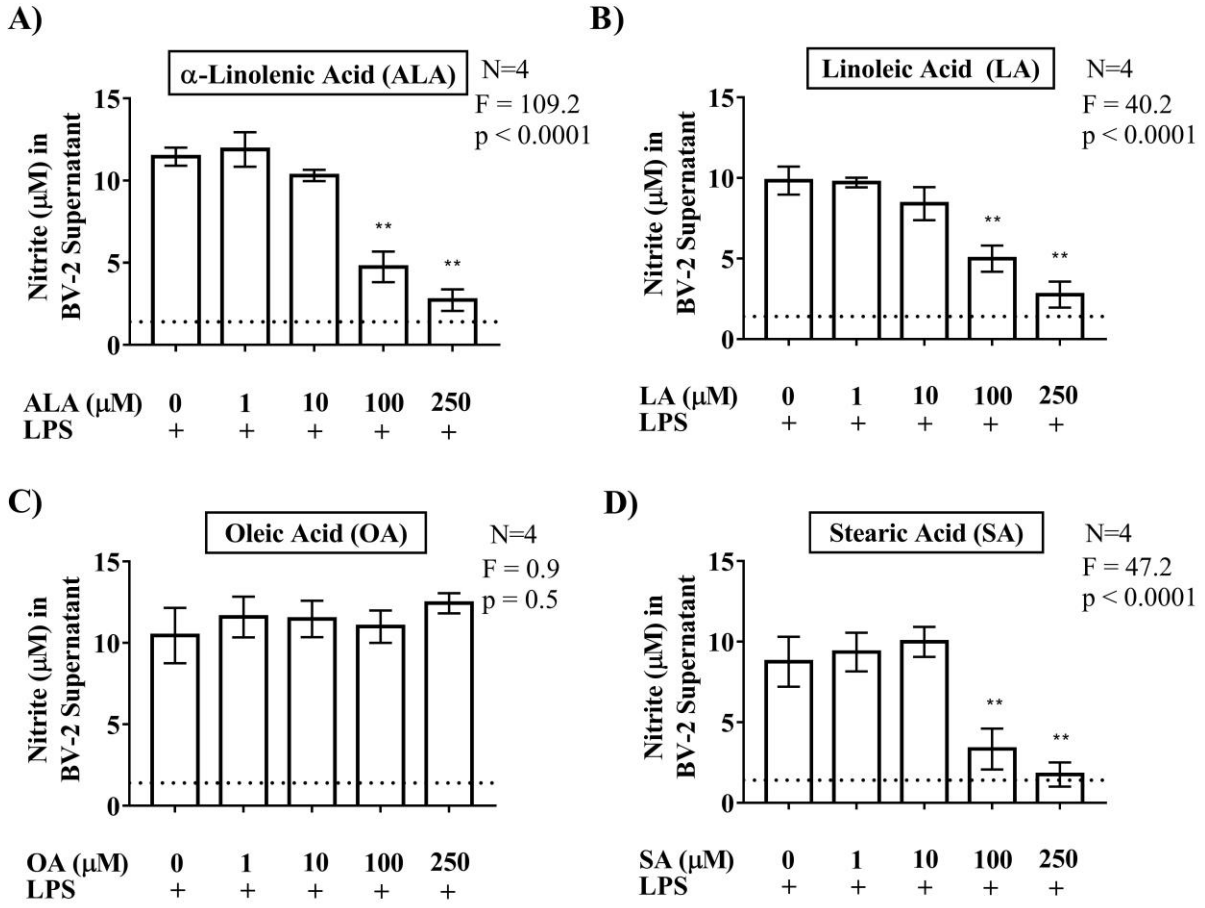

Figure 9. The effects of α-linolenic acid (ALA), linoleic acid (LA), oleic acid (OA), and stearic acid (SA) on the nitrite concentration in supernatants from LPS-stimulated BV-2 mouse microglia cultured in high glucose medium (17.0 mM). BV-2 cells were treated for 24 h with ALA, LA, OA, SA or their vehicle solution (0 μM fatty acid, 0.18% v/v ethanol and 0.6% w/v BSA). After incubation, cells were left unstimulated or were stimulated with LPS (0.5 μg/mL) for 24 h. Concentrations of fatty acids (μM) are shown on the abscissa: (A) ALA, (B) LA, (C) OA, and (D) SA. Nitrite (μM) in supernatants of cells stimulated with LPS was measured by the Griess assay. Data (means ± S.E.M.) from four independent experiments are presented. The detection limit of the Griess assay is shown as a dotted line (1.2 ± 1.1 μM). ** $p < 0.01$, different from cells stimulated in the absence of fatty acids (0 μM), according to randomized block design one-way ANOVA (F and p values indicated), followed by Dunnett's multiple comparison procedure.

3.3.1.3 Nitrite Concentration in the Presence of the GPR-120 Receptor Antagonist

Nitrite concentration (μM) was assessed by the Griess assay as described in 2.8.3. BV-2 cells that were cultured in high glucose media (17.0 mM) were pre-treated with 1 μM of the GPR-120 antagonist AH-7614 or its vehicle solution. After 30 min, cells were treated with 100

μM of ALA or LA, or 25 μM of DHA for 24 h. After incubation, cells were stimulated with LPS (0.5 μg/mL) for 24 h, followed by the measurement of nitrite in cell supernatants, as well as the MTT assay.

Nitrite levels in supernatants of cells that were stimulated in the absence of fatty acid and AH-7614 (0 μM) were compared to the nitrite levels in supernatants of cells treated with fatty acid in the absence of AH-7614, and stimulated with LPS. A significant reduction in nitrite was observed for each fatty acid treatment, when compared to cells not treated with fatty acids (Figure 10; A-C; cells cultured in 17.0 mM glucose media).

Supernatant nitrite levels of ALA-treated BV-2 cells pre-treated with AH-7614 were compared to supernatant nitrite levels from ALA-treated cells that received only the AH-7614 vehicle pre-treatment. This pre-treatment with AH-7614 did not significantly affect the nitrite concentration in BV-2 supernatants of cells treated with 100 μM of ALA (Figure 10; A). Pre-treatment with AH-7614 significantly increased nitrite levels in supernatants of cells that were treated with either 100 μM of LA or 25 μM of DHA prior to LPS stimulation (Figure 10; B, C; cells cultured in 17.0 mM glucose media).

No significant differences in cell viability were observed for BV-2 cells pre-treated with AH-7614 or its vehicle solution alone, followed by treatment with 100 μM of ALA (data not shown) or 25 μM of DHA (Figure 10; E), and stimulation with LPS. Cell viability was significantly reduced when cells were treated with LA in the absence of AH-7614, compared to the viability of cells that were stimulated in the absence of both fatty acid and AH-7614 (Figure 10; D). Cell viability was significantly increased when BV-2 cells were pre-treated with AH-7614, followed by the addition of LA and stimulation with LPS, compared to cells that did not receive AH-7614 before the addition of LA and LPS (cells cultured in 17.0 mM glucose media).

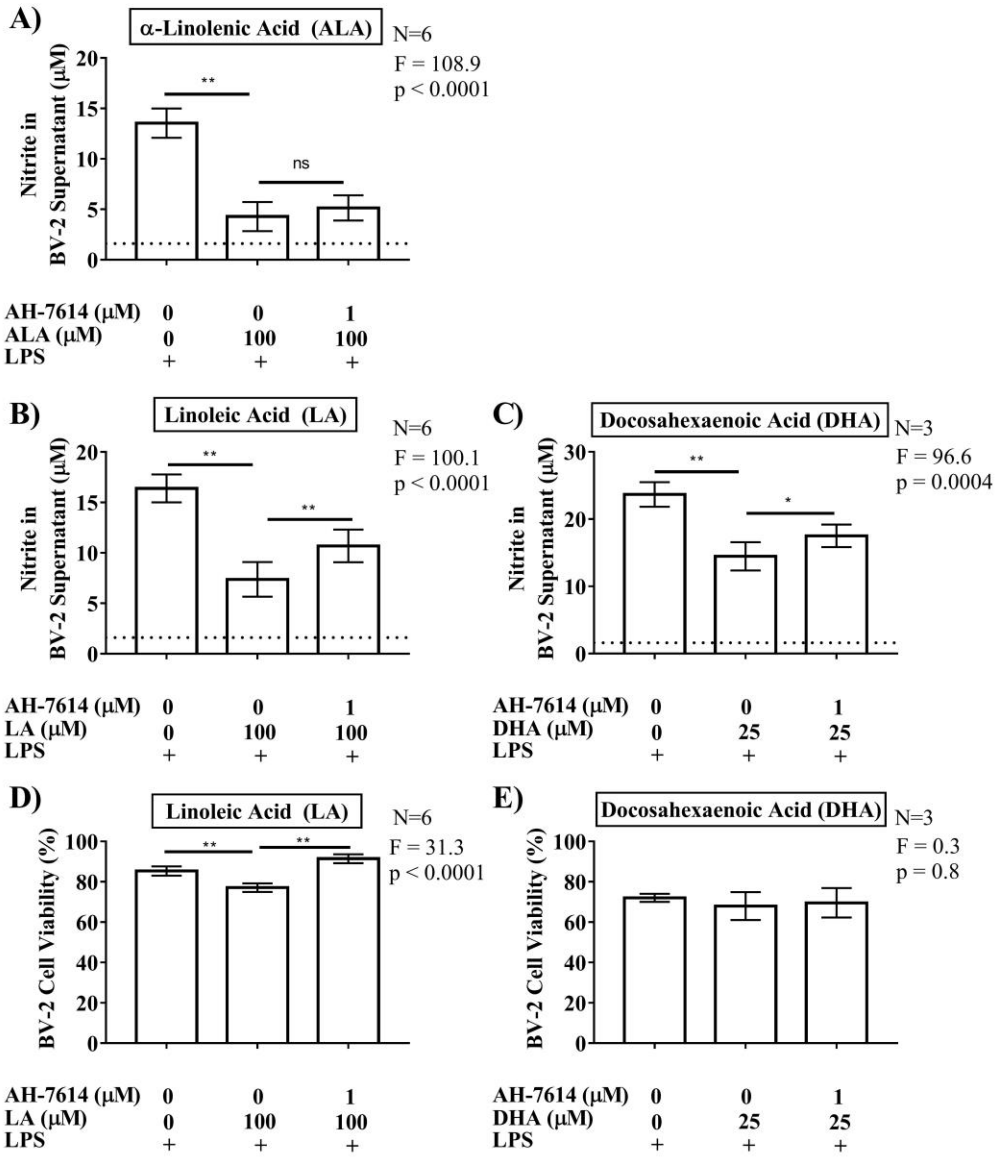

Figure 10. The effects of α-linolenic acid (ALA), linoleic acid (LA), and docosahexaenoic acid (DHA) on the nitrite concentration in supernatants from LPS-stimulated BV-2 mouse microglia cultured in high glucose medium (17.0 mM) in the presence of the GPR-120 antagonist AH-7614. BV-2 cells were treated for 30 min with AH-7614 or its vehicle alone (0.05% DMSO). After incubation, cells were treated for 24 h with ALA, LA, DHA or their vehicle alone (0 μM fatty acid; ALA and LA vehicle: 0.07% v/v ethanol and 0.2% w/v BSA; DHA vehicle: 0.01% v/v ethanol). After incubation, cells were stimulated with LPS (0.5 μg/mL) for 24 h. Concentrations of fatty acids are shown on the abscissa: (A) ALA, (B) LA, and (C) DHA. Nitrite (μM) in supernatants of cells stimulated with LPS was measured by the Griess assay. The MTT assay assessed viability of cells treated with (D) LA or (E) DHA in the presence or absence of AH-7614, followed by LPS stimulation. Viability data are expressed as a percentage of the viability of untreated and unstimulated BV-2 cells. Data (means ± S.E.M.) from three to six independent experiments are presented. The detection limit of the Griess assay is shown as a dotted line (1.6 ± 1.2 μM). * $p < 0.05$, ** $p < 0.01$, different from LPS-stimulated cells treated with fatty acids, in the absence of inhibitor (0 μM), according to randomized block design one-way ANOVA (F and p values are indicated) with Dunnett's multiple comparison procedure; ns, not significant.

3.3.1.4 iNOS Expression in LPS-stimulated BV-2 Cells

Experiments were performed as described in 2.9. BV-2 cells were cultured in high glucose media (17.0 mM) and were treated with 100 μM and 250 μM of ALA or LA, or vehicle solution (0 μM) alone for 24 h. After incubation, cells were left unstimulated or were stimulated with LPS (0.5 μg/mL) for an additional 24 h. iNOS protein was measured by western blotting techniques, and the protein expression data were presented as the ratio of iNOS to total protein quantified by Ponceau staining. The MTT assay was used to measure the viability of BV-2 cells that were treated with ALA, LA, or their vehicle solution (0 μM), followed by LPS stimulation.

Each iNOS expression result was compared to the expression in cells stimulated with LPS in the absence of fatty acid (0 μM). ALA at 250 μM significantly decreased the level of iNOS protein in BV-2 cells cultured in high glucose media (17.0 mM; Figure 11; A). Each viability result was compared to the viability of cells stimulated with LPS in the absence of fatty acid (0 μM). Cells treated with 250 μM of ALA showed significantly reduced viability (Figure 11; C). Cells treated with 100 μM and 250 μM of LA had a significantly decreased iNOS protein expression (Figure 11; D). Viability of LPS-stimulated cells was not altered at in the presence of 100 μM of LA, but was significantly decreased by 250 μM of LA, when compared to the viability of cells stimulated in the absence of fatty acid (Figure 11; D, F). Representative immunoblots are shown in Figure 11; B, E.

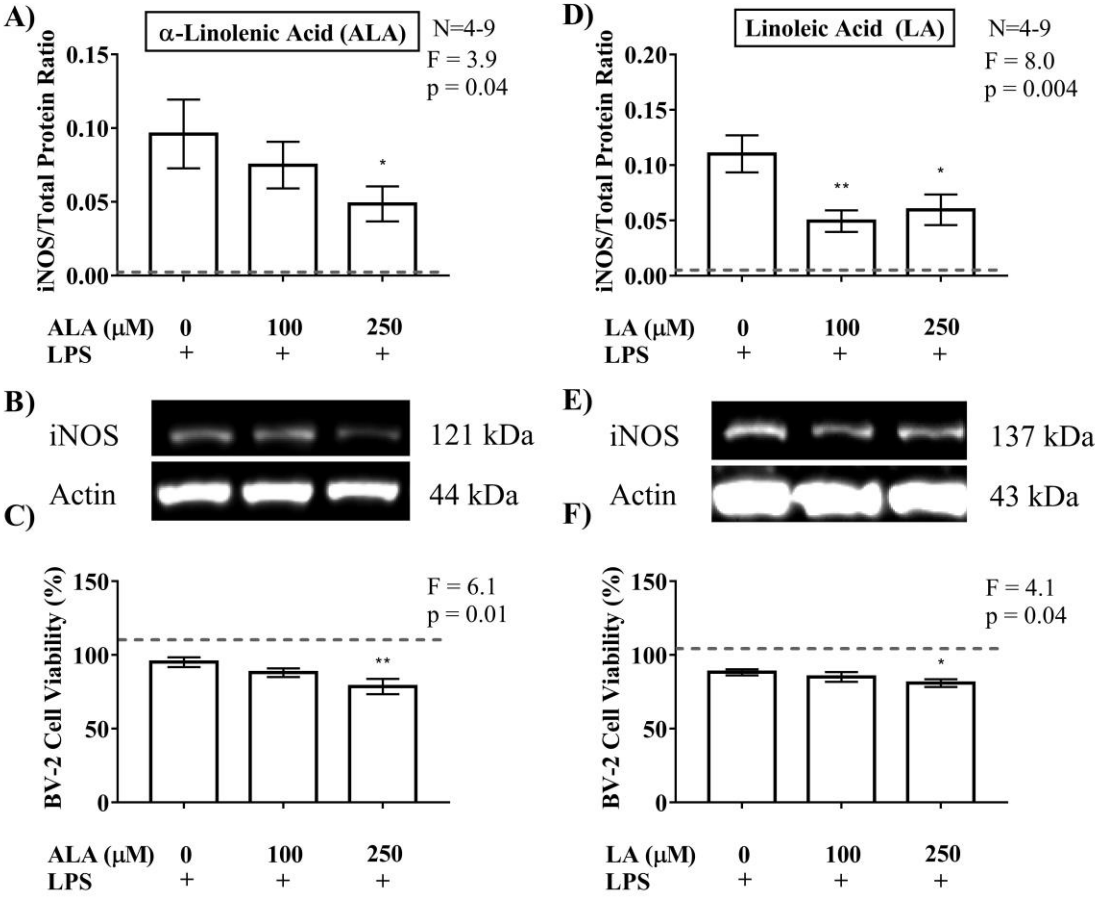

Figure 11. The effects of α-linolenic acid (ALA) and linoleic acid (LA) on the protein levels of inducible nitric oxide synthase (iNOS) in LPS-stimulated BV-2 mouse microglia cultured in high glucose medium (17.0 mM). BV-2 cells were treated for 24 h with ALA, LA or their vehicle solution (0 μM fatty acid, 0.18% v/v ethanol and 0.6% w/v BSA). After incubation, cells were left unstimulated or were stimulated with LPS (0.5 μg/mL) for 24 h. Concentrations of fatty acids (μM) are shown on the abscissa. Immunoblotting was used to measure iNOS, and total protein was assessed by Ponceau staining. Data are presented as the ratio of iNOS / total protein for BV-2 cells treated with (A) ALA and (D) LA, with representative chemiluminescence immunoblots shown in (B) and (E), respectively. Viability of ALA- and LA-treated BV-2 cells after LPS stimulation was assessed by the MTT assay and data in (C) and (F) are expressed as a percentage of the viability of unstimulated BV-2 cells without fatty acid or vehicle treatment. Data (means ± S.E.M.) from four to nine independent experiments are presented. The dashed lines represent iNOS expression or viability of unstimulated BV-2 cells treated with both fatty acid vehicle and LPS vehicle solutions. * $p < 0.05$, ** $p < 0.01$, different from cells stimulated in the absence of fatty acids (0 μM), according to randomized block design one-way ANOVA (F and p values indicated), followed by Dunnett's multiple comparison procedure.

3.3.1.5 IFN-γ-Stimulated BV-2 Cell Viability and Cell Death

Experiments were performed as described in 2.8.1. Different concentrations (1 μM, 10 μM, 25 μM, 100 μM, or 250 μM) of each fatty acid (ALA, LA, OA and SA), or its vehicle

solution (0 μM), were added for 24 h to BV-2 cells cultured in high glucose media (17.0 mM). After incubation, cells were left unstimulated or were stimulated with IFN-γ (50 U/mL) for 24 h, followed by the MTT and LDH assays to measure viability and death of BV-2 cells.

Results were compared to the data obtained from IFN-γ-stimulated cells cultured in high glucose media (17.0 mM) without fatty acid treatment (0 μM). No significant differences in viability were observed in cells treated with ALA and stimulated with IFN-γ (Figure 12; A). LA at 10 μM significantly reduced BV-2 cell viability (Figure 12; B). OA at 250 μM significantly increased BV-2 cell viability (Figure 12; C). 100 μM and 250 μM of SA reduced cell viability of stimulated BV-2 cells (Figure 12; D).

3.3.1.6 Nitrite Concentration from Supernatants of BV-2 Cells Stimulated by IFN-γ

Nitrite concentration (μM) was measured by the Griess assay as described in 2.8.1 to 2.8.2 using BV-2 cells cultured in high glucose media (17.0 mM). Results were compared to the data obtained from supernatants of cells stimulated with IFN-γ in the absence of fatty acid (0 μM). BV-2 cells treated with the highest 250 μM concentration of ALA prior to IFN-γ stimulation displayed significantly reduced nitrite levels over two-fold (Figure 13; A). At 100 μM and 250 μM, LA-treated and stimulated cells demonstrated significantly decreased supernatant nitrite levels (Figure 13; B). OA treatment in BV-2 cells followed by IFN-γ stimulation did not affect the supernatant nitrite concentration (Figure 13; C). SA at 250 μM significantly reduced nitrite in supernatants of stimulated BV-2 cells (Figure 13; D). Supernatant nitrite levels from cells treated with fatty acid vehicle and IFN-γ vehicle were 1.9 ± 0.7 μM associated with OA, and 0.7 ± 0.3 μM associated with LA treatments (data not shown). Nitrite

was undetected in all other supernatants from BV-2 cells treated with fatty acid vehicle and IFN-γ vehicle alone (cells cultured in high glucose of 17.0 mM; data not shown).

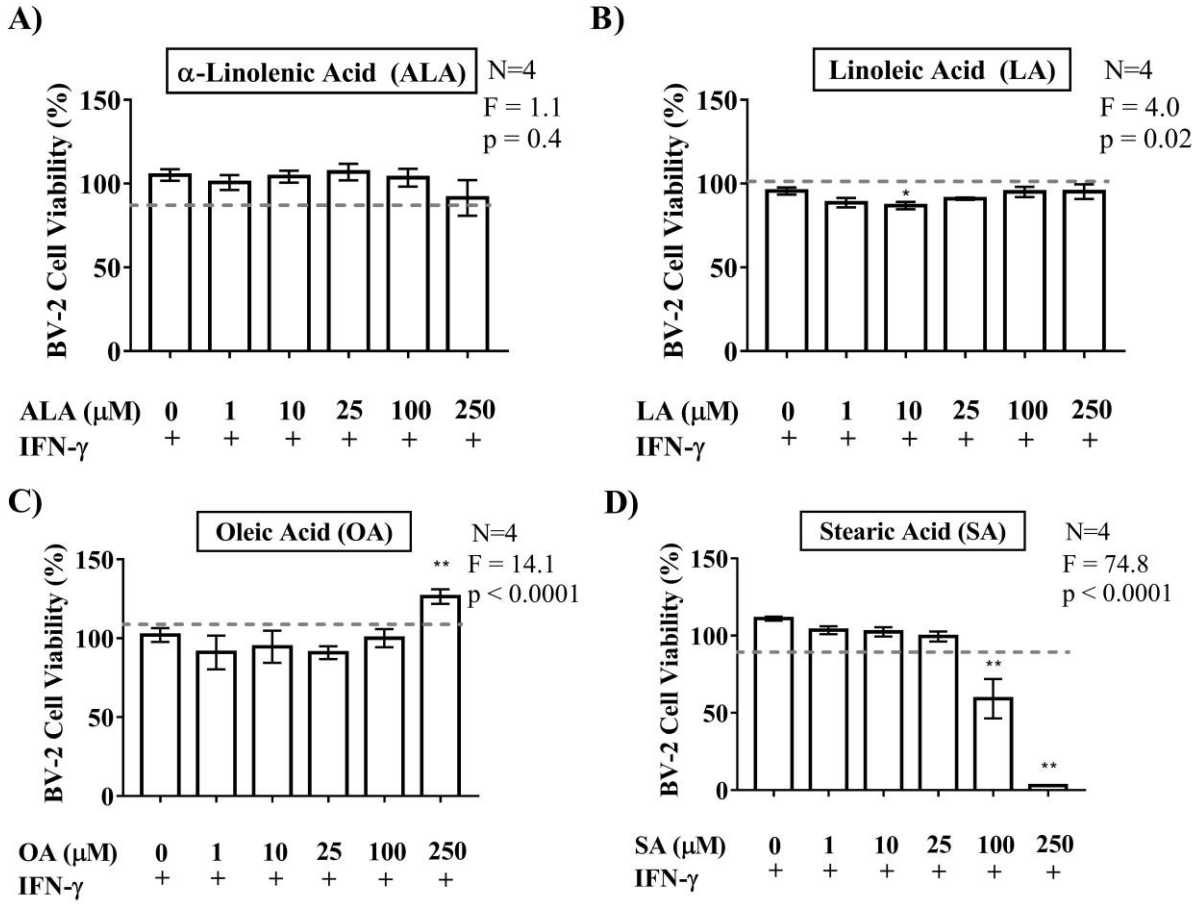

Figure 12. The effects of α-linolenic acid (ALA), linoleic acid (LA), oleic acid (OA), and stearic acid (SA) on the cell viability of IFN-γ-stimulated BV-2 mouse microglia cultured in high glucose medium (17.0 mM). BV-2 cells were treated for 24 h with ALA, LA, OA, SA or their vehicle solution (0 μM fatty acid, 0.18% v/v ethanol and 0.6 w/v BSA). After incubation, cells were left unstimulated or were stimulated with IFN-γ (50 U/mL) for 24 h. Concentrations of fatty acids (μM) are shown on the abscissa. The MTT assay assessed viability of BV-2 cells treated with (A) ALA, (B) LA, (C) OA, and (D) SA, followed by IFN-γ stimulation. Data are expressed as a percentage of the viability of unstimulated BV-2 cells without fatty acid or vehicle treatment. Data (means ± S.E.M.) from four independent experiments are presented. The dashed line represents viability of unstimulated BV-2 cells treated with both fatty acid vehicle and IFN-γ vehicle solutions. * $p < 0.05$, ** $p < 0.01$, different from cells stimulated in the absence of fatty acids (0 μM), according to randomized block design one-way ANOVA (F and p values indicated), followed by Dunnett's multiple comparison procedure.

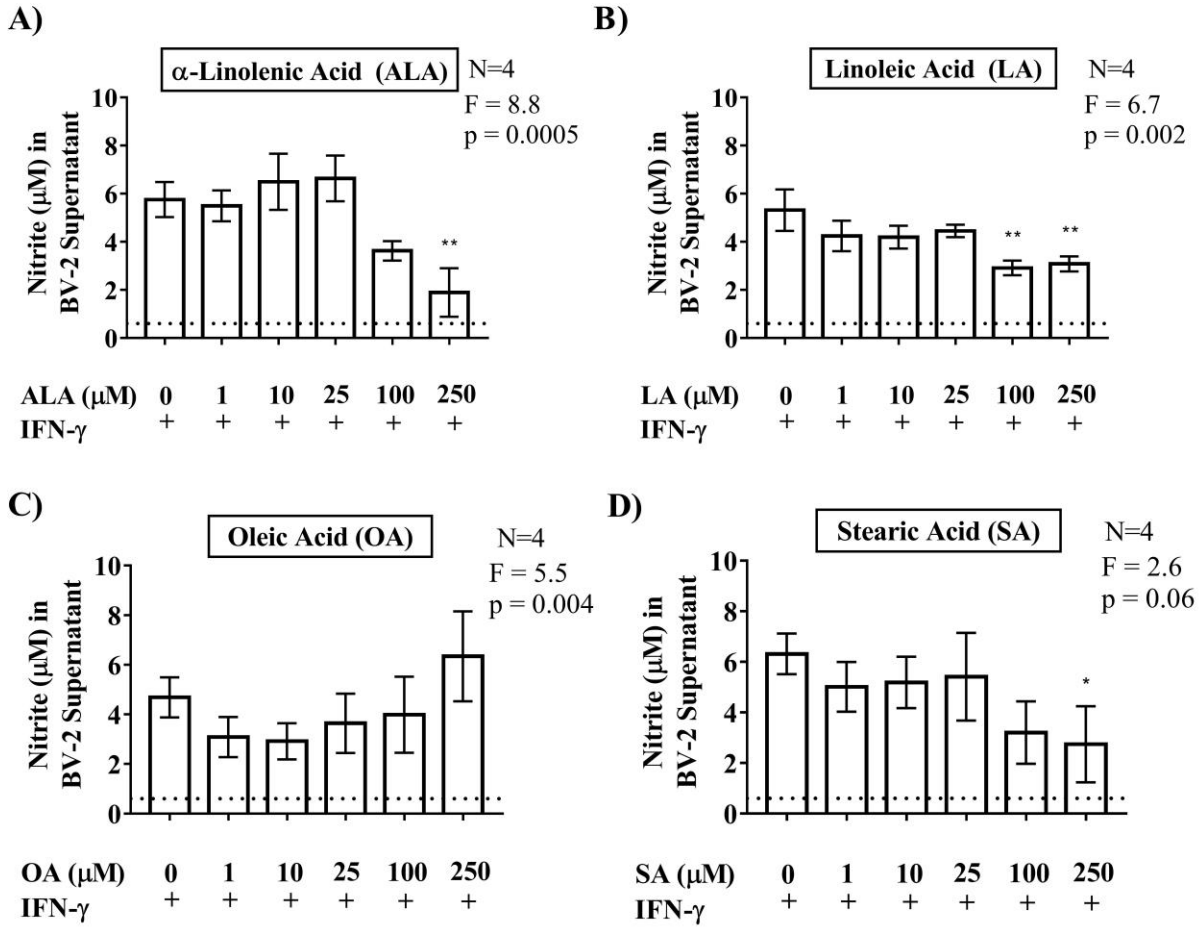

Figure 13. The effects of α-linolenic acid (ALA), linoleic acid (LA), oleic acid (OA), and stearic acid (SA) on the nitrite concentration in supernatants from IFN-γ-stimulated BV-2 mouse microglia cultured in high glucose medium (17.0 mM). BV-2 cells were treated for 24 h with ALA, LA, OA, SA or their vehicle solution (0 μM fatty acid, 0.18% v/v ethanol and 0.6% w/v BSA). After incubation, cells were left unstimulated or were stimulated with IFN-γ (50 U/mL) for 24 h. Concentrations of fatty acids (μM) are shown on the abscissa: (A) ALA, (B) LA, (C) OA, and (D) SA. Nitrite (μM) released after IFN-γ stimulation was measured by the Griess assay. Data (means ± S.E.M.) from four independent experiments are presented. The detection limit of the Griess assay is shown as a dotted line (0.6 ± 0.4 μM). * $p < 0.05$, ** $p < 0.01$, different from cells stimulated in the absence of fatty acids (0 μM), according to randomized block design one-way ANOVA (F and p values indicated), followed by Dunnett's multiple comparison procedure.

3.3.1.7 iNOS Expression in IFN-γ-stimulated BV-2 Cells

Experiments were performed as described in 2.9. BV-2 cells that were cultured in high glucose media (17.0 mM) were treated with different concentrations of ALA or LA (25 μM, 100

μM, and 250 μM), or their vehicle solution (0 μM) for 24 h. After incubation, cells were left unstimulated or were stimulated with IFN-γ (50 U/mL) for an additional 24 h. iNOS protein was measured by western blotting techniques. The protein expression data were presented as the ratio of iNOS to total protein quantified by Ponceau staining. The MTT assay was used to measure the viability of BV-2 cells cultured in high glucose media (17.0 mM) that were treated with ALA, LA, or their vehicle solution (0 μM), followed by IFN-γ stimulation.

Each iNOS expression result was compared to the expression in cells stimulated with IFN-γ in the absence of fatty acid (0 μM). No significant differences in iNOS expression were observed in cells treated with ALA or LA (Figure 14; A, D). Each viability result was compared to the viability of cells stimulated with IFN-γ in the absence of fatty acid (0 μM). Cells treated with 250 μM of ALA or LA had significantly reduced cell viability (Figure 14; C, F). Representative immunoblots are shown in Figure 14; B, E.

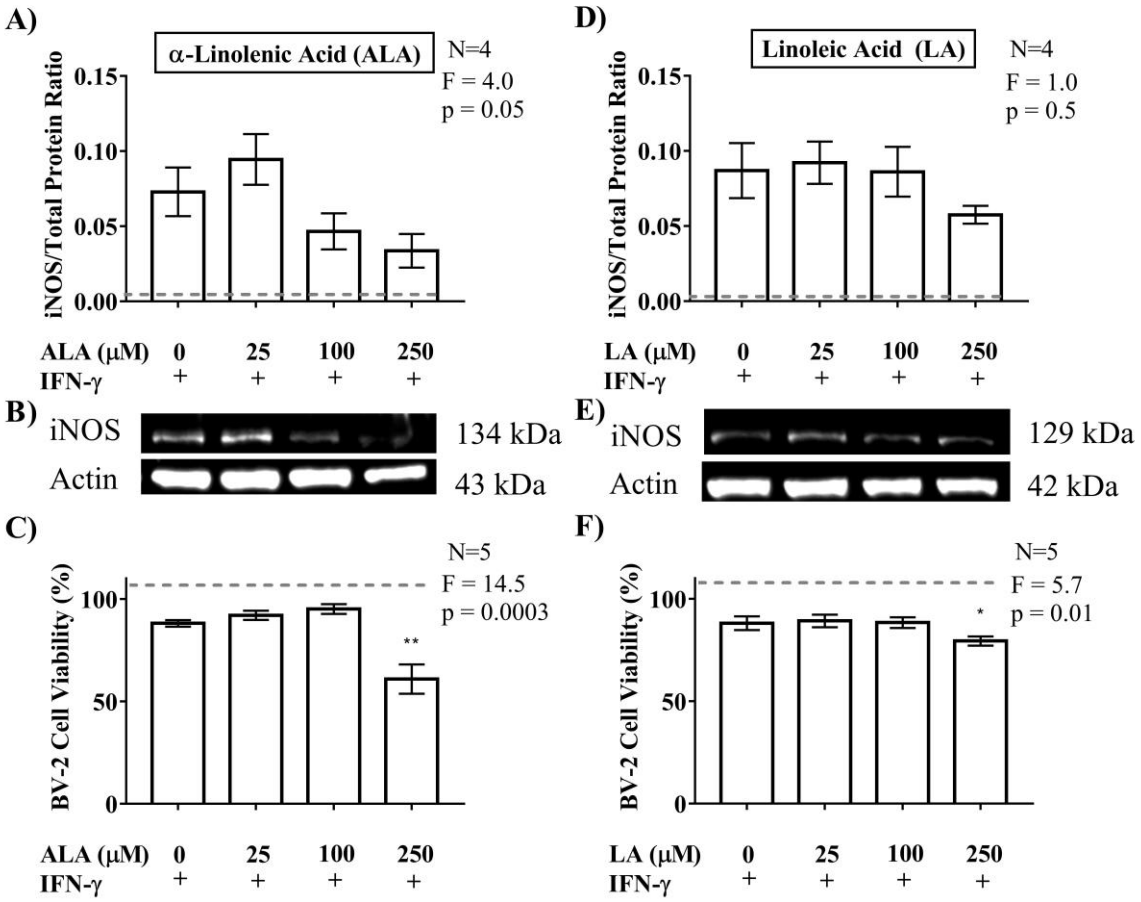

Figure 14. The effects of α-linolenic acid (ALA) and linoleic acid (LA) on the protein levels of inducible nitric oxide synthase (iNOS) in IFN-γ-stimulated BV-2 mouse microglia cultured in high glucose medium (17.0 mM). BV-2 cells were treated for 24 h with ALA, LA or their vehicle solution (0 μM fatty acid, 0.18% v/v ethanol and 0.6% w/v BSA). After incubation, cells were left unstimulated or were stimulated with IFN-γ (50 U/mL) for 24 h. Concentrations of fatty acids (μM) are shown on the abscissa. At the end of the experiment, BV-2 cells were washed with sterile PBS and lysed. Immunoblotting was used to measure iNOS, and total protein was assessed by Ponceau staining. Data are presented as the ratio of iNOS / total protein for BV-2 cells treated with (A) ALA and (D) LA, with representative chemiluminescence immunoblots shown in (B) and (E), respectively. Viability of ALA- and LA-treated BV-2 cells after IFN-γ stimulation was assessed by the MTT assay and data in (C) and (F) are expressed as a percentage of the viability of unstimulated BV-2 cells without fatty acid or vehicle treatment. Data (means ± S.E.M.) from four or five independent experiments are presented. The dashed lines represent iNOS expression or viability of unstimulated BV-2 cells treated with both fatty acid vehicle and IFN-γ vehicle solutions. * $p < 0.05$, ** $p < 0.01$, different from cells stimulated in the absence of fatty acids (0 μM), according to randomized block design one-way ANOVA (F and p values indicated), followed by Dunnett's multiple comparison procedure.

3.3.2 The Effects of Fatty Acids on the Release of ROS by HL-60 Cells

3.3.2.1 Cell Viability of Differentiated and LPS-Primed HL-60 Cells

Experiments were performed as described in 2.10. HL-60 cells were differentiated by DMSO to induce the expression of NADPH oxidase components (Levy et al., 1990). These cells were cultured in high glucose media (17.3 mM) and were then treated with different concentrations (1 μM, 10 μM, and 100 μM) of each fatty acid (ALA, LA, OA, and SA) or its vehicle solution (0 μM) alone for 24 h. After incubation, LPS (0.5 μg/mL) was added for 24 h. Viability results were compared to the viability of LPS-primed cells stimulated with FMLP in the absence of fatty acid (0 μM). Viability was reduced in cells treated with the highest 100 μM concentration of ALA or OA (Figure 15; A, C). LA increased cell viability at 1 and 10 μM (Figure 15; B). Treatment with SA showed no effect on cell viability (Figure 15; D).

3.3.2.2 Differentiated and LPS-Primed HL-60 Cells Stimulated by FMLP to Induce Respiratory Burst

Differentiated HL-60 cells were cultured in high glucose (17.3 mM), and treated with different concentrations (1 μM, 10 μM, and 100 μM) of each fatty acid (ALA, LA, OA, and SA) or its vehicle solution (0 μM) for 24 h. After incubation, LPS (0.5 μg/mL) was added to prime cells for 24 h, which leads to a 10-fold greater respiratory burst upon FMLP stimulation when compared to unprimed cells (see Figure 18).

Data in Figure 16 are presented as a measure of the luminol-dependent chemiluminescence response following stimulation with FMLP.

Results were compared to the data obtained from the chemiluminescence response of the LPS-primed cells stimulated in the absence of fatty acid (0 μM). Only the 100 μM treatment with

ALA decreased the HL-60 chemiluminescence response (Figure 16; A). LA at 1 μM significantly increased chemiluminescence of these cells (Figure 16; B). No other significant differences in chemiluminescence response upon priming and stimulation of the respiratory burst were observed in HL-60 cells cultured in high glucose media (17.3 mM) and treated with OA, or SA (Figure 16; C, D).

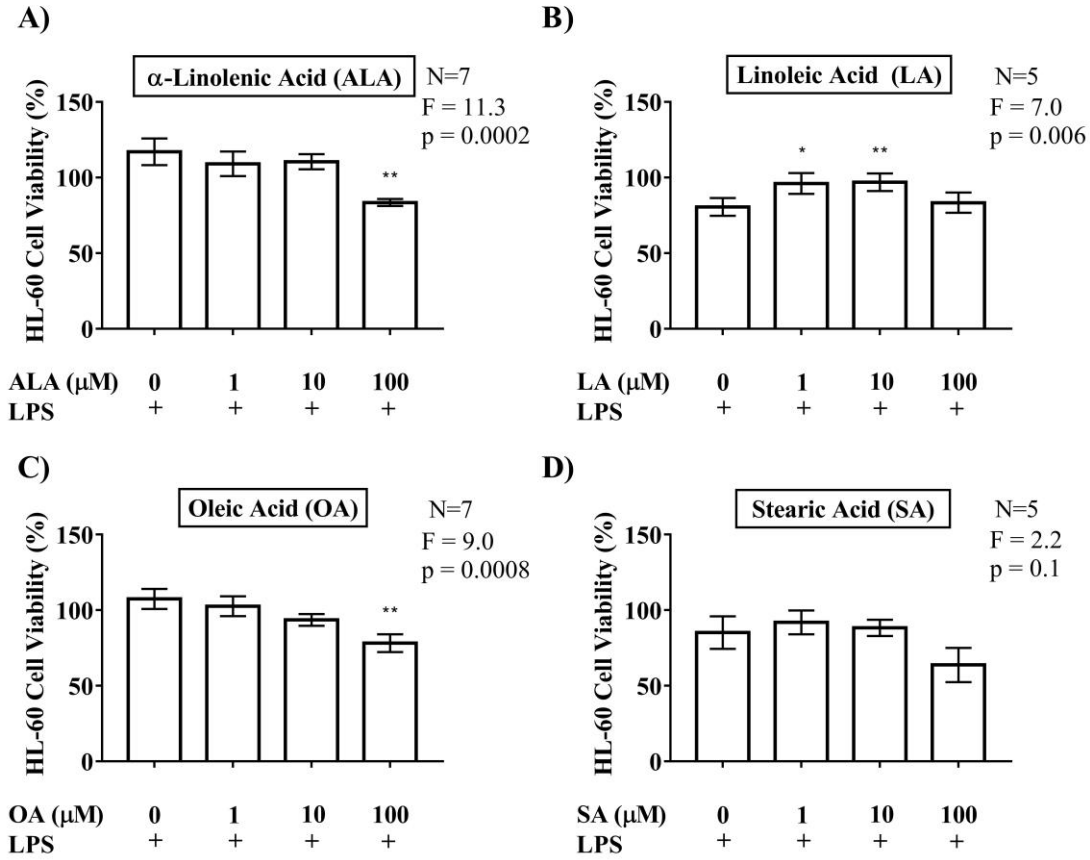

Figure 15. The effects of α-linolenic acid (ALA), linoleic acid (LA), oleic acid (OA), and stearic acid (SA) on the viability of LPS-primed HL-60 cells cultured in high glucose medium (17.3 mM). DMSO was used to differentiate HL-60 cells to induce the expression of NADPH oxidase components. Differentiated HL-60 cells were treated with ALA, LA, OA, SA or their vehicle solution (0 μM fatty acid, 0.18% v/v ethanol and 0.6% w/v BSA). Concentrations of fatty acids (μM) are shown on the abscissa. After incubation, cells were left unprimed or were primed with LPS (0.5 μg/mL) for 24 h. Viability of cells treated with (A) ALA, (B) LA, (C) OA, and (D) SA was assessed by the MTT assay. Data are expressed as a percentage of the viability of differentiated and unprimed HL-60 cells without fatty acid or vehicle treatment. Data (means ± S.E.M.) from five to seven independent experiments are presented. * $p < 0.05$, ** $p < 0.01$, different from LPS-primed cells in the absence of fatty acids (0 μM), according to the randomized block design one-way ANOVA (F and p values indicated), followed by Dunnett's multiple comparison procedure.

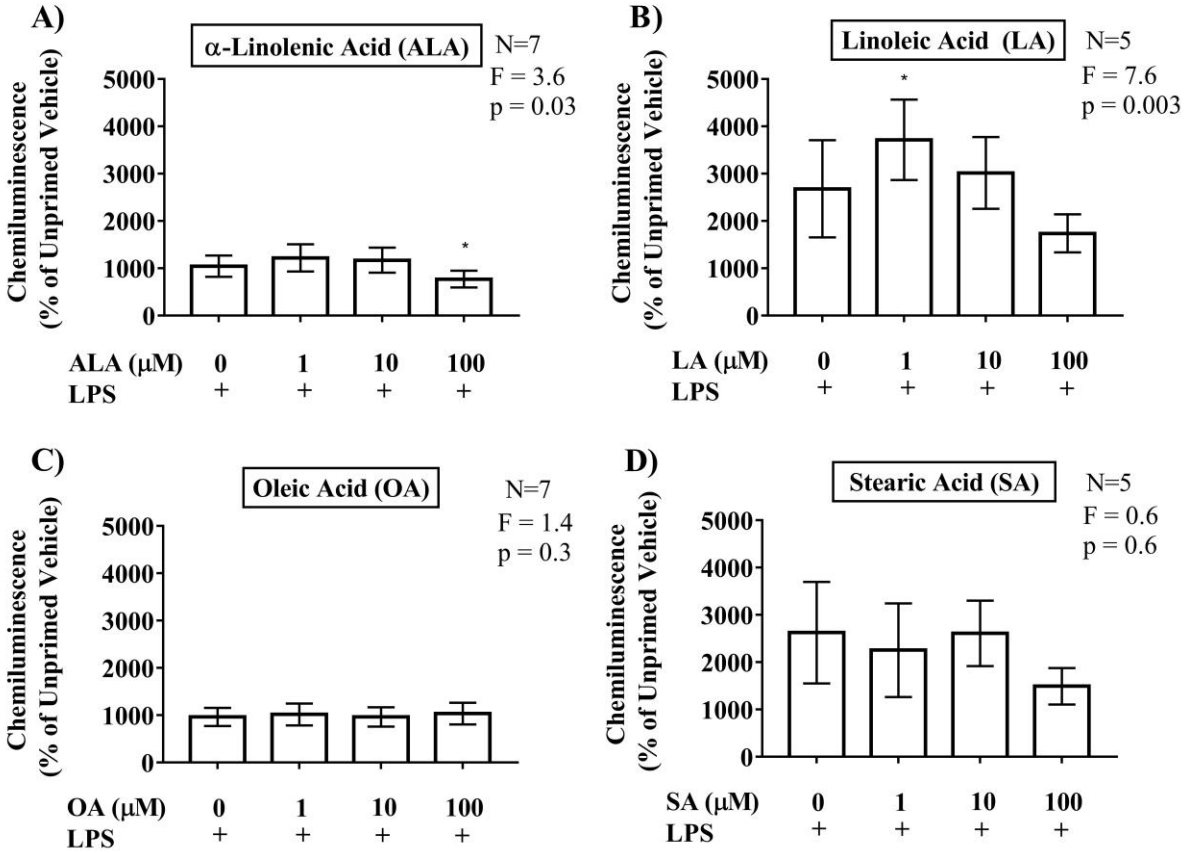

Figure 16. The effects of α-linolenic acid (ALA), linoleic acid (LA), oleic acid (OA), and stearic acid (SA) on the FMLP-stimulated respiratory burst of LPS-primed HL-60 cells cultured in high glucose medium (17.3 mM). DMSO was used to differentiate HL-60 cells to induce the expression of NADPH oxidase components. Differentiated HL-60 cells were treated with ALA, LA, OA, SA or their vehicle solution (0 μM fatty acid, 0.18% v/v ethanol and 0.6% w/v BSA). Concentrations of fatty acids (μM) are shown on the abscissa. After incubation, cells were left unprimed or were primed with LPS (0.5 μg/mL) for 24 h. After 24 h, cells were stimulated with FMLP to induce a respiratory burst, which was measured by the luminol-dependent chemiluminescence response. Chemiluminescence of cells treated with (A) ALA, (B) LA, (C) OA, and (D) SA is presented, and data are expressed as a percentage of the chemiluminescence response by differentiated and unprimed HL-60 cells treated with vehicle solution. Data (means ± S.E.M.) from five to seven independent experiments are presented. * $p < 0.05$, different from LPS-primed cells in the absence of fatty acids (0 μM), according to the randomized block design one-way ANOVA performed on chemiluminescence values before normalization (F and p values indicated), followed by Dunnett's multiple comparison procedure.

3.3.2.3 Cell Viability of Differentiated and Unprimed HL-60 Cells

Experiments were performed as described in 2.10. HL-60 cells were differentiated by DMSO, and then were treated with different concentrations (1 μM, 10 μM, and 100 μM) of each fatty acid (ALA, LA, OA, and SA) or its vehicle solution (0 μM) for 24 h.

Each result was compared to the data obtained from cells cultured in the absence of fatty acid (0 μM). At 1 μM, ALA and OA significantly increased HL-60 cell viability, and at 100 μM, they decreased cell viability (Figure 17; A, C). No difference in cell viability was observed for LA- and SA-treated HL-60 cells that were cultured in high glucose media (17.3 mM; Figure 17; B, D).

3.3.2.4 Unprimed HL-60 Cells Stimulated by FMLP to Induce Respiratory Burst

Differentiated HL-60 cells were cultured in high glucose media (17.3 mM) and then treated with different concentrations (1 μM, 10 μM, and 100 μM) of each fatty acid (ALA, LA, OA, and SA). FMLP was used to stimulate respiratory bursts. Data are presented as a measure of the luminol-dependent chemiluminescence response. Each response was compared to data obtained from cells stimulated in the absence of fatty acid (0 μM). These responses were approximately 10-fold lower than the responses observed in the LPS-primed HL-60 respiratory burst described in 3.3.2.2 (see Figure 16).

The only significant change in respiratory burst chemiluminescence was a significant increase for 1 μM, 10 μM, and 100 μM of OA treatment (Figure 18; C). No other significant difference in chemiluminescence of fatty acid treated HL-60 cells cultured in high glucose (17.3 mM) was observed (Figure 18; A, B, D).

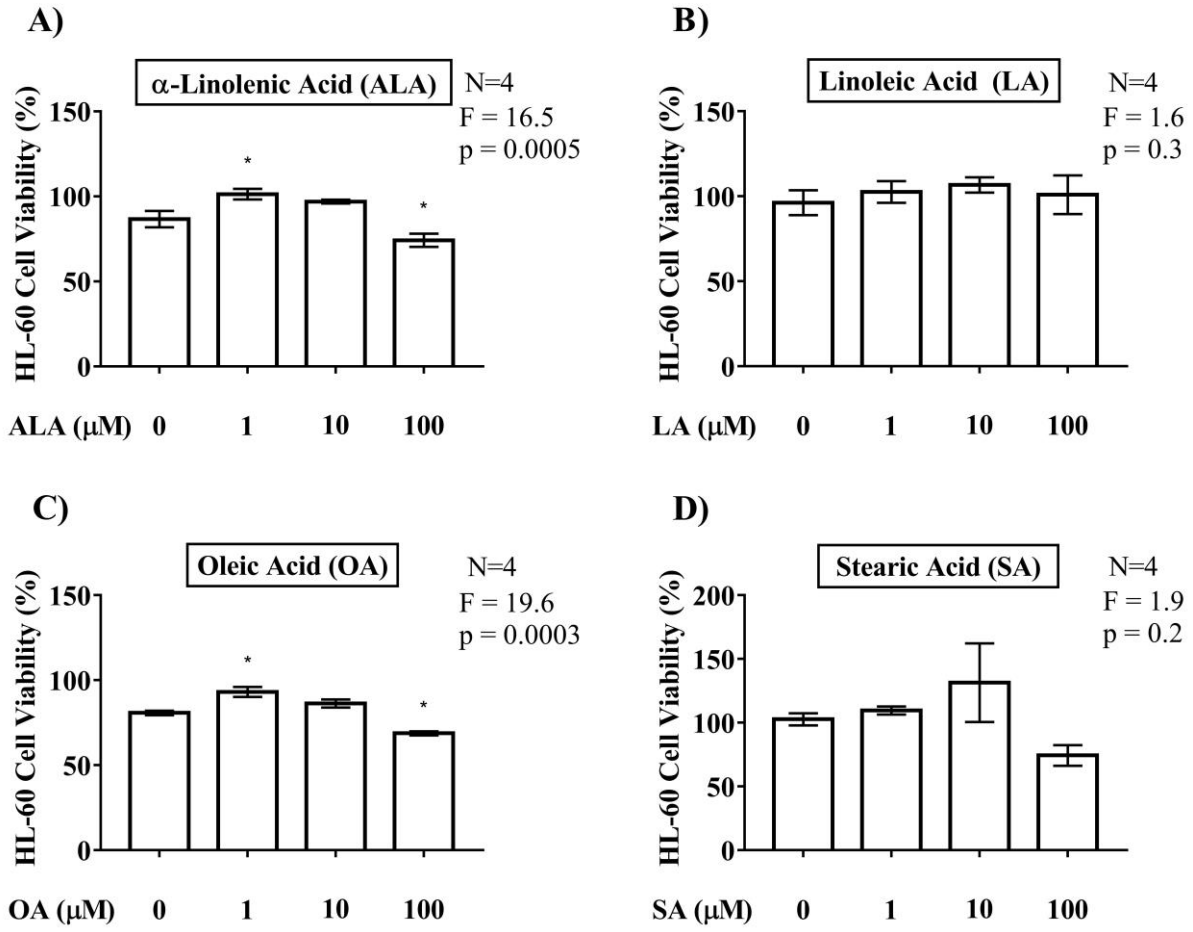

Figure 17. The effects of α-linolenic acid (ALA), linoleic acid (LA), oleic acid (OA), and stearic acid (SA) on the viability of unprimed HL-60 cells cultured in high glucose medium (17.3 mM). DMSO was used to differentiate HL-60 cells to induce the expression of NADPH oxidase components. Differentiated HL-60 cells were treated with ALA, LA, OA, SA or their vehicle solution (0 μM fatty acid, 0.18% v/v ethanol and 0.6% w/v BSA). Concentrations of fatty acids (μM) are shown on the abscissa. Viability of cells treated with (A) ALA, (B) LA, (C) OA, and (D) SA was assessed by the MTT assay. Data are expressed as a percentage of the viability of unprimed HL-60 cells without fatty acid or vehicle treatment. Data (means ± S.E.M.) from four independent experiments are presented. * $p < 0.05$, different from cells in the absence of fatty acids (0 μM), according to the randomized block design one-way ANOVA (F and p values indicated), followed by Dunnett's multiple comparison procedure.

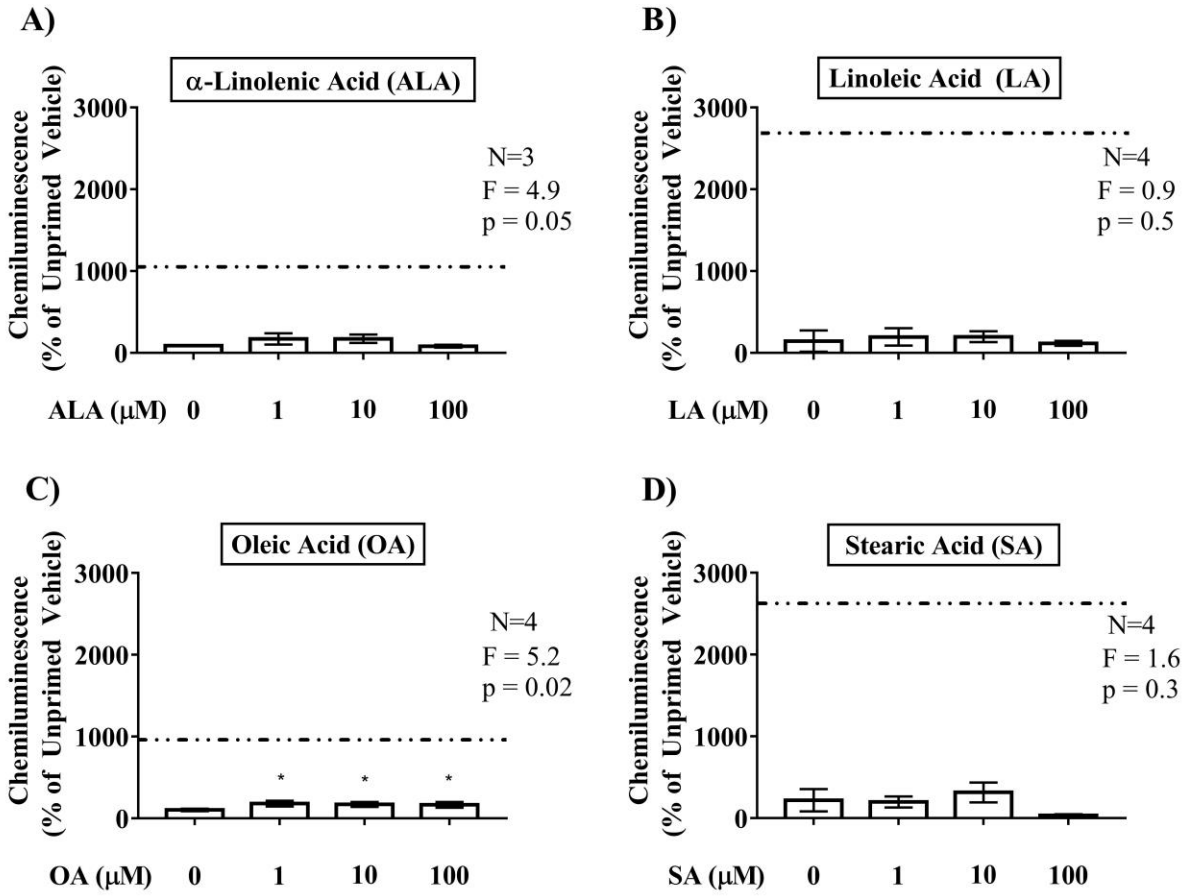

Figure 18. The effects of α-linolenic acid (ALA), linoleic acid (LA), oleic acid (OA), and stearic acid (SA) on the FMLP-stimulated respiratory burst of unprimed HL-60 cells cultured in high glucose medium (17.3 mM). DMSO was used to differentiate HL-60 cells. Differentiated HL-60 cells were treated with ALA, LA, OA, SA or their vehicle solution (0 μM fatty acid, 0.18% v/v ethanol and 0.6% w/v BSA). Concentrations of fatty acids (μM) are shown on the abscissa. After incubation, unprimed cells were stimulated with FMLP to induce a respiratory burst, which was measured by the luminol-dependent chemiluminescence response. Chemiluminescence of cells treated with (A) ALA, (B) LA, (C) OA, and (D) SA is expressed as a percentage of the chemiluminescence response by unprimed HL-60 cells treated with vehicle solution. Data (means ± S.E.M.) from four independent experiments are presented. Chemiluminescence levels from LPS-primed and FMLP-stimulated HL-60 cells treated with vehicle solution are shown as a dotted line for comparison. * $p < 0.05$, different from cells in the absence of fatty acids (0 μM), according to the randomized block design one-way ANOVA performed on chemiluminescence values before normalization (F and p values indicated), followed by Dunnett's multiple comparison procedure.

3.3.3 The Effects of Fatty Acids on MCP-1 Release by THP-1 Cells

Experiments were performed as described in 2.11. THP-1 cells that were cultured in high glucose media (17.0 mM) were treated with different concentrations (1 μM, 10 μM, 100 μM, and 250 μM) of each fatty acid (ALA, LA, OA, or SA) or its vehicle solution (0 μM) for 24 h. After incubation, a combination of LPS (0.5 ng/mL) and IFN-γ (150 U/mL) was used to stimulate the cells for 48 h. The MCP-1 concentration in THP-1 supernatants was quantified by ELISA. Two-way ANOVA assessed the main effects of 1) MCP-1 release from THP-1 cells that were left unstimulated or were stimulated with LPS and IFN-γ and 2) MCP-1 release from THP-1 cells treated with different fatty acid concentrations.

Unstimulated THP-1 cells cultured in high glucose media (17.0 mM) showed significantly lower levels of MCP-1 when compared to MCP-1 levels from cells stimulated with LPS and IFN-γ (Figure 19; A-D). No significant differences in MCP-1 release were observed with different fatty acid treatments by two-way ANOVA (Figure 19; A-D).

3.4 The Effects of Exogenous Fatty Acids on Microglial-Mediated Neurotoxicity

3.4.1 THP-1 Cell Viability and Death

Experiments were performed as described in 2.12. THP-1 cells that were cultured in high glucose media (17.0 mM) were treated with different concentrations (1 μM, 10 μM, 100 μM, and 250 μM) of each fatty acid (ALA, LA, OA, or SA) or its vehicle solution (0 μM) for 24 h. After incubation, a combination of LPS (0.5 ng/mL) and IFN-γ (150 U/mL) was used to stimulate the cells for 48 h. Two-way ANOVA assessed the main effects of 1) viability/cell death of THP-1 cells that were left unstimulated or were stimulated with LPS and IFN-γ and 2) viability/cell death of THP-1 cells treated with different fatty acid concentrations.

All two-way ANOVAs confirmed the significant difference between viability and cell death of unstimulated when compared to stimulated THP-1 cells cultured in high glucose media (17.0 mM; Figure 20, 21, 22, 23). No significant differences by two-way ANOVA of THP-1 cell viability or THP-1 cell death were observed when cells were treated with ALA (Figure 20). Only the 1 μM treatment of LA displayed significantly reduced cell viability of THP-1 cells (Figure 21; A), but cell death was unaffected at all LA concentrations (Figure 21; B). In cells that were either left unstimulated or were stimulated, 1 μM and 10 μM of OA significantly reduced cell viability, but 250 μM of OA increased cell viability (Figure 22; A). OA at 100 μM significantly increased cell viability of stimulated cells. OA did not significantly affect cell death at any concentration (Figure 22; B). All concentrations of SA significantly reduced cell viability of both unstimulated and stimulated cells (Figure 23, A). 250 μM of SA increased cell death of unstimulated cells (Figure 23; B). 100 μM and 250 μM of SA increased death of stimulated THP-1 cells.

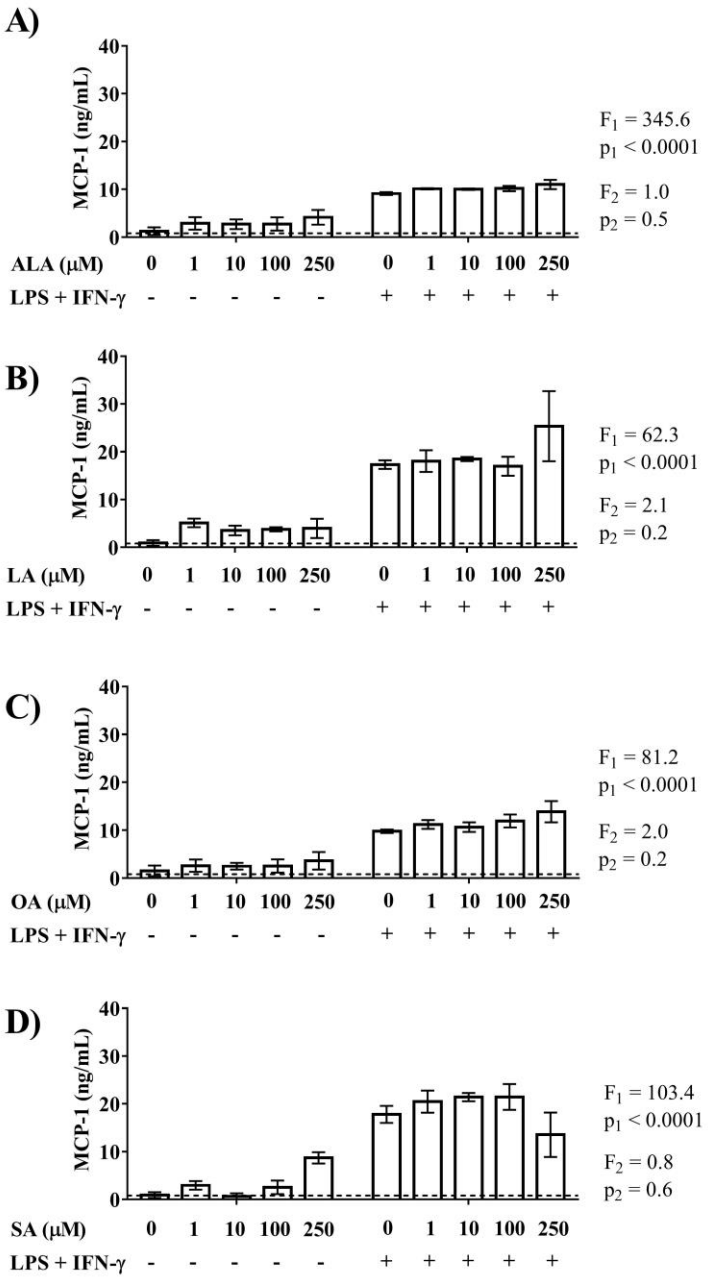

Figure 19. The effects of α-linolenic acid (ALA), linoleic acid (LA), oleic acid (OA), and stearic acid (SA) on the release of MCP-1 by THP-1 cells cultured in high glucose medium (17.0 mM). THP-1 cells were treated for 24 h with ALA, LA, OA, SA or their vehicle solution (0 μM fatty acid, 0.18% v/v ethanol and 0.6% w/v BSA). Concentrations of fatty acids (μM) are shown on the abscissa. After incubation, cells were left unstimulated or were stimulated with a combination of LPS (0.5 ng/mL) and IFN-γ (150 U/mL) for 48 h. After 48 h, ELISA was used to measure the release of MCP-1 (ng/mL) by THP-1 cells treated with (A) ALA, (B) LA, (C) OA, and (D) SA. Data (means ± S.E.M.) from three independent experiments are presented. The detection limit of the ELISA is shown as a dotted line (0.8 ± 0.9 ng/mL). No statistically significant differences within ALA, LA, OA, or SA treatments were observed using a two-way ANOVA. From the two-way ANOVA, F_1 and p_1 values represent the main effect of stimulation, and F_2 and p_2 values represent the main effect of the fatty acid treatment.

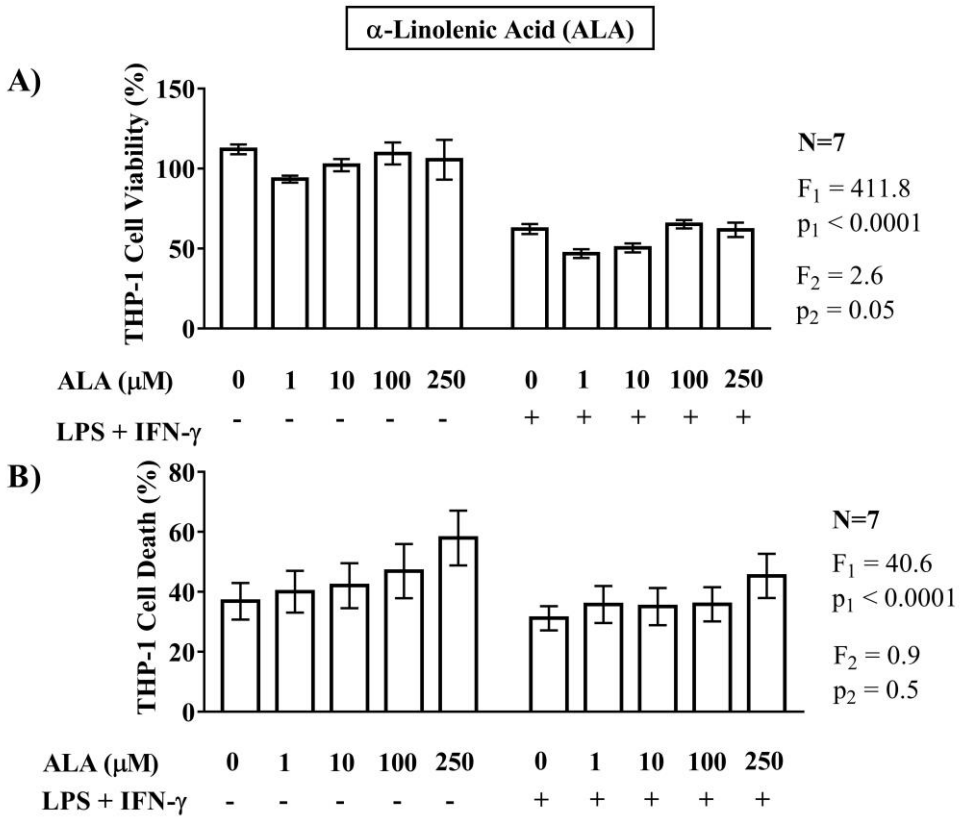

Figure 20. The effects of α-linolenic acid (ALA) on viability and death of THP-1 cells cultured in high glucose medium (17.0 mM). THP-1 cells were treated for 24 h with ALA or its vehicle solution (0 μM fatty acid, 0.18% v/v ethanol and 0.6% w/v BSA). Concentrations of ALA (μM) are shown on the abscissa. After incubation, cells were left unstimulated or were stimulated with a combination of LPS (0.5 ng/mL) and IFN-γ (150 U/mL) for 48 h. Viability and death of ALA-treated THP-1 cells after stimulation were assessed by the (A) MTT assay and (B) LDH assay. Viability data are expressed as a percentage of the viability of unstimulated THP-1 cells without fatty acid or vehicle treatment. Cell death data are expressed as a percentage of the LDH released from untreated, unstimulated and lysed THP-1 cells. Data (means ± S.E.M.) from seven independent experiments are presented. No statistically significant differences between ALA treatments were observed using a two-way ANOVA. From the two-way ANOVA, F_1 and p_1 values represent the main effect of stimulation, and F_2 and p_2 values represent the main effect of the fatty acid treatment.

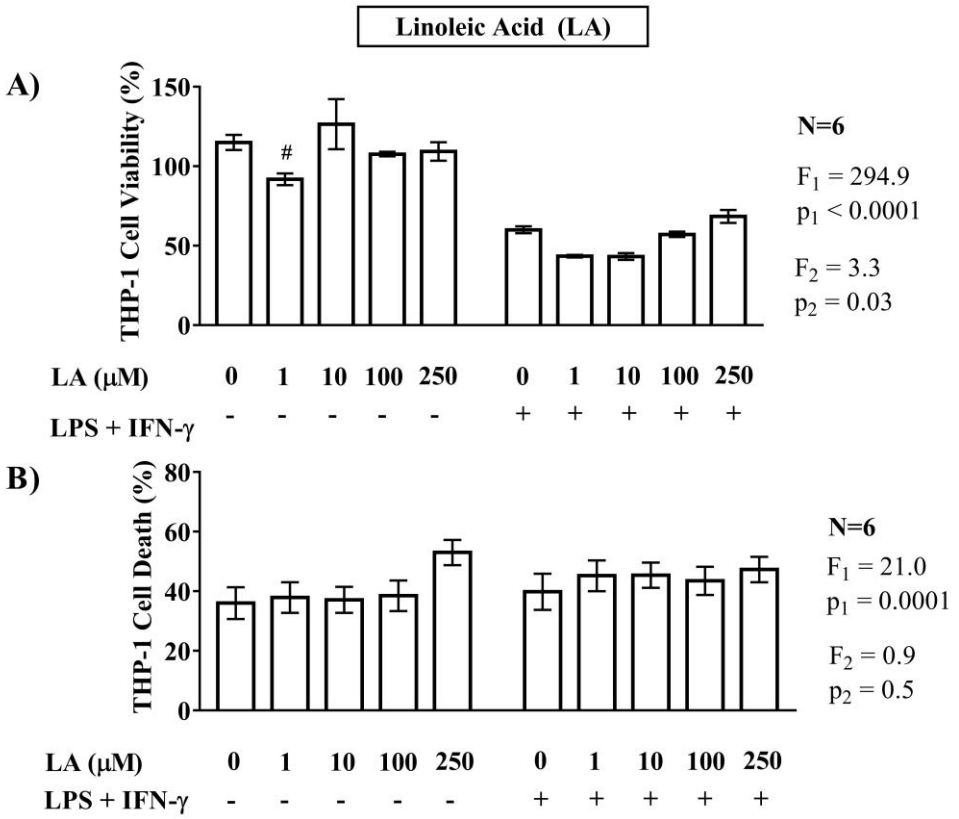

Figure 21. The effects of linoleic acid (LA) on viability and death of THP-1 cells cultured in high glucose medium (17.0 mM). THP-1 cells were treated for 24 h with LA or its vehicle solution (0 μM fatty acid, 0.18% v/v ethanol and 0.6% w/v BSA). Concentrations of LA (μM) are shown on the abscissa. After incubation, cells were left unstimulated or were stimulated with a combination of LPS (0.5 ng/mL) and IFN-γ (150 U/mL) for 48 h. Viability and cell death of LA-treated THP-1 cells after stimulation were assessed by the (A) MTT assay and (B) LDH assay. Viability data are expressed as a percentage of the viability of unstimulated THP-1 cells without fatty acid or vehicle treatment. Cell death data are expressed as a percentage of the LDH released from untreated, unstimulated and lysed THP-1 cells. Data (means ± S.E.M.) from six independent experiments are presented. # $p < 0.05$, different from unstimulated cells in the absence of fatty acid (0 μM), assessed by a two-way ANOVA followed by Dunnett's multiple comparison procedure. From the two-way ANOVA, F_1 and p_1 values represent the main effect of stimulation, and F_2 and p_2 values represent the main effect of the fatty acid treatment.

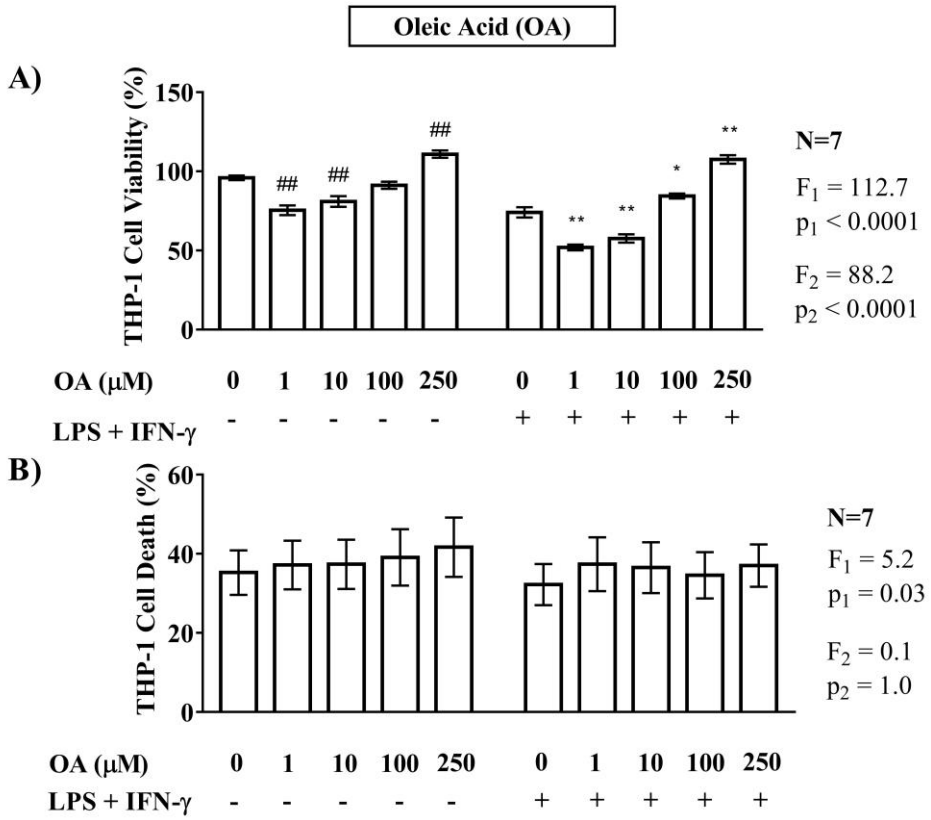

Figure 22. The effects of oleic acid (OA) on viability and death of THP-1 cells cultured in high glucose medium (17.0 mM). THP-1 cells were treated for 24 h with OA or its vehicle solution (0 μM fatty acid, 0.18% v/v ethanol and 0.6% w/v BSA). Concentrations of OA (μM) are shown on the abscissa. After incubation, cells were left unstimulated or were stimulated with a combination of LPS (0.5 ng/mL) and IFN-γ (150 U/mL) for 48 h. Viability and cell death of OA-treated THP-1 cells after stimulation were assessed by the (A) MTT assay, and (B) LDH assay. Viability data are expressed as a percentage of the viability of unstimulated THP-1 cells without fatty acid or vehicle treatment. Cell death data are expressed as a percentage of the LDH released from untreated, unstimulated and lysed THP-1 cells. Data (means ± S.E.M.) from seven independent experiments are presented. ## $p < 0.01$, different from unstimulated cells in the absence of fatty acid (0 μM); * $p < 0.05$, ** $p < 0.01$, different from cells stimulated in the absence of fatty acid (0 μM). Significance was assessed by a two-way ANOVA followed by Dunnett's multiple comparison procedure. From the two-way ANOVA, F_1 and p_1 values represent the main effect of stimulation, and F_2 and p_2 values represent the main effect of the fatty acid treatment.

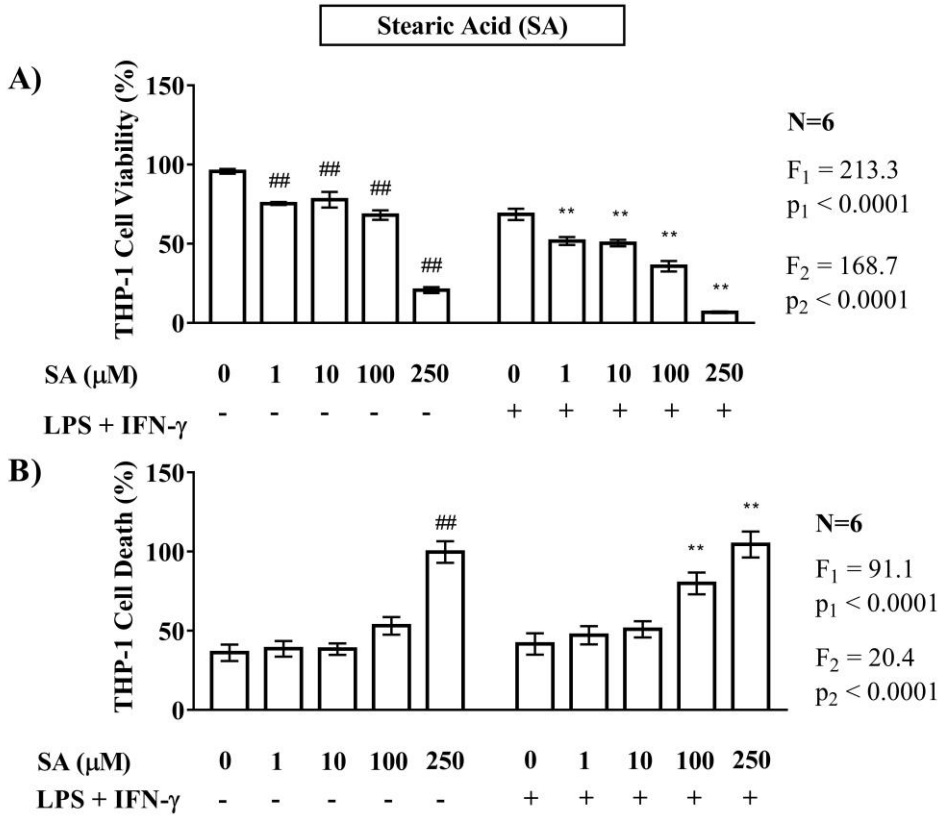

Figure 23. The effects of stearic acid (SA) on viability and death of THP-1 cells microglia cultured in high glucose medium (17.0 mM). THP-1 cells were treated for 24 h with SA or its vehicle solution (0 μM fatty acid, 0.18% v/v ethanol and 0.6% w/v BSA). Concentrations of SA (μM) are shown on the abscissa. After incubation, cells were left unstimulated or were stimulated with a combination of LPS (0.5 ng/mL) and IFN-γ (150 U/mL) for 48 h. Viability and cell death of SA-treated THP-1 cells after stimulation were assessed by the (A) MTT assay, and (B) LDH assay. Viability data are expressed as a percentage of the viability of unstimulated THP-1 cells without fatty acid or vehicle treatment. Cell death data are expressed as a percentage of the LDH released from untreated, unstimulated and lysed THP-1 cells. Data (means ± S.E.M.) from six independent experiments are presented. ## $p < 0.01$, different from vehicle-only treated unstimulated cells; ** $p < 0.01$, different from cells stimulated in the absence of fatty acid (0 μM). Significance was assessed by a two-way ANOVA followed by Dunnett's multiple comparison procedure. From the two-way ANOVA, F_1 and p_1 values represent the main effect of stimulation, and F_2 and p_2 values represent the main effect of the fatty acid treatment.

3.4.2 SH-SY5Y Cell Viability and Death

THP-1 supernatants were collected following treatment with fatty acids and stimulation with a combination of LPS and IFN-γ. SH-SY5Y cells cultured in high glucose media (17.0 mM) were exposed to THP-1 supernatants for 72 h. Following incubation, the effects of THP-1

supernatant exposure on SH-SY5Y cell viability and SH-SY5Y cell death were assessed by the MTT assay and LDH assay. All two-way ANOVAs confirmed the significant difference between SH-SY5Y cells exposed to supernatants from stimulated THP-1 cells when compared to SH-SY5Y cells exposed to unstimulated THP-1 cell supernatants (Figure 24, 25, 26, 27).

No significant differences in SH-SY5Y cell viability or cell death were observed when SH-SY5Y cells cultured in high glucose (17.0 mM) were exposed to supernatant from THP-1 cells that were previously treated with ALA (Figure 24). Similarly, there were no significant differences in viability or death upon SH-SY5Y cell exposure to supernatant from THP-1 cells previously treated with LA (Figure 25). Exposure to supernatants from THP-1 cells treated with OA also did not significantly affect SH-SY5Y cell viability or death (Figure 26). However, supernatants from SA-treated (250 μM) and stimulated THP-1 cells significantly increased the cell death of SH-SY5Y cells exposed to these supernatants without affecting cell viability (Figure 27; cells cultured in 17.0 mM glucose).

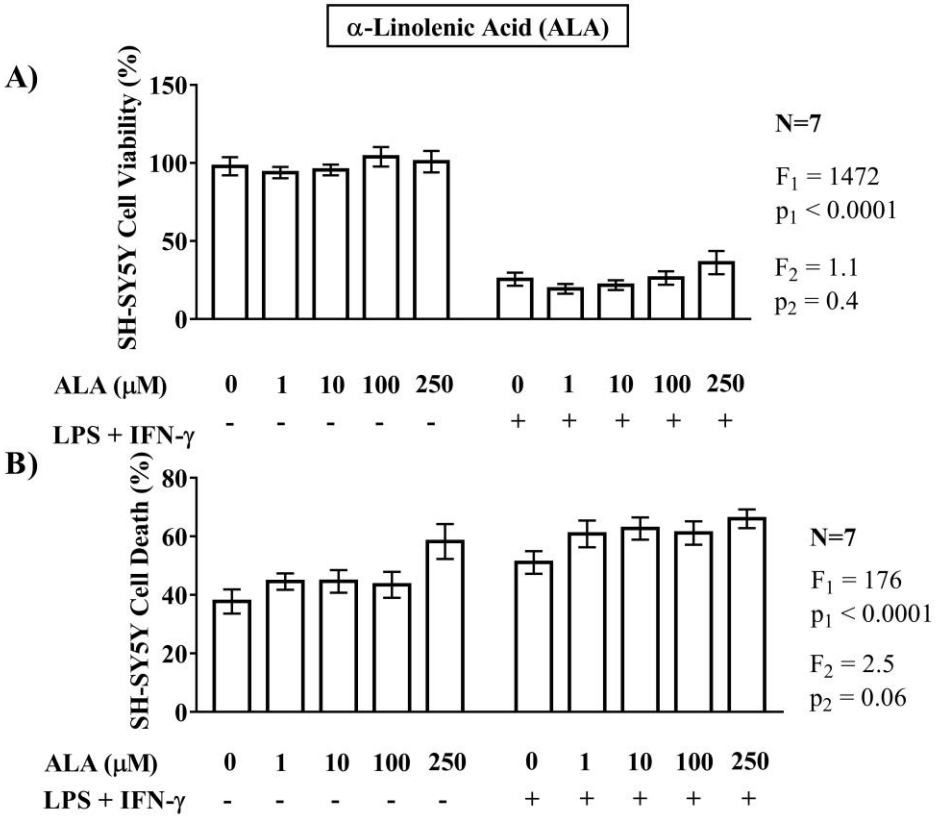

Figure 24. The effects of α-linolenic acid (ALA)-treated THP-1 cell supernatants on viability and death of SH-SY5Y cells cultured in high glucose medium (17.0 mM). THP-1 cells were treated for 24 h with ALA or its vehicle solution (0 μM fatty acid, 0.18% v/v ethanol and 0.6% w/v BSA). Concentrations of ALA (μM) are shown on the abscissa. After incubation, THP-1 cells were left unstimulated or were stimulated with a combination of LPS (0.5 ng/mL) and IFN-γ (150 U/mL) for 48 h. After 48 h, SH-SY5Y cells were exposed to THP-1 supernatants for 72 h. Viability and death of SH-SY5Y cells exposed to supernatants of ALA-treated THP-1 cells were assessed by the (A) MTT assay and (B) LDH assay. Viability data are expressed as a percentage of the viability of unstimulated SH-SY5Y cells without fatty acid or vehicle treatment. Cell death data are expressed as a percentage of the LDH released from untreated, unstimulated and lysed SH-SY5Y cells. Data (means ± S.E.M.) from seven independent experiments are presented. No statistically significant differences between ALA treatments were observed using a two-way ANOVA. From the two-way ANOVA, F_1 and p_1 values represent the main effect of stimulation, and F_2 and p_2 values represent the main effect of the fatty acid treatment.

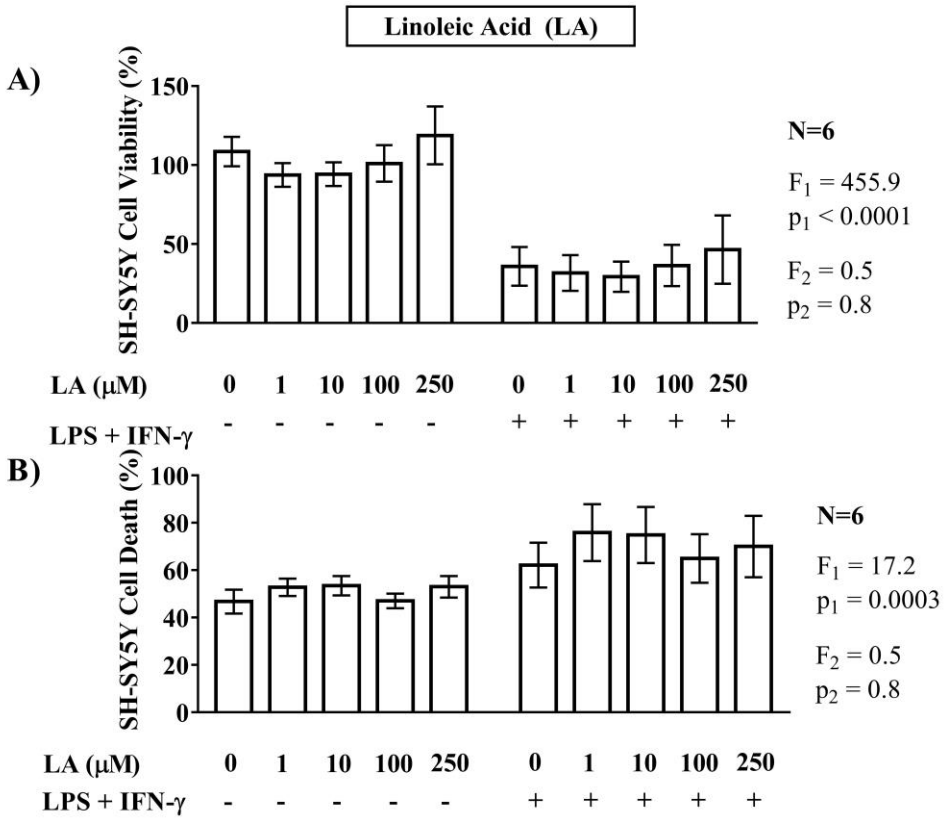

Figure 25. The effects of linoleic acid (LA)-treated THP-1 cell supernatants on viability and death of SH-SY5Y cells cultured in high glucose medium (17.0 mM). THP-1 cells were treated for 24 h with LA or its vehicle solution (0 μM fatty acid, 0.18% v/v ethanol and 0.6% w/v BSA). Concentrations of LA (μM) are shown on the abscissa. After incubation, THP-1 cells were left unstimulated or were stimulated with a combination of LPS (0.5 ng/mL) and IFN-γ (150 U/mL) for 48 h. After 48 h, SH-SY5Y cells were exposed to THP-1 supernatants for 72 h. Viability and death of SH-SY5Y cells exposed to supernatants of LA-treated THP-1 cells were assessed by the (A) MTT assay and (B) LDH assay. Viability data are expressed as a percentage of the viability of unstimulated SH-SY5Y cells without fatty acid or vehicle treatment. Cell death data are expressed as a percentage of the LDH released from untreated, unstimulated and lysed SH-SY5Y cells. Data (means ± S.E.M.) from six independent experiments are presented. No statistically significant differences between LA treatments were observed using a two-way ANOVA. From the two-way ANOVA, F_1 and p_1 values represent the main effect of stimulation, and F_2 and p_2 values represent the main effect of the fatty acid treatment.

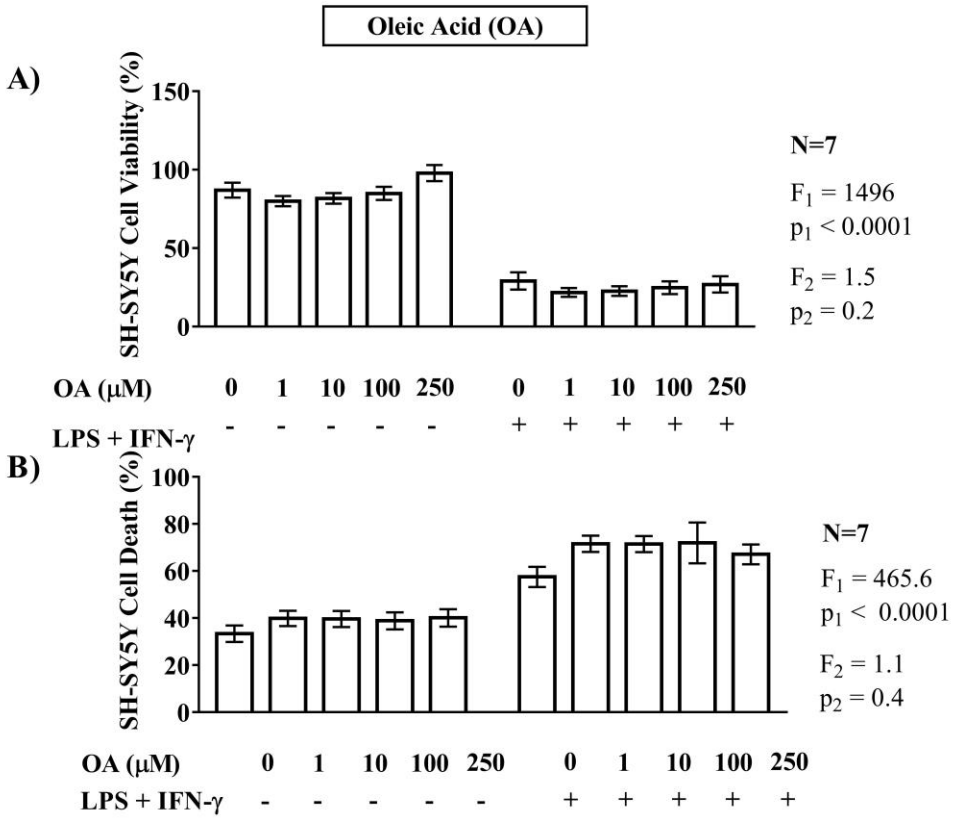

Figure 26. The effects of oleic acid (OA)-treated THP-1 cell supernatants on viability and death of SH-SY5Y cells cultured in high glucose medium (17.0 mM). THP-1 cells were treated for 24 h with OA or its vehicle solution (0 μM fatty acid, 0.18% v/v ethanol and 0.6% w/v BSA). Concentrations of OA (μM) are shown on the abscissa. After incubation, THP-1 cells were left unstimulated or were stimulated with a combination of LPS (0.5 ng/mL) and IFN-γ (150 U/mL) for 48 h. After 48 h, SH-SY5Y cells were exposed to THP-1 supernatants for 72 h. Viability and death of SH-SY5Y cells exposed to supernatants of OA-treated THP-1 cells were assessed by the (A) MTT assay and (B) LDH assay. Viability data are expressed as a percentage of the viability of unstimulated SH-SY5Y cells without fatty acid or vehicle treatment. Cell death data are expressed as a percentage of the LDH released from untreated, unstimulated and lysed SH-SY5Y cells. Data (means ± S.E.M.) from seven independent experiments are presented. No statistically significant differences between OA treatments were observed using a two-way ANOVA. From the two-way ANOVA, F_1 and p_1 values represent the main effect of stimulation, and F_2 and p_2 values represent the main effect of the fatty acid treatment.

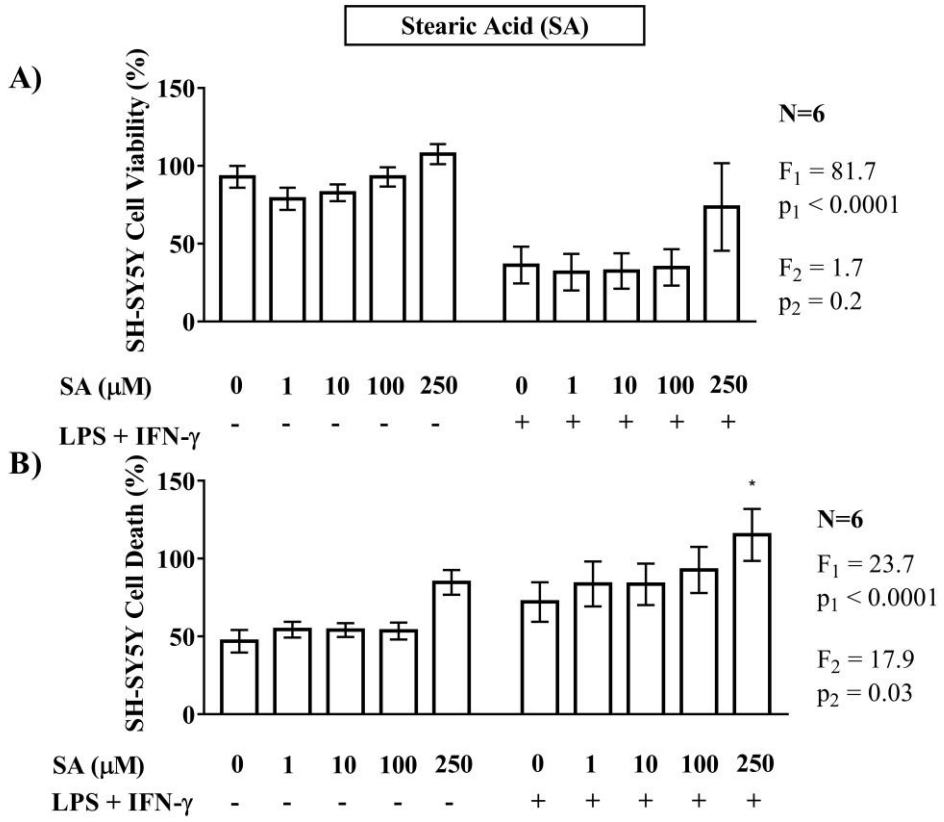

Figure 27. **The effects of stearic acid (SA)-treated THP-1 cell supernatants on viability and death of SH-SY5Y cells cultured in high glucose medium (17.0 mM).** THP-1 cells were treated for 24 h with SA or its vehicle solution (0 μM fatty acid, 0.18% v/v ethanol and 0.6% w/v BSA). Concentrations of SA (μM) are shown on the abscissa. After incubation, THP-1 cells were left unstimulated or were stimulated with a combination of LPS (0.5 ng/mL) and IFN-γ (150 U/mL) for 48 h. After 48 h, SH-SY5Y cells were exposed to THP-1 supernatants for 72 h. Viability and death of SH-SY5Y cells exposed to supernatants of SA-treated THP-1 cells were assessed by the (A) MTT assay and (B) LDH assay. Viability data are expressed as a percentage of the viability of unstimulated SH-SY5Y cells without fatty acid or vehicle treatment. Cell death data are expressed as a percentage of the LDH released from untreated, unstimulated and lysed SH-SY5Y cells. Data (means ± S.E.M.) from six independent experiments are presented. * $P < 0.05$, different from cells stimulated in the absence of fatty acid (0 μM), as assessed by a two-way ANOVA followed by Dunnett's multiple comparison procedure. From the two-way ANOVA, F_1 and p_1 values represent the main effect of stimulation, and F_2 and p_2 values represent the main effect of the fatty acid treatment.

Chapter 4: Discussion

4.1 Treatment of Microglia with Exogenous Fatty Acids Does Not Alter their Fatty Acid Content

Fatty acids can be transported into the cell by fatty acid binding proteins and transporters, and they can also interact with the cell surface receptors (Oh et al., 2010a; Schwenk et al., 2010). Once inside the cell, fatty acids can be metabolized for energy, used in synthesis pathways to produce bioactive mediators, incorporated into phospholipids, or stored in lipid droplets (Auestad et al., 1991; Bazinet and Laye, 2014; Daemen et al., 2016; Langelier et al., 2010). A commonly used method for determining the fatty acid content of a cell is gas chromatography, which can be used to establish a full profile of intracellular fatty acids. Some studies have already investigated the incorporation of fatty acids in mouse macrophage cells (Schroit and Gallily, 1979). Button et al. (2014) used gas chromatography to describe the fatty acid content of BV-2 microglia before and after stimulation with LPS. However, the incorporation of fatty acids into microglia following extracellular application of dietary fatty acids has not been studied.

During this thesis work, the fatty acid content of BV-2 cells that were cultured in low or high glucose media (5.6 mM or 17.0 mM, respectively) was investigated after their exposure to the dietary fatty acids ALA, LA, OA, and SA. During these studies, the baseline fatty acid content of untreated BV-2 cells showed similar relative abundances of fatty acid groups when compared to the composition previously reported in literature (Button et al., 2014). SFAs and MUFAs are highly abundant in BV-2 cells, with lower proportions of n-6 and n-3 PUFAs, in decreasing order. Although MUFAs were expected to be the most abundant group, it was observed that SFA levels exceeded those of MUFAs.

Treatment of BV-2 cells that were cultured in low and high glucose media (5.6 mM and 17.0 mM) with ALA, LA, OA, and SA was hypothesized to increase the fatty acid content of n-3 PUFAs, n-6 PUFAs, MUFAs, and SFAs, respectively. No significant effect of fatty acid treatment on the fatty acid content of BV-2 cells was observed. Therefore, treating cells with 25 μM of fatty acid for 24 h was not sufficient to induce a detectable change in the fatty acid content of microglia, due to lack of effect or an effect that was below the detection limit of GC-FID. This exposure to fatty acids may not be affecting immune responses in unstimulated BV-2 cells by modifying membrane composition.

In human monocyte-derived macrophages, high concentrations of glucose inhibited fatty acid transporter expression, such as the CD36 transporter (Moheimani et al., 2011). The high level of glucose in cell culture was hypothesized to prevent fatty acid uptake in microglia, which could result in the observed lack of effect on microglial fatty acid content. When gas chromatography experiments were repeated in low glucose media (5.6 mM), results were similar to data obtained in high glucose media (17.0 mM). Thus, reducing glucose levels did not promote the entry of fatty acids into BV-2 microglia. Based on previous findings with other CNS cell types, it was expected that BV-2 cells cultured over 24 h in media containing glucose at concentrations lower than 5.6 mM were not viable (Bahniwal, 2014). Thus, changes in fatty acid composition in the presence of even lower glucose concentrations were not studied.

Transcriptomics studies have described the presence of multiple fatty acid receptors and transporters in mouse microglia, including the scavenger receptor CD36, and fatty acid transport protein 4 (Zhang et al., 2014). CD36 is also an important mediator in LPS-induced activation of BV-2 mouse microglia, and primary mouse microglia. CD36 is involved in the phagocytosis of apoptotic material from neurons (Stolzing and Grune, 2004; Xia et al., 2017; Zhang et al., 2013).

Activating microglial cells to a pro-inflammatory state may allow for the increased uptake of fatty acids due to CD36 upregulation.

4.2 Fatty Acids Do Not Alter Phagocytic Activity of Primary Microglia

Microglia use phagocytosis to eliminate synapses, cellular debris and pathogens from the CNS (Brown and Neher, 2014; Floden and Combs, 2011; Petersen and Dailey, 2004). Increased phagocytosis is a pro-resolving action of microglia and is beneficial for maintenance of CNS homeostasis. Cell surface receptors bind molecules expressed by apoptotic cells such as phosphatidylserine to initiate engulfment, and the plasma membrane encapsulates this stimulus (reviewed by Sokolowski and Mandell, 2011). Phagocytosis can remove abnormal structures, but this process is impaired in aged and AD-like disease states (Floden and Combs, 2011; Sokolowski and Mandell, 2011). Phagocytosis can be impacted by membrane fluidity, which is affected by membrane composition (reviewed by Schumann, 2016). Fluidity of the phospholipid membrane can be increased by the presence of unsaturated fatty acids. Higher unsaturation of membrane fatty acids was previously associated with increased phagocytic activity (Calder et al., 1990). In this study, the treatment of macrophages with unsaturated fatty acids, including ALA and LA, induced a higher phagocytic response, while OA did not affect phagocytosis. Additionally, SA treatment did not alter phagocytic activity.

Based on the degree of unsaturation of the fatty acids studied, I hypothesized that ALA, LA and OA would increase phagocytic activity of primary mouse microglia. Conversely, SA was hypothesized to decrease microglial phagocytosis. My preliminary data showed that 25 μM of ALA, LA, OA, and SA had no effect on the phagocytic activity of primary microglia. This contrasts previous studies that determined a role for ALA and LA in promoting the phagocytic

response of RAW264.7 peripheral macrophages (Adolph et al., 2012). Lack of effect of OA on the phagocytosis of $A\beta_{1\text{-}42}$ by primary mouse microglia has previously been demonstrated (Ano et al., 2015), which is similar to the data obtained in experiments described in this thesis. The lack of effect of SA on phagocytosis has previously been documented in mouse macrophages (Calder et al., 1990), but not in microglia. The results described in this thesis indicate that SA does not impact phagocytosis in primary microglia. A previous study determined that OA did not alter BV-2 microglial membrane fluidity (Debbabi et al., 2017). This is consistent with our data, as we would expect that increased membrane fluidity would promote phagocytosis, whereas the lack of effect on phagocytic activity was observed.

Phagocytosis by microglia is critical for clearing pathogens as well as abnormal proteins in the CNS. The inability of fatty acids to promote a positive effect on phagocytosis would have no beneficial effect on the microglial neuroinflammatory response; however, if an effect to increase phagocytosis was observed, cells would have a greater ability to clear proteins including toxic Aβ protein. Compounds that can modulate phagocytic activity are of particular interest as potential treatment options for AD, among other neurodegenerative diseases (reviewed in Fu et al., 2014).

I cannot conclusively comment on the interaction of membrane composition and phagocytosis, as fatty acid content and phagocytosis were assessed using identical treatment parameters but different cell types: BV-2 mouse microglia and primary mouse microglia. Primary adult mouse microglia were chosen for physiological relevance in the phagocytosis assay, and the procedure to measure phagocytosis by fluorescence has been previously described using primary microglia (Pointer, 2017). In primary microglia, the effect of fatty acid treatment on intracellular fatty acid composition remains to be determined since experiments were

performed with BV-2 microglia only. Interestingly, there is evidence that NO may impair phagocytosis in BV-2 mouse microglia (Kopec and Carroll, 2000). Thus, a further direction for my studies would be to determine if microglial phagocytosis is altered at concentrations of fatty acid that impact NO release by microglia.

4.3 Fatty Acids Reduce BV-2 Microglia Nitrite and iNOS Levels

NO, a reactive nitrogen species that can diffuse freely through cellular membranes, is an important regulatory molecule in the immune system (Aktan, 2004). BV-2 microglia constitutively express endothelial NO synthase (eNOS), which may generate low levels of NO (Kopec and Carroll, 2000). At low concentrations, NO can be beneficial to the brain as a neurotransmitter (reviewed by Balez and Ooi, 2016; Paul and Ekambaram, 2011). Upregulation of iNOS enzyme can produce high levels of NO, which occurs in BV-2 microglia stimulated by LPS and IFN-γ (Kopec and Carroll, 2000). Such high levels of NO released by microglia cause pro-inflammatory outcomes that are detrimental to neurons, including nitroxidative stress and necrotic damage (Bal-Price and Brown, 2001; Brown, 2010). Nitrite, which is a breakdown product of NO, as well as iNOS were measured in BV-2 microglia cultures. In these experiments, nitrite was present in significantly increased levels in supernatants from BV-2 microglia (cultured in high glucose media of 17.0 mM) after stimulation with LPS as well as IFN-γ. Similarly, the expression of iNOS was induced by stimulation using LPS or IFN-γ.

ALA was previously shown to reduce nitrite and iNOS in LPS-stimulated RAW 264.7 macrophages (Ren and Chung, 2007). Thus, we hypothesized that ALA treatment would also reduce nitrite and iNOS in microglia. ALA reduced nitrite released by BV-2 cells that were cultured in high glucose (17.0 mM) and stimulated with LPS; however, ALA did not reduce the

expression of iNOS upon LPS stimulation without causing reduced cell viability. Similar data have been obtained by using peripheral macrophages. One study using RAW 264.7 macrophages found that ALA reduced nitrite in supernatant, but did not significantly reduce iNOS (Ambrozova et al., 2010). These results may seem contradictory; however, several factors need to be considered when interpreting the result that ALA treatment reduced nitrite levels without reducing iNOS protein expression. Firstly, a control experiment determined that at the highest concentration used, none of the fatty acids significantly altered nitrite levels in the absence of cells, which indicated that none of the fatty acids (ALA, LA, OA, or SA) exhibited NO-scavenging activity or interfered with the Griess assay (data not shown). The discrepancy in the effects of ALA on nitrite levels and iNOS expression could be due to indirect interactions with NO. NO reacts with multiple chemical species, which could be affected by the fatty acids in cell culture. NO interacts with signalling factors, such as thiols, to form additional chemical species, known as nitrosothiols (Broniowska and Hogg, 2012; Wolhuter et al., 2018). For example, S-nitrosoglutathione is formed from a reaction of NO with glutathione (Keszler et al., 2011). ALA has been shown to increase glutathione in smooth muscle (Hamilton et al., 2003). Further studies should assess the changes in expression of NO-reactive groups induced by ALA to address this research gap.

OA was hypothesized to inhibit the NO release by microglia leading to reduced nitrite levels in BV-2 supernatant. However, no significant differences in nitrite levels were observed; thus, at the concentrations tested, OA did not affect this microglial immune response. These results did not support the observed reduction of nitrite in the supernatant of BV-2 microglia and primary rat microglia reported in one available study (Oh et al., 2009). This could be due to differences in study design including incubation times, as the study had an acute exposure to OA

(1 h), whereas in this thesis a different exposure time was used (24 h). OA administered *in vivo* did not affect the level of nitrite in the supernatant of peritoneal macrophages obtained from these animals (Magdalon et al., 2012). While a different cell type was used, this lack of effect *in vivo* was similar to the lack of *in vitro* effects described in this thesis.

Since metabolites of LA are known for having pro-inflammatory functions, LA was hypothesized to increase nitrite release and upregulate the expression of iNOS. In LPS-stimulated BV-2 cells, LA treatment was found to decrease nitrite release as well as the expression of iNOS. In support of these results, another study determined that in a species of mushroom that exhibits medicinal properties, LA is the primary component that is responsible for reducing the BV-2 cell expression of iNOS (Nallathamby et al., 2016). The observed reduction in nitrite levels may reduce inflammatory responses, as the excessive release of NO leads to nitroxidative stress in the CNS. Nitrosative and oxidative stress are implicated in neurodegenerative pathways and could induce microglial responses to increase neuroinflammation and alter CNS status (reviewed in Brown, 2010; Zhang et al., 2012; Bordt and Polster, 2014).

SA was previously shown to induce the production of iNOS mRNA in BV-2 microglia, among other enhanced pro-inflammatory outcomes (Wang et al., 2012). We hypothesized that SA would enhance pro-inflammatory responses in microglia, resulting in increased levels of nitrite in BV-2 supernatant when experiments were performed in high glucose media (17.0 mM). However, in our experiments SA significantly reduced levels of nitrite in BV-2 supernatant. Increased toxicity upon SA treatment was likely responsible for this effect, which was demonstrated as reduced viability and increased death of BV-2 cells cultured in the presence of high glucose (17.0 mM) and SA.

n-3 PUFAs interact with the GPR-120 receptor to exert immunomodulatory responses (Oh et al., 2010a; Han et al., 2017). For instance, in mice fed high-fat diet, treatment with DHA reduced mRNA expression of pro-inflammatory markers such as iNOS (Oh et al., 2010a). This effect was abrogated when the gene for the GPR-120 receptor was deleted, demonstrating that some anti-inflammatory functions of PUFAs are mediated through GPR-120. In human embryonic kidney cells, ALA was the most potent agonist of this receptor among the fatty acids tested; however, DHA as well as the product of the LA desaturation reaction, γ-linolenic acid, also interacted with GPR-120 (Hirasawa et al., 2005). The GPR-120 receptor mRNA is expressed by BV-2 cells (Dragano et al., 2017).

The mechanism by which ALA and LA reduce nitrite in microglial supernatant is currently unknown. The fatty acids ALA and LA were hypothesized to interact with GPR-120 to cause the observed decreases in nitrite levels. DHA was used as a positive control to demonstrate that AH-7614 was effectively blocking activity of the GPR-120 receptor. We hypothesized that DHA would bind GPR-120 to reduce BV-2 nitrite levels. We can provide no evidence that ALA operates through a GPR-120-dependent mechanism. Pre-treatment with 1 μM of the GPR-120 antagonist, AH-7614, did not significantly block the reduction in nitrite levels by ALA treatment at 100 μM in the presence of high glucose (17.0 mM). Conversely, the nitrite-reducing effect of the 25 μM treatment with DHA was significantly inhibited by 1 μM of AH-7614. LA may also operate through a GPR-120-dependent mechanism. This interaction with GPR-120 was demonstrated by a significant increase in nitrite after 1 μM of AH-7614 antagonist was used in combination with 100 μM of LA treatment, when compared to BV-2 cells treated with LA alone. These data must be interpreted with caution as a significant increase in viability was observed for

these LA-treated cells (all experiments were performed in media containing 17.0 mM glucose). Thus, further studies are required to elucidate this mechanism.

IFN-γ was used as another stimulus to test whether the observed effects of fatty acids on nitrite were specific to LPS stimulatory pathways. LPS exerts its effects through the TLR-4/nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB) pathway, whereas IFN-γ exerts its effects through the Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway (Aktan, 2004). We hypothesized that ALA and LA treatment could reduce iNOS expression induced by both pathways. However, treatment with ALA and LA reduced the levels of nitrite in IFN-γ-stimulated BV-2 cells without downregulating iNOS expression.

The GPR-120 receptor has been shown to operate through downstream inhibition of NF-κB in RAW 264.7 macrophages (Oh et al., 2010a). LA reduced the expression of iNOS in LPS-stimulated BV-2 cells and may regulate its transcription, potentially through activation of the GPR-120 receptor. This receptor activation could inhibit LPS-stimulated iNOS production, which is dependent on NF-κB, resulting in lower nitrite levels in the supernatant. Additionally, we did not find evidence to suggest that the nitrite reduction seen in cells cultured in high glucose media (17.0 mM), treated with ALA or LA and stimulated with IFN-γ occurs by iNOS-dependent regulation. Further experiments should be performed to clarify the regulation of NO in IFN-γ-stimulated BV-2 cells.

4.4 Fatty Acids Do Not Affect HL-60 ROS Release

The release of ROS is an important microglial immune response to eliminate pathogens. ROS can be generated by the respiratory burst where cell stimulation (for example, with FMLP) leads to assembly of NADPH-oxidase machinery and release of a large burst of ROS (Forehand

et al., 1989). Use of LPS prior to this stimulation primes the respiratory burst response, which leads to enhanced production of ROS.

ALA and OA were hypothesized to reduce the ROS release of LPS-primed and FMLP-stimulated HL-60 cells. The respiratory burst in differentiated HL-60 cells cultured in high glucose media (17.3 mM) was studied to model human microglia. LA and SA were hypothesized to increase the ROS release by respiratory burst. For the LPS-primed and FMLP-induced respiratory burst, only ALA treatment reduced ROS release at the highest concentration, which corresponded to a reduction in cell viability. Thus, this thesis does not describe significant effects of any fatty acid on ROS release that could not be explained by their effects on cell viability. These data indicate that the fatty acids studied would not reduce oxidative damage caused by activated microglia in the presence of high glucose (17.3 mM).

The unprimed and FMLP-stimulated respiratory burst was assessed by luminol-dependent chemiluminescence measurement. ALA did not significantly affect the unprimed HL-60 respiratory burst. OA only slightly but significantly increased the unprimed respiratory burst at 1 μM, 10 μM and 100 μM. At 1 μM, but not 10 μM, OA also significantly increased cell viability, and at 100 μM OA significantly reduced cell viability. The result described herein may indicate immunomodulatory effects of OA at these specific concentrations in the presence of high glucose (17.3 mM). Increased LA content of primary human neutrophils has been previously shown to correlate with the reduced FMLP-stimulated respiratory burst by these cells (Bellavite et al., 1995). This study also failed to find a correlation between the SA content of human neutrophils and ROS release via their respiratory burst response. However, both LA and SA did not affect unprimed respiratory burst of FMLP-stimulated HL-60 cells in the presence of high glucose (17.3 mM) described by this thesis. It is important to note that the unprimed

respiratory burst induced by FMLP shows large variability between vehicle-treated cells, and can be 10-fold lower than the LPS-primed HL-60 respiratory burst. Thus, respiratory bursts generated by unprimed cells may not be sufficiently strong and reliable to observe any changes.

4.5 Fatty Acids Do Not Exhibit Effects on MCP-1 Release from THP-1 Cells

MCP-1 is a chemokine involved in the microglial immune response. ALA has been shown to reduce the mRNA expression of pro-inflammatory cytokines TNF-α and IL-1β in THP-1 cells (Zhao et al., 2005). LA treatment has been shown to reduce the chemoattractant chemokine IL-8 protein secreted from THP-1 cells (Håversen et al., 2009), in addition to decreasing mRNA expression of pro-inflammatory cytokines TNF-α and IL-1β (Zhao et al., 2005). SA treatment has been shown to increase the release of pro-inflammatory mediators including IL-1β and TNF-α by THP-1 cells (Håversen et al., 2009). We hypothesized that MCP-1 is reduced upon treatment with ALA and OA, and increased upon treatment with LA and SA. No effects of any fatty acid treatment on MCP-1 release by unstimulated or LPS- and IFN-γ-stimulated THP-1 cells were observed.

As MCP-1 is a chemokine, it can promote the resolution of acute inflammation, but it was also found at increased levels in CSF of AD-afflicted individuals; additionally, MCP-1 was detected within Aβ plaques in human brains afflicted with AD (Galimberti et al., 2006; Ishizuka et al., 1997). MCP-1 did not affect the levels of pro-inflammatory mediators in rat microglia, indicating that MCP-1 does not exacerbate inflammation but contributes to inflammation resolution by recruiting monocytes and microglia (Hinojosa et al., 2011; Sheehan et al., 2007). Accordingly, MCP-1 release may be a beneficial response that is not affected by fatty acid treatment as described in this thesis. Inflammatory conditions may promote neurodegeneration,

and as MCP-1 was unaltered following fatty acid treatment, these findings do not support a pro-inflammatory role of the fatty acids studied. Further, lack of effect of OA on the release of MCP-1 by unstimulated THP-1 cells has been shown previously (Cullberg et al., 2014). Similarly, another study found no effect of LA on levels of MCP-1 released by THP-1 cells that had been differentiated to a macrophage phenotype (Wang et al., 2009). Our findings support these results in the THP-1 microglia model, and are first to suggest the lack of effect of ALA and SA on MCP-1 release from THP-1 cells cultured in high glucose media (17.0 mM).

4.6 Fatty Acids Do Not Affect Microglia-Mediated Neurotoxicity

ALA and OA were hypothesized to inhibit microglial immune responses, and lead to reduced microglial neurotoxicity. Both ALA and OA showed no effect on microglia-mediated neurotoxicity when assessed by measuring SH-SY5Y neuronal viability and cell death following exposure to supernatants from THP-1 cells cultured with fatty acid in the presence of high glucose (17.0 mM). Previous studies showed that neurotoxicity was unaltered upon exposure of neuronal cells to media from THP-1 cells treated with OA (Little et al., 2012). However, ALA had not been previously studied using this *in vitro* model. The data obtained indicated that exposure to this fatty acid did not affect the overall inflammatory status of the THP-1 cells sufficiently to impart an effect on the secretion of neurotoxic molecules in the presence of high glucose (17.0 mM). A previous study has also shown no effect of ALA treatment on count and viability of unstimulated THP-1 cells (Vangaveti et al., 2014).

LA and SA were hypothesized to increase microglial immune responses, and lead to increased neurotoxicity upon incubation of neuronal cells with supernatants of THP-1 cells cultured with fatty acid. Neurotoxicity was previously found upon incubation of neurons with

supernatants from THP-1 cells that had been cultured with the SFA palmitate (Little et al., 2012). Metabolites of LA, but not LA itself, were also found to decrease viability of THP-1 cells (Vangaveti et al., 2014). However, in this thesis work no concentration-dependent effect of the LA treatment was observed on viability and death of THP-1 cells, which corroborates these previous findings. No effect was seen for viability or death of SH-SY5Y neuronal cells after they were exposed to supernatants from LA-treated THP-1 cells in the presence of high glucose (17.0 mM), which suggests that LA treatment did not promote neurotoxicity of microglia at the concentrations studied.

The toxicity of 100 μM of SA on THP-1 cells in culture has been previously described (Håversen et al., 2009). In this thesis work, SA toxicity at all concentrations was observed as reduced THP-1 cell viability, and as increased cell death at high SA concentrations. When neuronal cell viability and death were assessed following exposure to these THP-1 supernatants, a significant increase in neuronal death was determined for the highest concentration of SA. These data indicate that SA inhibits immune functions of THP-1 cells cultured in high glucose (17.0 mM) due to its toxic effect on this cell type. As widespread destruction of immune cells is not desired for homeostasis of the CNS, the toxic effects of SA may be detrimental when interacting with microglia in the CNS.

Chapter 5: Conclusion

5.1 Research Hypotheses Addressed

5.1.1 Hypothesis 1: The Fatty Acid Composition of BV-2 Microglia Changes Following Fatty Acid Treatment

Contrary to my hypothesis, treatment with four different types of dietary fatty acids added extracellularly at a concentration of 25 μM for 24 h did not affect the fatty acid composition of BV-2 microglia cultured in either low or high glucose media (5.6 mM or 17.0 mM, respectively). Future experiments could explore different fatty acid concentrations and microglia exposure times.

5.1.2 Hypothesis 2: Fatty Acids Affect the Phagocytic Activity of Primary Microglia

None of the dietary fatty acids studied were found to affect the phagocytic activity of primary mouse microglia upon incubation with 25 μM of fatty acid for 24 h in the presence of high glucose (17.0 mM). Future experiments could explore different fatty acid concentrations and microglia exposure times.

5.1.3 Hypothesis 3: Fatty Acids Regulate Select Microglial Responses

Aligned with my hypothesis, in the presence of high glucose (17.0 mM) the dietary fatty acids ALA and LA regulated microglial RNS release and iNOS expression. However, none of the fatty acids studied affected microglial secretion of ROS, MCP-1, and neurotoxins in the presence of high glucose (17.0 mM to 17.3 mM). Future studies could elucidate the mechanism behind the effects of dietary fatty acids on RNS release and iNOS expression.

5.2 Limitations of the Research

One important limitation in collecting *in vitro* data is the well-established differences between human and mouse cells. Firstly, BV-2 microglia and primary microglia used in this thesis work are mouse cells. The ability to extrapolate data from mouse cells to human cells has recently come into question. A study by Galatro et al. (2017) found some similarities between human and mouse microglia; however, there were many gene transcripts that were expressed differentially in aged human and mouse microglia. Further, mouse cells have altered patterns of fatty acid uptake, when compared to the uptake of fatty acids demonstrated for humans (Fritsche, 2007). For instance, an analysis of multiple mouse and human studies found that mouse lymphocytes have higher levels of DHA, compared to human cells. PUFA uptake in lymphocytes after supplementation of mouse diets and human diets differs as well, with the accumulation of higher percentages of DHA in mouse cells. Therefore, the effects shown in mouse cells may have a different magnitude when compared to human cells.

An important caveat in this thesis work is that mouse cells have been used to determine the effects of fatty acids on microglial fatty acid content, phagocytosis, and iNOS expression. Our data regarding nitrite and iNOS levels require caution to interpret, as it is currently a source of controversy whether human macrophages release NO in quantities large enough to promote inflammation (Thomas and Mattila, 2014). iNOS has been detected in human monocytes and macrophages isolated from various disease states and in one human microglial cell line (Hjorth et al., 2010; Thomas and Mattila, 2014). Mouse and rat glia release large amounts of NO when activated, which promote inflammation and neuron death (Bal-Price and Brown, 2001; Brown, 2010; Liberatore et al., 1999). The impact of iNOS-dependent production of NO by human

microglia is unknown. Thus, inferences made may not be applicable to human cells, and some degree of caution must be used when interpreting data discussed in this thesis.

To resolve these important species limitations, where possible, human cell lines have been used instead of mouse cell lines. Unfortunately, there are very few cell lines of commercially available immortalized human microglia, which are based on viral transfection (Garcia-Mesa et al., 2017). Based on the limitations of immortalized human microglia, this research instead relied on immortalized human cell models of microglia. All of the human cell types used were previously obtained from cancerous cell lines (Collins et al., 1977; Påhlman et al., 1990; Tsuchiya et al., 1980). Mouse microglial BV-2 cells were also immortalized by viral transfection (Blasi et al., 1990).

Another limitation comes from the selected *in vitro* method of fatty acid treatment. An experimental design that provides a continuous supply of fatty acid and glucose would improve the physiological relevance of the findings. Since this type of experimental design was not practical for this thesis work, fatty acid was instead added to cell culture at a single time point. Fatty acid-BSA complexes were prepared, which improves the physiological relevance of these findings when compared to the use of free fatty acids alone in cell culture. To maintain consistency between fatty acids studied, all fatty acid-BSA complexes were prepared in the same manner. However, BSA does not bind each fatty acid with the same affinity. For instance, among the fatty acids studied, SA was shown to have lower affinity for BSA compared to LA and OA, but ALA was not tested (Spector et al., 1969). Thus, cell culture medium containing BSA and fatty acids with low BSA-binding affinity may contain a higher concentration of free fatty acids compared to cell culture media containing BSA and fatty acids with high BSA-binding affinity.

Finally, the concentration of glucose in media used throughout the experiments is an important consideration of my thesis work. Although studies in the field of my thesis work frequently use conditions of high glucose (with varying concentrations between 17 mM and 35 mM), it would be ideal to limit glucose levels within experimental media to obtain more physiological glucose concentrations. However, most of the experiments described by this thesis also use microglia activated by LPS, and LPS activation relies on glycolysis to sustain pro-inflammatory microglial responses (Shen et al., 2017). Thus, low glucose media (5.6 mM) was only used in experiments measuring fatty acid composition where microglia were not activated.

5.3 Future Directions

5.3.1 Objective 1

Data presented in this thesis indicate that ALA reduces nitrite levels in BV-2 supernatant, but not through GPR-120-dependent mechanisms. When GPR-120 was inhibited by its antagonist AH-7614, the effect of ALA on nitrite secretion was not blocked. Observations made in this thesis cannot rule out GPR-120-dependent effects of LA on microglia. The increase in nitrite upon inhibition of GPR-120 with AH-7614, followed by the addition of LA, suggests that LA could be operating through GPR-120-dependent mechanisms. However, further studies that include additional metrics of cell viability and cell death are required to determine conclusively whether LA operates through GPR-120-dependent mechanisms.

Another future direction should be to investigate the iNOS-independent effect of ALA. I determined that ALA reduced nitrite independent of GPR-120 and iNOS. The reduction of nitrite that I observed may be due to the effects of ALA on NO-reactive factors in microglia, which could be determined using proteomics experiments or additional targeted protein analysis.

Finally, other fatty acid binding receptors are potential candidates that could elicit the nitrite and iNOS-reducing response to LA that I observed. GPR-40 is a free fatty acid receptor that is expressed in primary mouse microglia and BV-2 microglia, and has been shown to interact with PUFAs such as ALA in peripheral cells (Dragano et al., 2017; Ohue-Kitano et al., 2018; Zhang et al., 2014). Experiments with GPR-40 antagonists will clarify possible involvement of this receptor in LA-mediated inhibition of NO secretion or iNOS expression by microglia.

5.3.2 Objective 2

Experiments performed with human and mouse cells indicate that the addition of unsaturated fatty acid to cell culture reduces the pro-inflammatory response of microglia to SFAs. For instance, OA has been shown to reduce toxicity of mouse neuronal and macrophage cells that was associated with palmitate treatment (Kim et al., 2017; Kwon et al., 2014). In primary astrocytes, increasing concentrations of DHA reduced the palmitate-induced production of TNF-α (Gupta et al., 2012). In my thesis, I have described the effects of four 18 carbon fatty acids in isolation. However, cells *in vivo* interact with many fatty acids simultaneously, which could alter their biological activities. Therefore, to approximate dietary exposure more closely *in vitro*, further studies should test combinations of fatty acids at physiological concentrations.

5.3.3 Objective 3

In this thesis work, fatty acid concentrations were used at a concentration range exceeding their physiological ranges in the CNS (Fonteh et al., 2014). Further studies are needed to address uptake of the dietary fatty acids in the brain and to determine fatty acid concentrations

available to cells of the CNS during pathological states. After dietary lipid content is altered, the rat brain does not exhibit a high degree of membrane lipid fluctuation when compared to other tissues (Abbott et al., 2012). This may be due to the restricted passage of fatty acids across the BBB; however, BBB leakage occurs in some pathological states, allowing the passage of serum molecules. Disruption of the BBB was noted for some AD-afflicted individuals, which was correlated with high plasma triglyceride content (Bowman et al., 2012). *In vivo* experiments can be used to measure the levels of CNS fatty acids in these disease states, and also clarify the effects of altering dietary fatty acids on their uptake into the CNS.

5.3.4 Significance of the Research

This thesis discusses the key roles of dietary fatty acids in regulating microglial inflammatory responses in the presence of high glucose (17.0 mM to 17.3 mM glucose used in these experiments). Select functions of microglia are altered by their exposure to PUFAs, including the reduced release of RNS, which was assessed by nitrite levels in microglial supernatants, and expression of iNOS protein. The 18 carbon fatty acids studied did not affect other inflammatory functions of microglia, which included phagocytosis, as well as the release of ROS by respiratory burst, MCP-1, and neurotoxic mediators.

Excessively activated microglia promote neuroinflammation, which is a key factor in AD pathogenesis. Currently there is a lack of viable options for delaying the progression of this debilitating disease. Recommendations for disease prevention through dietary intervention require a thorough understanding of how dietary components affect immune cells of the brain. This research contributes to a much larger field studying the effects of modifiable and dietary risk factors for AD on inflammatory mechanisms of the brain.

I demonstrated that the n-3 PUFA ALA and the n-6 PUFA LA reduced the release of microglial RNS in the presence of high glucose (17.0 mM), and specifically LA reduced the secretion of RNS in an iNOS-dependent manner. As high levels of extracellular RNS may be cytotoxic to nearby cells, ALA and LA may be able to reduce the cytotoxicity inflicted by the microglial secretion of RNS. Therefore, increasing dietary intake of PUFAs, in a balanced ratio, while reducing the dietary intake of SA may decrease the cytotoxicity associated with activated microglia, which may subsequently reduce neuroinflammation associated with neurodegenerative disease. This recommendation is in agreement with the literature that suggests that following the dietary patterns of the MeDi, a diet balanced in n-3 PUFAs and n-6 PUFAs, may have neuroprotective outcomes (Debbabi et al., 2017; Van De Rest et al., 2015). My thesis concludes that both ALA and LA modify microglial immune responses in an anti-inflammatory manner. Further research of the mechanisms governing the neuroprotective effects of these PUFAs is required to develop accurate dietary recommendations for the prevention of neurodegenerative disease.

Works Cited

Abbott, S.K., Else, P.L., Atkins, T.A., Hulbert, A.J., 2012. Fatty acid composition of membrane bilayers: importance of diet polyunsaturated fat balance. Biochim. Biophys. Acta 1818, 1309–1317. doi:10.1016/j.bbamem.2012.01.011

Adolph, S., Fuhrmann, H., Schumann, J., 2012. Unsaturated Fatty Acids Promote the Phagocytosis of *P. aeruginosa* and *R. equi* by RAW264.7 Macrophages. Curr. Microbiol. 65, 649–655. doi:10.1007/s00284-012-0207-3

Aeberhard, E., Menkes, J.H., 1968. Biosynthesis of long chain fatty acids by subcellular particles of mature brain. J. Biol. Chem. 243, 3834–3840.

Aktan, F., 2004. iNOS-mediated nitric oxide production and its regulation. Life Sci. 75, 639–653. doi:10.1016/j.lfs.2003.10.042

Alashmali, S.M., Hopperton, K.E., Bazinet, R.P., 2016. Lowering dietary n-6 polyunsaturated fatty acids. Curr. Opin. Lipidol. 27, 54–66. doi:10.1097/MOL.0000000000000255

Ambrozova, G., Pekarova, M., Lojek, A., 2010. Effect of polyunsaturated fatty acids on the reactive oxygen and nitrogen species production by raw 264.7 macrophages. Eur. J. Nutr. 49, 133–139. doi:10.1007/s00394-009-0057-3

Anderson, E.K., Hill, A.A., Hasty, A.H., 2012. Stearic acid accumulation in macrophages induces toll-like receptor 4/2-independent inflammation leading to endoplasmic reticulum stress-mediated apoptosis. Arterioscler. Thromb. Vasc. Biol. 32, 1687–1695. doi:10.1161/ATVBAHA.112.250142

Ano, Y., Ozawa, M., Kutsukake, T., Sugiyama, S., Uchida, K., Yoshida, A., Nakayama, H., 2015. Preventive effects of a fermented dairy product against Alzheimer's disease and identification of a novel oleamide with enhanced microglial phagocytosis and anti-inflammatory activity. PLoS One 10, e0118512. doi:10.1371/journal.pone.0118512

Araldi, E., Fernández-Fuertes, M., Canfrán-Duque, A., Tang, W., Cline, G.W., Madrigal-Matute, J., Pober, J.S., Lasunción, M.A., Wu, D., Fernández-Hernando, C., Suárez, Y., 2017. Lanosterol modulates TLR4-mediated innate immune responses in macrophages. Cell Rep. 19, 2743–2755. doi:10.1016/j.celrep.2017.05.093

Auestad, N., Korsak, R.A., Morrow, J.W., Edmond, J., 1991. Fatty acid oxidation and ketogenesis by astrocytes in primary culture. J. Neurochem. 56, 1376–1386. doi:10.1111/j.1471-4159.1991.tb11435.x

Bahniwal, M. K. 2014. Chronic inflammation as the mechanistic link between type 2 diabetes mellitus and Alzheimer's disease. University of British Columbia (Okanagan). cIRcle, doi: 10.14288/1.0074350

Baierle, M., Vencato, P.H., Oldenburg, L., Bordignon, S., Zibetti, M., Trentini, C.M., Duarte, M.M.M.F., Veit, J.C., Somacal, S., Emanuelli, T., Grune, T., Breusing, N., Garcia, S.C., 2014. Fatty acid status and its relationship to cognitive decline and homocysteine levels in the elderly. Nutrients 6, 3624–3640. doi:10.3390/nu6093624

Bal-Price, A., Brown, G.C., 2001. Inflammatory neurodegeneration mediated by nitric oxide from activated glia-inhibiting neuronal respiration, causing glutamate release and excitotoxicity. J. Neurosci. 21, 6480–6491. doi:10.1523/JNEUROSCI.21-17-06480.2001

Balez, R., Ooi, L., 2016. Getting to NO Alzheimer's disease: neuroprotection versus neurotoxicity mediated by nitric oxide. Oxid. Med. Cell. Longev. 2016, 3806157. doi:10.1155/2016/3806157

Banks, W.A., Farr, S.A., Salameh, T.S., Niehoff, M.L., Rhea, E.M., Morley, J.E., Hanson, A.J., Hansen, K.M., Craft, S., 2018. Triglycerides cross the blood-brain barrier and induce central leptin and insulin receptor resistance. Int. J. Obes. (Lond). 42, 391–397. doi:10.1038/ijo.2017.231

Bazinet, R.P., Laye, S., 2014. Polyunsaturated fatty acids and their metabolites in brain function and disease. Nat. Rev. Neurosci. 15, 771–785. doi:10.1038/nrn3820

Bellavite, P., Guarini, P., Biasi, D., Carletto, A., Trevisan, M.T., Caramaschi, P., Bambara, L.M., Corrocher, D.R., 1995. Correlations between the intensity of WLP-dependent respiratory burst and cellular fatty acid composition in human neutrophils. Br. J. Haematol. 89, 271–276.

Ben Haim, L., Carrillo-de Sauvage, M.A., Ceyzériat, K., Escartin, C., 2015. Elusive roles for reactive astrocytes in neurodegenerative diseases. Front. Cell. Neurosci. 9, 278. doi:10.3389/fncel.2015.00278

Benakis, C., Garcia-Bonilla, L., Iadecola, C., Anrather, J., 2015. The role of microglia and myeloid immune cells in acute cerebral ischemia. Front. Cell. Neurosci. 8, 461. doi:10.3389/fncel.2014.00461

Blasi, E., Barluzzi, R., Bocchini, V., Mazzolla, R., Bistoni, F., 1990. Immortalization of murine microglial cells by a v-raf/v-myc carrying retrovirus. J. Neuroimmunol. 27, 229–237.

Bordt, E.A., Polster, B.M., 2014. NADPH oxidase- and mitochondria-derived reactive oxygen species in proinflammatory microglial activation: a bipartisan affair? Free Radic. Biol. Med. 76, 34–46. doi:10.1016/j.freeradbiomed.2014.07.033

Bourre, J.M., Pascal, G., Durand, G., Masson, M., Dumont, O., Piciotti, M., 1984. Alterations in the fatty acid composition of rat brain cells (neurons, astrocytes, and oligodendrocytes) and of subcellular fractions (myelin and synaptosomes) induced by a diet devoid of n-3 fatty acids. J. Neurochem. 43, 342–348. doi:10.1111/j.1471-4159.1984.tb00906.x

Bowman, G.L., Kaye, J.A., Quinn, J.F., 2012. Dyslipidemia and blood-brain barrier integrity in Alzheimer's disease. Curr. Gerontol. Geriatr. Res. 2012, 184042. doi:10.1155/2012/184042

Broniowska, K.A., Hogg, N., 2012. The chemical biology of S-nitrosothiols. Antioxid. Redox Signal. 17, 969–980. doi:10.1089/ars.2012.4590

Bronzuoli, M.R., Iacomino, A., Steardo, L., Scuderi, C., 2016. Targeting neuroinflammation in Alzheimer's disease. J. Inflamm. Res. 9, 199–208. doi:10.2147/JIR.S86958

Brown, G.C., 2010. Nitric oxide and neuronal death. Nitric Oxide 23, 153–165. doi:10.1016/j.niox.2010.06.001

Brown, G.C., Neher, J.J., 2014. Microglial phagocytosis of live neurons. Nat. Rev. Neurosci. 15, 209–216. doi:10.1038/nrn3710

Brown, G.C., Vilalta, A., 2015. How microglia kill neurons. Brain Res. 1628, 288–297. doi:10.1016/j.brainres.2015.08.031

Bryan, N.S., Grisham, M.B., 2007. Methods to detect nitric oxide and its metabolites in biological samples. Free Radic. Biol. Med. 43, 645–657. doi:10.1016/j.freeradbiomed.2007.04.026

Burckhardt, M., Herke, M., Wustmann, T., Watzke, S., Langer, G., Fink, A., 2016. Omega-3 fatty acids for the treatment of dementia. Cochrane database Syst. Rev. 4, CD009002. doi:10.1002/14651858.CD009002.pub3

Burd, J.F., Usategui-Gomez, M., 1973. A colorimetric assay for serum lactate dehydrogenase. Clin. Chim. Acta 46, 223–227. doi:10.1016/0009-8981(73)90174-5

Button, E.B., Mitchell, A.S., Domingos, M.M., Chung, J.H.J., Bradley, R.M., Hashemi, A., Marvyn, P.M., Patterson, A.C., Stark, K.D., Quadrilatero, J., Duncan, R.E., 2014. Microglial cell activation increases saturated and decreases monounsaturated fatty acid content, but both lipid species are proinflammatory. Lipids 49, 305–316. doi:10.1007/s11745-014-3882-y

Caccamo, A., Branca, C., Piras, I.S., Ferreira, E., Huentelman, M.J., Liang, W.S., Readhead, B., Dudley, J.T., Spangenberg, E.E., Green, K.N., Belfiore, R., Winslow, W., Oddo, S., 2017. Necroptosis activation in Alzheimer's disease. Nat. Neurosci. 20, 1236–1246. doi:10.1038/nn.4608

Calder, P.C., Bond, J.A., Harvey, D.J., Gordon, S., Newsholme, E.A., 1990. Uptake and incorporation of saturated and unsaturated fatty acids into macrophage lipids and their effect upon macrophage adhesion and phagocytosis. Biochem. J. 269, 807–814.

Capozzi, M.E., McCollum, G.W., Cousins, D.B., Penn, J.S., 2016. Linoleic acid is a diabetes-relevant stimulator of retinal inflammation in human retinal muller cells and microvascular endothelial cells. J. Diabetes Metab. 7, 718. doi:10.4172/2155-6156.1000718

Chan, F.K.M., Moriwaki, K., De Rosa, M.J., 2013. Detection of necrosis by release of lactate dehydrogenase (LDH) activity. Methods Mol. Biol. 979, 65–70. doi:10.1007/978-1-62703-290-2_7

Christon, R., Haloui, R.B., Durand, G., 1995. Dietary polyunsaturated fatty acids and aging modulate glutathione-related antioxidants in rat liver. J. Nutr. 125, 3062–3070. doi:10.1093/jn/125.12.3062

Chung, W.S., Clarke, L.E., Wang, G.X., Stafford, B.K., Sher, A., Chakraborty, C., Joung, J., Foo, L.C., Thompson, A., Chen, C., Smith, S.J., Barres, B.A., 2013. Astrocytes mediate synapse elimination through MEGF10 and MERTK pathways. Nature 504, 394–400. doi:10.1038/nature12776

Clark, A.K., Gruber-Schoffnegger, D., Drdla-Schutting, R., Gerhold, K.J., Malcangio, M., Sandkuhler, J., 2015. Selective activation of microglia facilitates synaptic strength. J. Neurosci. 35, 4552-4570. doi:10.1523/JNEUROSCI.2061-14.2015

Collins, S.J., Gallo, R.C., Gallagher, R.E., 1977. Continuous growth and differentiation of human myeloid leukaemic cells in suspension culture. Nature 270, 347–349. doi:10.1038/270347a0

Cooper, C., Sommerlad, A., Lyketsos, C.G., Livingston, G., 2015. Modifiable predictors of dementia in mild cognitive impairment: a systematic review and meta-analysis. Am. J. Psychiatry 172, 323–334. doi:10.1176/appi.ajp.2014.14070878

Cordain, L., Eaton, S.B., Sebastian, A., Mann, N., Lindeberg, S., Watkins, B.A., O'Keefe, J.H., Brand-Miller, J., 2005. Origins and evolution of the Western diet: health implications for the 21st century. Am. J. Clin. Nutr. 81, 341–354. doi:10.1093/ajcn.81.2.341

Cullberg, K.B., Larsen, J.Ø., Pedersen, S.B., Richelsen, B., 2014. Effects of LPS and dietary free fatty acids on MCP-1 in 3T3-L1 adipocytes and macrophages *in vitro*. Nutr. Diabetes 4, e113. doi:10.1038/nutd.2014.10

Czapski, G.A., Czubowicz, K., Strosznajder, J.B., Strosznajder, R.P., 2016. The lipoxygenases: their regulation and implication in Alzheimer's disease. Neurochem. Res. 41, 243–257. doi:10.1007/s11064-015-1776-x

Daemen, S., van Zandvoort, M.A.M.J., Parekh, S.H., Hesselink, M.K.C., 2016. Microscopy tools for the investigation of intracellular lipid storage and dynamics. Mol. Metab. 5, 153–163. doi:10.1016/j.molmet.2015.12.005

Das, A., Kim, S.H., Arifuzzaman, S., Yoon, T., Chai, J.C., Lee, Y.S., Park, K.S., Jung, K.H., Chai, Y.G., 2016. Transcriptome sequencing reveals that LPS-triggered transcriptional responses in established microglia BV2 cell lines are poorly representative of primary microglia. J. Neuroinflammation 13, 182. doi:10.1186/s12974-016-0644-1

Debbabi, M., Zarrouk, A., Bezine, M., Meddeb, W., Nury, T., Badreddine, A., Karym, E.M., Sghaier, R., Bretillon, L., Guyot, S., Samadi, M., Cherkaoui-Malki, M., Nasser, B., Mejri, M., Ben-Hammou, S., Hammami, M., Lizard, G., 2017. Comparison of the effects of major fatty acids present in the Mediterranean diet (oleic acid, docosahexaenoic acid) and in hydrogenated oils (elaidic acid) on 7-ketocholesterol-induced oxiapoptophagy in microglial BV-2 cells. Chem. Phys. Lipids 207, 151–170. doi:10.1016/j.chemphyslip.2017.04.002

Decker, T., Lohmann-Matthes, M.L., 1988. A quick and simple method for the quantitation of lactate dehydrogenase release in measurements of cellular cytotoxicity and tumor necrosis factor (TNF) activity. J. Immunol. Methods 115, 61–69. doi:10.1016/0022-1759(88)90310-9

Desai, A., Park, T., Barnes, J., Kevala, K., Chen, H., Kim, H.Y., 2016. Reduced acute neuroinflammation and improved functional recovery after traumatic brain injury by α-linolenic acid supplementation in mice. J. Neuroinflammation 13, 253. doi:10.1186/s12974-016-0714-4

Dragano, N.R.V., Solon, C., Ramalho, A.F., de Moura, R.F., Razolli, D.S., Christiansen, E., Azevedo, C., Ulven, T., Velloso, L.A., 2017. Polyunsaturated fatty acid receptors, GPR40 and GPR120, are expressed in the hypothalamus and control energy homeostasis and inflammation. J. Neuroinflammation 14, 91. doi:10.1186/s12974-017-0869-7

Eskelinen, M.H., Ngandu, T., Helkala, E.L., Tuomilehto, J., Nissinen, A., Soininen, H., Kivipelto, M., 2008. Fat intake at midlife and cognitive impairment later in life: a population-based CAIDE study. Int. J. Geriatr. Psychiatry 23, 741–747. doi:10.1002/gps.1969

Eskilsson, A., Matsuwaki, T., Shionoya, K., Mirrasekhian, E., Zajdel, J., Schwaninger, M., Engblom, D., Blomqvist, A., 2017. Immune-induced fever is dependent on local but not generalized prostaglandin E_2 Synthesis in the Brain. J. Neurosci. 37, 5035–5044. doi:10.1523/JNEUROSCI.3846-16.2017

Esposito, G., Giovacchini, G., Liow, J.-S., Bhattacharjee, A.K., Greenstein, D., Schapiro, M., Hallett, M., Herscovitch, P., Eckelman, W.C., Carson, R.E., Rapoport, S.I., 2008. Imaging neuroinflammation in Alzheimer's disease with radiolabeled arachidonic acid and PET. J. Nucl. Med. 49, 1414–1421. doi:10.2967/jnumed.107.049619

Feart, C., Alles, B., Merle, B., Samieri, C., Barberger-Gateau, P., 2012. Adherence to a Mediterranean diet and energy, macro-, and micronutrient intakes in older persons. J. Physiol. Biochem. 68, 691–700. doi:10.1007/s13105-012-0190-y

Ferreira, S.T., Klein, W.L., 2011. The Aβ oligomer hypothesis for synapse failure and memory loss in Alzheimer's disease. Neurobiol. Learn Mem. 96, 529–543. doi:10.1016/j.nlm.2011.08.003

Floden, A.M., Combs, C.K., 2011. Microglia demonstrate age-dependent interaction with amyloid-β fibrils. J. Alzheimer's Dis. 25, 279–293. doi:10.3233/JAD-2011-101014

Fonteh, A.N., Cipolla, M., Chiang, J., Arakaki, X., Harrington, M.G., 2014. Human cerebrospinal fluid fatty acid levels differ between supernatant fluid and brain-derived nanoparticle fractions, and are altered in Alzheimer's disease. PLoS One 9, e100519. doi:10.1371/journal.pone.0100519

Forehand, J.R., Pabst, M.J., Phillips, W.A., Johnston, R.B., 1989. Lipopolysaccharide priming of human neutrophils for an enhanced respiratory burst. Role of intracellular free calcium. J. Clin. Invest. 83, 74–83. doi:10.1172/JCI113887

Fotakis, G., Timbrell, J.A., 2006. In vitro cytotoxicity assays: comparison of LDH, neutral red, MTT and protein assay in hepatoma cell lines following exposure to cadmium chloride. Toxicol. Lett. 160, 171–177. doi:10.1016/j.toxlet.2005.07.001

Fraser, T., Tayler, H., Love, S., 2010. Fatty Acid Composition of frontal, temporal and parietal neocortex in the normal human brain and in Alzheimer's disease. Neurochem. Res. 35, 503–513. doi:10.1007/s11064-009-0087-5

Freeman, L.R., Haley-Zitlin, V., Rosenberger, D.S., Granholm, A.C., 2014. Damaging effects of a high-fat diet to the brain and cognition: a review of proposed mechanisms. Nutr. Neurosci. 17, 241–251. doi:10.1179/1476830513Y.0000000092

Fritsche, K., 2007. Important differences exist in the dose–response relationship between diet and immune cell fatty acids in humans and rodents. Lipids 42, 961–979. doi:10.1007/s11745-007-3106-9

Fu, R., Shen, Q., Xu, P., Luo, J.J., Tang, Y., 2014. Phagocytosis of microglia in the central nervous system diseases. Mol. Neurobiol. 49, 1422–1434. doi:10.1007/s12035-013-8620-6

Galatro, T.F., Holtman, I.R., Lerario, A.M., Vainchtein, I.D., Brouwer, N., Sola, P.R., Veras, M.M., Pereira, T.F., Leite, R.E.P., Möller, T., Wes, P.D., Sogayar, M.C., Laman, J.D., den Dunnen, W., Pasqualucci, C.A., Oba-Shinjo, S.M., Boddeke, E.W.G.M., Marie, S.K.N., Eggen, B.J.L., 2017. Transcriptomic analysis of purified human cortical microglia reveals age-associated changes. Nat. Neurosci. 20, 1162–1171. doi:10.1038/nn.4597

Galimberti, D., Schoonenboom, N., Scheltens, P., Fenoglio, C., Bouwman, F., Venturelli, E., Guidi, I., Blankenstein, M.A., Bresolin, N., Scarpini, E., 2006. Intrathecal chemokine synthesis in mild cognitive impairment and Alzheimer disease. Arch. Neurol. 63, 538-543. doi:10.1001/archneur.63.4.538

Garcia-Mesa, Y., Jay, T.R., Checkley, M.A., Luttge, B., Dobrowolski, C., Valadkhan, S., Landreth, G.E., Karn, J., Alvarez-Carbonell, D., 2017. Immortalization of primary microglia: a new platform to study HIV regulation in the central nervous system. J. Neurovirol. 23, 47–66. doi:10.1007/s13365-016-0499-3

Gouveia, A., Bajwa, E., Klegeris, A., 2017. Extracellular cytochrome c as an intercellular signaling molecule regulating microglial functions. Biochim. Biophys. Acta 1861, 2274–2281. doi:10.1016/j.bbagen.2017.06.017

Graham, L.C., Harder, J.M., Soto, I., de Vries, W.N., John, S.W.M., Howell, G.R., 2016. Chronic consumption of a western diet induces robust glial activation in aging mice and in a mouse model of Alzheimer's disease. Sci. Rep. 6, 21568. doi:10.1038/srep21568

Guillemot-Legris, O., Muccioli, G.G., 2017. Obesity-induced neuroinflammation: beyond the hypothalamus. Trends Neurosci. 40, 237–253. doi:10.1016/j.tins.2017.02.005

Gupta, S., Knight, A.G., Gupta, S., Keller, J.N., Bruce-Keller, A.J., 2012. Saturated long-chain fatty acids activate inflammatory signaling in astrocytes. J. Neurochem. 120, 1060–1071. doi:10.1111/j.1471-4159.2012.07660.x

Guruswamy, R., ElAli, A., 2017. Complex roles of microglial cells in ischemic stroke pathobiology: new insights and future directions. Int. J. Mol. Sci. 18, 496. doi:10.3390/ijms18030496

Hamilton, J.S., Powell, L.A., McMaster, C., McMaster, D., Trimble, E.R., 2003. Interaction of glucose and long chain fatty acids (C18) on antioxidant defences and free radical damage in porcine vascular smooth muscle cells in vitro. Diabetologia 46, 106–114. doi:10.1007/s00125-002-1003-6

Han, L., Song, S., Niu, Y., Meng, M., Wang, C., 2017. Eicosapentaenoic acid (EPA) induced macrophages activation through GPR120-mediated Raf-ERK1/2-IKKβ-NF-κB p65 signaling pathways. Nutrients 9, 937. doi:10.3390/nu9090937

Hanisch, U.K., Kettenmann, H., 2007. Microglia: active sensor and versatile effector cells in the normal and pathologic brain. Nat. Neurosci. 10, 1387–1394. doi:10.1038/nn1997

Hansen, M.B., Nielsen, S.E., Berg, K., 1989. Re-examination and further development of a precise and rapid dye method for measuring cell growth/cell kill. J. Immunol. Methods 119, 203–210.

Harvey, L.D., Yin, Y., Attarwala, I.Y., Begum, G., Deng, J., Yan, H.Q., Dixon, C.E., Sun, D., 2015. Administration of DHA reduces endoplasmic reticulum stress-associated inflammation and alters microglial or macrophage activation in traumatic brain injury. ASN Neuro 7, 1759091415618969. doi:10.1177/1759091415618969

Hatanaka, E., Levada-Pires, A.C., Pithon-Curi, T.C., Curi, R., 2006. Systematic study on ROS production induced by oleic, linoleic, and γ-linolenic acids in human and rat neutrophils. Free Radic. Biol. Med. 41, 1124–1132. doi:10.1016/j.freeradbiomed.2006.06.014

Håversen, L., Danielsson, K.N., Fogelstrand, L., Wiklund, O., 2009. Induction of proinflammatory cytokines by long-chain saturated fatty acids in human macrophages. Atherosclerosis 202, 382–393. doi:10.1016/j.atherosclerosis.2008.05.033

Hellwing, C., Tigistu-Sahle, F., Fuhrmann, H., Käkelä, R., Schumann, J., 2018. Lipid composition of membrane microdomains isolated detergent-free from PUFA supplemented RAW264.7 macrophages. J. Cell. Physiol. 233, 2602–2612. doi:10.1002/jcp.26138

Henn, A., Lund, S., Hedtjärn, M., Schrattenholz, A., Pörzgen, P., Leist, M., 2009. The suitability of BV2 cells as alternative model system for primary microglia cultures or for animal experiments examining brain inflammation. ALTEX 26, 83–94. doi:10.14573/altex.2009.2.83

Hennig, B., Watkins, B.A., 1989. Linoleic acid and linolenic acid: effect on permeability properties of cultured endothelial cell monolayers. Am. J. Clin. Nutr. 49, 301–305. doi:10.1093/ajcn/49.2.301

Heras-Sandoval, D., Pedraza-Chaverri, J., Pérez-Rojas, J.M., 2016. Role of docosahexaenoic acid in the modulation of glial cells in Alzheimer's disease. J. Neuroinflammation 13, 61. doi:10.1186/s12974-016-0525-7

Hinojosa, A.E., Garcia-Bueno, B., Leza, J.C., Madrigal, J.L.M., 2011. CCL2/MCP-1 modulation of microglial activation and proliferation. J. Neuroinflammation 8, 77. doi:10.1186/1742-2094-8-77

Hirasawa, A., Tsumaya, K., Awaji, T., Katsuma, S., Adachi, T., Yamada, M., Sugimoto, Y., Miyazaki, S., Tsujimoto, G., 2005. Free fatty acids regulate gut incretin glucagon-like peptide-1 secretion through GPR120. Nat. Med. 11, 90–94. doi:10.1038/nm1168

Hjorth, E., Frenkel, D., Weiner, H., Schultzberg, M., 2010. Effects of immunomodulatory substances on phagocytosis of $A\beta_{1-42}$ by human microglia. Int. J. Alzheimers. Dis. 2010. doi:10.4061/2010/798424

Hussain, R.F., Nouri, A.M., Oliver, R.T., 1993. A new approach for measurement of cytotoxicity using colorimetric assay. J. Immunol. Methods 160, 89–96.

Ishizuka, K., Kimura, T., Igata-Yi, R., Katsuragi, S., Takamatsu, J., Miyakawa, T., 1997. Identification of monocyte chemoattractant protein-1 in senile plaques and reactive microglia of Alzheimer's disease. Psychiatry Clin. Neurosci. 51, 135–138. doi:10.1111/j.1440-1819.1997.tb02375.x

Janssen, B., Vugts, D., Windhorst, A., Mach, R., 2018. PET Imaging of Microglial Activation—Beyond Targeting TSPO. Molecules 23, 607. doi:10.3390/molecules23030607

Jessen, K.R., 2004. Glial cells. Int. J. Biochem. Cell Biol. 36, 1861–1867. doi:10.1016/J.BIOCEL.2004.02.023

Johnson, V.E., Stewart, W., Smith, D.H., 2010. Traumatic brain injury and amyloid-β pathology: a link to Alzheimer's disease? Nat. Rev. Neurosci. 11, 361–370. doi:10.1038/nrn2808

Kametani, F., Hasegawa, M., 2018. Reconsideration of amyloid hypothesis and tau hypothesis in Alzheimer's disease. Front. Neurosci. 12, 25. doi:10.3389/fnins.2018.00025

Kang, J.X., Wang, J., 2005. A simplified method for analysis of polyunsaturated fatty acids. BMC Biochem. 6, 5. doi:10.1186/1471-2091-6-5

Keszler, A., Zhang, Y., Hogg, N., 2010. The Reaction between nitric oxide, glutathione and oxygen in the presence and absence of protein: how are S-nitrosothiols formed? Free Radic. Biol. Med. 48, 55-64. doi:10.1016/j.freeradbiomed.2009.10.026

Kim, D.H., Cho, Y.M., Lee, K.H., Jeong, S.W., Kwon, O.J., 2017. Oleate protects macrophages from palmitate-induced apoptosis through the downregulation of CD36 expression. Biochem. Biophys. Res. Commun. 488, 477–482. doi:10.1016/j.bbrc.2017.05.066

Klegeris, A., Bissonnette, C.J., McGeer, P.L., 2005. Modulation of human microglia and THP-1 cell toxicity by cytokines endogenous to the nervous system. Neurobiol. Aging 26, 673–682. doi:10.1016/j.neurobiolaging.2004.06.012

Klegeris, A., McGeer, P.L., 2003. Toxicity of human monocytic THP-1 cells and microglia toward SH-SY5Y neuroblastoma cells is reduced by inhibitors of 5-lipoxygenase and its activating protein FLAP. J. Leukoc. Biol. 73, 369–378. doi:10.1189/jlb.1002482

Kopec, K.K., Carroll, R.T., 2000. Phagocytosis is regulated by nitric oxide in murine microglia. Nitric Oxide 4, 103–111. doi:10.1006/niox.2000.0280

Korecka, J.A., van Kesteren, R.E., Blaas, E., Spitzer, S.O., Kamstra, J.H., Smit, A.B., Swaab, D.F., Verhaagen, J., Bossers, K., 2013. Phenotypic characterization of retinoic acid differentiated SH-SY5Y cells by transcriptional profiling. PLoS One 8, e63862. doi:10.1371/journal.pone.0063862

Kovalevich, J., Langford, D., 2013. Considerations for the use of SH-SY5Y neuroblastoma cells in neurobiology. Methods Mol. Biol. 1078, 9–21. doi:10.1007/978-1-62703-640-5_2

Kwon, B., Lee, H.-K., Querfurth, H.W., 2014. Oleate prevents palmitate-induced mitochondrial dysfunction, insulin resistance and inflammatory signaling in neuronal cells. Biochim. Biophys. Acta 1843, 1402–1413. doi:10.1016/j.bbamcr.2014.04.004

Langelier, B., Linard, A., Bordat, C., Lavialle, M., Heberden, C., 2010. Long chain polyunsaturated fatty acids modulate membrane phospholipid composition and protein localization in lipid rafts of neural stem cell cultures. J. Cell Biochem. 110, 1356-1364. doi:10.1002/jcb.22652

Layé, S., Nadjar, A., Joffre, C., Bazinet, R.P., 2018. Anti-inflammatory effects of omega-3 fatty acids in the brain: physiological mechanisms and relevance to pharmacology. Pharmacol. Rev. 70, 12–38. doi:10.1124/pr.117.014092

Lecrux, C., Hamel, E., 2011. The neurovascular unit in brain function and disease. Acta Physiol. 203, 47–59. doi:10.1111/j.1748-1716.2011.02256.x

Lee, J.K., Tansey, M.G., 2013. Microglia isolation from adult mouse brain. Methods Mol. Biol. 1041, 17-23. doi:10.1007/978-1-62703-520-0_3

Lee, J.Y., Sohn, K.H., Rhee, S.H., Hwang, D., 2001. Saturated fatty acids, but not unsaturated fatty acids, induce the expression of cyclooxygenase-2 mediated through toll-like receptor 4. J. Biol. Chem. 276, 16683–16689. doi:10.1074/jbc.M011695200

Lee, K.M., MacLean, A.G., 2015. New advances on glial activation in health and disease. World J. Virol. 4, 42–55. doi:10.5501/wjv.v4.i2.42

Lee, Y., Back, J.H., Kim, J., Kim, S.H., Na, D.L., Cheong, H.K., Hong, C.H., Kim, Y.G., 2010. Systematic review of health behavioral risks and cognitive health in older adults. Int. Psychogeriatrics 22, 174–187. doi:10.1017/S1041610209991189

Lehert, P., Villaseca, P., Hogervorst, E., Maki, P.M., Henderson, V.W., 2015. Individually modifiable risk factors to ameliorate cognitive aging: a systematic review and meta-analysis. Climacteric 18, 678–689. doi:10.3109/13697137.2015.1078106

Levi, G., Minghetti, L., Aloisi, F., 1998. Regulation of prostanoid synthesis in microglial cells and effects of prostaglandin E2 on microglial functions. Biochimie 80, 899–904. doi:10.1016/S0300-9084(00)88886-0

Levy, R., Rotrosen, D., Nagauker, O., Leto, T.L., Malech, H.L., 1990. Induction of the respiratory burst in HL-60 cells. Correlation of function and protein expression. J. Immunol. 145, 2595–2601.

Li, Y., Lu, B., Sheng, L., Zhu, Z., Sun, H., Zhou, Y., Yang, Y., Xue, D., Chen, W., Tian, X., Du, Y., Yan, M., Zhu, W., Xing, F., Li, K., Lin, S., Qiu, P., Su, X., Huang, Y., Yan, G., Yin, W., 2018. Hexokinase 2-dependent hyperglycolysis driving microglial activation contributes to ischemic brain injury. J. Neurochem. 144, 186–200. doi:10.1111/jnc.14267

Liberatore, G.T., Jackson-Lewis, V., Vukosavic, S., Mandir, A.S., Vila, M., McAuliffe, W.G., Dawson, V.L., Dawson, T.M., Przedborski, S., 1999. Inducible nitric oxide synthase stimulates dopaminergic neurodegeneration in the MPTP model of Parkinson disease. Nat. Med. 5, 1403–1409. doi:10.1038/70978

Little, J.P., Madeira, J.M., Klegeris, A., 2012. The saturated fatty acid palmitate induces human monocytic cell toxicity toward neuronal cells: exploring a possible link between obesity-related metabolic impairments and neuroinflammation. J. Alzheimer's Dis. 30, 179–183. doi:10.3233/JAD-2011-111262

Little, J.P., Simtchouk, S., Schindler, S.M., Villanueva, E.B., Gill, N.E., Walker, D.G., Wolthers, K.R., Klegeris, A., 2014. Mitochondrial transcription factor A (Tfam) is a pro-inflammatory extracellular signaling molecule recognized by brain microglia. Mol. Cell. Neurosci. 60, 88–96. doi:10.1016/j.mcn.2014.04.003

Liu, Y., Peterson, D.A., Kimura, H., Schubert, D., 1997. Mechanism of cellular 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction. J. Neurochem. 69, 581–593. doi:10.1046/j.1471-4159.1997.69020581.x

Liu, Y., Sun, Q., Chen, X., Jing, L., Wang, W., Yu, Z., Zhang, G., Xie, M., 2014. Linolenic acid provides multi-cellular protective effects after photothrombotic cerebral ischemia in rats. Neurochem. Res. 39, 1797–1808. doi:10.1007/s11064-014-1390-3

Magdalon, J., Vinolo, M.A.R., Rodrigues, H.G., Paschoal, V.A., Torres, R.P., Mancini-Filho, J., Calder, P.C., Hatanaka, E., Curi, R., 2012. Oral administration of oleic or linoleic acids modulates the production of inflammatory mediators by rat macrophages. Lipids 47, 803–812. doi:10.1007/s11745-012-3687-9

Martín, V., Fabelo, N., Santpere, G., Puig, B., Marín, R., Ferrer, I., Díaz, M., 2010. Lipid alterations in lipid rafts from Alzheimer's disease human brain cortex. J. Alzheimers. Dis. 19, 489–502. doi:10.3233/JAD-2010-1242

Martínez-Lapiscina, E.H., Clavero, P., Toledo, E., Estruch, R., Salas-Salvadó, J., San Julián, B., Sanchez-Tainta, A., Ros, E., Valls-Pedret, C., Martinez-Gonzalez, M.Á., 2013. Mediterranean diet improves cognition: the PREDIMED-NAVARRA randomised trial. J. Neurol. Neurosurg. Psychiatry 84, 1318–1325. doi:10.1136/jnnp-2012-304792

Martins De Lima-Salgado, T., Sampaio, S.C., Cury-Boaventura, M.F., Curi, R., 2011. Modulatory effect of fatty acids on fungicidal activity, respiratory burst and TNF-a and IL-6 production in J774 murine macrophages. Br. J. Nutr. 105, 1173–1179. doi:10.1017/S0007114510004873

Massiera, F., Barbry, P., Guesnet, P., Joly, A., Luquet, S., Moreilhon-Brest, C., Mohsen-Kanson, T., Amri, E.Z., Ailhaud, G., 2010. A Western-like fat diet is sufficient to induce a gradual enhancement in fat mass over generations. J. Lipid Res. 51, 2352–2361. doi:10.1194/jlr.M006866

Matsuo, M., Hamasaki, Y., Fujiyama, F., Miyazaki, S., 1995. Eicosanoids are produced by microglia, not by astrocytes, in rat glial cell cultures. Brain Res. 685, 201–204. doi:10.1016/0006-8993(95)00490-H

McKenzie, J.A., Spielman, L.J., Pointer, C.B., Lowry, J.R., Bajwa, E., Lee, C.W., Klegeris, A., 2017. Neuroinflammation as a common mechanism associated with the modifiable risk factors for Alzheimer's and Parkinson's diseases. Curr. Aging Sci. 10, 158–176. doi:10.2174/1874609810666170315113244

Milanski, M., Degasperi, G., Coope, A., Morari, J., Denis, R., Cintra, D.E., Tsukumo, D.M.L., Anhe, G., Amaral, M.E., Takahashi, H.K., Curi, R., Oliveira, H.C., Carvalheira, J.B.C., Bordin, S., Saad, M.J., Velloso, L.A., 2009. Saturated fatty acids produce an inflammatory response predominantly through the activation of TLR4 signaling in hypothalamus: implications for the pathogenesis of obesity. J. Neurosci. 29, 359–370. doi:10.1523/JNEUROSCI.2760-08.2009

Moheimani, F., Tan, J.T.M., Brown, B.E., Heather, A.K., van Reyk, D.M., Davies, M.J., 2011. Effect of exposure of human monocyte-derived macrophages to high, versus normal, glucose on subsequent lipid accumulation from glycated and acetylated low-density lipoproteins. Exp. Diabetes Res. 2011, 851280. doi:10.1155/2011/851280

Mosmann, T., 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J. Immunol. Methods 65, 55–63. doi:10.1016/0022-1759(83)90303-4

Murphy, M.G., 1995. Effects of exogenous linoleic acid on fatty acid composition, receptor-mediated cAMP formation, and transport functions in rat astrocytes in primary culture. Neurochem. Res. 20, 1365–1375. doi:10.1007/BF00992513

Nallathamby, N., Serm, L.G., Raman, J., Malek, S.N.A., Vidyadaran, S., Naidu, M., Kuppusamy, U.R., Sabaratnam, V., 2016. Identification and *in vitro* evaluation of lipids from sclerotia of *Lignosus rhinocerotis* for antioxidant and anti-neuroinflammatory activities. Nat. Prod. Commun. 11, 1485–1490.

Nguyen, L.N., Ma, D., Shui, G., Wong, P., Cazenave-Gassiot, A., Zhang, X., Wenk, M.R., Goh, E.L.K., Silver, D.L., 2014. Mfsd2a is a transporter for the essential omega-3 fatty acid docosahexaenoic acid. Nature 509, 503–506. doi:10.1038/nature13241

Niki, E., 2009. Lipid peroxidation: physiological levels and dual biological effects. Free Radic. Biol. Med. 47, 469–484. doi: 10.1016/j.freeradbiomed.2009.05.032

Nowson, C.A., Service, C., Appleton, J., Grieger, J.A., 2018. The impact of dietary factors on indices of chronic disease in older people: A systematic review. J. Nutr. Health Aging 22, 282–296. doi:10.1007/s12603-017-0920-5

Oda, T., Maeda, H., 1986. A new simple fluorometric assay for phagocytosis. J. Immunol. Methods 88, 175–183.

Oh, D.Y., Talukdar, S., Bae, E.J., Imamura, T., Morinaga, H., Fan, W., Li, P., Lu, W.J., Watkins, S.M., Olefsky, J.M., 2010a. GPR120 is an omega-3 fatty acid receptor mediating potent anti-inflammatory and insulin-sensitizing effects. Cell 142, 687–698. doi:10.1016/j.cell.2010.07.041

Oh, Y.T., Lee, J.Y., Lee, J., Kim, H., Yoon, K.S., Choe, W., Kang, I., 2009. Oleic acid reduces lipopolysaccharide-induced expression of iNOS and COX-2 in BV2 murine microglial cells: possible involvement of reactive oxygen species, p38 MAPK, and IKK/NF-κB signaling pathways. Neurosci. Lett. 464, 93–97. doi:10.1016/j.neulet.2009.08.040

Oh, Y.T., Lee, J.Y., Lee, J., Lee, J.H., Kim, J.E., Ha, J., Kang, I., 2010b. Oleamide suppresses lipopolysaccharide-induced expression of iNOS and COX-2 through inhibition of NF-kappaB activation in BV2 murine microglial cells. Neurosci. Lett. 474, 148–53. doi:10.1016/j.neulet.2010.03.026

Ohue-Kitano, R., Yasuoka, Y., Goto, T., Kitamura, N., Park, S.B., Kishino, S., Kimura, I., Kasubuchi, M., Takahashi, H., Li, Y., Yeh, Y.S., Jheng, H.F., Iwase, M., Tanaka, M., Masuda, S., Inoue, T., Yamakage, H., Kusakabe, T., Tani, F., Shimatsu, A., Takahashi, N., Ogawa, J., Satoh-Asahara, N., Kawada, T., 2018. α-Linolenic acid–derived metabolites from gut lactic acid bacteria induce differentiation of anti-inflammatory M2 macrophages through G protein-coupled receptor 40. FASEB J. 32, 304–318. doi:10.1096/fj.201700273R

Olsen, K.S., Fenton, C., Frøyland, L., Waaseth, M., Paulssen, R.H., Lund, E., 2013. Plasma fatty acid ratios affect blood gene expression profiles—a cross-sectional study of the Norwegian women and cancer post-genome cohort. PLoS One 8, e67270. doi:10.1371/journal.pone.0067270

Påhlman, S., Mamaeva, S., Meyerson, G., Mattsson, M.E., Bjelfman, C., Ortoft, E., Hammerling, U., 1990. Human neuroblastoma cells in culture: a model for neuronal cell differentiation and function. Acta Physiol. Scand. Suppl. 592, 25–37.

Palomba, L., Cerioni, L., Cantoni, O., 2009. Arachidonic acid: a key molecule for astrocyte survival to peroxynitrite. Glia 57, 1672–1679. doi:10.1002/glia.20879

Paul, V., Ekambaram, P., 2011. Involvement of nitric oxide in learning and memory processes. Indian J. Med. Res. 133, 471–478.

Petersen, M.A., Dailey, M.E., 2004. Diverse microglial motility behaviors during clearance of dead cells in hippocampal slices. Glia 46, 195–206. doi:10.1002/glia.10362

Phillips, M.A., Childs, C.E., Calder, P.C., Rogers, P.J., 2012. Lower omega-3 fatty acid intake and status are associated with poorer cognitive function in older age: a comparison of individuals with and without cognitive impairment and Alzheimer's disease. Nutr. Neurosci. 15, 271–277. doi:10.1179/1476830512Y.0000000026

Pointer, C. B. 2017. The novel role of extracellular cardiolipin in microglial activation and neuroinflammation. University of British Columbia (Okanagan). cIRcle, doi:10.14288/1.0347222

Preik-Steinhoff, H., Kelm, M., 1996. Determination of nitrite in human blood by combination of a specific sample preparation with high-performance anion-exchange chromatography and electrochemical detection. J. Chromatogr. B. 685, 348–352.

Rademacher, D., Kearn, C.S., Carrier, E.J., Patel, S., Delgado, M.A., Barkmeier, A., Klick, D.E., Breese, N.M., Pfister, S.L., Nithipatikom, K., Campbell, W.B., Hillard, C.J., 2004. Production of hydroxyeicosatetraenoic acids and prostaglandins by a novel rat microglial cell line. J. Neuroimmunol. 149, 130–141. doi:10.1016/j.jneuroim.2003.12.022

Ramlackhansingh, A.F., Brooks, D.J., Greenwood, R.J., Bose, S.K., Turkheimer, F.E., Kinnunen, K.M., Gentleman, S., Heckemann, R.A., Gunanayagam, K., Gelosa, G., Sharp, D.J., 2011. Inflammation after trauma: microglial activation and traumatic brain injury. Ann. Neurol. 70, 374–383. doi:10.1002/ana.22455

Ransohoff, R.M., 2016. How neuroinflammation contributes to neurodegeneration. Science 353, 777–783. doi:10.1126/science.aag2590

Ren, J., Chung, S.H., 2007. Anti-inflammatory effect of α-linolenic acid and its mode of action through the inhibition of nitric oxide production and inducible nitric oxide synthase gene expression via NF-κB and mitogen-activated protein kinase pathways. J. Agric. Food Chem. 55, 5073–5080. doi:10.1021/jf0702693

Richieri, G. V, Kleinfeld, A.M., 1995. Unbound free fatty acid levels in human serum. J. Lipid Res. 36, 229–240.

Ridnour, L.A., Sim, J.E., Hayward, M.A., Wink, D.A., Martin, S.M., Buettner, G.R., Spitz, D.R., 2000. A spectrophotometric method for the direct detection and quantitation of nitric oxide, nitrite, and nitrate in cell culture media. Anal. Biochem. 281, 223–229. doi:10.1006/abio.2000.4583

Ross, R.A., Biedler, J.L., Spengler, B.A., Reis, D.J., 1981. Neurotransmitter-synthesizing enzymes in 14 human neuroblastoma cell lines. Cell. Mol. Neurobiol. 1, 301–312. doi:10.1007/BF00710685

Saab, A.S., Nave, K.-A., 2017. Myelin dynamics: protecting and shaping neuronal functions. Curr. Opin. Neurobiol. 47, 104–112. doi:10.1016/j.conb.2017.09.013

Salem, N., Litman, B., Kim, H.Y., Gawrisch, K., 2001. Mechanisms of action of docosahexaenoic acid in the nervous system. Lipids 36, 945–959. doi:10.1007/s11745-001-0805-6

Schiessel, D.L., Yamazaki, R.K., Kryczyk, M., Coelho De Castro, I., Yamaguchi, A.A., Pequito, D.C.T., Brito, G.A.P., Borghetti, G., Ulia Aikawa, J., Nunes, E.A., Atia Naliwaiko, K., Fernandes, L.C., 2016. Does oil rich in α-linolenic fatty acid cause the same immune modulation as fish oil in Walker 256 tumor-bearing rats? Nutr. Cancer 68, 1369–1380. doi:10.1080/01635581.2016.1224364

Schrauwen, P., Westerterp, K.R., 2000. The role of high-fat diets and physical activity in the regulation of body weight. Br. J. Nutr. 84, 417–427. doi:10.1017/S0007114500001720

Schroit, A.J., Gallily, R., 1979. Macrophage fatty acid composition and phagocytosis: effect of unsaturation on cellular phagocytic activity. Immunology 36, 199–205.

Schumann, J., 2016. It is all about fluidity: fatty acids and macrophage phagocytosis. Eur. J. Pharmacol. 785, 18–23. doi:10.1016/j.ejphar.2015.04.057

Schwenk, R.W., Holloway, G.P., Luiken, J.J.F.P., Bonen, A., Glatz, J.F.C., 2010. Fatty acid transport across the cell membrane: regulation by fatty acid transporters. Prostaglandins, Leukot. Essent. Fat. Acids 82, 149–154. doi:10.1016/J.PLEFA.2010.02.029

Schwingshackl, L., Hoffmann, G., 2013. Comparison of effects of long-term low-rat vs high-fat diets on blood lipid levels in overweight or obese patients: a systematic review and meta-analysis. J. Acad. Nutr. Diet. 113, 1640–1661. doi:10.1016/j.jand.2013.07.010

Shaikh, S.R., Teague, H., 2012. N-3 fatty acids and membrane microdomains: from model membranes to lymphocyte function. Prostaglandins, Leukot. Essent. Fat. Acids 87, 205–208. doi:10.1016/j.plefa.2012.09.007

Sharif, O., Bolshakov, V.N., Raines, S., Newham, P., Perkins, N.D., 2007. Transcriptional profiling of the LPS induced NF-κB response in macrophages. BMC Immunol. 8, 1. doi:10.1186/1471-2172-8-1

Sheehan, J.J., Zhou, C., Gravanis, I., Rogove, A.D., Wu, Y.P., Bogenhagen, D.F., Tsirka, S.E., 2007. Cellular/molecular proteolytic activation of monocyte chemoattractant protein-1 by plasmin underlies excitotoxic neurodegeneration in mice. J. Neurosci. 27, 1738–1745. doi:10.1523/JNEUROSCI.4987-06.2007

Shen, Y., Kapfhamer, D., Minnella, A.M., Kim, J.-E., Won, S.J., Chen, Y., Huang, Y., Low, L.H., Massa, S.M., Swanson, R.A., 2017. Bioenergenic state regulates innate inflammatory responses through the transcriptional co-repressor CtBP. Nat. Commun. 8, 624. doi: 10.1038/s41467-017-00707-0

Sigalet, D.L., Martin, G., 1999. Lymphatic absorption of glucose and fatty acids as determined by direct measurement. J. Pediatr. Surg. 34, 39–43. doi:10.1016/S0022-3468(99)90225-7

Simopoulos, A.P., 2016. An increase in the omega-6/omega-3 fatty acid ratio increases the risk for obesity. Nutrients 8, 128. doi:10.3390/nu8030128

Simopoulos, A.P., 2011. Evolutionary aspects of diet: the omega-6/omega-3 ratio and the brain. Mol. Neurobiol. 44, 203–215. doi:10.1007/s12035-010-8162-0

Slepko, N., Minghetti, L., Polazzi, E., Nicolini, A., Levi, G., 1997. Reorientation of prostanoid production accompanies “activation” of adult microglial cells in culture. J. Neurosci. Res. 49, 292–300. doi:10.1002/(SICI)1097-4547(19970801)49:3<292::AID-JNR4>3.0.CO;2-7

Snowden, S.G., Ebshiana, A.A., Hye, A., An, Y., Pletnikova, O., O’Brien, R., Troncoso, J., Legido-Quigley, C., Thambisetty, M., 2017. Association between fatty acid metabolism in the brain and Alzheimer disease neuropathology and cognitive performance: a nontargeted metabolomic study. PLoS Med. 14, e1002266. doi:10.1371/journal.pmed.1002266

Sokolowski, J.D., Mandell, J.W., 2011. Phagocytic clearance in neurodegeneration. Am. J. Pathol. 178, 1416–1428. doi:10.1016/j.ajpath.2010.12.051

Spector, A.A., 1975. Fatty acid binding to plasma albumin. J. Lipid Res. 16, 165–79.

Spector, A.A., John, K., Fletcher, J.E., 1969. Binding of long-chain fatty acids to bovine serum albumin. J. Lipid Res. 10, 56–67.

Spielman, L.J., Little, J.P., Klegeris, A., 2014. Inflammation and insulin/IGF-1 resistance as the possible link between obesity and neurodegeneration. J Neuroimmunol 273, 8–21. doi:10.1016/j.jneuroim.2014.06.004

Stolzing, A., Grune, T., 2004. Neuronal apoptotic bodies: phagocytosis and degradation by primary microglial cells. FASEB J. 18, 743–745. doi:10.1096/fj.03-0374fje

Sydenham, E., Dangour, A.D., Lim, W.-S., 2012. Omega 3 fatty acid for the prevention of cognitive decline and dementia. Cochrane Database Syst. Rev. CD005379. doi:10.1002/14651858.CD005379.pub3

Tabe, S., Hikiji, H., Ariyoshi, W., Hashidate-Yoshida, T., Shindou, H., Okinaga, T., Shimizu, T., Tominaga, K., Nishihara, T., 2016. Lysophosphatidylethanolamine acyltransferase 1/membrane-bound O-acyltransferase 1 regulates morphology and function of P19C6 cell–derived neurons. FASEB J. 30, 2591–2601. doi:10.1096/fj.201500097R

Tabernero, A., Velasco, A., Granda, B., Lavado, E.M., Medina, J.M., 2002. Transcytosis of albumin in astrocytes activates the sterol regulatory element-binding protein-1, which promotes the synthesis of the neurotrophic factor oleic acid. J. Biol. Chem. 277, 4240–4246. doi:10.1074/jbc.M108760200

Thomas, A.C., Mattila, J.T., 2014. "Of mice and men": arginine metabolism in macrophages. Front. Immunol. 5, 479. doi:10.3389/fimmu.2014.00479

Thomas, D.C., 2017. The phagocyte respiratory burst: historical perspectives and recent advances. Immunol. Lett. 192, 88–96. doi:10.1016/j.imlet.2017.08.016

Tremblay, M.-E., Zhang, I., Bisht, K., Savage, J.C., Lecours, C., Parent, M., Titorenko, V., Maysinger, D., 2016. Remodeling of lipid bodies by docosahexaenoic acid in activated microglial cells. J. Neuroinflammation 13, 116. doi:10.1186/s12974-016-0580-0

Tsuchiya, S., Yamabe, M., Yamaguchi, Y., Kobayashi, Y., Konno, T., Tada, K., 1980. Establishment and characterization of a human acute monocytic leukemia cell line (THP-1). Int. J. Cancer 26, 171–176. doi:10.1002/ijc.2910260208

Ukropec, J., Reseland, J.E., Gasperikova, D., Demcakova, E., Madsen, L., Berge, R.K., Rustan, A.C., Klimes, I., Drevon, C.A., Sebökova, E., 2003. The hypotriglyceridemic effect of dietary n−3 FA is associated with increased β-oxidation and reduced leptin expression. Lipids 38, 1023–1029. doi:10.1007/s11745-006-1156-z

Van De Rest, O., Berendsen, A.A., Haveman-Nies, A., De Groot, L.C., 2015. Dietary patterns, cognitive decline, and dementia: a systematic review. Adv. Nutr. 6, 154–168. doi:10.3945/an.114.007617

Vane, J.R., Bakhle, Y.S., Botting, R.M., 1998. Cyclooxygenases 1 and 2. Annu. Rev. Pharmacol. Toxicol. 38, 97–120. doi:10.1146/annurev.pharmtox.38.1.97

Vangaveti, V.N., Jansen, H., Kennedy, R.L., Malabu, U.H., 2016. Hydroxyoctadecadienoic acids: oxidised derivatives of linoleic acid and their role in inflammation associated with metabolic syndrome and cancer. Eur. J. Pharmacol. 785, 70–76. doi:10.1016/j.ejphar.2015.03.096

Vangaveti, V.N., Shashidhar, V.M., Rush, C., Malabu, U.H., Rasalam, R.R., Collier, F., Baune, B.T., Kennedy, R.L., 2014. Hydroxyoctadecadienoic acids regulate apoptosis in human THP-1 cells in a PPARγ-dependent manner. Lipids 49, 1181–1192. doi:10.1007/s11745-014-3954-z

Wang, J., Yu, L., Schmidt, R.E., Su, C., Huang, X., Gould, K., Cao, G., 2005. Characterization of HSCD5, a novel human stearoyl-CoA desaturase unique to primates. Biochem. Biophys. Res. Commun. 332, 735–742. doi:10.1016/J.BBRC.2005.05.013

Wang, S., Wu, D., Lamon-Fava, S., Matthan, N.R., Honda, K.L., Lichtenstein, A.H., 2009. In vitro fatty acid enrichment of macrophages alters inflammatory response and net cholesterol accumulation. Br. J. Nutr. 102, 497–501. doi:10.1017/S0007114509231758

Wang, T.Y., Liu, M., Portincasa, P., Wang, D.Q.-H., 2013. New insights into the molecular mechanism of intestinal fatty acid absorption. Eur. J. Clin. Invest. 43, 1203-1223. doi:10.1111/eci.12161

Wang, Z., Liu, D., Wang, F., Liu, S., Zhao, S., Ling, E.A., Hao, A., 2012. Saturated fatty acids activate microglia via toll-like receptor 4/NF-κB signalling. Br. J. Nutr. 107, 229–241. doi:10.1017/S0007114511002868

Weylandt, K.H., Chiu, C.Y., Gomolka, B., Waechter, S.F., Wiedenmann, B., 2012. Omega-3 fatty acids and their lipid mediators: towards an understanding of resolvin and protectin formation. Prostaglandins Other Lipid Mediat. 97, 73–82. doi:10.1016/j.prostaglandins.2012.01.005

Whittington, R.A., Planel, E., Terrando, N., 2017. Impaired resolution of inflammation in Alzheimer's disease: a review. Front. Immunol. 8, 1464. doi:10.3389/fimmu.2017.01464

Wolhuter, K., Whitwell, H.J., Switzer, C.H., Burgoyne, J.R., Timms, J.F., Eaton, P., 2018. Evidence against stable protein S-nitrosylation as a widespread mechanism of post-translational regulation. Mol. Cell 69, 438–450.e5. doi:10.1016/j.molcel.2017.12.019

Xia, M., Zhao, Q., Zhang, H., Chen, Y., Yuan, Z., Xu, Y., Zhang, M., 2017. Proteomic analysis of HDAC3 selective inhibitor in the regulation of inflammatory response of primary microglia. Neural Plast. 2017, 6237351. doi:10.1155/2017/6237351

Xiong, Y., Mahmood, A., Chopp, M., 2013. Animal models of traumatic brain injury. Nat. Rev. Neurosci. 14, 128–42. doi:10.1038/nrn3407

Xu, D.M., Omura, T., Masaki, N., Arima, H., Banno, T., Okamoto, A., Hanada, M., Takei, S., Matsushita, S., Sugiyama, E., Setou, M., Matsuyama, Y., 2016. Increased arachidonic acid-containing phosphatidylcholine is associated with reactive microglia and astrocytes in the spinal cord after peripheral nerve injury. Sci. Rep. 6, 26427. doi:10.1038/srep26427

Xu, W., Tan, L., Wang, H.F., Jiang, T., Tan, M.S., Zhao, Q.F., Li, J.Q., Wang, J., Yu, J.T., 2015. Meta-analysis of modifiable risk factors for Alzheimer's disease. J. Neurol. Neurosurg. Psychiatry 86, 1299–1306. doi:10.1136/jnnp-2015-310548

Xu, Z.Z., Berta, T., Ji, R.R., 2013. Resolvin E1 inhibits neuropathic pain and spinal cord microglial activation following peripheral nerve injury. J. Neuroimmune Pharmacol. 8, 37–41. doi:10.1007/s11481-012-9394-8

Yoshida, Y., Umeno, A., Akazawa, Y., Shichiri, M., Murotomi, K., Horie, M., 2015. Chemistry of lipid peroxidation products and their use as biomarkers in early detection of diseases. J. Oleo Sci. J. Oleo Sci 64, 347–356. doi:10.5650/jos.ess14281

Yoshida, Y., Yoshikawa, A., Kinumi, T., Ogawa, Y., Saito, Y., Ohara, K., Yamamoto, H., Imai, Y., Niki, E., 2009. Hydroxyoctadecadienoic acid and oxidatively modified peroxiredoxins in the blood of Alzheimer's disease patients and their potential as biomarkers. Neurobiol. Aging 30, 174–185. doi:10.1016/j.neurobiolaging.2007.06.012

Zazpe, I., Sanchez-Tainta, A., Estruch, R., Lamuela-Raventos, R.M., Schröder, H., Salas-Salvado, J., Corella, D., Fiol, M., Gomez-Gracia, E., Aros, F., Ros, E., Ruíz-Gutierrez, V., Iglesias, P., Conde-Herrera, M., Martinez-Gonzalez, M.A., 2008. A large randomized individual and group intervention conducted by registered dietitians increased adherence to Mediterranean-type diets: the PREDIMED study. J. Am. Diet. Assoc. 108, 1134–44. doi:10.1016/j.jada.2008.04.011

Zhang, Q.-G., Laird, M.D., Han, D., Nguyen, K., Scott, E., Dong, Y., Dhandapani, K.M., Brann, D.W., 2012. Critical role of NADPH oxidase in neuronal oxidative damage and microglia activation following traumatic brain injury. PLoS One 7, e34504. doi:10.1371/journal.pone.0034504

Zhang, S., Yang, L., Kouadir, M., Tan, R., Lu, Y., Chang, J., Xu, B., Yin, X., Zhou, X., Zhao, D., 2013. PP2 and piceatannol inhibit PrP106-126-induced iNOS activation mediated by CD36 in BV2 microglia. Acta Biochim. Biophys. Sin. (Shanghai). 45, 763–772. doi:10.1093/abbs/gmt074

Zhang, Y., Chen, K., Sloan, S.A., Bennett, M.L., Scholze, A.R., O'Keeffe, S., Phatnani, H.P., Guarnieri, P., Caneda, C., Ruderisch, N., Deng, S., Liddelow, S.A., Zhang, C., Daneman, R., Maniatis, T., Barres, B.A., Wu, J.Q., 2014. An RNA-sequencing transcriptome and splicing database of glia, neurons, and vascular cells of the cerebral cortex. J. Neurosci. 34, 11929–11947. doi:10.1523/JNEUROSCI.1860-14.2014

Zhao, G., Etherton, T.D., Martin, K.R., Vanden Heuvel, J.P., Gillies, P.J., West, S.G., Kris-Etherton, P.M., 2005. Anti-inflammatory effects of polyunsaturated fatty acids in THP-1 cells. Biochem. Biophys. Res. Commun. 336, 909–917. doi:10.1016/j.bbrc.2005.08.204

Zhao, Y., Calon, F., Julien, C., Winkler, J.W., Petasis, N.A., Lukiw, W.J., Bazan, N.G., 2011. Docosahexaenoic acid-derived neuroprotectin D1 induces neuronal survival via secretase- and PPARγ-mediated mechanisms in Alzheimer's disease models. PLoS One 6, e15816. doi:10.1371/journal.pone.0015816

Zlokovic, B. V, 2011. Neurovascular pathways to neurodegeneration in Alzheimer's disease and other disorders. Nat. Rev. Neurosci. 12, 723–738. doi:10.1038/nrn3114

Appendices

Appendix A

A.1 Reagents Used in Immunoblotting Experiments

RIPA Buffer: 150 mM sodium chloride, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 50 mM Tris buffer, dH_2O, corrected to pH 8.0

Laemmli Sample Buffer: 0.125 M Tris buffer, 20% glycerol, 10% mercaptoethanol, 4 % sodium dodecyl sulfate, 0.004% bromophenol blue, dH_2O

10 X Towbin Transfer Buffer: 1.9 M glycine, 0.2 M Tris buffer, dH_2O

1 X Towbin Transfer Buffer: 20% methanol, 10% of 10 X Towbin Transfer Buffer, dH_2O

TBS-T: 150 mM NaCl, 10 mM Tris buffer, 0.2% Tween-20, dH_2O, corrected to pH 8.0

A.2 Reagents Used in ELISA Experiments

Coating solution: 0.1 M sodium bicarbonate buffer, dH2O, corrected to pH 9.6

PBS-T: 1 X PBS, 0.05% Tween-20, dH_2O

Appendix B

B.1 Methods for Primary Adult Mouse Microglial Phagocytosis

B.1.1 Extraction of Primary Mouse Microglia

Primary mouse microglia were extracted as previously described (Lee et al., 2013). Tissues from C57BL/6 mice (9 to 37 weeks old) were supplied by our collaborators Drs. Barker and Ghosh from the University of British Columbia Okanagan campus Biology Department. All F0 and F10 media in the primary mouse microglia extraction protocol were supplemented with 0.45 g/mL glucose. Three to four brains were placed in a six cm tissue culture dish. Tissues were minced with sterile razor blades in three mL F0, and added to three mL dissociation medium (one mg/mL papain, 0.13 M trehalose dihydrate, and 20 U/mL DNase1 in F0) in sterile 50 mL conical tubes. Mechanical dissociation was promoted by continuous inversion of the tubes for 20 to 50 min at 37°C. F10 was added to each tube to neutralize enzymes in the dissociation medium and tubes were centrifuged at 250 x g at room temperature for five min, followed by aspiration of supernatant and resuspension of the pellet in F0. Centrifugation was repeated at 250 x g, supernatant was aspirated and three mL of F10 was added to each tube. To disperse cells, cell pellets were thoroughly mixed with three decreasing sizes of pipette tips. Dispersion began with the 10 mL serological pipettes, followed by the addition of three mL of F10, and homogenization using P1000 pipette tips. Next two mL of F10 was added, and the mixture was homogenized using P200 pipette tips. Dispersed cells were passed through cell filters (100 μm; ThermoFisher Scientific) and centrifuged for four min at 250 x g. The cell pellet was then mixed with five mL of F10. Centrifugation was repeated for four min at 250 x g, and the cell pellets were collected. A density centrifugation gradient was required to separate microglia from myelin, other cells and

cellular debris. For this, stock isotonic percoll (SIP) was prepared by mixing nine volumes of percoll and one volume of 10 X HBSS. Each percoll density gradient was prepared in a 15 mL conical tube by resuspending the collected cell pellet in four mL of 37% SIP (diluted in 1 X HBSS), followed by underlaying four mL of 70% SIP (diluted in 1 X HBSS) and overlaying four mL of 30% SIP (diluted in 1 X HBSS), followed by adding two mL of 1 X HBSS on the top. From bottom to top of the 15 mL conical tube, the gradient included: 70% SIP, 37% SIP, 30% SIP, and 1 X HBSS. Each percoll gradient conical tube was centrifuged for 40 min at 300 x g, 8°C, during which time the myelin collected between the top 1 X HBSS and 30% SIP layers. The microglial cells formed a visible phase between the bottom 37% SIP and 70% SIP layers. Three mL of each microglial cell phase were collected. These cells were washed in 1 X HBSS, by mixing every one volume of the microglial cell phase with three volumes of 1 X HBSS. Each conical was centrifuged for seven min at 500 x g, 4°C, followed by three steps of resuspension in two mL of 1 X HBSS and centrifugation for four min at 800 x g, room temperature. Cell pellets were resuspended in F10, and incubated in 10 cm tissue culture dishes for 24 to 48 h to allow microglia to adhere. After incubation, adherent cells were washed once with PBS to dislodge any non-adherent cells, followed by standard cell maintenance procedures including the addition of fresh F10 media without additional supplemented glucose every 48 to 72 h.

B.1.2 Fatty Acid Treatment of Primary Microglia

Five to seven days post-extraction, primary mouse microglia were seeded in 8-well chamber slides at 15 000 to 40 000 cells in 350 μL of F5 media (40 000 to 120 000 cells/mL) for 24 h. The fatty acid/BSA solutions (2.5 mM fatty acid/6% BSA; see 2.4) were first diluted to 1.75 mM in F0 media followed by 50-fold dilution into cell cultures to achieve the final well

concentration of 25 μM for each fatty acid. Vehicle control was obtained by a 1.4-fold dilution of the vehicle solution (1.8% ethanol/6% BSA solution; see 2.4) in F0 media, and 50-fold dilution into cell cultures to achieve final concentrations of 0.03% (v/v) ethanol and 0.08% (w/v) BSA. After 24 h, fluorescent beads (Fluoresbright YG 1 μm microspheres; final concentration 0.25% v/v) were added to the primary mouse microglia cultures with agitation. A one-hour incubation in the CO_2 incubator at 37°C with 5% CO_2 and 95% air atmosphere was based on a published standard curve showing dependence of peripheral macrophage phagocytosis on incubation time (Oda and Maeda, 1986). The published curve found a linear trend during one hour of bead incubation, compared to two hours where bead phagocytosis became saturated. Microglial cells were then washed once with PBS.

B.1.3 Cell Fixation, Staining and Visualization

After washing, cells were fixed to the glass surface of each chamber for 30 min in 4% PFA, following which cells were washed once with PBS. DAPI with Fluoroshield™ (100 μL) was used to cover the cells prior to imaging under a Zeiss microscope (AxioObserver.Z1 widefield epifluorescence microscope). For DAPI, 350 nm excitation and 470 nm emission wavelengths were used, and for the fluorescent beads 470 nm excitation and 520 nm emission wavelengths were used. Zen image acquisition software was used for image capture. Each replicate consisted of a minimum of 15 to 25 cells per sample (refer to Table 6 for cell counts), with several cells per microscope field, and at least three pictures analysed. Phagocytosis was measured as the corrected total cell fluorescence (CTCF). Fluorescence of each cell analysed was compared to background area not containing any cells. The fluorescence background was the fluorescence value within a cell-free area multiplied by the area of the selected cell. The

integrated density of the cell was calculated by the ImageJ software (National Institute of Health, USA) as equal to fluorescence of the cell multiplied by the area of the cell, and this measurement was used for determination of the CTCF value. The CTCF was defined as the integrated density of the fluorescence divided by the fluorescence background according to the following formulae.

Fluorescence Background = Background Fluorescence Value x Selected Cell Area (4)

CTCF = Integrated Density / Fluorescence Background (5)

Where Integrated Density, Background Fluorescence Value, and Selected Cell Area are calculated by ImageJ software. Confocal microscopy (Olympus FluoView confocal microscope Model #FV1000, Olympus Inc., Richmond Hill, ON, Canada) was performed by Caitlin Pointer to confirm that fluorescent beads visualized and analysed by this method were engulfed by the cells as opposed to their adherence to cell surface (Pointer, 2017).

Table 6. The numbers of primary mouse microglial cells that were used to measure phagocytosis of fluorescent beads.

Fatty Acid Treatment	Replicate	Number of Cells	
		Vehicle Treated	Fatty Acid Treated
ALA	N1	27	31
	N2	26	36
	N3	46	41
LA	N1	27	25
	N2	26	25
	N3	46	58
OA	N1	44	27
	N2	63	27
	N3	30	25
SA	N1	44	29
	N2	63	39
	N3	30	29

B.2 The Effects of Fatty Acids on the Primary Microglial Phagocytosis of Microspheres

Microglia were extracted, as described in B.1, five to seven days prior to their use in experiments. 25 μM of each fatty acid was added to primary microglia for 24 h. After incubation, Fluoresbright® YG 1 μm microspheres were added to the microglia for one h. Fluorescence of microspheres was used to measure the phagocytic activity of the primary microglia (Figure 28). Phagocytosis associated with each fatty acid treatment was compared to the phagocytosis associated with vehicle treatment (0 μM) by Student's paired t-test. For primary mouse microglia treated with either ALA, LA, OA or SA, no significant differences were observed in phagocytosis of fluorescent microspheres (Figure 28; A, B, C, D).

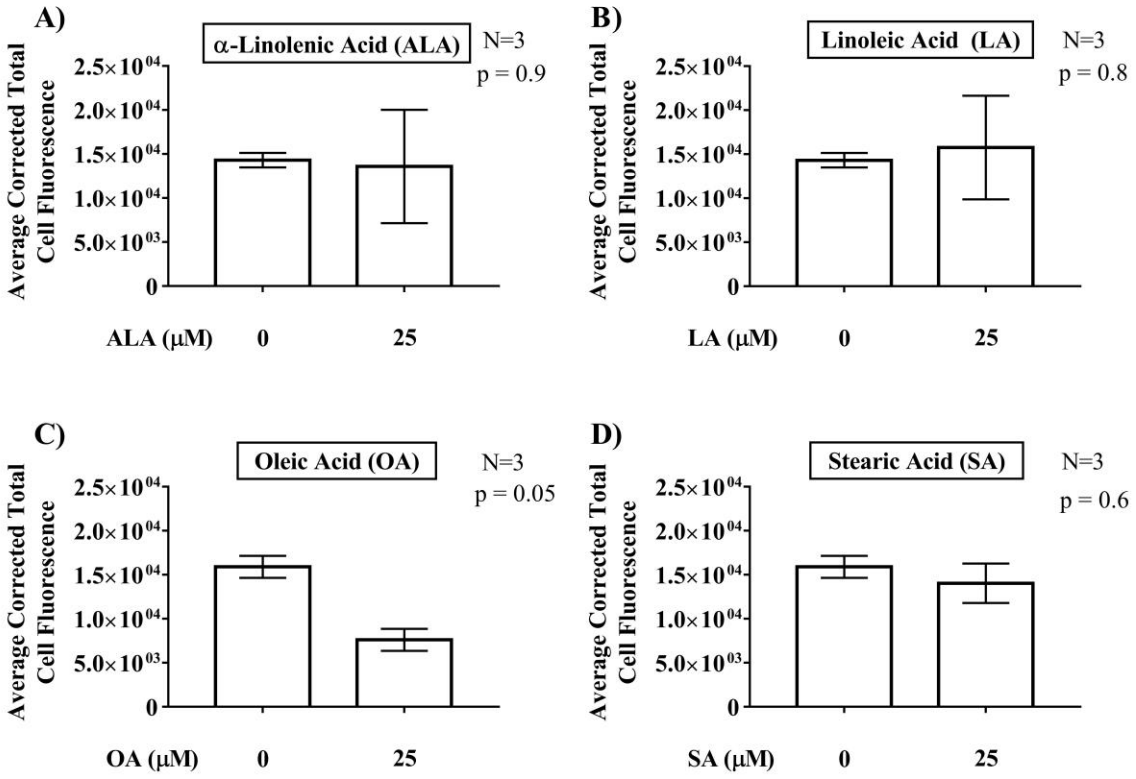

Figure 28. The effects of α-linolenic acid (ALA), linoleic acid (LA), oleic acid (OA), and stearic acid (SA) on the phagocytic activity of primary adult mouse microglia cultured in high glucose medium (17.0 mM). Primary mouse microglia were treated for 24 h with 25 μM of ALA, LA, OA, SA or their vehicle alone (0 μM fatty acid, 0.03% v/v ethanol and 0.08% w/v BSA). Phagocytosis of Fluoresbright® YG 1 μm microspheres (0.25% v/v), which were incubated with primary microglia for one h, was assessed using light microscopy. Data are presented as the average corrected total cell fluorescence following treatment with (A) ALA, (B) LA, (C) OA, and (D) SA. Data (means ± S.E.M.) from three independent experiments are presented. No statistically significant differences were detected by Student's paired t-test followed by Holm's step-down correction (F and P values indicated on figure).