CHARCTERIZING PREVIOUSLY UNRECOGNIZED EFFECTS OF ENDOGENOUS MOLECULES ON THE PHAGOCYTIC ACTIVITY OF GLIA, AS WELL AS THEIR SECRETOME

by

Tyler Joseph Richard Wenzel

B.Sc., Simon Fraser University, 2013
B.Ed., Simon Fraser University, 2014

A THESIS IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

In

THE COLLEGE OF GRADUATE STUDIES

(Biochemistry and Molecular Biology)

THE UNIVERSITY OF BRITISH COLUMBIA
(Okanagan)

November 2020

Thesis Committee

The following individuals certify that they have read, and recommend to the College of Graduate for acceptance, a thesis entitled:

CHARCTERIZING PREVIOUSLY UNRECOGNIZED EFFECTS OF ENDOGENOUS MOLECULES ON THE PHAGOCYTIC ACTIVITY OF GLIA, AS WELL AS THEIR SECRETOME

submitted by Tyler Joseph Richard Wenzel in partial fulfillment of the requirements for

the degree of **Doctorate of Philosophy** (Ph.D.)

Dr. Andis Klegeris, Department of Biology

Supervisor

Dr. Frederic Menard, Department of Chemistry

Supervisory Committee Member

Dr. Julien Gibon, Department of Biology

Supervisory Committee Member

Dr. David Jack, Department of Chemistry

University Examiner

Dr. Tiina Kauppinen, University of Manitoba

External Examiner

Additional Committee Members include:

Dr. Mark Rheault, Department of Biology

Supervisory Committee Member

Thesis Abstract

Neuroinflammation driven by non-neuronal cells, such as microglia and astrocytes, contributes to the progression of neurological disorders, including Alzheimer's disease. When released from cells, mitochondrial phospholipid cardiolipin (CL), soluble mitochondrial protein cytochrome c (CytC), and gut bacterial metabolites short-chain fatty acids (SCFAs) may interact with microglia and astrocytes, modulating their neuroinflammatory functions. In this thesis, I characterize previously unknown molecular mechanisms and functional consequences of microglia and astrocyte activation by extracellular CL, CytC and SCFAs.

In chapter two, I demonstrate that cardiolipin (CL) induces the secretion of monocyte chemoattractant protein (MCP)-1, interferon-γ-induced protein 10 and nitric oxide by microglia, while inhibiting secretion of inflammatory molecules by stimulated microglia-like cells. CL also upregulates microglial phagocytosis. I determine that toll-like receptor (TLR) 4 mediates the effects of CL on microglial phagocytosis and MCP-1 secretion.

In chapter three, I demonstrate that microglia and astrocytes release CytC when exposed to cytotoxins. Extracellular CytC induces the secretion of cytotoxins, interleukin (IL)-12 p70, IL-8, IL-1β, and granulocyte-macrophage colony-stimulating factor (GM-CSF) by astrocytic cells, and TLR 4 antagonists inhibit the secretion of the latter three cytokines.

In chapter four, I show SCFAs acetate, propionate, butyrate, formate and valerate alone or as a mixture decrease IL-1β, MCP-1, tumor necrosis factor (TNF)-α, and cytotoxin secretions by stimulated microglia-like cells. GLPG 0974, a free fatty acid receptor (FFAR) 2 and 3 antagonist, blocks the inhibitory effect of formate alone, but not the SCFA mixture, on IL-1β secretion by microglia-like cells. Formate and valerate alone decrease the phagocytic activity of stimulated microglia-like cells. Formate, but not valerate, alone inhibits the respiratory burst of microglia-like cells, reducing the production of reactive oxygen species.

Extracellularly released CL and CytC regulate immune responses of microglia and astrocytes, respectively, in a TLR 4-dependent manner. Due to its anti-inflammatory properties, cardiolipin could be used to reduce neuroinflammation, while TLR 4 antagonists may reduce the adverse neuroinflammatory effects of extracellular cytochrome c. In addition, SCFAs may regulate the immune responses of microglia. Therefore, therapies that promote the colonization and proliferation of SCFA-producing bacteria may reduce neuroinflammation, and subsequently slow the progression of Alzheimer's disease.

Lay Summary

Neurodegenerative disorders, such as Alzheimer's disease, are incurable. Prolonged inflammation in the brain contributes to the death of neurons, which are cells critical for normal cognition. I tested the ability of three different types of molecules native to the human body to decrease or increase brain inflammation. I determined that cardiolipin and short-chain fatty acids can reduce brain inflammation. Cardiolipin and short-chain fatty acids could be developed as drugs that reduce brain inflammation and possibly slow the progression of neurodegenerative diseases. Additionally, I determined that cytochrome c induces inflammation in the brain. Therefore, it is possible that cytochrome c contributes to the progression of neurodegenerative diseases and blocking the activity of extracellular cytochrome c could slow the progression of neurodegenerative diseases.

Preface

All of the work in this thesis was conducted at the University of British Columbia Okanagan campus. I was responsible for the conceptualization, investigation, formal analysis, and writing for the majority of the research reported in this thesis. The laboratory of Dr. Andis Klegeris is licensed under the UBC Clinical Research Ethics Board application number H10-00202, as well as the UBC Biosafety Permit B18-0103. I have completed the Tri-Council Policy Statement: Ethical Conduct for Research Involving Humans Course on Research Ethics (TCPS2: CORE) training.

Parts of chapter 1 (section 1.3 and 1.4) have been published in peer-reviewed journals [**Wenzel TJ**, Kwong E, Bajwa E, Klegeris A., 2020. Review: Resolution-associated molecular patterns (RAMPs) as endogenous regulators of glia functions in neuroinflammatory disease. C.N.S. Neurol. Disord. Drug Targets 10, 483–494. https://doi.org/10.2174/1871527319666200702143719; Leitner G, **Wenzel TJ**, Marshall N, Gates E, Klegeris A., 2019. Review: Targeting toll-like receptor 4 to modulate neuroinflammation in central nervous system disorders. Expert Opin. Ther. Targets 23, 865-82. https://doi.org/10.1080/14728222.2019.1676416]. I wrote the passages and designed the figures used in this thesis, with editorial comments from my co-authors.

A version of chapter 2 is in revision [**Wenzel TJ**, Ranger AL, McRae S, Klegeris A., 2020. Extracellular cardiolipin modulates microglial phagocytosis and cytokine secretion in a toll-like receptor (TLR) 4-dependent manner. J. Neuroimmunol. In Revision. (Reference: JNI_2020_322)]. I was the lead contributor for this primary research article. For this research project, I was responsible for the conceptualization, investigation and manuscript writing. The experiments described by figures 2.3 and 2.4 were assisted by my co-authors S. McRae and A.L. Ranger, respectively. All my co-authors contributed to manuscript edits.

A version of chapter 3 has been published in Biochimica et Biophysica Acta - General Subjects [**Wenzel TJ**, Bajwa E, Klegeris A., 2019. Cytochrome c can be released into extracellular space and modulate functions of human astrocytes in a toll-like receptor 4-

dependent manner. Biochim. Biophys. Acta - Gen. Subj. 1863, 129400. https://doi.org/10.1016/j.bbagen.2019.07.009]. I was the co-lead contributor for this primary research article. For this research project, I was responsible for the conceptualization, investigation and manuscript writing, with the exception of the introduction and a subsection of the discussion which was written by E. Bajwa. The experiments described by figures 3.4D were performed by E. Bajwa, and the data was formally analyzed by me. E. Bajwa also assisted in collecting the samples in the experiments described by table 3.1 and figure 3.1, which were subsequently analyzed by Eve Technologies. All my co-authors contributed to manuscript edits.

A version of chapter 4 has been published in Molecular and Cellular Neuroscience [**Wenzel TJ**, Gates EJ, Ranger AL, Klegeris A., 2020. Short-chain fatty acids (SCFAs) alone or in combination regulate select immune functions of microglia-like cells. Mol. Cell. Neurosci. 105, 103493. https://doi.org/10.1016/j.mcn.2020.103493]. I was the lead contributor for this primary research article. For this research project, I was responsible for the conceptualization, investigation and manuscript writing. E.J. Gates performed the experiments described by figures 4.5 and 4.6, and the data was formally analyzed by me. A. Ranger performed the experiments described by figure 4.3 and formally analyzed the data. All my co-authors contributed to manuscript edits.

I either authored or co-authored the following publications during my doctoral program:

1. **Wenzel TJ,** Kwong E, Bajwa E, Klegeris A., 2020. Review: Resolution-associated molecular patterns (RAMPs) as endogenous regulators of glia functions in neuroinflammatory disease. C.N.S. Neurol. Disord. Drug Targets 10, 483–494. https://doi.org/10.2174/1871527319666200702143719
2. **Wenzel TJ,** Gates EJ, Ranger AL, Klegeris A., 2020. Original Research: Short-chain fatty acids (SCFAs) alone or in combination regulate select immune functions of microglia-like cells. Mol. Cell. Neurosci. 105, 103493. https://doi.org/10.1016/j.mcn.2020.103493
3. **Wenzel TJ**, Bajwa E, Klegeris A., 2019. Original Research: Cytochrome c can be released into extracellular space and modulate functions of human astrocytes in a toll-like receptor 4-dependent manner. Biochim. Biophys. Acta - Gen. Subj. 1863, 129400. https://doi.org/10.1016/j.bbagen.2019.07.009
4. Leitner G, **Wenzel TJ,** Marshall N, Gates E, Klegeris A., 2019. Review: Targeting toll-like receptor 4 to modulate neuroinflammation in central nervous system

disorders. Expert Opin. Ther. Targets 23, 865-82. https://doi.org/10.1080/14728222.2019.1676416

5. Pointer C, **Wenzel TJ**, Klegeris A., 2018. Original Research: Extracellular cardiolipin regulates select immune functions of microglia and microglia-like cells. Brain Res. Bullet. 146, 153-63. https://doi.org/10.1016/j.brainresbull.2019.01.002

In addition, I have authored or co-authored the following manuscripts, which are in revision:

1. **Wenzel TJ**, Ranger AL, McRae S, Klegeris A., 2020. Original Research: Extracellular cardiolipin modulates microglial phagocytosis and cytokine secretion in a toll-like receptor (TLR) 4-dependent manner. J. Neuroimmunol. In Revision. (Reference: JNI_2020_322)

Table of Contents

List of Tables

List of Figures

List of Abbreviations

α-syn..... α-synuclein
Aβ..... Amyloid-β peptide
Aβ42..... Amyloid-β peptide 1-42
ABC ..... ATP-binding cassette
AD..... Alzheimer's disease
AHR ..... Aryl hydrocarbon receptor
ANOVA ..... Analysis of variance
ApoER..... Apolipoprotein E receptor
BBB..... Blood-brain barrier
BCA ..... Bicinchoninic acid
BDNF ..... Brain-derived neurotrophic factor
BMDM..... Bone-marrow derived macrophages
BSA..... Bovine serum albumin
C57BL/6..... C57 black 6
Cas9..... Clustered regularly interspaced short palindromic repeats associated protein 9
CBS ..... Calf bovine serum
CD ..... Cluster of differentiation
CHL..... Chemiluminescence
CL ..... Cardiolipin
CNS..... Central nervous system
CRISPR..... Clustered regularly interspaced short palindromic repeats
CytC ..... Cytochrome c
DAMP ..... Damage-associated molecular pattern
DMEM-F12... Dulbecco's modified Eagle medium F12/Ham
EAAT ..... Excitatory amino acid transporter
EDTA ..... Ethylenediaminetetraacetic acid
ELISA ..... Enzyme-linked immunosorbent assay

F4/80 ..... Epidermal growth factor-like module-containing mucin-like hormone receptor-like 1

FFAR..... Free fatty acid receptor

FITC..... Fluorescein isothiocyanate

fMLP..... N-formylmethionine-leucyl-phenylalanine

GDNF..... Glial cell line-derived neurotrophic factor

GF ..... Germ-free

GFAP ..... Glial fibrillary acidic protein

GLUT..... Glucose transporters

Gpr109a..... Niacin receptor 1

GM-CSF..... Granulocyte-macrophage colony-stimulating factor

HMGB1..... High-mobility group box 1

HSP ..... Heat-shock protein

IBA..... Ionized calcium binding adaptor molecule

ICAM ..... Intercellular adhesion molecule

IFN ..... Interferon

IgG ..... Immunoglobulin G

IL..... Interleukin

iNOS ..... Inducible nitric oxide synthase

IP ..... Interferon-γ-induced protein

iPSC ..... Inducible pluripotent stem cells

IRAK..... Interleukin-1 receptor associated kinases

IRF3 ..... Interferon regulatory factor 3

KO..... Knockout

LAT..... Large neutral amino acid transporters

LCMV..... Lymphocytic choriomeningitis virus

LDL..... Low-density lipoproteins

LPS..... Lipopolysaccharide

LRP ..... Low-density lipoprotein receptor related protein

LLME..... L-leucyl-L-leucine methyl ester

MALP-2 ..... Macrophage-activating lipopeptides, 2 kDa

MAPK..... Mitogen-activated protein kinase

MCP ..... Monocyte chemoattractant protein

MD ..... Myeloid differentiation factor

MFI ..... Mean fluorescence intensity

MS..... Multiple sclerosis

MTT ..... 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenytetrazolium bromide

MyD88 ..... Myeloid differentiation primary-response protein 88

NF-κB ..... Nuclear factor kappa-light-chain-enhancer of activated B cells

NFT ..... Neurofibrillary tangle

NLRP3 ..... Nucleotide-binding domain, leucine-rich repeat family pyrin domain containing 3

PA181 ..... 1,2-dilinoleoyl-*sn*-glycero-3-phosphate

PA182 ..... 1,2-dioleoyl-*sn*-glycero-3-phosphate

PAMP..... Pathogen-associated molecular pattern

PBS ..... Phosphate-buffered saline

PD ..... Parkinson's disease

PI..... Propidium iodide

Poly(I:C) ..... Polyinosinic-polycytidylic acid

PRR..... Pattern recognition receptor

RAGE..... Receptor for advanced glycation end products

RAMP ..... Resolution-associated molecular pattern

ROS..... Reactive oxygen species

S100B..... S100 calcium-binding protein B

SCFA..... Short-chain fatty acid

SEM ..... Standard error of mean

SOCS..... Suppressor of cytokine signaling

SOD..... Superoxide dismutase

SPF..... Specific-pathogen free

TIR ..... Toll/interleukin-1 receptor

TIRAP ..... Toll/interleukin-1 receptor homology domain-containing adaptor protein

TFAM ..... Mitochondrial transcription factor A

TGF ..... Transforming growth factor

TLR ..... Toll-like receptor

TNF ..... Tumor necrosis factor

TRAF ..... Tumor necrosis factor receptor associated factor

TRAM ..... Toll/interleukin-1 receptor domain-containing adaptor protein inducing interferon-β-related adaptor molecule

TRIF ..... Toll/interleukin-1 receptor domain-containing adaptor protein inducing interferon-β

UBC ..... University of British Columbia

ZVAD-FMK . Carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]-fluoromethylketone

Acknowledgements

I would like to thank several key individuals who enhanced my learning throughout my doctoral program.

First, I would like to thank my supervisor Dr. Andis Klegeris, whose mentorship made me the scientist I am today. In an extremely short period of time, I learned a lot about experimental design, statistics, as well as verbal and written science communication. I have no doubt that these fundamental skills will contribute to my future success as an independent, Canadian scientist. I have already begun to experience the benefits your mentorship provided, including awards, job offers and once-in-a-lifetime experiences. I look forward to working with you in the future as a neuroscience colleague.

Secondly, I would like to thank my committee members, Drs. Fred Menard, Julien Gibon and Mark Rheault. Each of you contributed to my development as an independent scientist in different ways. Dr. Menard: thank you for always challenging my understanding of biochemistry principles. Dr. Gibon: thank you for always being so engaged in my learning and taking every opportunity to check in on me. Dr. Rheault: thank you for always having your door open, despite how busy you are, to have hour long discussions about statistics, careers in science, or experimental designs.

Thirdly, I would like to personally thank Barb Lucente, who contributed to the completion of this thesis in many ways. Whether it was as important as promptly giving me information required for the successful completion of an experiment, or just checking in on how I was doing, you were always there to help me. There are likely countless things that you did for me that I am not even aware of, and no amount of space in my thesis could properly describe my gratitude for all the work you do for graduate students.

Lastly, I would like to thank every other aspiring scientist who I met along this journey, including fellow lab members, other graduate students, or undergraduate students at UBCO.

Each of you contributed to my growth in different ways. All the challenging questions and scientific discussions led to many learning opportunities for me. I was disheartened that our in-person time was abruptly ended by a global pandemic, but I will remember our interactions for years to come.

I hope that you all will be proud of the scientist I become.

Chapter 1: Introduction

1.1 Thesis overview

Neurons and glia are the main cell types in the brain. Glia, which include microglia, astrocytes and oligodendrocytes, protect and support neurons (Jensen et al., 2013; Lannes et al., 2017). Together, neurons and glia cooperate to enable brain functions, including motor control, behavior and emotions (Perea et al., 2014). These vital brain functions are often disrupted by neurodegenerative diseases, such as Alzheimer's disease (AD)**.**

At a cellular level, several functions of glia become dysregulated in AD. This thesis focusses on functions of microglia and astrocytes that are disrupted by AD, namely phagocytosis and the secretion of inflammatory mediators and cytotoxins (Brown and Vilalta, 2015; Fu et al., 2014; Galloway et al., 2019; Meraz-Ríos et al., 2013). Glia secrete inflammatory mediators that normally support neuron survival by, for example, recruiting microglia to a site of injury where they can remove pathogens and unwanted tissue debris (Fan et al., 2017). However, dysregulated secretion of inflammatory mediators and cytotoxins by glia causes neurons to die (Brown and Vilalta, 2015). Under certain pathological conditions, glia become chronically activated, secreting cytotoxins (e.g. cathepsins, Fas ligand, glutamate, matrix metalloproteinases and reactive oxygen species (ROS)), as well as adverse levels of inflammatory cytokines (Brown and Vilalta, 2015). Inflammatory cytokines, such as tumour necrosis factor (TNF)-α and interleukin (IL)-1β activate nearby microglia and astrocytes, initiating positive feedback leading to exacerbated neuron death. Therefore, molecules that reduce the secretion of inflammatory mediators and cytotoxins by glia may lessen excessive neuronal death observed in neurodegenerative diseases. Phagocytosis is commonly viewed as a protective function because it removes tissue debris and pathological proteins (Galloway et al., 2019). Yet, under certain pathological conditions, glia can phagocytose live neurons, causing their death (Brown and Neher, 2014; Hornik et al., 2016). Therefore, molecules that modulate the phagocytic activity of glia could be neuroprotective in specific disease states, and slow the progression of neurodegenerative diseases, including AD.

In this Introduction (chapter 1), I elaborate on several topics that are relevant to the experiments described in chapters 2, 3 and 4. In section 1.2, I describe the structure and function of the blood-brain barrier (BBB). It is important to understand the characteristics of the BBB that make this structure one of the primary roadblocks for developing CNS disease-modifying therapies, and how researchers can overcome these roadblocks. In section 1.3, I elaborate on glial signaling induced by endogenous molecules that can contribute to the progression of AD. I hypothesize that the molecules studied in this thesis, namely cardiolipin (CL), cytochrome c (CytC) and short-chain fatty acids (SCFAs), modulate these signaling events. In section 1.4, I briefly describe the structure of toll-like receptor (TLR) 4, as well as the intracellular signaling this receptor activates. The experiments described in chapters 2 and 3 demonstrate that CL and CytC interact with TLR 4. In section 1.5, I describe the cell lines used in this thesis to model functions of microglia and astrocytes that become dysregulated in AD. I end this introduction stating the hypotheses tested in thesis, and the specific research objectives I investigated.

The main discoveries of the experimental work presented in this thesis include: TLR 4 mediates the effects of extracellular CL on microglia and microglia-like cells (chapter 2), CytC is released from damaged glia, extracellular CytC acts as a TLR 4-dependent endogenous modulator of astrocyte neuroimmune functions, (chapter 3), and SCFAs mixed at physiological serum concentrations modulate immune functions of microglia (chapter 4).

1.2 Structure and function of the blood-brain barrier

The BBB requires the support of several different cell types to maintain brain homeostasis. Cells required to maintain normal BBB functions include endothelial cells, pericytes, and astrocytes (Daneman and Prat, 2015; Rhea and Banks, 2019). Microglia (Haruwaka et al., 2019) and neurons (Savettieri et al., 2000) also regulate the integrity of the BBB, but to a lesser extent. Altogether, these cells enable the BBB to act as a highly selective, semipermeable structure that separates the circulatory system and the parenchyma of central nervous system (CNS).

Endothelial cells envelop the blood vessels and represent the boundary between the blood and interstitial fluid of the brain. Unlike many other endothelial tissues throughout the body, blood vessels formed by endothelial cells of the BBB lack fenestra (pores). Many tight junction proteins exist in between adjacent endothelial cells of the BBB. The primary tight junction proteins include occludin, and the claudin family of proteins. These tight junction proteins are linked to the actin cytoskeleton of endothelial cells by the zonus occludin group of proteins (Daneman and Prat, 2015; Komarova et al., 2017; Sweeney et al., 2018). These tight junction proteins prevent the passive paracellular diffusion of molecules larger than four nm in diameter (Daneman and Prat, 2015). In particular, claudin 5 has been implicated as the primary tight junction protein that prevents molecules from crossing the BBB, as the absence of claudin 5 increases the permeability of the BBB (Nitta et al., 2003). While the exact function of other tight junction proteins remains to be revealed (Saitou et al., 2000), it has been suggested that occludin prevents BBB breakdown by regulating calcium trafficking (Brown and Davis, 2002). Endothelial cells also secrete a variety of extracellular matrix proteins into the perivascular space that make up a BBB structure called the basement membrane, which provides cells, such as endothelial cells and astrocytes, with anchoring points, as well as aids in proper signaling between the BBB cells (Baeten and Akassoglou, 2011; Daneman and Prat, 2015).

Under physiological conditions, endothelial cells that form the BBB express less adhesion molecules, compared to the endothelial cells that form peripheral capillary beds (Frohman et al., 1991). Adhesion molecules include many families of cell surface proteins, such as selectins and cell adhesion molecules. Cell adhesion molecules allow peripheral immune cells to exit the blood, move across the endothelial boundary, and enter the interstitial fluid (Bohatschek et al., 2001; Lyck et al., 2003). Once in the interstitial fluid, peripheral immune cells may work together with native brain immune cells, namely microglia, to remove pathogens and toxins (Cao and Zheng, 2018; Prinz and Priller, 2017).

Pericytes, which are anchored to the basement membrane of the CNS, are also required for the formation of the BBB (Daneman et al., 2010). In a series of experiments on BBB integrity, Daneman *et al.* (2010) demonstrate that the selective permeability of the BBB is compromised until mesenchymal progenitor cells differentiate into pericytes during

development. This study also shows that pericytes inhibit the endothelial cell expression of many proteins, such as intercellular adhesion molecule (ICAM)-1, that increase BBB permeability. Additionally, the same study illustrates that the expression of tight junction proteins, such as occludin and claudin 5, by CNS endothelial cells is significantly lower when cultured without CNS pericytes. Pericytes also control the dilation of capillaries. Pericyte-mediated dilation of capillaries and its effects on neurological disorders, such as AD, have been reviewed (Hamilton et al., 2010).

Anchored to the basement membrane, astrocytes have many diverse functions that aid in the formation and maintenance of the BBB (Jensen et al., 2013; Wang and Bordey, 2009). Astrocytes secrete sonic hedgehog, a signaling molecule in the brain, as well as laminin, a component of the basement membrane. Astrocytes also have aquaporins 1, 4, and 9, which are responsible for maintaining water homeostasis in the CNS (Potokar et al., 2016; Sweeney et al., 2018). Astrocytes aid in the structural integrity of the BBB by trapping the pericytes and basement membrane in between endothelial cells and astrocytic end-feet (Sweeney et al., 2018).

Astrocytes secrete important signaling molecules that regulate the permeability of the BBB. For example, previous studies demonstrate that monocyte chemoattractant protein (MCP)-1 secreted by astrocytes reduces the expression of claudin 5 (Paul et al., 2014), a common marker for BBB integrity (Lv et al., 2018). While the role of claudin 5 in BBB integrity has been established in claudin 5 knockout mice (Nitta et al., 2003), it is unknown whether reducing the expression of claudin 5 alone increases the permeability of the BBB.

Several different processes enable a wide range of molecules to cross the BBB. In general, small (approximately < 400 Da), uncharged, lipophilic compounds can passively diffuse across the BBB in a transcellular manner. Drugs that target the CNS are often designed with these properties (Nau et al., 2010). As they move across the BBB, these drugs are often transformed by enzymes into a more water-soluble chemical structure. This transformation prevents drugs from being sequestered by endothelial cells (Banks, 2009). Other mechanisms prevent the entry of small lipophilic molecules into the interstitial fluid of the brain. For example, endothelial cells express many efflux transporters, such as multidrug resistant transport

proteins, on their luminal (the plasma membrane exposed to blood) surface. These efflux transporters prevent some small lipophilic molecules from entering brain tissue by actively pumping these compounds back into the blood (Banks, 2009; Nau et al., 2010). Individual variations of multidrug resistant transporter proteins have been implicated in patient-dependent drug insensitivity (Fellay et al., 2002; Löscher and Potschka, 2002).

Many nutrients that the brain needs to function properly cannot diffuse across the BBB. These nutrients include molecules such as glucose (McAllister et al., 2001), amino acids (Smith, 2000; Tărlungeanu et al., 2016), ketone bodies (White and Venkatesh, 2011), select hormones (Hampl et al., 2015) and insulin (Blázquez et al., 2014). These molecules use a range of transmembrane transporters to cross the BBB into the brain. For example, glucose engages glucose transporters (GLUT) 1 (Patching, 2017); amino acids engage several transporters, including large neutral amino acid transporters (LAT) and excitatory amino-acid transporters (EAAT*)* (Smith, 2000); ketone bodies engage monocarboxylate transporters (Vijay and Morris, 2014); and hormones engage transporters, such as organic anion transporters (Morris et al., 2017). Other molecules cross the BBB in a vesicle-mediated manner. For example, insulin and low-density lipoproteins (LDLs) bind and activate insulin receptor and LDL receptor, respectively. When activated, these receptors are endocytosed into vesicles and subsequently activate native sorting machinery. This vesicle-sorting machinery will either send the vesicle across the BBB, or to be degraded (Pulgar, 2019).

Additionally, disease-related proteins, such as amyloid-ß peptides (Aß) which are associated with AD, actively cross the BBB using various transport proteins. For example, receptor for advanced glycation end products (RAGE) transports peripheral Aß into the brain, while low-density lipoprotein receptor related protein (LRP)-1, also known as apolipoprotein E receptor (ApoER), transports Aß out of the brain (Deane et al., 2008). The expression of LRP-1 is lower in the frontal cortex of AD patients, compared to healthy age-matched humans (Kang et al., 2000; Shinohara et al., 2014). However, this association between LRP-1 expression and AD remains controversial (Causevic et al., 2003; Qiu et al., 2001).

The BBB is an integral structure that contributes to the health and homeostasis of the brain. This boundary prevents the movement of many toxic molecules from the blood into the brain. However, the selective permeability of a normal BBB is one of the primary roadblocks to overcome when developing drugs for CNS disorders (Nau et al., 2010). The BBB integrity may be reduced in patients with neurological disorders, such as AD (Greene et al., 2019; Kang et al., 2000; Shinohara et al., 2014).

1.3 Select extracellularly released endogenous molecules contribute to chronic neuroinflammation and subsequent exacerbated neuronal death

Damage-associated molecular patterns (DAMPs) are endogenous molecules released from stressed, damaged or dying cells into the extracellular space. The best characterized DAMPs include high mobility group box 1 (HMGB1), calgranulins A and B, and heat-shock proteins (HSPs) 60 and 70. Several recent reviews have summarized the immunoregulating effects of these DAMPs (Dukay et al., 2019; Paudel et al., 2018; Wang et al., 2018). Their biological activity is well characterized in peripheral tissues where DAMPs have been shown to induce secretion of inflammatory mediators and upregulate phagocytic activity of several different types of immune cells (Dukay et al., 2019; Paudel et al., 2018; Wang et al., 2018). CNS effects of DAMPs have also been studied where they have been shown to interact with glial cells, including microglia and astrocytes (Gouveia et al., 2017; Pointer et al., 2019). DAMPs can induce microglial secretion of cytotoxins and inflammatory cytokines, such as IL-1β, IL-6, IL-8, and TNF-α, as well as upregulate the phagocytic activity of microglia (Amor et al., 2014). Astrocytes, which support a broad range of neuronal functions, similarly respond to extracellularly released DAMPs by increasing secretion of cytotoxins and upregulating phagocytic activity (Wakida et al., 2018).

1.4 Activation of microglia and astrocytes

The transition of microglia and astrocytes into a functional state characterized by the upregulated secretion of cytokines and cytotoxins, as well as altered phagocytic activity,

indicates these cells are displaying an activated phenotype. The activated phenotypes of microglia and astrocytes have been described as M1/M2 and A1/A2, respectively.

The term ‘M1 microglia’ represents the pro-inflammatory phenotype, while ‘M2 microglia’ describes the anti-inflammatory phenotype of these cells (Tang and Le, 2016). The secretory profile characteristic of M1 microglia includes IL-1β, TNF-α, MCP-1, nitric oxide, proteases, and superoxide anion (Orihuela et al., 2016; Tang and Le, 2016). Many molecules secreted by M1 microglia are known cytotoxins (Madeira et al., 2012; Wenzel et al., 2020a). M1 microglia also exhibit decreased phagocytic activity (Orihuela et al., 2016). The primary function of M1 microglia is to recruit immune cells to the site of injury and to secrete toxins that kill pathogens and aberrant cells. In contrast, M2 microglia exhibit upregulated secretion of IL-4, IL-10, IL-13, TGF-β, and neurotrophic factors (e.g. insulin-like growth factor 1), as well as increased phagocytic activity (Tang and Le, 2016). The M2 microglia remove cellular debris and misfolded proteins, as well as promote tissue repair and neuronal survival. Similar to microglia, terms ‘A1 astrocytes’ and ‘A2 astrocytes’ describe the pro- and anti-inflammatory phenotypes, respectively (Li et al., 2019). Also similar to microglia, A1 astrocytes secrete toxins and immune cell-recruiting molecules, while A2 astrocytes promote tissue repair and the survival of neurons.

It is critical to point out that this binary categorization of microglia and astrocyte phenotypes is likely an oversimplification (Cunningham et al., 2019; Ransohoff, 2016; Stratoulias et al., 2019). For example, microglia treated with the TLR 4 agonist LPS exhibit upregulated mRNA expression of molecules that are characteristic of both M1 and M2 phenotypes, such as TNF-α, IL-1β, IL-4 and IL-10, as well as display higher phagocytic activity (Leitner et al., 2019; Lively and Schlichter, 2018). Therefore, in studies that aim to classify glia as pro- or anti-inflammatory, it is beneficial to measure a broad set of inflammatory markers and to consider additional activation states that deviate from the M1/M2 polarization.

Chronic activation of microglia and astrocytes can contribute to the progression of neurodegenerative diseases, such as AD and Parkinson’s disease (PD) (Thompson and Tsirka, 2017). For example, the activation of microglia and astrocytes leads to an increased secretion of

inflammatory molecules and cytotoxins that further activate these cells in an autocrine or paracrine fashion, causing positive feedback, as well as neuronal and glial damage (Thompson and Tsirka, 2017). Damaged glia lose their homeostatic function and cease their metabolic support of neurons further exacerbating cell death.

1.5 Neurodegenerative diseases

Neurodegeneration is defined as an exacerbated loss of neurons or neuronal function (Han et al., 2018). AD and PD are the most prevalent neurodegenerative disorders, affecting approximately 30 million and 6 million people worldwide. As implied by AD and PD being categorized as neurodegenerative disorders, both these pathological conditions are characterized by exacerbated neuron death. Thus, experiments described in this thesis aim to characterize the indirect effects that CL, CytC, and SCFAs could have on neuron death by regulating glial functions. Therefore, my studies implicate these endogenous molecules in the pathogenesis of AD and PD.

1.5.1 AD

AD is the most prevalent neurological disorder worldwide (Han et al., 2018). AD is clinically characterized by a progressive decline in memory and other cognitive functions. Pathological features of AD include brain atrophy as well as the accumulation of Aβ deposits and neurofibrillary tau tangles. Aβ is a neurotoxic protein (Tanokashira et al., 2017). The toxic properties of Aβ may be partly mediated by the ability of this protein to activate microglial TLR 4 (Cho et al., 2013; Shi et al., 2016).

1.5.2 PD

PD is the second most common neurological disorder, after AD (Balestrino and Schapira, 2020). Similar to AD, PD is also a progressive neurodegenerative disorder, but patients with PD exhibit impaired cognition and motor function. The defining motor symptoms of PD include

tremors, rigidity, bradykinesia, and postural instability. There are two primary pathological hallmarks of PD: 1) the loss of dopaminergic neurons in the substantia nigra (area in the midbrain), and 2) aggregates of α-syn called Lewy bodies. Similar to Aβ, α-syn is a neurotoxic protein that activates microglial TLR 4 (Fellner et al., 2013; Ingelsson, 2016; Roodveldt et al., 2013).

1.6 Toll-like receptors

The TLR family in humans includes ten different receptors, all of which are transmembrane proteins. TLRs recognize pathogen-associated molecular patterns (PAMPs), such as lipopolysaccharide (LPS), and DAMPs, such as HMGB1 and mitochondrial transcription factor A (TFAM).

1.6.1 Toll-like receptor 4

TLR 4 agonists activate downstream myeloid differentiation primary-response protein 88 (MyD88)-dependent and MyD88-independent pathways through the dimerization of TLR 4/myeloid differentiation factor (MD) 2 with agonist complexes. TLR 4 agonists engage these pathways by interacting with MD 2 to form a complex with TLR 4. For example, the lipid A component of LPS binds MD 2 and activates TLR4 in a MyD88-dependent manner on human immune cells, such as monocytes and macrophages (Oblak and Jerala, 2015). The formation of the TLR 4/MD 2 and agonist complex can be CD 14-dependent or CD 14-independent. In the MyD88-dependent pathway, the adaptor proteins include toll/IL-1 receptor (TIR) homology domain-containing adaptor protein (TIRAP), IL-1 receptor associated kinases (IRAKs) and TNF receptor associated factor (TRAF) 6. The engagement of adaptor molecules in the MyD88-dependent pathway initiates signal transduction pathways that lead to the activation of mitogen-activated protein kinases (MAPKs) and subsequent production of several different cytokines and chemokines. In the MyD88-independent pathway, the recruited adaptor proteins include TIR domain-containing adaptor protein inducing interferon (IFN)-β (TRIF), TRIF-related adaptor molecule (TRAM), and TRAF3. The engagement of adaptor molecules in the MyD88-

independent pathway initiates signal transduction pathways that lead to the activation of interferon regulatory factor 3 (IRF3), promoting the production of IFN-β and endocytosis of the TLR 4/MD 2 and agonist complex (Fig. 1.1). Both MyD88-dependent and MyD88-independent pathways have been shown to regulate phagocytosis in several different types of immune cells (Esen and Kielian, 2014; Fu et al., 2014); however, neither of these two pathways are absolutely required for phagocytosis since TRIF and MyD88 double knockout (KO) dendritic cells exposed to LPS have been shown to retain phagocytic activity (Zanoni et al., 2011).

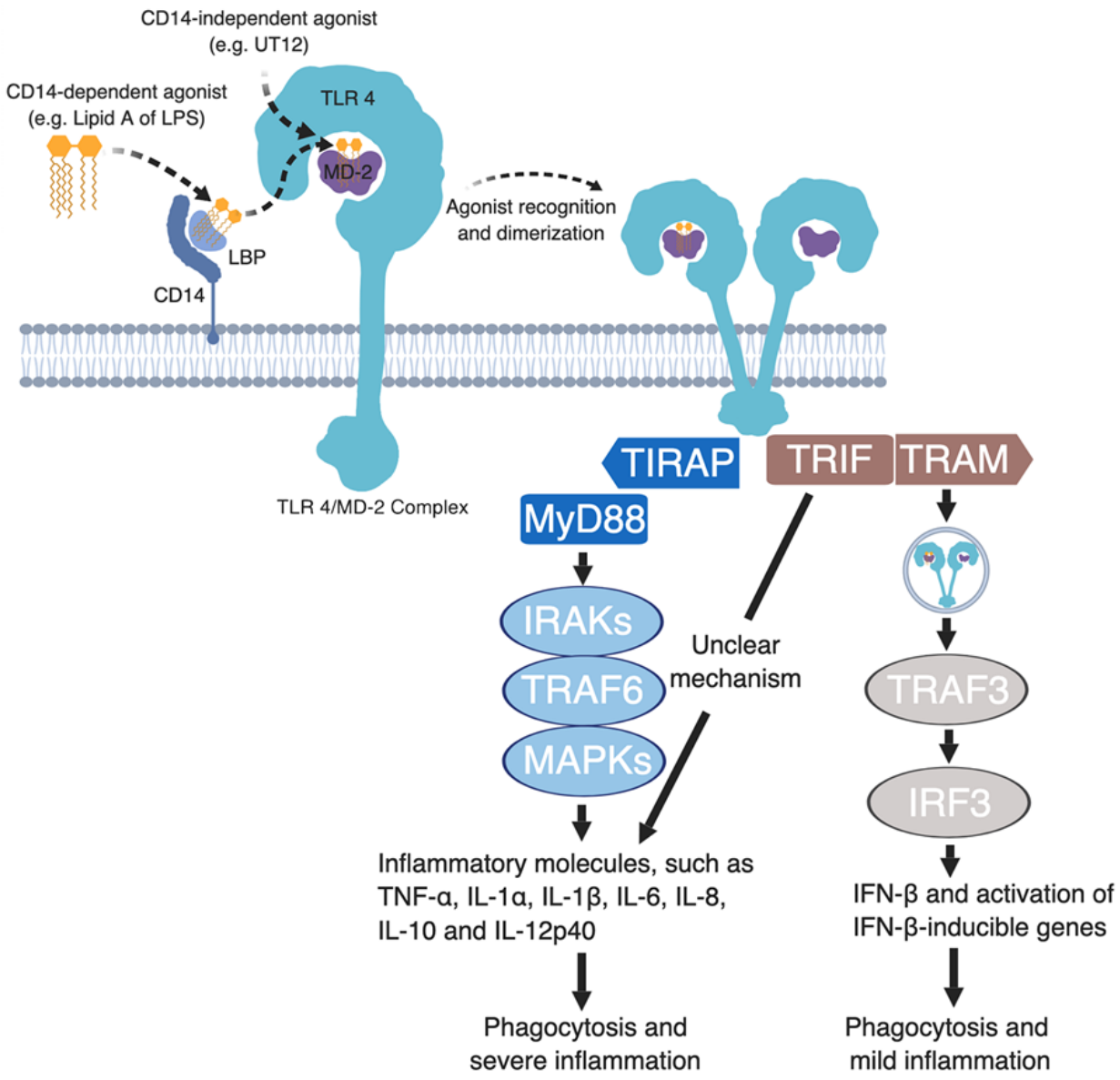


Figure 1.1. TLR 4 is activated after a complex of agonist with TLR 4/MD 2 is formed. Once activated, TLR 4/MD 2 and agonist complexes dimerize and engage two separate downstream signaling pathways: the MyD88-dependent and MyD88-independent pathways. The MyD88-dependent pathway leads to the secretion of inflammatory molecules and increased phagocytic activity, and the MyD88-independent pathway leads to the secretion of IFN-β and increased phagocytic activity. IFN-β activates several genes, such as suppressor of cytokine signaling (SOCS)-1 and SOCS-3 in astrocytes (Qin et al., 2008). Upon activation of the MyD88-

independent pathway, the TLR 4/MD 2 and agonist complex becomes internalized by endocytosis. The exact mechanisms of interaction between TRIF and MyD88-dependent pathway are unknown, but it is established that TRIF influences the MyD88-dependent secretory profile of immune cells. Both pathways have been shown to increase the phagocytic activity of immune cells, such as microglia. However, they may not increase phagocytic activity through the same mechanisms.

1.7 Effects of the gut microbiota on microglia and astrocytes

Gut microbes co-exist with humans in a mutualistic relationship. These microbes benefit the host in several ways. For example, gut microbes are necessary for the synthesis of vitamin K and B. They are also involved in the metabolism of many compounds, such as bile acids, sterols, and xenobiotics (Roberfroid et al., 1995). Recent evidence suggests that the gut microbiota regulates host health. For example, altered composition of the human gut microbiota is suggested to be linked to the onset of multiple sclerosis (MS) (Gandy et al., 2019), PD (Sampson et al., 2016; Scheperjans et al., 2015) and AD (Harach et al., 2017; Haran et al., 2019).

The effects of absent or altered gut microbiota on neurological disorders have been studied using germ-free (GF) mice. For example, relapsing-remitting MS-model mice kept in specific-pathogen free (SPF) conditions spontaneously develop MS-like symptoms (Berer et al., 2011). Interestingly, when these MS-model mice are housed in a sterile environment, they do not develop as severe MS-like symptoms. Other studies show that housing AD- (Harach et al., 2017) or PD- (Sampson et al., 2016) model mice in GF conditions reduces disease-related proteins or symptoms. For example, brain tissues from eight-month-old AD-model mice in GF conditions have reduced levels of amyloid-β peptides with 42 residues (Aβ42, hallmark protein of AD), compared to age-matched brain tissues from AD-model mice raised in SPF conditions. While the aforementioned study did not measure the cognitive impairment of these AD-model mice, other studies show that the cognition of these mice declines between seven and eight months of age (Gengler et al., 2010; Radde et al., 2006; Serneels et al., 2009). Interestingly, neuron loss is not observed in these AD-model mice until they are 17 months old (Rupp et al., 2011). PD-model mice in GF conditions show decreased motor dysfunction and lower insoluble α-synuclein (α-

syn) (hallmark protein of PD) levels compared to PD-mode mice in SPF conditions (Sampson et al., 2016).

Previous studies show the immune response of microglia from GF mice treated with LPS decreases, compared to microglia from SPF mice treated with LPS (Erny et al., 2015). For instance, the mRNA expression of IL-1β, IL-6, TNF-α, and MCP-1 is 10 to 20 fold lower in microglia from GF mice treated with LPS, compared to microglia from LPS-treated SPF mice (Erny et al., 2015). In the same study, similar significant results are shown using microglia from GF mice exposed to lymphocytic choriomeningitis virus (LCMV), instead of LPS. The reduced immune response of LCMV-activated microglia from GF mice is also accompanied with a reduced expression of ionized calcium binding adaptor molecule (IBA)-1 (Erny et al., 2015), a well-established marker of microglia activation (Hovens et al., 2014). However, it is important to note that IBA-1 expression is upregulated in microglia throughout GF mice brains, compared to microglia populations from of SPF mice. This observation that microglia in GF mice exist in a more activated state compared to the microglia in SPF mice may be due to their sensitivity to stimuli. To elaborate, conventionally raised SPF mice exhibit higher levels of LPS in portal blood compared to GF mice (Molinaro et al., 2017). When primary murine microglia were exposed to three LPS challenges in 24 h intervals, microglia secreted significantly less TNF-α on the second and third dose of LPS (Ajmone-Cat et al., 2003). Therefore, it is plausible that immune cells in GF mice are sensitive to stimuli, such as LPS, and gut microbes are required to train immune cells, including microglia, to appropriately respond to immune stimuli. Interestingly, recolonizing the gut of GF mice reduces the upregulated expression of IBA-1 by microglia in these animals (Erny et al., 2015). It is possible that microglia in GF mice are more activated compared to microglia from SPF mice; therefore, the capacity of microglia from GF mice to mount an immune response when stimulated by LPS or LCMV may be limited. The same study also shows that adding a mixture of acetate, butyrate and propionate, which are bacterial metabolites, to the drinking water of GF mice reduces the upregulated expression of IBA-1 by microglia in these mice (Erny et al., 2015). Altogether, the reduced immune response by microglia in GF mice may compromise the ability of microglia to maintain brain homeostasis.

Microglia are malformed in the brains of GF mice. For example, microglia from GF mice express higher levels of colony-stimulating factor-1 receptor, epidermal growth factor-like module-containing mucin-like hormone receptor-like 1 (F4/80), and cluster of differentiation (CD) 31 (Erny et al., 2015). Higher levels of these proteins are reminiscent of immature microglia, as the expression of colony-stimulating factor-1, F4/80 and CD 31 are downregulated in adult murine microglia, compared to embryonic murine microglia (Kierdorf et al., 2013). Previous studies show a higher density of immature microglia in GF mice, compared to mice with a normal gut microbiota. Additionally, the behavior of microglia in GF mice is significantly different from microglia populations in SPF mice. For example, microglia from GF mice often directly contact other microglia, crossing the territorial boundaries exhibited by microglia from SPF mice (Erny et al., 2015). Therefore, a normal gut microbiota regulates the phenotype and behavior of adult murine microglia.

Few studies have tested the effects of an absent or altered gut microbiota on the morphology or functions of astrocytes. Unlike microglial populations, the density of astrocytes does not change in GF mice, compared to mice with a normal gut microbiota (Erny et al., 2015). Interestingly, a previous study demonstrates that metabolites of tryptophan reduce the immune response of primary murine astrocytes (Rothhammer et al., 2016). It is important to point out that tryptophan, an essential amino acid for humans acquired through their diet, is catabolized into indole, indoxyl-3-sulfate, indole-3-propionic acid, and indole-3-aldehyde by gut microbes (Roager and Licht, 2018). The mRNA expression of MCP-1 and nitric oxide synthase 2 by primary murine astrocytes decreases when antibiotic-treated mice are administered orally with indole, indole-3-propionic acid, or indole-3-aldehyde. Similar trends are observed when antibiotic-treated mice are injected intraperitoneally with indoxyl-3-sulfate (Rothhammer et al., 2016). Therefore, tryptophan or its metabolites may regulate the activation of astrocytes. These anti-inflammatory properties of tryptophan or its metabolites may also have clinical significance in neurodegenerative diseases. For example, disease scores are reduced in MS-model mice fed tryptophan, compared to MS-model mice fed a diet without tryptophan (Rothhammer et al., 2016). This ability of tryptophan to improve disease scores is blocked in MS-model mice with

aryl hydrocarbon receptor (AHR) knocked out (Rothhammer et al., 2016), suggesting that the anti-inflammatory properties of tryptophan or its metabolites may be partially regulated by AHR.

It is important to note that the microbiota of mice and humans are significantly different (Nguyen et al., 2015), so the clinical significance of the observations in mouse models needs to be determined. Many published studies demonstrate association between the composition of the gut microbiota and diseases, such as PD (Scheperjans et al., 2015) or AD (Haran et al., 2019). However, it is currently unknown whether diseases, such as AD, initiate a change in gut microbiota composition, or the progression of the disorder is regulated by changes in the composition of gut bacteria.

1.8 Research rationales

Many structural components of mitochondria, when released into the extracellular space, activate microglia and astrocytes (Culmsee et al., 2019; Joshi et al., 2019). For example, Joshi *et al.* (2019) demonstrate activated primary murine microglia release mitochondrial fragments, and these fragments induce the secretion of neurotoxins by primary murine astrocytes. The same study demonstrates that treating microglia with mitochondrial fission inhibitor P110 prevents the secretion of neurotoxins by astrocytes. Joshi *et al.* (2019) did not identify the secreted mitochondrial components that activated glial cells, and thus the experiments in chapters 2 and 3 were conducted to fill this knowledge gap. In particular, in chapter 2, I investigated the effects of the extracellular cardiolipin (mitochondrial phospholipid) on select immune functions of microglia, and in chapter 3, I studied the effects of extracellular CytC (mitochondrial protein) on astrocytic immune functions. Cardiolipin and CytC have been reported to modulate the immune responses of immune cells, including microglia (Gouveia et al., 2017; Pizzuto et al., 2019; Pointer et al., 2019), providing rationale to further investigate the extracellular effects of these mitochondrial components on glia. Since several components of mitochondria are reported to act as TLR 4 ligands, including cytochrome c and cardiolipin (Balasubramanian et al., 2015; Cloonan and Choi, 2013; Gouveia et al., 2017), I tested whether the effects of cardiolipin and cytochrome c on select immune functions of glia were mediated by TLR 4. Since TLR 4 agonists

induce phenotypes representative of both M1/M2 microglia and A1/A2 astrocytes, the research in this thesis tested hypothesis that TLR 4 is the target receptor of CL and CytC. I did not attempt to classify glia with regard to the M1/M2 or A1/A2 phenotypes they acquired after interacting with CL and CytC.

Metabolites released by gut microbes reduce the secretion of inflammatory mediators by immune cells, including microglia (Spielman et al., 2018). SCFAs are one family of molecules, among many, that are released by gut microbes and possess inflammation-modulating properties. For example, acetate (5 mM) reduces the secretion of IL-1β and MCP-1 by LPS-stimulated human monocytes (Ang et al., 2016), and butyrate (2.5-5 mM) reduces the secretion of TNF-α by primary rat microglia (Huuskonen et al., 2004). Yet, serum concentrations of individual SCFAs are less than 500 μM (Hanzlik et al., 2005; Ktsoyan et al., 2016; Zhao et al., 2007). Therefore, the millimolar **concentrations** of SCFAs used in previous studies are well above **levels** that microglia would encounter *in vivo*. The effects of SCFAs at micromolar concentrations on the immune functions of microglia are unknown. Therefore, to fill this knowledge gap, I tested the effects of SCFAs acetate, butyrate, propionate, valerate and formate mixed at a physiologically-relevant ratio applied at the total concentrations of the mixture between 5 – 500 μM on microglia. Specifically, since high concentrations of single SCFAs reduce the secretion of IL-1β, MCP-1 and TNF-α by immune cells, including microglia (Ang et al., 2016; Huuskonen et al., 2004), I investigated the effects of individual SCFAs or their mixture at physiological concentrations on these cytokines.

1.8.1 Cell culture models

Cell lines used in this thesis respond to molecules in a fashion similar to primary cells and were utilized to model cells of the CNS. Immortalized cells used were derived from cancerous tissue or experimentally created. Cell lines often express genes and display morphologies different from their primary cell counterparts (Maqsood et al., 2013). In this thesis, several significant findings obtained using cell lines were confirmed with primary cells.

Murine BV-2 microglia, human THP-1 monocytic cells, and differentiated human HL-60 myelomonocytic cells were used to model microglia. Due to interspecies differences in immune responses and receptor expression of microglia (Bhattacherjee et al., 2019; Davis et al., 2018; Du et al., 2017), HL-60 and THP-1 cells were used to model specific functions of human microglia, such as phagocytosis, secretion of cytokines, and respiratory burst response. Unlike murine microglia, primary human glia and THP-1 cells express known SCFA receptors free fatty acid receptor (FFAR) 2 and 3 (Uhlén et al., 2015). THP-1 cells enabled me to investigate the effects of SCFAs on select microglial functions, and whether these effects were mediated by FFAR 2 and 3.

Differentiated human HL-60 myelomonocytic cells were used to model the respiratory burst response since these cells, similar to microglia, express functional NADPH-dependent oxidase (Levy et al., 1990). BV-2 cells were used to study functions characteristic of microglia, including phagocytosis and the secretion of cytokines (Kapellos et al., 2016; Subedi et al., 2019). THP-1 cells were also used to study microglial functions, as these cells were previously described to secrete cytotoxins at levels similar to primary human microglia (Klegeris et al., 1999).

U118 MG cells, which present with both glioblastoma and astrocytoma cell morphologies, were used as models of human astrocytes. U118 MG cells express low levels of astrocyte marker glial fibrillary acidic protein (GFAP) (Restrepo et al., 2011). Similar to their primary cell counterpart, U118 MG cells secrete cytokines and cytotoxins in response to immune stimuli (Hashioka et al., 2009).

Human SH-SY5Y neuroblastomas were used to model neurons. These cells express markers characteristic of neuronal cells, including microtubule associated protein 2 and neuron specific endonuclease. Importantly, human SH-SY5Y cells are widely used to study neurotoxicity *in vitro* (Hashioka et al., 2009; Heusinkveld and Westerink, 2017).

1.9 Research hypotheses and objectives

Neuroinflammation driven by microglia and astrocytes contributes to the pathogenesis of neurodegenerative diseases, such as AD. Previous studies demonstrated that CL, CytC and SCFAs can exist extracellularly (Balasubramanian et al., 2015; Dalile et al., 2019; Renz et al., 2001; Sorice et al., 2004). Most of the research on these molecules has focussed on their role in immune signaling in the periphery (Balasubramanian et al., 2015; Nastasi et al., 2015; Pullerits et al., 2004). However, the effects of CL, CytC and SCFAs in the CNS are unknown. Therefore, **I hypothesize** that extracellularly-released endogenous molecules CL, CytC and SCFAs regulate select immune responses of glia. The **overall objective** of this thesis is to discover and characterize previously unknown effects of CL, CytC and SCFAs on select functions of glia.

In chapter two, I hypothesize that CL, a phospholipid primarily located in mitochondrial membranes that may be released into the extracellular space upon cell death, modulates the secretion of cytokines and cytotoxins by microglia, as well as their phagocytic activity, in a TLR 4-dependent manner. To address this hypothesis, I focussed on the following specific research objectives:

1) Determine whether CL regulates the secretion of inflammatory mediators and cytotoxins by microglia and microglia-like cells.
2) Establish whether select lipids that are structurally similar to CL regulate the secretion of inflammatory mediators and cytotoxins by microglia-like cells.
3) Examine whether TLR 4 mediates the effect of CL on secretion of inflammatory mediators and cytotoxins by microglia and microglia-like cells.
4) Establish whether cardiolipin reduces the cytotoxic secretions induced by Aβ42 and α-syn.
5) Identify whether TLR 4 mediates the ability of CL to upregulate microglial phagocytosis.

In chapter three, I hypothesize that CytC, a soluble protein associated with the mitochondrial electron transport chain, is released into the extracellular space upon glial cell

death and modulates the cytokine secretion of astrocytes in a TLR 4-dependent manner. To address this hypothesis, I focussed on the following specific research objectives:

1) Examine whether cytotoxins, such as staurosporine, Aβ42, and TNF-α induce release of CytC from microglia and astrocytes into the culture medium.
2) Establish whether CytC induces the secretion of cytokines and cytotoxins by astrocytes.
3) Identify whether astrocyte TLR 4 is engaged by CytC.
4) Determine whether CytC modulates the expression of astrocyte TLR 4.

In chapter four, I hypothesize that physiological concentrations of SCFAs alone and in combination modulate the secretion of cytokines and cytotoxins by microglia, as well as their phagocytic activity. To address this hypothesis, I focussed on the following specific research objectives:

1) Characterize whether SCFAs decrease the secretion of inflammatory cytokines and cytotoxins by microglial cells.
2) Examine whether SCFAs reduce microglia-mediated cytotoxicity towards neurons.
3) Establish whether the cytokine-modulating effects of SCFAs are mediated by the known SCFA receptors: FFAR 2 and 3.
4) Determine whether select SCFAs modulate the phagocytic activity of microglial cells.

Chapter 2: Extracellular cardiolipin modulates microglial phagocytosis and cytokine secretion in a toll-like receptor 4-dependent manner

2.1 Chapter introduction

Neuroinflammation contributes to the pathogenesis of neurological disorders, including AD and PD. Under normal brain conditions, cytokines are released by microglia, the innate immune cells of the brain, to correct altered physiological states and maintain homeostasis of the CNS. Pathology-associated proteins, like Aβ in AD or α-syn in PD, activate microglia, upregulating their secretion of cytotoxins and inflammatory cytokines (Fellner et al., 2013; Shi et al., 2016). Some of these cytokines, such as TNF-α and IFN-γ, provide positive feedback by activating nearby microglia (Kuno et al., 2005; Rock et al., 2005). This leads to excessive secretion of microglial cytotoxins and inflammatory mediators, propagating neurodegeneration (Clayton et al., 2017).

DAMPs and resolution-associated molecular patterns (RAMPs) are endogenous molecules that are released into the extracellular space from injured cells. Some of the most extensively studied pro-inflammatory DAMPs include ATP and HMGB1 (Gong et al., 2020; Klegeris, 2020; Venegas and Heneka, 2017). αB-crystallin and HSP 10 are often characterized as RAMPs due to their anti-inflammatory and pro-resolving properties (Shields et al., 2011). The biological activity of these molecular patterns is well characterized in peripheral tissues, where they have been shown to regulate phagocytic activity and the secretion of cytokines by several different immune cell types (Roh and Sohn, 2018). Their CNS effects are less understood (for reviews, see Klegeris, 2020; Shields et al., 2011; Wenzel et al., 2020b)

Depending on the experimental outcomes, CL has been identified as either a DAMP or a RAMP by different studies (Chakraborty et al., 2017; Grazioli and Pugin, 2018; Pointer et al., 2019; Wenzel et al., 2020b). CL is a mitochondrial phospholipid primarily found in the inner mitochondrial membrane of mammalian cells. Unlike most phospholipids forming cellular membranes, CL possesses a dimeric structure that includes a double glycerophosphate backbone

and four acyl chains (Pointer and Klegeris, 2017). During cellular death, CL migrates to the outer layer of the plasma membrane, where it can interact with nearby cells (Sorice et al., 2004). In addition, extracellular CL has been detected in human sera, where it binds to β2-glycoprotein (also known as apolipoprotein H) (Deguchi et al., 2000; Kertesz et al., 1995).

Extracellular CL has been shown to regulate select responses of immune cells. For example, it increases the phagocytic activity of primary murine microglia, as well as their expression of brain-derived neurotrophic factor (BDNF) and glial cell-line derived neurotrophic factor (GDNF) (Pointer et al., 2019). Furthermore, LPS-stimulated peripheral immune cells display reduced secretion of cytokines when exposed to CL-containing liposomes, compared to cells exposed to liposomes without CL (Balasubramanian et al., 2015; Pizzuto et al., 2019). In addition, CL-containing liposomes increase the phagocytic activity of peripheral immune cells (Balasubramanian et al., 2015). TLR 4 and its associated receptors, including CD 36, CD 14, and MD 2, have been implicated as the molecular targets of CL in immune cells (Balasubramanian et al., 2015; Pizzuto et al., 2019).

Most of the research on extracellular CL has focussed on its role in immune signaling in the periphery (Balasubramanian et al., 2015; Iyer et al., 2013; Pizzuto et al., 2019); only a limited number of studies have examined the effects of extracellular CL in the CNS (Pointer et al., 2019; Pointer and Klegeris, 2017). I hypothesized that CL regulates microglial secretion of inflammatory mediators in a TLR 4-dependent manner. Due to the reported differences between the TLR 4-ligand recognition domains in humans and mice (Vaure and Liu, 2014), I used murine and human cell lines, in addition to primary murine cells, to model select immune functions of microglia. I demonstrate that secretion of MCP-1, IFN-γ-induced protein (IP)-10, and nitric oxide is induced by exposure of human microglia-like cells to extracellular CL; however, when CL is added to immune-stimulated primary murine microglia, their secretion of MCP-1 and TNF-α is inhibited.

2.2 Materials and methods

2.2.1 Reagents

3-(4,5-dimethyl-2-thiazoyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), N-(1-naphthyl)ethylenediamine dihydrochloride, N,N-dimethylformamide, N-formylmethionine-leucyl-phenylalanine (fMLP), α-syn, bisBenzimide (Hoechst 33258), CL (from bovine heart, Cat. No. C1649), LPS, propidium iodide (PI) and sulfanilamide were obtained from Sigma Aldrich (Oakville, ON, Canada). 1X phosphate-buffered saline (PBS) tablets were purchased from VWR International (Mississauga, ON, Canada). Human and mouse recombinant IFN-γ, as well as the enzyme-linked immunosorbent assay (ELISA) development kits for human and MCP-1, mouse TNF-α, and human IP-10 were purchased from PeproTech (Embrun, ON, Canada). Fluorescein isothiocyanate (FITC) externally-labelled one μm fluorescent polystyrene latex beads were purchased from Bangs Laboratories (Fishers, IN, USA). 1,2-dilinoleoyl-*sn*-glycero-3-phosphate (PA182) and 1,2-dioleoyl-*sn*-glycero-3-phosphate (PA181) were from Avanti Polar Lipids (Alabaster, AL, USA). Aβ42 was purchased from California Peptide Research (Salt Lake City, UT, USA). All other reagents were from Thermofisher Scientific (Ottawa, ON, Canada). α-syn and Aβ42 were prepared as described in previously published studies (Hashioka et al., 2011; Klegeris et al., 2006). CL from Sigma Aldrich (Cat. No. C1649) is representative of the most common isotypes in mice brain cells and cell lines (Zhang et al., 2011). There are additional CL species that can be found in brain parenchyma, but are not included in the commercially available solution I used.

2.2.2 Cell culture models

Primary murine microglia were extracted from the brains of eight-week old C57BL/6 mice as described in a previously published study (Lee and Tansey, 2013), except that dispase II was omitted from the cell dissociation medium. Primary microglia were cultured *in vitro* for seven days before being used in experiments. The murine microglia BV-2 cell line was donated by Dr. G. Garden (Department of Neurology, University of Washington, Seattle, WA, USA). The human THP-1 monocytic cell line was purchased from the American Type Culture

Collection (ATCC, Manassas, VA, USA). The human neuroblastoma SH-SY5Y cell line was donated by Dr. R. Ross (Department of Biological Sciences, Fordham University, Bronx, NY, USA). Cells were cultured in Dulbecco's modified Eagle medium nutrient mixture F-12 Ham (DMEM-F12) supplemented with 10% calf bovine serum (CBS), penicillin (100 U/ml) and streptomycin (100 μg/ml) in T-75 flasks incubated at 37 °C in humidified 5% CO_2 and 95% air atmosphere.

2.2.3 Secretion of cytokines by primary murine microglia, murine BV-2 microglia and human THP-1 microglia-like cells

Primary murine microglia were plated in 96-well plates at a concentration of 5 x 10^4 cells/ml in 250 μl DMEM-F12 containing 5% CBS, penicillin (100 U/ml) and streptomycin (100 μg/ml). One well was used for each experimental condition. CL (0.4-14 μM) at concentrations similar to levels detected in plasma from fasting humans (Deguchi et al., 2000) or its vehicle solution (ethanol) was added to culture media, followed by a 15 min incubation period. Primary microglia were treated with LPS (20 ng/ml), Aβ42 (5 μM), α-syn (10 μg/ml), IFN-γ (150 U/ml) or their vehicle solutions (PBS for all stimulants, except Aβ42 which is dissolved in 8% acetonitrile in a solution of 28% endotoxin-free water:72% PBS). Supernatants were collected 48 h later and centrifuged at 1000 x *g* for 10 min. Concentrations of MCP-1 and TNF-α in primary murine microglia supernatants were measured using PeproTech ELISA development kits according to the manufacturer's instructions.

This experiment was repeated to measure the concentrations of MCP-1 in the supernatants of BV-2 microglia and THP-1 monocytic cells, with modifications. BV-2 microglia and THP-1 cells were plated in 24-well plates at a concentration of 2 x 10^5 cells/ml in 500 μl DMEM-F12 containing 5% CBS, penicillin (100 U/ml) and streptomycin (100 μg/ml), and 5 x 10^5 cells/ml in one ml DMEM-F12 containing 5% CBS, penicillin (100 U/ml) and streptomycin (100 μg/ml), respectively. 30 min prior to the addition of CL, TLR 4-blocking antibodies (10 μg/ml, clone: MTS510 for BV-2 microglia, clone: HTA125 for THP-1 cells) were added to the culture medium of cells. In addition to MCP-1, concentrations of IP-10 were measured in THP-1

supernatants using PeproTech ELISA development kits according to the manufacturer's instructions. BV-2 microglia and THP-1 cells were not stimulated with Aβ42, α-syn or IFN-γ, and only THP-1 cells were stimulated with LPS.

2.2.4 Measurement of nitric oxide release by the Griess assay

The secretion of nitric oxide was measured by the Griess assay as described in a previously published study (Pointer et al., 2019), with minor modifications. Human THP-1 cells were plated as described in 2.3. One well was used for each experimental condition. CL or its vehicle solution was added to the culture medium of cells. Following a 15 min incubation period, cells were treated with LPS (400 ng/ml) or its vehicle solution. Following a 24 h incubation period, nitrite concentration was measured by collecting supernatants, adding an equal volume of Griess reagent (1% sulfanilamide, 2.5% phosphoric acid, 0.1% N-(1-naphthyl)ethylenediamine dihydrochloride in H_2O), and measuring the optical density at 550 nm using the FLUOstar Omega microplate reader.

2.2.5 Secretion of cytotoxins by human THP-1 microglia-like cells

The experiments were performed as described in a previously published study (Wenzel et al., 2020a), with minor modification. Human THP-1 monocytic cells were plated as described in 2.3. One well was used for each experimental condition. CL, PA182 (0.4-14 μM), PA181 (0.4-14 μM) or their vehicle solutions (ethanol) were added to culture media, followed by a 15 min incubation period. THP-1 cells were treated with a combination of LPS (20 ng/ml) plus IFN-γ (150 U/ml) or vehicle solution. Supernatants were collected 48 h later. 0.4 ml of the collected THP-1 supernatants were transferred to each well containing SH-SY5Y neuronal cells that had been plated 24 h earlier at a concentration of 3 x 10^5 cells/ml in 0.4 ml of DMEM-F12 containing 5% CBS, penicillin (100 U/ml) and streptomycin (100 μg/ml). After 72 h incubation, neuronal cell viability was assessed by the MTT assay and cell death was assessed by the PI assay. Viability of unstimulated and stimulated THP-1 cells was also assessed by the MTT and PI assays at the end of the 48 h incubation period with CL

2.2.6 Phagocytic activity of primary murine microglia and human THP-1 microglia-like cells

Phagocytic activity was assessed as described in a previously published study (Wenzel et al., 2020a), with minor modifications. Primary murine microglia were plated in poly-D-lysine-coated, eight-chambered coverglass slides at a density of 5 x 10^4 cells/ml in 500 µl DMEM-F12 containing 5% CBS, penicillin (100 U/ml) and streptomycin (100 µg/ml), while THP-1 cells were plated at a concentration of 1 x 10^5 cells/ml in 500 µl DMEM-F12 containing 5% CBS, penicillin (100 U/ml) and streptomycin (100 µg/ml). One chamber was used for each experimental condition. CL or its vehicle solution was added to culture media, followed by a 15 min incubation period. In select wells, cells were incubated for 30 min with anti-TLR 4 antibodies (10 µg/ml, clone MTS510 for primary murine microglia, or clone HTA125 for THP-1 cells) prior to the addition of CL. One µm diameter externally FITC-labelled latex beads were added in a 10:1 bead:cell ratio. After one h incubation, light from non-internalized beads was quenched by adding trypan blue at a final concentration of 0.05% to the culture medium. Supernatant, trypan blue, and beads were removed from all wells by washing two times with one ml PBS. Prior to imaging, bisBenzimide (2 µg/ml) nuclear stain was added to all wells in 0.5 ml PBS.

Cells were visualized using a Zeiss AxioObserver.Z1 widefield epifluorescence microscope with Zen image acquisition software (version 2.0) at an excitation/emission of 350/470 nm for bisBenzimide, and 470/520 nm for the fluorescent beads. Images of a minimum of 50 cells per well were acquired and analyzed by another researcher, and thus data were collected in a blinded manner. The mean fluorescence intensity (MFI) was measured using ImageJ (version 1.53a, National Institute of Health, USA). Only viable cells, identified by trypan blue exclusion, were examined. In addition, primary murine microglia were visualized using an Olympus FluoView FV1000 confocal microscope and imaged using FV10-ASW software (version 3.0) to confirm that fluorescing beads were engulfed by cells.

2.2.7 Assessing cell viability/death

Cell viability was monitored by the MTT assay, which measured the reduction of water-soluble tetrazolium dye MTT to an insoluble purple formazan product by viable cells (Hansen et al., 1989; Wenzel et al., 2019). Cells were incubated with MTT (0.5 mg/ml) at 37 °C for one h in a 5% CO_2 incubator. The resulting formazan crystals were dissolved by adding a volume of sodium dodecyl sulfate (20% w/v)/N,N-dimethylformamide (50% v/v) solution equal to that of the culture medium present in the well, then incubating the plates for three h. Optical densities were measured at 570 nm using the FLUOstar Omega microplate reader. Cell viability data are presented as percent compared to values obtained from cells incubated in growth medium only.

To assess cell death, PI, a fluorescent DNA dye which cannot penetrate membranes of live cells, was used (Nieminen et al., 1992; Wenzel et al., 2019). PI (20 µg/ml) was added to the cell cultures and the plates were incubated for 20 min at 37 °C in a 5% CO_2 incubator. Fluorescence intensity was measured by the FLUOstar Omega microplate reader, using 485 nm excitation and 625 nm emission filters. Cell death data are presented as percent compared to values obtained from cells lysed in growth medium only.

2.2.8 Data analysis

Data obtained were analyzed using (1) the randomized block design one-way analysis of variance (ANOVA), followed by the Dunnett's or Tukey's post-hoc test, or (2) the paired Student's *t*-test with the Holm-Bonferroni correction for multiple comparisons. Independent experiments are defined herein as assays performed on different days. Data are presented as means ± standard error of the mean (SEM). Significance was established at $P<0.05$. Detection limits were calculated using the following formula:

$$\boldsymbol{Detection\ limit} = \frac{[(\text{ODmean } of\ six\ sample\ blanks)+3*(standard\ deviation\ of\ ODmean\ comprising\ six\ sample\ blanks)]-b}{m}$$

Where:

ODmean = mean optical density

m = slope value obtained from the linear standard curve

b = intercept value with the optical density axis obtained from the linear standard curve

2.3 Results

2.3.1 CL regulates the secretion of MCP-1 and TNF-α by primary murine microglia

We assessed the effects of extracellular CL on the secretion of inflammatory mediators MCP-1 and TNF-α by primary murine microglia. Previous studies have shown that microglial secretion of these two inflammatory mediators can be induced by a diverse set of stimuli, including LPS (McManus et al., 1998; Welser-Alves and Milner, 2013), IFN-γ (Rock et al., 2005; Welser-Alves and Milner, 2013), Aβ42 (Cho et al., 2013; Shi et al., 2016), and α-syn (Fellner et al., 2013; Roodveldt et al., 2013), which were also selected for the first series of experiments (Fig. 2.1). Treating resting primary microglia with extracellular CL only (14 μM) induced their secretion of MCP-1 (Fig. 2.1A), but not TNF-α (Fig. 2.1F). Figure 2.1 illustrates that LPS, Aβ42, α-syn, and IFN-γ increased secretion of MCP-1 and TNF-α by primary murine microglia. When cells were treated with CL prior to stimulation with LPS or Aβ42, their secretion of MCP-1 and TNF-α decreased, compared to stimulated microglia treated with CL vehicle solution only (Fig. 2.2B,C,G,H). CL reduced α-syn-induced secretion of TNF-α (Fig. 2.1I), while only a trend towards significant inhibition was observed for MCP-1 secretion (P=0.06, Fig. 2.1D). CL had no effect on the secretion of MCP-1 and TNF-α by cells stimulated with IFN-γ (Fig. 2.2E,J). According to the MTT assay, no significant changes in cell viability were induced by CL, the stimuli alone, or any combinations of CL and stimuli used (Fig. S2.2 in Appendix).

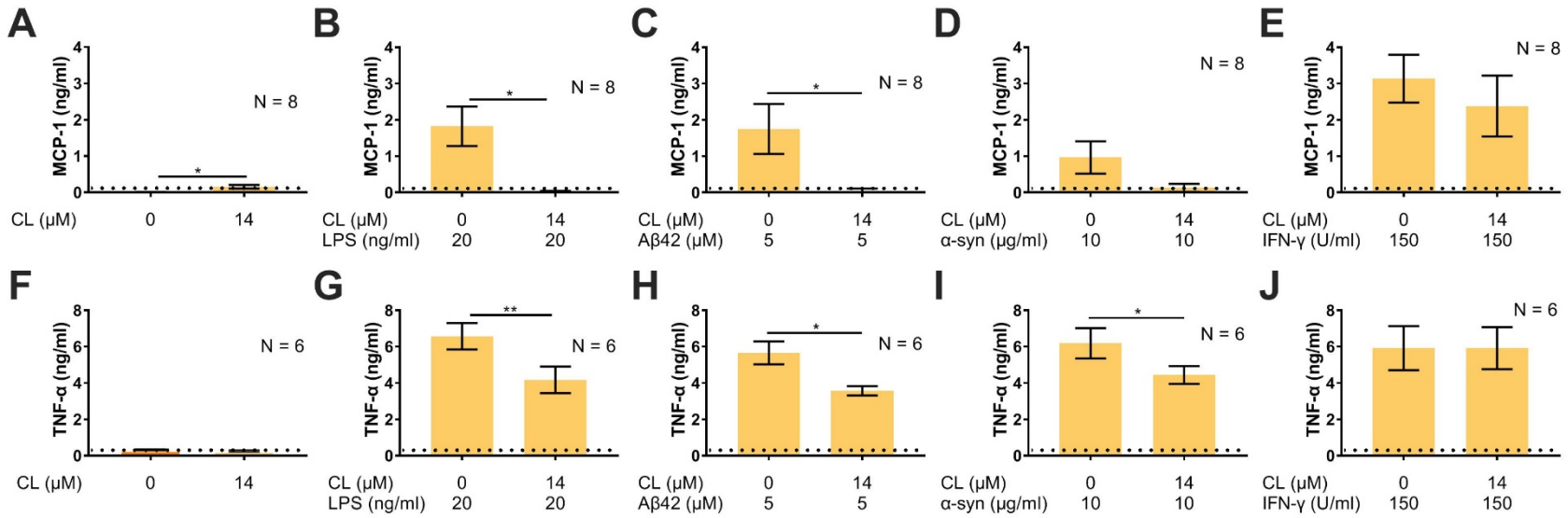


Figure 2.1. Effects of CL on MCP-1 (**A-E**) and TNF-α (**F-J**) secretion by unstimulated and stimulated primary murine microglia. CL was added to cell cultures to reach a final concentration of 14 μM. Cells were left unstimulated (**A,F**) or were stimulated with 20 ng/ml LPS (**B,G**), 5 μM Aβ42 (**C,H**), 10 μg/ml α-syn (**D,I**), or 150 U/ml IFN-γ (**E,J**) 15 min later. After 48 h incubation, concentrations of MCP-1 and TNF-α in cell-free supernatants were measured by ELISAs. Data (means ± SEM) from eight (**A-E**) or six (**F-J**) independent experiments performed on separate days are presented. *P<0.05, **P<0.01 according to the paired Student's *t*-test with Holm-Bonferroni correction for multiple comparisons. The detection limits of the MCP-1 and TNF-α ELISAs are shown as dotted lines.

2.3.2 CL, but not structurally similar phospholipids, induces the secretion of MCP-1 by murine BV-2 microglia and human THP-1 microglia-like cells in a TLR 4-dependent manner

In silico studies have implicated TLR 4 as one of the molecular targets of CL (Balasubramanian et al., 2015). Activation of TLR 4 has also been shown to increase the secretion of MCP-1 by immune cells, including microglia (Rustenhoven et al., 2016). Therefore, I tested whether the effects of CL alone on MCP-1 secretion by BV-2 microglia and THP-1 monocytic cells were mediated by this receptor. TLR 4-blocking antibodies (10 μg/ml) or their solvent was added to cell cultures, followed by CL (14 μM) or its vehicle solution (Fig. 2.2A,B). CL alone increased the secretion of MCP-1 by BV-2 microglia and THP-1 cells. This effect of CL was inhibited by TLR 4-blocking antibodies, which on their own did not affect MCP-1 secretion by BV-2 microglia or THP-1 cells. CL, TLR 4-blocking antibodies, or their solvents did not affect viability of BV-2 or THP-1 cells according to the MTT assay (Fig. S2.3 in Appendix and 2.5D,F).

Next, I assessed whether phospholipids that are structurally similar to CL, namely PA182 or PA181 (Fig. S2.1 in Appendix), could increase the secretion of MCP-1 by THP-1 monocytic cells. PA182 and PA181 have only one phosphate head group and two acyl chains, which is different from CL possessing two phosphate heads and four acyl chains; however, the 18-carbon length and two degrees of acyl chain unsaturation make PA182 and PA181 similar to the CL species used in this study. Neither PA182 nor PA181 at the concentration range used for CL (0.4-14 μM) induced secretion of MCP-1 by THP-1 monocytic cells (Fig. 2.2C,D).

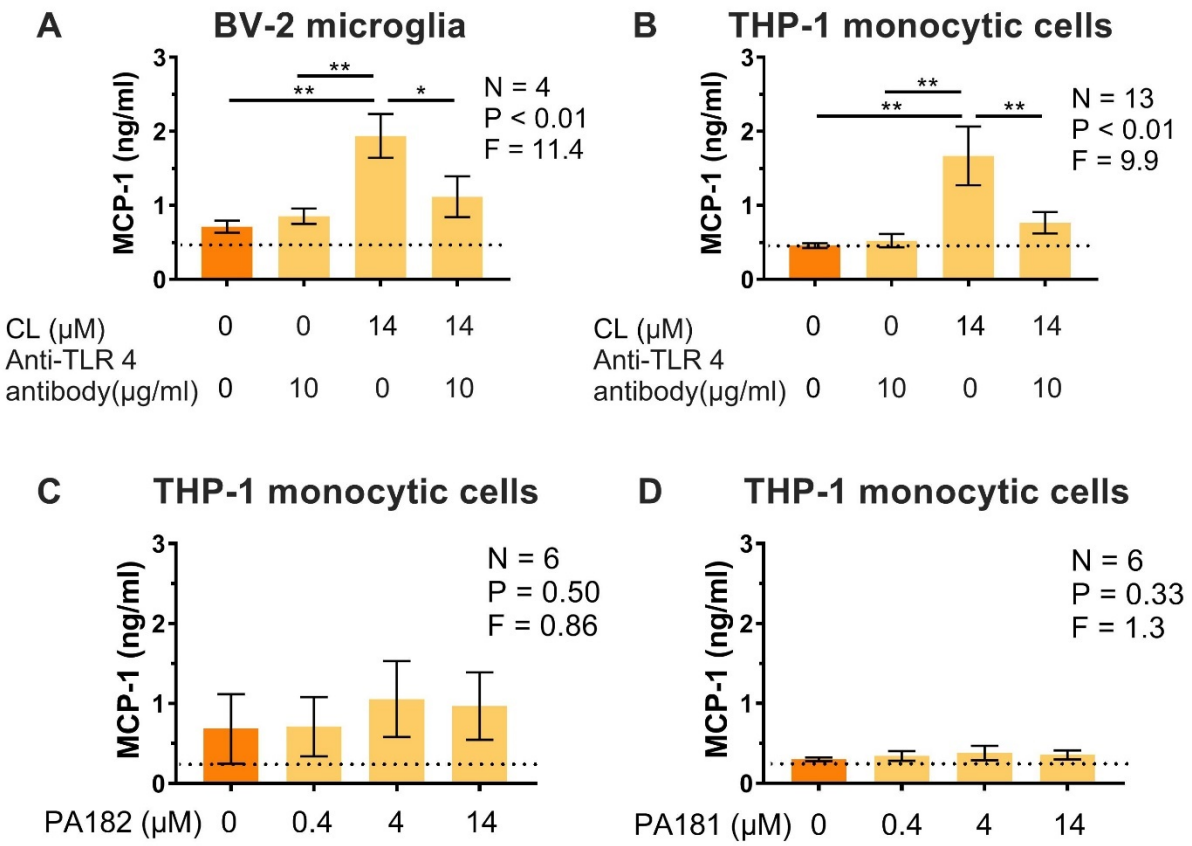


Figure 2.2. Effects of CL on MCP-1 secretion by murine BV-2 microglia (**A**), and human THP-1 monocytic cells (**B**) in the presence or absence of TLR 4-blocking antibodies, as well as the effects of phospholipids with two 18-carbon acyl chains, PA182 (**C**) and PA181 (**D**)**,** on MCP-1 secretion by THP-1 cells. 10 μg/ml of anti-TLR 4 antibodies were added to cell cultures 30 min before stimulation with CL (**A,B**). Different concentrations of PA182 (**C**) and PA181 (**D**) on their own were added to cell cultures. After 48 h incubation, concentrations of MCP-1 in cell-free supernatants were measured by ELISA. Data (means ± SEM) from four to 13 independent experiments performed on separate days are presented. *P<0.05, **P<0.01 according to the Tukey's post-hoc test. P and F values calculated by the one-way randomized blocks ANOVA are shown, as well as the detection limits of the MCP-1 ELISAs as dotted lines.

2.3.3 CL regulates the secretion of IP-10 and nitric oxide by THP-1 microglia-like cells

Since CL alone increased the secretion of MCP-1 by BV-2 and THP-1 cells, I studied the effect of this phospholipid on secretion of two other inflammatory mediators, IP-10 and nitric oxide, by human monocytic THP-1 cells. Nitric oxide was measured through its major breakdown product, nitrite, using the Griess assay. CL (0.4-14 μM), when added to THP-1 cell cultures, induced the secretion of IP-10 at all concentrations tested (Fig. 2.3A), and the secretion of nitric oxide at the highest concentration tested (Fig. 2.3C). Control experiments showed no chemical interaction between CL and the Griess reagents (Fig. S2.4 in Appendix).

Next, I tested the effects of CL at the same concentrations on the secretion of IP-10 and nitric oxide by LPS-stimulated THP-1 cells. The secretion of IP-10 was reduced when CL (4 or 14 μM) was added to culture medium before LPS stimulation (Fig. 2.3B). Even at high 400 ng/ml concentrations, LPS alone did not induce the secretion of nitric oxide by THP-1 cells; instead, LPS likely inhibited the stimulatory effect of CL at 14 μM seen on fig. 2.3C since in the presence of LPS there was only a trend towards significant upregulation of nitric oxide by CL ($P=0.054$, Fig. 2.3D). Viability of THP-1 cells was not affected by CL, or its vehicle solution, according to the MTT assay (Fig. S2.5 in Appendix).

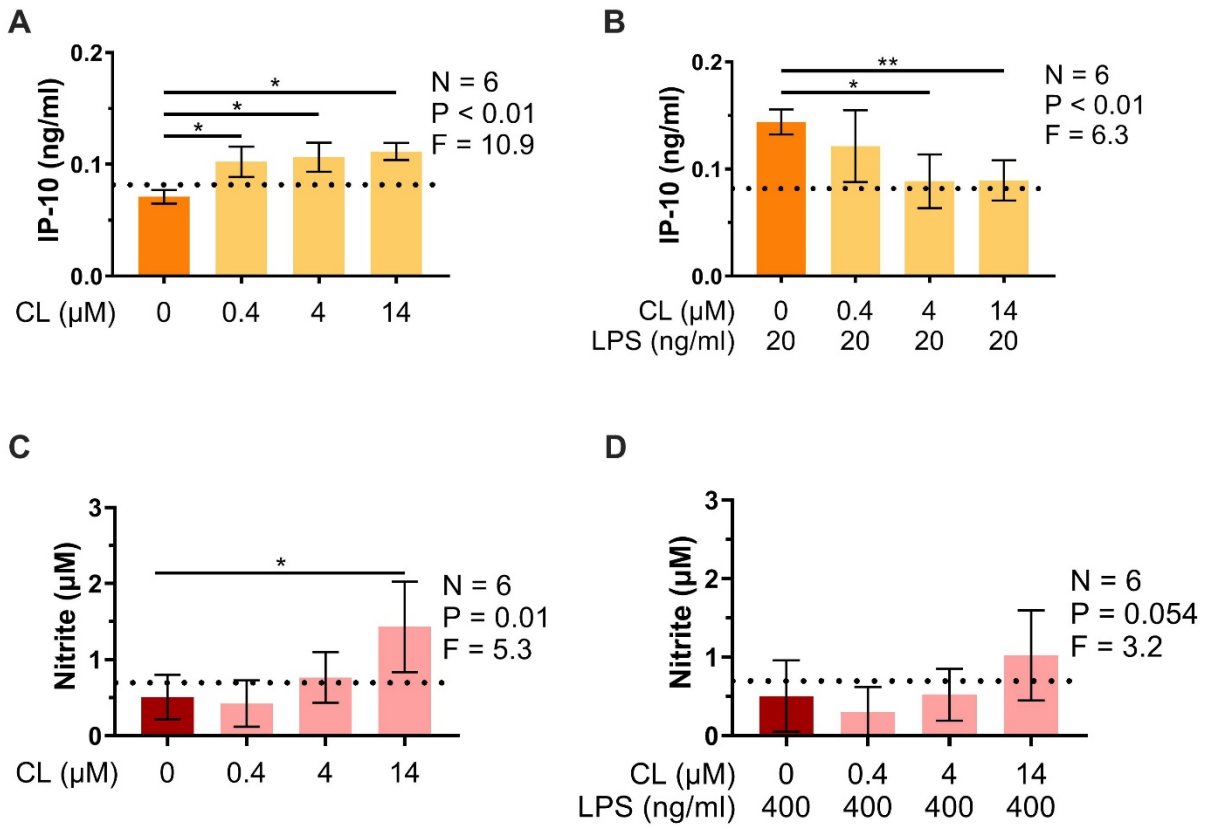


Figure 2.3. Effects of CL on IP-10 (**A,B**) and nitric oxide (**C,D**) secretion by human THP-1 monocytic cells in the presence or absence of LPS. Increasing concentrations of CL (0.4-14 μM) were added to cell cultures. Cells were left unstimulated (**A,C**) or were stimulated with 20 ng/ml (**B**) or 400 ng/ml (**D**) LPS 15 min later. After 48 h incubation, concentrations of IP-10 and nitrite in cell-free supernatants were measured by an ELISA and Griess assay respectively. Data (means ± SEM) from six independent experiments performed on separate days are presented. *P<0.05, **P<0.01 compared to cells not exposed to CL, according to the Dunnett's post-hoc test. P and F values calculated by the one-way randomized blocks ANOVA are shown, as well as the detection limits of the ELISA (**A,B**) or Griess assay (**C,D**) as dotted lines.

2.3.4 CL, but not structurally similar phospholipids PA182 and PA181, regulates the secretion of cytotoxins by THP-1 microglia-like cells

Since CL regulated the secretion of inflammatory cytokines and nitric oxide, a reactive nitrogen species, by THP-1 cells, I studied the effect of CL on secretion of cytotoxins by these microglia-like cells. I also tested the structurally similar phospholipids PA182 and PA181. Increasing concentrations of CL (0.4-14 μM), PA182 (0.4-14 μM) or PA181 (0.4-14 μM) were added to THP-1 cell cultures prior to their stimulation with LPS plus IFN-γ (Fig. 2.4). Figures 2.4A,C,E demonstrate that LPS plus IFN-γ stimulation induced the cytotoxicity of THP-1 supernatants towards SH-SY5Y neuronal cells, which could be observed as reduced viability of SH-SY5Y recorded by the MTT assay. At the highest concentration tested, CL (14 μM) reduced the toxicity of THP-1 supernatants towards SH-SY5Y neuronal cells, compared to supernatants from THP-1 cells stimulated in the absence of CL (Fig. 2.4A). PA182 and PA181 did not significantly affect the cytotoxicity of supernatants from stimulated THP-1 cells (Fig. 2.4C,E). No significant changes in THP-1 cell viability were detected in this experiment according to the MTT assay (Fig. 2.4B,D,F). In addition, when CL, PA182, or PA181 (0.4-14 μM) was added to unstimulated THP-1 cells, cytotoxicity towards SH-SY5Y cells was not induced (Fig. 2.5).

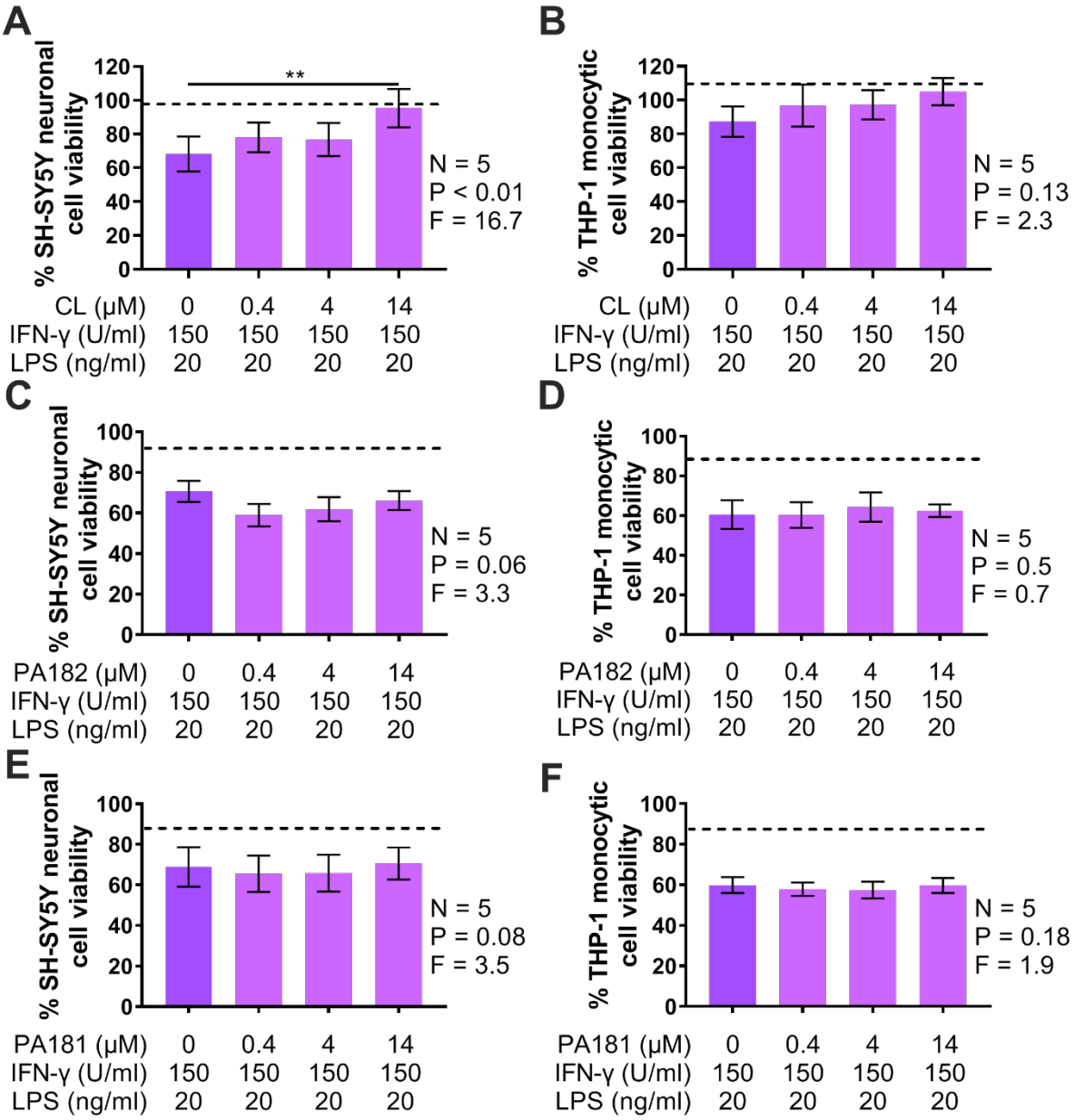


Figure 2.4. Effect of CL (**A**), PA182, (**C**) and PA181 (**E**) on cytotoxicity of stimulated human THP-1 monocytic cells towards human SH-SY5Y neuronal cells. Increasing concentrations of CL (**B**), PA182 (**D**), or PA181 (**F**) were added to THP-1 cell cultures 15 min before their stimulation with LPS (20 ng/ml) plus IFN-γ (150 U/ml). After 48 h incubation, THP-1 supernatants were transferred onto SH-SY5Y cell cultures and viability of THP-1 cells measured by the MTT assay (**B,D,F**). Following 72 h incubation with supernatants from THP-1 cells, viability of SH-SY5Y cells was measured by the MTT assay (**A,C,E**). Data (means ± SEM) from

five independent experiments performed on different days are presented. ** P<0.01 compared to SH-SY5Y cells exposed to supernatants from THP-1 cells stimulated in the absence of CL, according to the Dunnett's post-hoc test. P and F values calculated by the one-way randomized blocks ANOVA are shown. Dashed lines represent the mean signal from SH-SY5Y cells incubated in tissue culture medium only and not exposed to THP-1 supernatants (**A,C,E**), or the mean signal from unstimulated THP-1 cells (**B,D,F**).

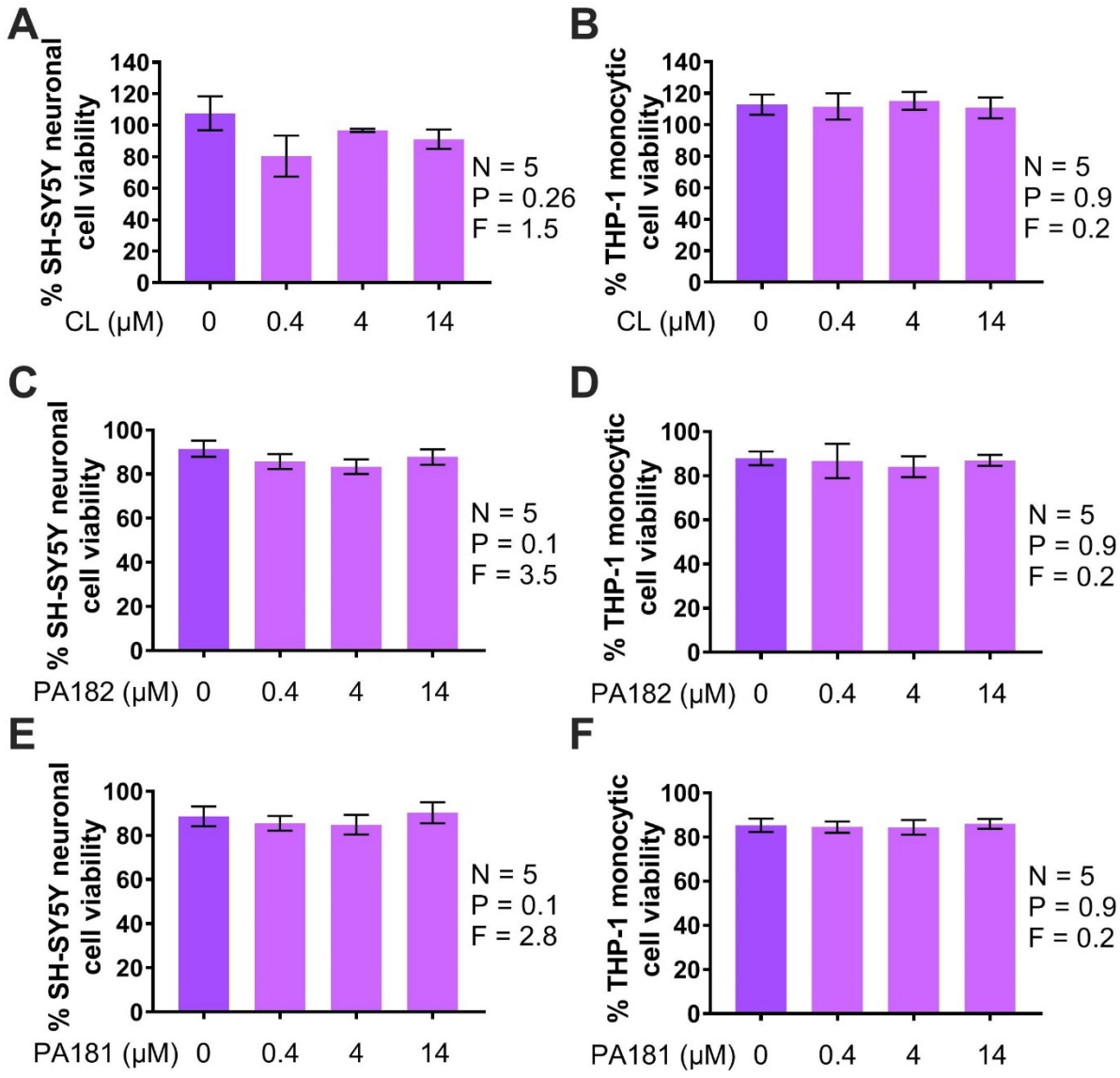


Figure 2.5. Effect of CL (**A**), PA182, (**C**) and PA181 (**E**) on cytotoxicity of unstimulated human THP-1 monocytic cells towards human SH-SY5Y neuronal cells. Increasing concentrations of

CL (**B**), PA182 (**D**), or PA181 (**F**) were added to THP-1 cell cultures. After 48 h incubation, THP-1 supernatants were transferred onto SH-SY5Y cell cultures and viability of THP-1 cells measured by the MTT assay (**B,D,F**). Following 72 h incubation with supernatants from THP-1 cells, viability of SH-SY5Y cells was measured by the MTT assay (**A,C,E**). Data (means ± SEM) from five independent experiments performed on different days are presented. P and F values calculated by the one-way randomized blocks ANOVA are shown.

2.3.5 CL-induced increase of phagocytosis by primary murine microglia and THP-1 microglia-like cells is mediated by TLR 4

The activation of TLR 4 has been shown to increase the phagocytic activity of immune cells, including microglia (Michaud et al., 2013). Since CL reduced the secretion of cytokines by stimulated microglia and microglia-like cells in a TLR 4-dependent manner, I used the effective concentration of CL (14 μM) to evaluate its effect on the phagocytic activity of primary murine microglia and THP-1 cells. TLR 4-blocking antibodies (10 μg/ml) or their solvent was added to primary murine microglia or THP-1 cell cultures, followed by CL or its vehicle solution. The phagocytosis of fluorescent latex beads by primary murine microglia and THP-1 cells was studied 24 h later. Figure 2.6 demonstrates CL alone upregulated the phagocytic activity of primary murine microglia and THP-1 cells. TLR 4-blocking antibodies significantly reduced this effect of CL in both cell types. TLR 4-blocking antibodies on their own did not affect the phagocytic activity of primary murine microglia or THP-1 cells. No significant changes in cell viability were detected in this experiment according to the MTT assay (Fig. S2.6 in Appendix).

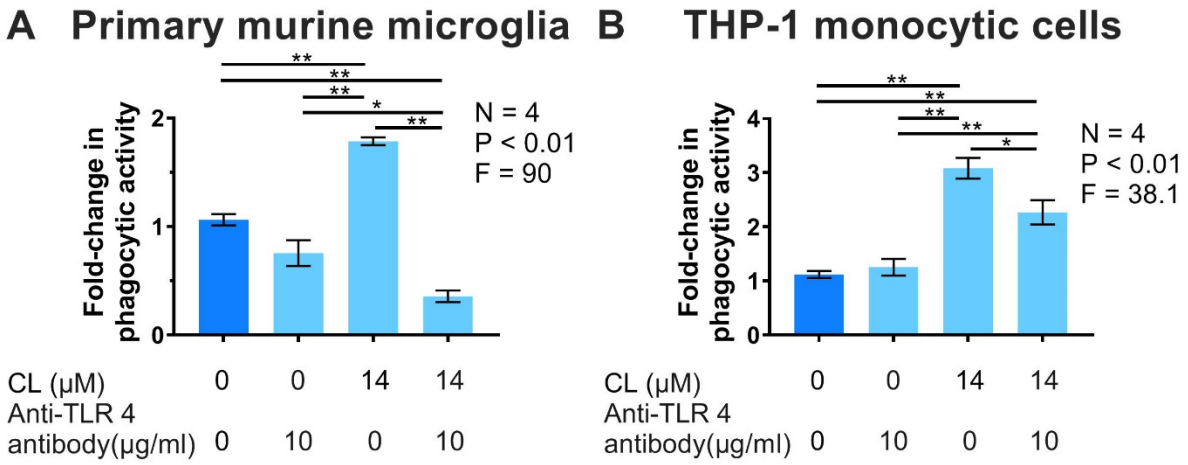


Figure 2.6. Effects of CL on phagocytosis of latex beads by primary murine microglia (**A**) or human THP-1 monocytic cells (**B**) in the presence or absence of TLR 4-blocking antibodies. 10 μg/ml of anti-TLR 4 antibodies were added to cell cultures first. After 30 min incubation, CL was added to reach the final concentrations of 14 μM. After a 24 h incubation period, fluorescent latex beads were added to cell cultures for one h, followed by quenching non-internalized beads with 0.05% trypan blue. Fluorescence intensity of unquenched latex beads in live cells was measured using a widefield microscope. Data (means ± SEM) from four independent experiments performed on separate days are presented as a fold change of phagocytosed bead fluorescence in live cells, compared to cells exposed to latex beads in growth medium only. *P<0.05, **P<0.01 according to the Tukey's post-hoc test. P and F values calculated by the one-way randomized blocks ANOVA are shown.

2.4 Chapter discussion

Extracellular CL has recently been shown to regulate the functions of immune cells, including microglia and peripheral macrophages (Balasubramanian et al., 2015; Pizzuto et al., 2019; Pointer et al., 2019). For example, extracellular CL upregulated murine microglia phagocytic activity and expression of two different neurotrophic factors (Pointer et al., 2019). Phagocytic activity of cell lines from human and murine hosts was also upregulated by treatment with CL-containing liposomes (Balasubramanian et al., 2015). Initially, *in silico* studies identified TLR 4 as the candidate cell surface receptor CL could interact with (Balasubramanian

et al., 2015). Subsequently, *in vitro* experiments using human and murine peripheral immune cells demonstrated that CL species with saturated acyl chains activated TLR 4, while species with unsaturated acyl chains blocked the effects of TLR 4 agonists (Pizzuto et al., 2019). It is important to note that the innate and acquired immune responses of humans and mice are not identical (Mestas and Hughes, 2004). Specifically, the coding sequences for the extracellular ligand-recognition domain of TLR 4 are different between humans and mice; although, the intracellular TLR 4 signalling domain is highly conserved between these two species (Lizundia et al., 2008; Schroder et al., 2012; Vaure and Liu, 2014). Therefore, I investigated the effects of extracellular CL on microglia by using both human and murine cells. Upon activation, microglia release a mixture of inflammatory cytokines and cytotoxins, possibly contributing to the progression of neurodegenerative diseases (Brabazon et al., 2018; Clayton et al., 2017; Klegeris et al., 2005). In addition, microglial phagocytosis, which is essential to maintaining brain homeostasis, becomes dysregulated in AD (Galloway et al., 2019); therefore, I studied whether extracellular CL could regulate these immune functions of microglia *in vitro*.

Our data, showing inhibitory effect of CL on the secretion of TNF-α and MCP-1 by activated primary murine microglia, were consistent with previous observations demonstrating that CL reduced the secretion of TNF-α by LPS-activated immortalized murine bone marrow-derived macrophages (BMDMs), BV-2 microglia, primary human monocyte-derived macrophages, human peripheral blood mononuclear cells and THP-1 cells (Balasubramanian et al., 2015; Pizzuto et al., 2019; Pointer et al., 2019). Similar to our observations with LPS-activated THP-1 cells, Pizzuto *et al.* (2019) also demonstrated that mono- and di-unsaturated CL downregulated the secretion of IP-10 by LPS-activated murine BMDMs.

We were able to repeat experiments by Pointer *et al.* (2019) who showed that extracellular CL inhibited secretion of cytotoxins by LPS plus IFN-γ-stimulated THP-1 cells without reducing their viability. Herein, I extended this previous observation by demonstrating that CL inhibited secretion of several potentially neurotoxic molecules, including MCP-1, IP-10, and TNF-α (Annis et al., 2013; Sui et al., 2004; Yang et al., 2011), by activated microglia or microglia-like cells. Since the cytotoxicity of microglial supernatants can be mediated by a mixture of toxins (Clayton et al., 2017), the overall anti-cytotoxic activity of CL on microglia-

like THP-1 cells is likely due to its ability to lower concentrations of these toxins in supernatants from immune-stimulated cells. I also showed that the anti-cytotoxic activity of CL was not mediated by its phospholipid fragments.

We demonstrated that PA182 and PA181, which are similar to the phospholipids forming CL, did not inhibit secretion of cytotoxins by immune-stimulated THP-1 cells. Furthermore, unlike CL, neither of these two phospholipids induced secretion of MCP-1 by THP-1 cells. These observations indicated that the overall four acyl chain structure of CL was required for its extracellular activity. Interestingly, liposomes containing both phosphatidylcholine and phosphatidylserine were shown to downregulate the secretion of TNF-α and nitric oxide by Aβ plus IFN-γ-stimulated primary rat microglia, as well as to reduce their production of reactive oxygen species (Hashioka et al., 2007). The fact that PA182 and PA181 were ineffective in our study demonstrated that not all phospholipids regulate the secretion of cytokines and cytotoxins by microglia and microglia-like cells and that the cellular effects of extracellular CL are specific for this diphosphatidylglycerol lipid.

Our observation that CL alone induced the secretion MCP-1 and IP-10, but not TNF-α, by microglia and microglia-like cells was partially supported by a previous study demonstrating that liposomes containing CL activated nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) in RAW-Blue cells derived from murine RAW 264.7 macrophages (Pizzuto et al., 2019). NF-κB activation in immune cells commonly leads to upregulated secretion of a broad range of cytokines, including IP-10 (CXCL10), MCP-1, TNF-α (Liu et al., 2017). Interestingly, Pizzuto *et al.* (2019) showed that CL-containing liposomes induced the secretion of TNF-α by murine BMDMs and human monocytic cells, which is different from our observations with murine primary microglia; however, it should also be noted that this study used liposomes containing CL with saturated acyl chains, while the CL used in our experiments had all acyl chains unsaturated. It is important to point out that our data showed MCP-1 levels in the supernatants of primary murine microglia were lower than BV-2 microglia after CL treatment alone. Interestingly, previous studies demonstrated that primary murine microglia produced higher levels of MCP-1 in response to LPS compared to BV-2 microglia (Chhor et al., 2013; Das et al., 2016). However, it is important to note that these studies either did not report the density

of cells plated or used different densities for each cell type. It is possible that low levels of MCP-1 secretion by primary murine microglia I observed was due to these cells being plated at a density four times less than BV-2 microglia.

Our data, showing CL alone induced the secretion of nitric oxide by undifferentiated THP-1 cells, were unique since several previous studies had failed to induce nitric oxide productions by human microglia or microglia-like cells, even when such potent stimuli as LPS and IFN-γ were used (Chen et al., 1996; Klegeris et al., 2005). Interestingly, Pointer et al. (2019) reported that CL alone did not induce the secretion of nitric oxide, or the expression of inducible nitric oxide synthase (iNOS), by murine BV-2 microglia. Therefore, our observation that CL induces the secretion of nitric oxide by THP-1 cells may be cell type-specific. Even though nitric oxide is commonly considered a microglial cytotoxin, it is important to point out that its neuroprotective properties have also been reported (Garry et al., 2015). Even though I demonstrated CL alone upregulated the secretion of several potentially cytotoxic molecules, including MCP-1, IP-10 and nitric oxide, by THP-1 microglia-like cells, supernatants from CL-stimulated THP-1 cells were not toxic towards SH-SY5Y neuronal cells. This observation could indicate that CL alone was unable to induce the release of monocytic cell cytotoxins at sufficiently high concentrations to show toxic effects.

Exogenous CL and liposomes containing CL have already been shown to upregulate the phagocytic activity of immune cells, including primary murine microglia, RAW264.7 murine macrophages, and differentiated THP-1 cells (Balasubramanian et al., 2015; Pointer et al., 2019). Balasubramanian *et al.* (2015) demonstrated that liposomes containing CL with monounsaturated acyl chains were internalized with efficiency similar to liposomes containing CL with two degrees of acyl chain unsaturation. The same study showed that liposomes containing a mixture of CL species with different length and saturation of acyl chains upregulated the phagocytic activity of RAW 264.7 cells. I extended these previous studies by demonstrating that extracellular CL upregulated phagocytic activity of primary murine microglia and human THP-1 cells in TLR 4-dependent manner.

By using TLR 4 blocking antibodies, I showed that this receptor mediated the extracellular CL-induced phagocytosis as well as cytokine secretion of microglia and microglia-like cells of murine and human origin. Supporting our observations, Pizzuto *et al.* (2019) demonstrated that the entire LPS receptor-activation pathway, including CD 14, MD 2, and TLR 4, was required for CL to induce the secretion of cytokines. Interestingly, Balasubramanian *et al.* (2015) reported that CD 36, another receptor often associated with TLR 4, could also mediate the CL-induced increase in phagocytic activity. Their data demonstrating reduced, but not absent, phagocytic activity in macrophages with CD 36 knocked out indicated that TLR 4 and CD 36 could cooperate to regulate the effects of CL on microglial phagocytosis.

Additional indirect support for TLR 4 as the key microglial surface receptor that binds CL came from our observations that this phospholipid alone induced cytokine production by microglia and microglia-like cells, while inhibiting their cytokine secretion induced by TLR 4 agonist LPS, but not IFN-γ which does not bind to TLRs (Ottum et al., 2015). Very similar observations were reported by Pizzuto *et al.* (2019) who demonstrated that CL alone induced the secretion of TNF-α and IL-1β by primary human monocytes, but inhibited LPS-induced secretion of the same cytokines by primary human peripheral blood mononuclear cells. It is possible that CL enhances cell tolerance towards TLR 4 agonists through mechanisms described in previously published studies (Sato et al., 2000; Seeley and Ghosh, 2017). For example, pre-treating murine peritoneal macrophages with TLR 2/6 agonist macrophage-activating lipopeptides, 2 kDa (MALP-2) prior to LPS reduced their secretion of TNF-α by downregulating TLR 4 expression (Sato et al., 2000). Recent studies also showed that the activation of TLR 4 by LPS upregulated the production of microRNAs, which alter signaling events upon subsequent activation of this receptor (for a review, see Seeley and Ghosh, 2017). For instance, these microRNAs inhibited the activation of NF-κB and the secretion of cytokines including TNF-α.

Several other TLR 4 ligands have shown a similar ability to induce cytokine secretion by resting cells and inhibit the stimulatory effects of LPS (Hayashi et al., 2014; Tanaka et al., 2013). For example, the synthetic TLR 4 ligands 1Z105 and 1Z88 alone induced the production of IP-10 and IL-6 by bone marrow-derived dendritic cells. The same molecules inhibited LPS-induced secretion of IL-6 by bone marrow-derived macrophages (Hayashi et al., 2014). This study also

reported that the stimulatory effects of 1Z105 and 1Z88 on cytokine production were significantly weaker when compared to such potent TLR 4 agonists as LPS and monophosphoryl lipid A. Notably, our experiments also showed that CL induced low-level secretion of MCP-1 compared to other known TLR 4 agonists LPS, Aβ42 and α-syn.

2.5 Chapter conclusion

Since Aβ42 and α-syn have been previously reported to activate glia in a TLR 4-dependent manner (Fellner et al., 2013; Shi et al., 2016), I investigated whether CL inhibited cytokine secretion by primary microglia stimulated with these neuropathology-associated proteins. Our observation that CL reduced the secretion of the pro-inflammatory, and potentially cytotoxic, TNF-α by Aβ42- and α-syn-stimulated primary murine microglia is in line with several previous reports identifying protective effects of TLR 4 antagonists in experimental and animal models of AD and PD (Hughes et al., 2020, 2019; Kostuk et al., 2018; Rannikko et al., 2015). Even though CL induced low-level secretion of several inflammatory mediators, the fact that it also upregulated the beneficial phagocytic activity of microglia and reduced the secretion of cytotoxins by microglia stimulated with potent immune stimuli (LPS, Aβ42 and α-syn) indicated that extracellular CL might play an overall protective and neuroinflammation-resolving role in brain pathologies. Thus, our data support previous studies identifying CL as one of the RAMPs of the CNS (Klegeris, 2020; Wenzel et al., 2020b). Future *in vivo* experiments using brain-derived CL will need to be conducted to establish conclusively that extracellular CL can regulate microglial activation and ameliorate neuroinflammatory processes in AD and PD. Additional experiments also need to be conducted to determine physiologically-relevant concentrations of CL that exist in the intercellular space of the brain parenchyma under pathological conditions, such as AD.

Chapter 3: Cytochrome *c* can be released into extracellular space and modulate functions of human astrocytes in a toll-like receptor 4-dependent manner

3.1 Chapter introduction

CytC is a highly conserved 12-kDa protein consisting of 104 amino acids and a covalently attached heme group (Bushnell et al., 1990). The primary location of CytC is in the mitochondrial intermembrane space. CytC has multiple intracellular functions: (1) it serves as an electron carrier between complex III and IV of the electron transport chain; (2) it scavenges ROS generated by the electron transport chain; and (3) it has a key role in the initiation of apoptosis once CytC leaks from mitochondria into the cytosol (Atlante et al., 2000; Goodsell, 2004; Hüttemann et al., 2011). During oxidative stress, the release of CytC into the cytosol mediates apoptosis by initiating the caspase cascade through the activation of caspase 9 (Kagan et al., 2005).

Following cell death in the periphery and the CNS, elevated concentrations of CytC have been observed in the serum and cerebrospinal fluid, respectively, suggesting that CytC may also be released into the extracellular space by damaged or dying cells (Au et al., 2012; Radhakrishnan et al., 2007). For example, following the induction of necrosis in splenocytes, CytC was observed immediately in the extracellular space, while apoptotic insults caused a delayed release (Jemmerson et al., 2002). Similarly, damaged neurons have been shown to release CytC into cell culture medium (Ahlemeyer et al., 2002).

Several immunomodulatory properties of extracellular CytC have been reported in the periphery. Intra-articular injection of CytC in mice induced the accumulation of neutrophils and macrophages leading to chronic inflammation and symptoms characteristic of rheumatoid arthritis (Pullerits et al., 2004). The same study demonstrated extracellular CytC induced NF-κB activation and the release of inflammatory cytokines and chemokines such as IL-6, TNF-α, and MCP-1 from cultured mouse splenocytes. These studies indicate a possible role for CytC as a DAMP similar to HMGB1 and TFAM. DAMPs are endogenous molecules that can perform immunomodulatory functions following active secretion or passive release by damaged or dying

cells into the extracellular space (Bianchi et al., 2011; Faraco et al., 2007; Little et al., 2014; Lotze and Tracey, 2005; Štros, 2010).

Similar to other well-characterized DAMPs such as HMGB1 and TFAM, extracellular CytC has been shown to activate immune responses of microglia and microglia-like cells, which represent the innate immune system in the CNS. Studies using human microglia and neuron models have demonstrated that CytC in combination with bacterial LPS causes neurotoxic inflammatory responses *in vitro,* and these effects of CytC on microglia were at least partially mediated by its interaction with the TLR 4 (Gouveia et al., 2017). Since TLR 4 is also expressed by another CNS glial cell type, astrocytes, I hypothesized that CytC released into the extracellular space of the CNS may also be recognized by astrocytes and produce neuroinflammatory effects that are detrimental to neuronal survival.

Astrocytes are the most abundant subtype of glial cells in the CNS and they play numerous roles that support neuronal function including the initiation and modulation of synapse formation, transportation and storage of metabolic substrates, as well as maintenance of ion and water homeostasis (Forsyth et al., 1996; Simard and Nedergaard, 2004; Ullian et al., 2004). However, in response to specific pathological stimuli, astrocytes undergo a process of activation similar to microglia, termed reactive astrogliosis, in which they upregulate the production of cytotoxins and pro-inflammatory mediators (Johnstone et al., 1999; Li et al., 2011; Nagele et al., 2004). In this study, I used primary human astrocytes and U118 MG human astrocytic cells to show that extracellularly applied CytC modulated immune responses in this cell type. I demonstrated that CytC upregulated secretion of the inflammatory mediators granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-1β, IL-12 p70, and IL-8 as well as induced astrocyte-mediated cytotoxicity. The effects of CytC on astrocytes were shown to be at least partially mediated by the TLR 4 receptor. In addition, I demonstrated that damaged microglia and astrocytes could be another source of extracellular CytC in the brain. By elucidating the role of CytC and TLR 4 in the mechanisms leading to CNS neuroinflammation, I identified possible novel targets for the development of therapies for neurodegenerative disorders where immune activation of astrocytes contributes to the disease pathogenesis.

3.2 Materials and methods

3.2.1 Reagents

Bovine serum albumin (BSA), CBS, DMEM-F12, 0.05% and 0.25% trypsin with ethylenediaminetetraacetic acid (EDTA), diethanolamine, penicillin/streptomycin stock solutions, N, N-dimethylformamide, sodium dodecyl sulfate, and the Pierce bicinchoninic acid (BCA) protein assay kit were purchased from Thermofisher Scientific (Ottawa, ON, Canada). MTT, PBS, phosphatase substrate tablets, PI, and CytC (from bovine heart, Cat. No. C3131) were obtained from Sigma Aldrich (Oakville, ON, Canada). Human recombinant IFN-γ and IL-1β, as well as ELISA development kits for IL-8 and IL-1β, were purchased from PeproTech (Embrun, ON, Canada). TAK 242 was supplied by Tocris (Oakville, ON, Canada). The alkaline phosphatase-labeled anti-mouse antibody was supplied by Invitrogen Canada (Burlington, ON, Canada). Mouse CytC ELISA kits were supplied by Reddot Biotech (Ottawa, ON, Canada). Aβ42 was purchased from California Peptide Research (Salt Lake City, UT, USA). All other reagents were from Thermofisher Scientific (Ottawa, ON, Canada).

3.2.2 Cell cultures models

The human glioma-derived astrocytic U118 MG cell line was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). U118 MG cells, which present with both glioblastoma and astrocytoma cell morphologies, were used as models of human astrocytes. The human neuroblastoma SH-SY5Y cell line was donated by Dr. R. Ross (Department of Biological Sciences, Fordham University, Bronx, NY, USA). The murine microglia BV-2 cell line was donated by Dr. G. Garden (Department of Neurology, University of Washington, Seattle, WA, USA). GFAP-positive and S100 calcium-binding protein B (S100B)-positive primary human astrocytes isolated from surgically resected temporal lobe tissues were obtained from the Kinsmen Laboratory of Neurological Research, the University of British Columbia (UBC), Canada. Normal tissue overlying epileptic foci was collected from four different subjects. The use of human brain materials was approved by the UBC Clinical Screening Committee for Human Subjects. Cells were cultured in DMEM-F12 supplemented

with 10% CBS, penicillin (100 U/ml) and streptomycin (100 µg/ml) in T-75 flasks incubated at 37 °C in humidified 5% CO_2 and 95% air atmosphere.

3.2.3 Secretion of inflammatory mediators by U118 MG cells and human astrocytes

Primary human astrocytes were plated in 96-well plates at a concentration of 2 x 10^5 cells/ml in 0.25 ml DMEM-F12 medium containing 5% CBS, penicillin (100 U/ml) and streptomycin (100 µg/ml) and incubated for 24 h to allow their adherence. Cells were incubated with mouse anti-TLR 4 antibodies (five µg/ml, clone HTA125, Ebioscience, San Diego, CA, USA), isotype control mouse monoclonal anti-superoxide dismutase (SOD) 1 antibody (10 µg/ml, clone 10D5, from Sigma Aldrich), TAK 242 (10 µM), or equivalent volumes of solvents (PBS or dimethyl sulfoxide). Following one h incubation, 50 µg/ml CytC was added. After 48 h incubation, cell-free supernatants were collected and stored at -20°C. IL-8 concentration in the supernatants was measured by the ELISA kit according to the instructions provided by its manufacturer (PeproTech). Concentrations of all other inflammatory mediators in supernatant samples were measured by Eve Technologies (Calgary, AB, Canada) using multiplex assays. Detection limits were reported to be 0.13 – 0.35 pg/ml.

U118 MG astrocytic cells were plated in 24-well plates at a concentration of 2 x 10^5 cells/ml in one ml of DMEM-F12 containing 5% CBS, penicillin (100 U/ml) and streptomycin (100 µg/ml) and incubated for 24 h to allow their adherence. Cells were incubated with 10 µg/ml mouse anti-TLR 4 antibodies or an equivalent volume of solvent (PBS). Following 30 min incubation, CytC (1-50 µg/ml) was added. Heat-inactivated CytC (50 µg/ml) was also used since thermal denaturation leads to irreversible structural changes in this protein (Liu and Konermann, 2009); it was prepared by incubating CytC for 45 min at 90 °C. After 48 h incubation, cell-free supernatants were collected to measure IL-1β and IL-8 concentrations by ELISA.

3.2.4 Measurement of TLR 4 expression

Human U118 MG astrocytic cells were plated in 96-well plates at 2 x 10^5 cells/ml in 0.25 ml DMEM-F12 medium containing 5% CBS, penicillin (100 U/ml) and streptomycin (100

μg/ml) and incubated for 24 h to allow their adherence. Cells were incubated for 48 h with: solvent (PBS); IL-1β (100 U/ml) plus IFN-γ (150 U/ml); CytC (50 μg/ml) alone; and CytC in combination with IL-1β and IFN-γ. Expression of TLR 4 on the surface of U118 MG cells was measured as described previously for other cell surface receptors (Józefowski et al., 2011; Klegeris et al., 2006). First, cells were fixed by air drying for 30 min. The plates were incubated at room temperature with 3% BSA in PBS for two h, washed three times with PBS, incubated with monoclonal anti-TLR 4 antibodies diluted 1:100 in 3% BSA in PBS for one h, washed three times with PBS, and then incubated with goat anti-mouse immunoglobulin G (IgG) alkaline phosphatase conjugate diluted 1:3000 in 3% BSA in PBS for one h. After washing three more times with PBS, the presence of surface TLR 4 in each of the wells was determined using a microplate reader. Optical densities at 405 nm were measured after incubating the wells for two h with 1 mg/ml Sigma 104 phosphate substrate in 0.1 M diethanolamine buffer, pH 9.8. Optical density signal in each well was normalized to protein concentration in the same well measured with a BCA assay used as described by the manufacturer (Thermofisher Scientific).

3.2.5 Cytotoxicity of U118 MG cells and human astrocytes towards SH-SY5Y neuronal cells

To study CytC-induced cytotoxicity of astrocytic cells, supernatant transfer experiments were performed as described in a previously published study (Klegeris et al., 2003). Briefly, U118 MG cells were plated as outlined in section 2.3. Following 24 h incubation, the cells were stimulated with different concentrations of CytC (1-50 μg/ml). After 48 h incubation, 0.4 ml of cell-free supernatant were transferred to each well containing SH-SY5Y cells that had been plated 24 h earlier at a concentration of 2 x 10^5 cells/ml in 0.4 ml of DMEM-F12 containing 5% CBS, penicillin (100 U/ml) and streptomycin (100 μg/ml). After 72 h incubation, neuronal cell viability was assessed by the MTT assay and cell death was assessed by the PI assay.

Primary human astrocytes were plated as described in section 2.3. Following 24 h incubation, the cells were stimulated with CytC (5 μg/ml). After 24 h incubation, 0.15 ml of cell-free supernatant was transferred to each well containing SH-SY5Y cells that had been plated 24 h earlier at a concentration of 2 x 10^5 cells/ml in 0.15 ml of DMEM-F12 containing 5% CBS,

penicillin (100 U/ml) and streptomycin (100 μg/ml). After 72 h incubation, neuronal cell viability was assessed by the MTT assay.

3.2.6 Direct toxicity of CytC to SH-SY5Y neuronal cells

When neuronal cells are exposed to supernatants from CytC-stimulated astrocytes, some of the CytC could be transferred onto SH-SY5Y cells possibly causing the toxic effects; therefore, the direct toxicity of extracellular CytC applied to the culture media of SH-SY5Y cells was assessed as follows. Neuronal cells were plated at 2 x 10^5 cells/ml in 0.4 ml DMEM-F12 medium containing 5% CBS, penicillin (100 U/ml) and streptomycin (100 μg/ml) and incubated for 24 h to allow their adherence. Cells were then exposed to varying concentrations of CytC (1-50 μg/ml) for 72 h and the MTT assay was used to measure neuronal cell viability.

3.2.7 Release of CytC into the extracellular space by human astrocytes and BV-2 murine microglia

Concentration of extracellular CytC in cell supernatants was measured using an ELISA as described previously for staurosporine-treated cells with modifications (Ahlemeyer et al., 2002). Murine BV-2 microglia and primary human astrocytes were plated in a 6-well plate at a concentration of 2 x 10^5 in one ml of serum-free DMEM-F12 and incubated for 24 h to allow their adherence. Cells were exposed to the following experimental conditions for eight and 24 h: 200 nM staurosporine or its vehicle solution (0.1% dimethyl sulfoxide); 25 μM Aβ42 or its vehicle solution (PBS); 250 μg/ml TNF-α or its vehicle solution (PBS). Cell-free supernatants were collected and concentrated approximately three-fold using centrifugal filters with a 10-kDa molecular weight cutoff and centrifugation (4000 x *g* for 10 min at room temperature).

3.2.8 ELISA

Concentrations of IL-8 and IL-1β in cell-free supernatants from U118 MG cells stimulated for 48 h with CytC were measured using PeproTech ELISA development kits according to the manufacturer's instructions. The detection limits for the IL-8 and IL-1β ELISAs

were experimentally determined to be between 20 and 80 pg/ml. Concentrations of CytC in cell-free supernatants from BV-2 murine microglia and primary human astrocytes treated with staurosporine, Aβ42, or TNF-α were measured using Reddot Biotech CytC ELISA kits according to the manufacturer's instructions. The detection limit for the CytC ELISA was experimentally determined to be 0.13 ng/ml.

3.2.9 MTT cell viability assay

Viability of the cells was monitored by the MTT assay as described previously (Hansen et al., 1989; Mosmann, 1983). This assay measures the reduction of water-soluble tetrazolium dye MTT to an insoluble purple formazan product by viable cells. MTT (0.5 mg/ml) was added to the cell cultures and the plates were incubated for one h at 37 °C in a CO_2 incubator. An equal volume of 20% w/v sodium dodecyl sulfate in 1:1 N,N-dimethylformamide:H_2O solution was then added to each well to solubilize the formazan crystals. The plates were incubated at 37 °C for three h and optical densities were measured at 570 nm using the FLUOstar Omega microplate reader (BMG Labtech, Nepean, ON, Canada).

3.2.10 Propidium iodide cell death assay

The percentage of dead cells was evaluated using a fluorescent microplate reader as described previously with modifications (Nieminen et al., 1992; Zhang et al., 1999). PI, which cannot penetrate membranes of live cells, was added to each well at 20 μg/ml. After 20 min incubation at 37ºC, the fluorescence intensity from the dead cells was measured by a microplate reader using 485 nm excitation and 625 nm emission filters.

3.2.11 Data analysis

Data obtained were analyzed using (1) the randomized block design one-way ANOVA, followed by the Dunnett's or Tukey's post-hoc test, or (2) the paired Student's t-test, followed by the Holm-Bonferroni correction for multiple comparisons. Data are presented as means ± SEM.

Significance was established at $P<0.05$. Detection limits were calculated using the following formula:

$$\boldsymbol{Detection\ limit} = \frac{[(\text{ODmean } of\ six\ sample\ blanks)+3*(standard\ deviation\ of\ ODmean\ comprising\ six\ sample\ blanks)]-b}{m}$$

Where:

ODmean = mean optical density

m = slope value obtained from the linear standard curve

b = intercept value with the optical density axis obtained from the linear standard curve

3.3 Results

3.3.1 CytC induces secretion of inflammatory mediators by human astrocytes

The concentrations of eight different inflammatory mediators in the supernatant of primary human astrocytes exposed to 50 μg/ml CytC for 48 h were measured and the data obtained are summarized in table 3.1 and figure 3.1. Human astrocytes exposed to CytC secreted significantly elevated concentrations of GM-CSF, IL-1β, and IL-12 p70 when compared to astrocytes exposed to PBS vehicle solution alone.

Table 3.1. Concentration of inflammatory mediators in the supernatants of primary human astrocytes.

Inflammatory mediators	Vehicle-treated astrocytes (pg/ml)	CytC-treated astrocytes (pg/ml)	P-values
GM-CSF	0.27 ± 0.05	0.85 ± 0.23	P<0.01 (**)
IL-1β	0.19 ± 0.04	0.53 ± 0.17	P<0.01 (**)
IL-12 p70	0.43 ± 0.02	0.93 ± 0.03	P<0.01 (**)
IFN-γ	0.33 ± 0.03	0.33 ± 0.03	P=0.4
IL-6	0.36 ± 0.02	0.36 ± 0.01	P=0.9
MCP-1	0.69 ± 0.13	2.05 ± 0.78	P=0.3
TNF-α	0.30 ± 0.01	0.30 ± 0.01	P=0.6

Concentrations of inflammatory mediators in the supernatants of primary human astrocytes exposed to CytC for 48 h were measured using multiplex analysis. Data (means ± SEM) from cells extracted from three different subjects are presented. ** P<0.01, different from CytC vehicle-treated cells, according to two-tailed paired the Student's t-test with the Holm-Bonferroni correction for multiple comparisons.

3.3.2 Anti-TLR 4 antibody inhibits CytC-induced secretion of inflammatory mediators by human astrocytes

We investigated TLR 4 as a possible receptor engaged by CytC leading to the increased secretion of inflammatory mediators by primary human astrocytes. TLRs have been implicated in mediating the immune responses of microglia and microglia-like cells to several different DAMP molecules, including mitochondrial TFAM (Schindler et al., 2018). In addition, extracellular CytC was shown to prime the respiratory burst of microglia-like cells by engaging TLR 4 (Gouveia et al., 2017). Astrocytes also express several different TLRs, including TLR 4 (Bsibsi et al., 2007). I demonstrated that pre-incubation of primary human astrocytes with anti-

TLR 4 antibodies (5 µg/ml) significantly inhibited their CytC (50 µg/ml)-induced secretion of GM-CSF (Fig. 3.1A) and IL-1β (Fig. 3.1B), but not IL-12 p70 (Fig. 3.1C). Anti-TLR 4 antibodies also reduced CytC-induced IL-8 secretion more than two fold, but this effect showed only a trend towards significance with the limited numbers of experiments performed (Fig. 3.1D). Anti-TLR 4 antibodies on their own did not affect the secretion of these four inflammatory mediators by astrocytes (Fig. S3.1 in Appendix).

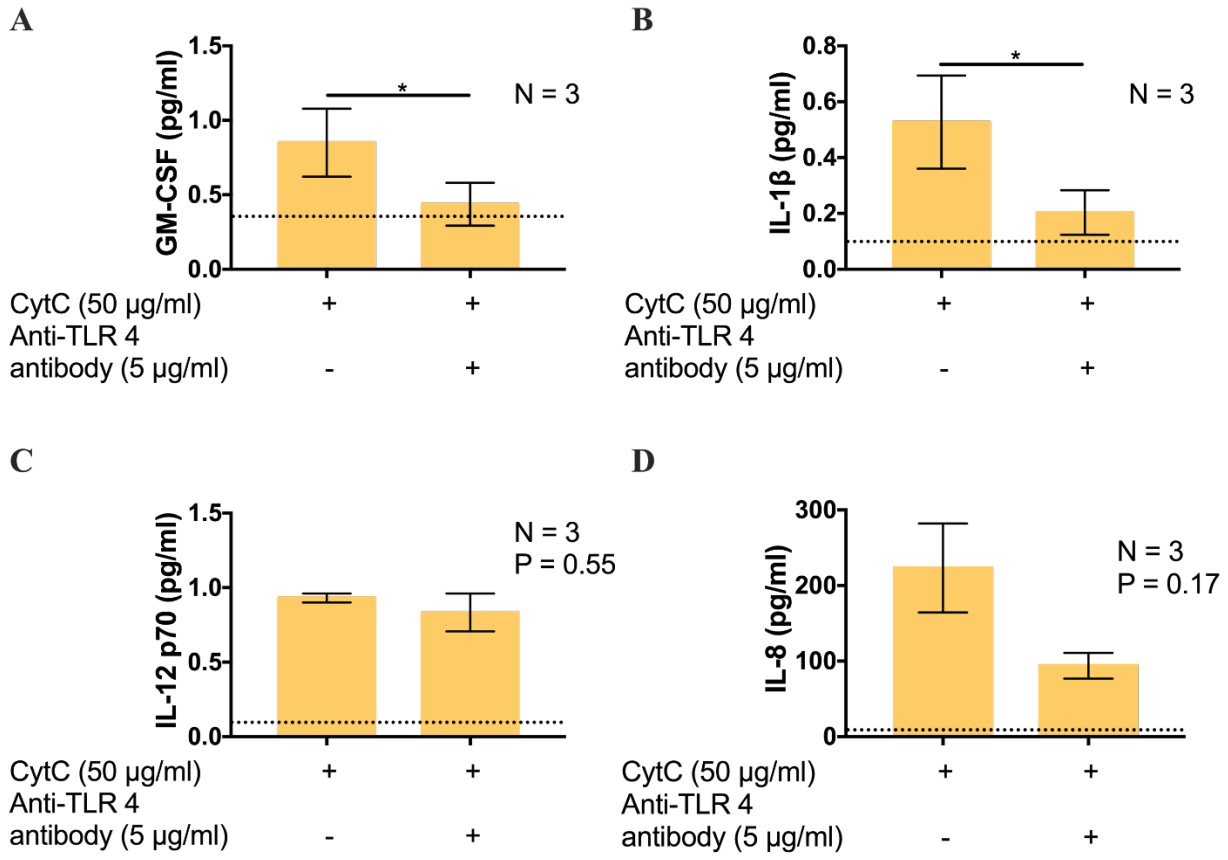


Figure 3.1. Anti-human TLR 4 antibodies inhibited CytC-induced upregulation of GM-CSF (**A**) and IL-1β (**B**), but not IL-12 p70 (**C**) or IL-8 (**D**) secretion by human primary astrocytes. Data (means ± SEM) from cells extracted from three different subjects are presented. * $P<0.05$ according to the paired Student's t-test with the Holm-Bonferroni correction for multiple comparisons. The detection limits of the ELISAs are shown as dotted lines.

3.3.3 CytC upregulates the secretion of IL-1β and IL-8 by U118 MG astrocytic cells

Next, I showed that exposure of human U118 MG astrocytic cells to extracellular CytC (1-50 μg/ml) for 48 h resulted in a significant increase of IL-1β (Fig. 3.2A) and IL-8 (Fig. 3.2B) concentrations in their supernatants compared to cells treated with the vehicle solution only. At the highest concentration tested, CytC induced an approximate three-fold increase in secretion of both these cytokines. To investigate whether the tertiary structure of CytC, including the heme group, was required for this biological activity, the experiment was repeated by using heat-inactivated CytC. Similar to the reported lack of effects on microglia-like cells (Gouveia et al., 2017), the exposure of U118 MG astrocytic cells to heated CytC (50 μg/ml) for 48 h did not induce secretion of IL-1β (Fig. 3.2A) or IL-8 (Fig. 3.2B). CytC (see Fig. 3.5C), or heated CytC (Fig. S3.2 in Appendix), at the concentrations used did not significantly affect the viability of U118 MG cells.

3.3.4 Anti-TLR 4 antibodies and TLR 4 signaling inhibitor TAK 242 block CytC-induced secretion of cytokines by U118 MG astrocytic cells

We also evaluated whether the CytC-induced secretion of IL-1β and IL-8 by U118 MG cells depended on TLR 4. Anti-TLR 4 antibodies (10 μg/ml) blocked CytC (50 μg/ml)-induced secretion of IL-1β (Fig. 3.2C) and IL-8 (Fig. 3.2D) by U118 MG cells. Anti-TLR 4 antibodies on their own did not affect the secretion of IL-1β (Fig. 3.2C) or IL-8 (Fig. 3.2D) by U118 MG cells. The anti-SOD 1 isotype control antibodies did not inhibit CytC-induced secretion of IL-1β by U118 MG cells (Fig. S3.3 in Appendix). In addition, I showed that the CytC-induced secretion of IL-1β was blocked by one h pre-incubation of U118 MG cells with 10 μM TAK 242 (Fig. 3.2E), which is a selective inhibitor of TLR 4 signaling (Hussey et al., 2012; Wei et al., 2016). At the concentrations used, the anti-TLR 4 or anti-SOD 1 antibodies, TAK 242, CytC, or their vehicle solutions did not affect the viability of U118 MG cells (Fig. S3.4 in Appendix).

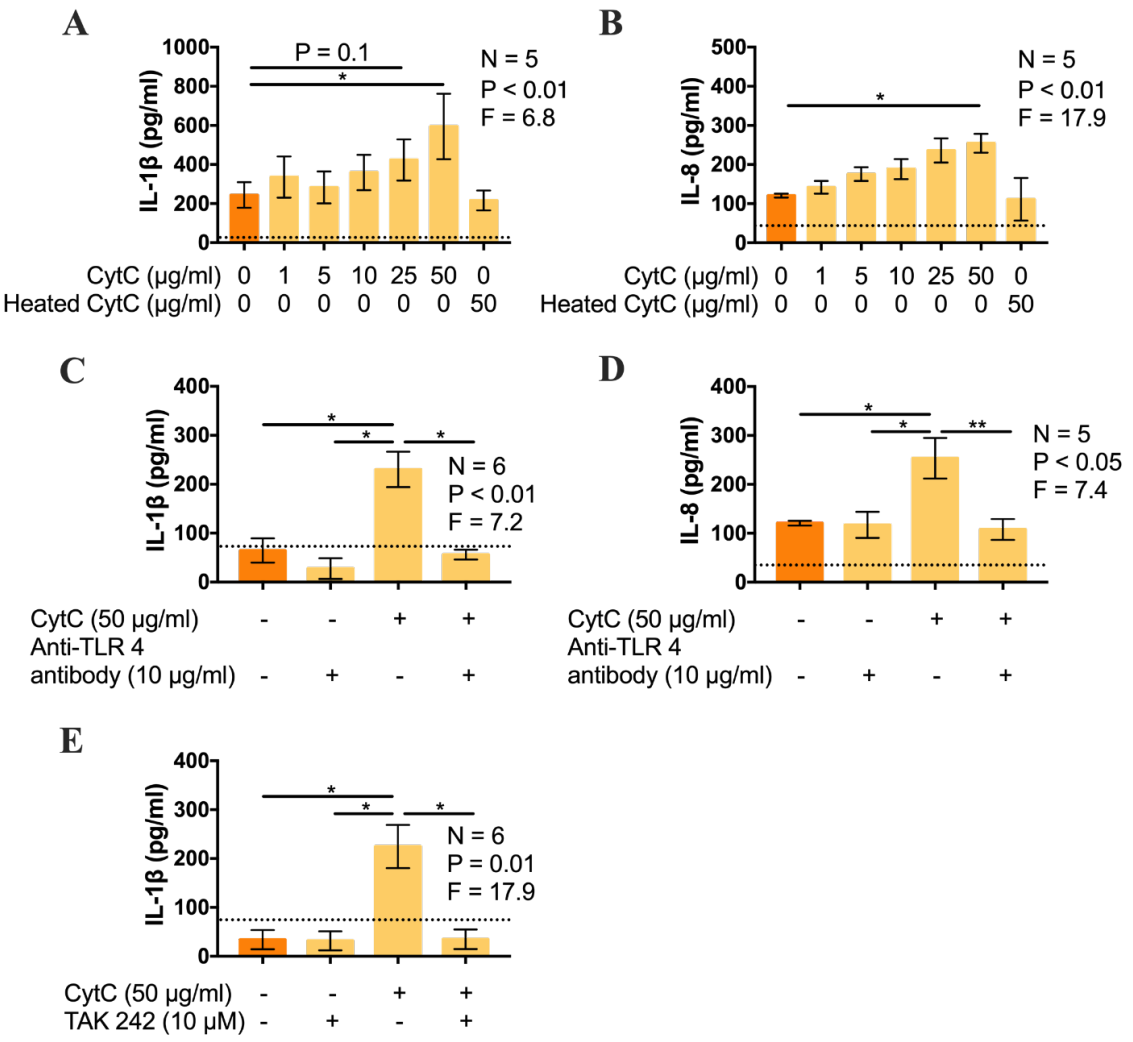


Figure 3.2. Extracellular CytC upregulated the secretion of IL-1β (**A**), and IL-8 (**B**) by human U118 MG astrocytic cells, while heated CytC applied at the highest concentration (50 µg/ml) was ineffective. Anti-human TLR 4 antibodies blocked CytC-induced secretion of IL-1β (**C**), and IL-8 (**D**) by U118 MG astrocytic cells. An inhibitor of TLR 4 signaling TAK 242 abolished CytC-induced secretion of IL-1β by U118 MG astrocytic cells (**E**). Anti-human TLR 4 antibodies, TAK 242, or the vehicle solution did not affect the concentration of IL-1β or IL-8 in supernatants of U118 MG astrocytic cells. Data (means ± SEM) from five to six independent experiments are presented. * P<0.05 according to the Dunnett's post-hoc test (**A,B**). * P<0.05, ** P<0.01 according to the Tukey's post-hoc test (**C-E**). P and F values for the one-way randomized blocks ANOVA are also shown, as well as the detection limit of the ELISAs as a dotted line.

3.3.5 CytC upregulates TLR 4 expression by U118 MG astrocytic cells

Immune stimuli acting through TLR 4 have been shown to upregulate expression of this receptor (Moser et al., 2016); therefore, I measured TLR 4 protein levels in U118 MG cells following their exposure to extracellular CytC. Figure 3.3 shows that TLR 4 levels were significantly higher in U118 MG cells exposed to extracellular CytC (50 µg/ml) compared to cells treated with the vehicle solution only. TLR 4 expression showed a trend towards further 1.5-fold increase when cells were co-stimulated with CytC, IFN-γ and IL-1β (Fig. 3.3).

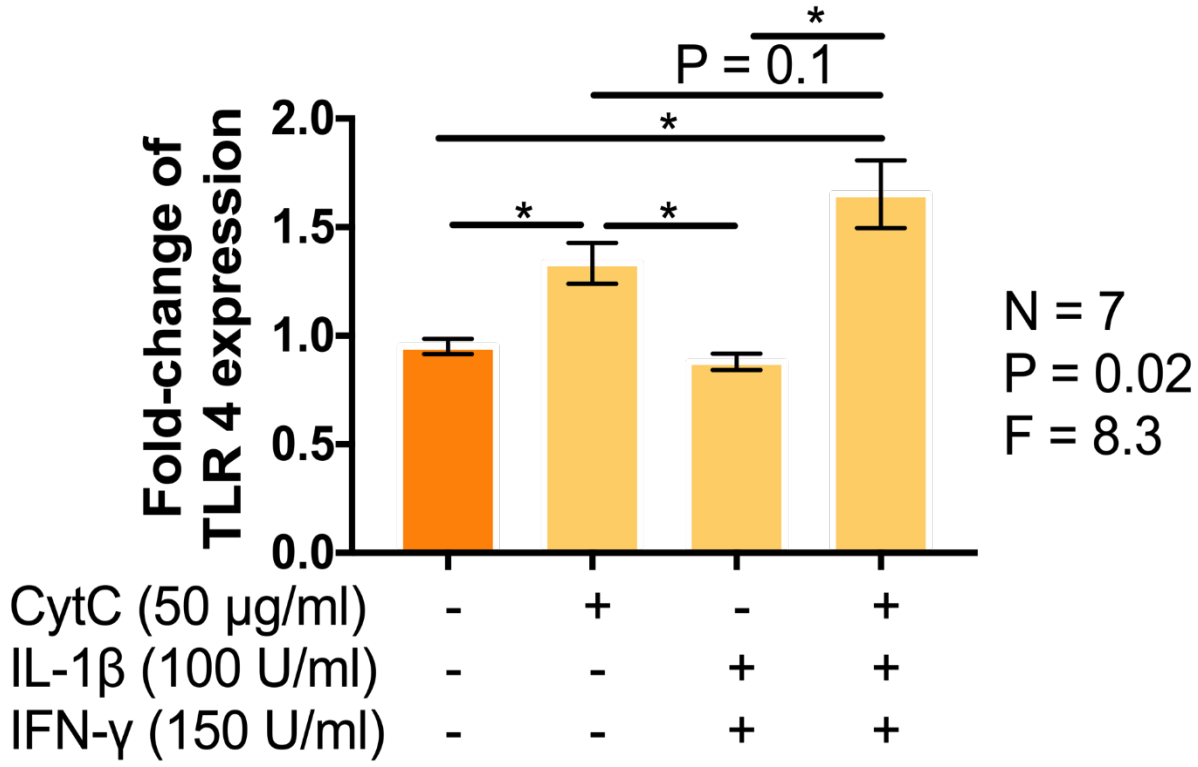


Figure 3.3. Extracellular CytC upregulated the cell surface expression of TLR 4 by human U118 MG astrocytic cells. CytC (50 µg/ml) alone and in combination with IFN-γ (150 U/ml) and IL-1β (100 U/ml) was added to U118 MG cell culture medium and TLR 4 levels were measured following 48 h incubation. Data (means ± SEM) from seven independent experiments are normalized to the protein concentration in each well. The results are expressed as the fold-change compared to cells incubated in the absence of vehicle solution or stimuli. * $P<0.05$ according to the Tukey's post-hoc test. P and F values for the one-way randomized blocks ANOVA are also shown.

3.3.6 CytC increases astrocyte-mediated cytotoxicity towards SH-SY5Y cells

When the brain is in a state of chronic neuroinflammation, as observed in AD, astrocytes can become adversely activated and continually release cytotoxic molecules (Bal-Price and Brown, 2001; Deshpande et al., 2005). These cytotoxins can cause damage or death to surrounding neurons (Hashioka et al., 2009; Kato and Svensson, 2015; Land, 2015). I examined whether extracellular CytC induces cytotoxicity of human astrocytes. I showed that supernatants from U118 MG cells that had been treated with CytC (1-50 μg/ml) reduced the viability of SH-SY5Y neuronal cells, measured by two different methods, in comparison to supernatants from U118 MG astrocytes exposed to vehicle alone (Fig. 3.4A,B). CytC had no significant effect on viability of U118 MG astrocytic cells (Fig. 3.4C). Since CytC could be transferred onto SH-SY5Y cells with the U118 MG cell supernatants, I tested the direct effects of extracellular CytC on SH-SY5Y viability. Incubation of neuronal cells with CytC (1-50 μg/ml) for 72 h had no significant effect on their viability (Fig. 3.4E) indicating that CytC induced secretion of toxins from U118 MG cells instead of being directly toxic to SH-SY5Y cells. Incubation of SH-SY5Y cells with supernatant from primary human astrocytes that had been exposed to CytC (5 μg/ml) led to a trend towards significant decrease in viability, compared to neuronal cells incubated with supernatants from primary astrocytes exposed to vehicle solution only (Fig. 3.4D).

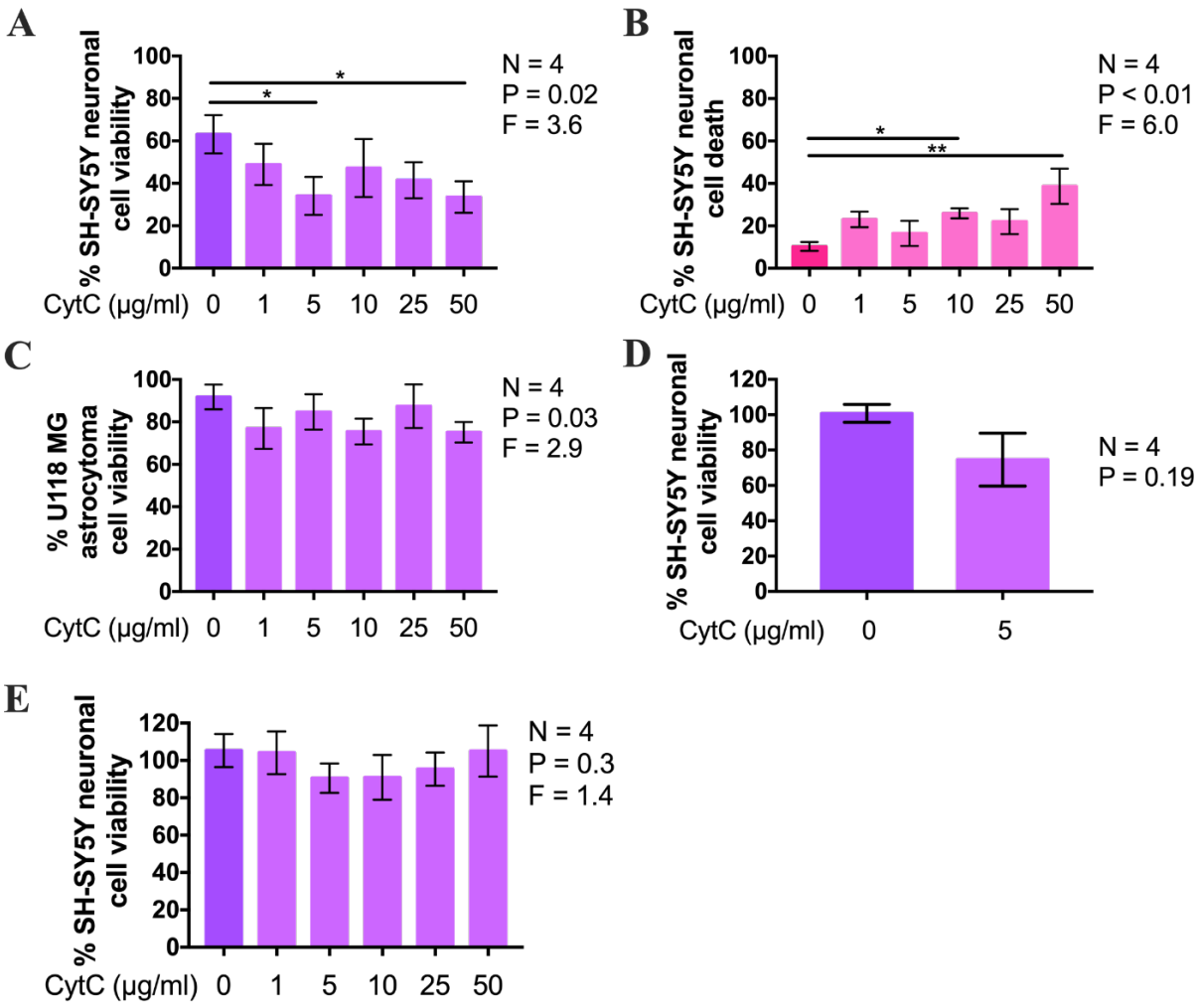


Figure 3.4. The supernatant from CytC-treated U118 MG astrocytic cells reduced viability of SH-SY5Y neuronal cells (**A**) as assessed by the MTT assay, and increased death of SH-SY5Y neuronal cells (**B**) as assessed by the PI assay following 48 h incubation. Viability of U118 MG astrocytic cells was not affected by CytC according to the MTT assay (**C**). Viability of SH-SY5Y neuronal cells treated with the supernatant from CytC-treated primary human astrocytes showed a trend towards significant decrease compared to SH-SY5Y neuronal cells treated with supernatant from unstimulated primary human astrocytes (**D**). Direct 72 h exposure of SH-SY5Y cells to CytC at the concentrations used to stimulate astrocytic cells did not reduce viability of neuronal cells (**E**). Data (means ± SEM) from four independent experiments are presented as percent values obtained from cells incubated in growth medium only (**A,C,D**). Cell death data are presented as percent values obtained from cells lysed in growth medium only (**B**). * P<0.05,

** P<0.01 according to the Dunnett's post-hoc test. P and F values for the one-way randomized blocks ANOVA are also shown.

3.3.7 CytC is released into the cell culture medium from human astrocytes and murine BV-2 microglia treated with staurosporine, Aβ42, or TNF-α

Staurosporine, Aβ42, and TNF-α have been used to induce death of cultured neurons (Ahlemeyer et al., 2002; Han et al., 2017; Neniskyte et al., 2014). Exposure of neurons to cytotoxic concentrations of staurosporine has been show to induce release of CytC into the extracellular space (Ahlemeyer et al., 2002). CytC was undetectable in supernatants from viable cultured astrocytes or BV-2 cells (Fig. 3.5 and 3.6). I demonstrated the presence of CytC in supernatants of primary human astrocytes after 24 h exposure to 200 nM staurosporine (Fig. 3.5A). Increased concentration of CytC in the supernatant of primary human astrocytes exposed to staurosporine was accompanied by a significant decrease in cell viability compared to primary human astrocytes treated with staurosporine vehicle solution (Fig. 3.5B). CytC was not released from primary astrocytes incubated for 24 h in the presence of staurosporine vehicle solution (Fig. 3.5A).

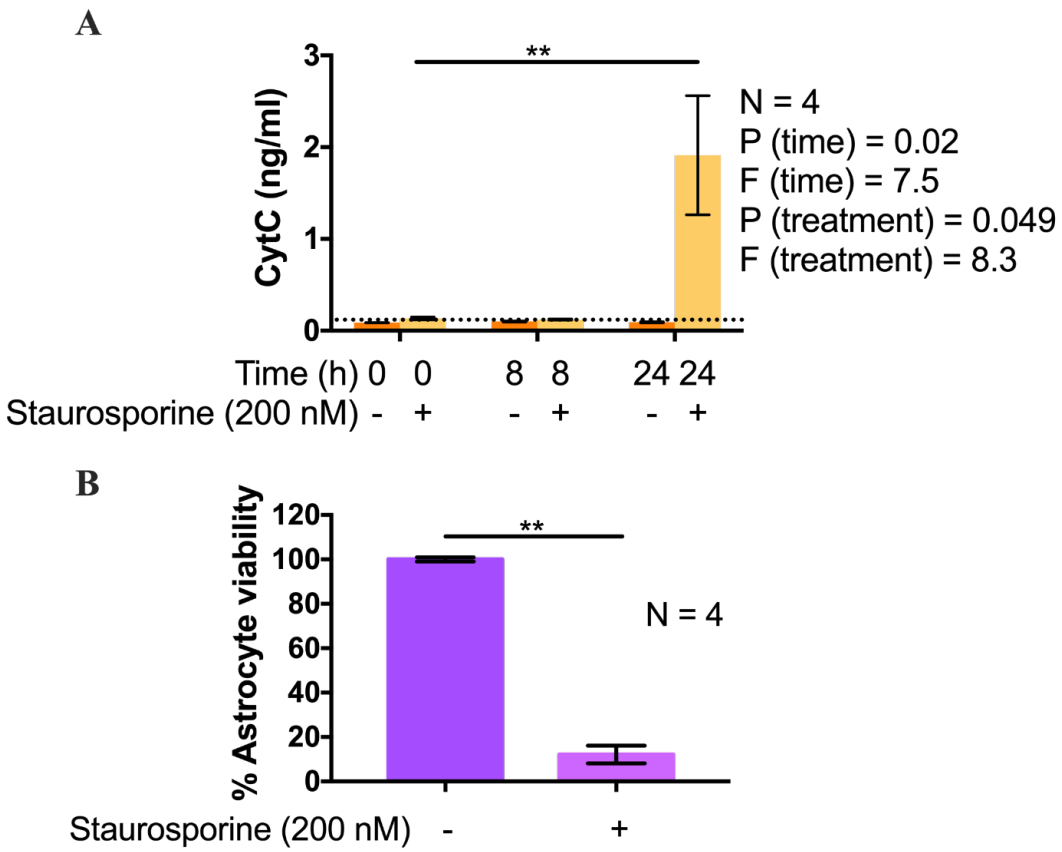


Figure 3.5. CytC was detected in culture medium from primary human astrocytes exposed to staurosporine, but not from untreated cells (**A**). Cell viability of primary human astrocytes exposed to staurosporine decreased after 24 h incubation (**B**). Data (means ± SEM) from cells extracted from four different subjects are presented. Cell viability is expressed as a percentage of MTT absorbance from primary human astrocytes not treated with staurosporine or its vehicle (**B**). ** $P<0.01$ according to the Dunnett's post-hoc test (**A**), or paired Student's t-test (**B**). P and F values for the two-way randomized blocks ANOVA are also shown, as well as the detection limit of the ELISA as a dotted line (**A**).

To determine if other glial cell types could be a source of extracellular CytC, ELISA was used to quantify CytC in the supernatants of murine BV-2 microglia. After a 24 h incubation period, there was a significant increase in the CytC concentration in the supernatants of BV-2 cells exposed to staurosporine (200 nM), Aβ42 (25 μM), or TNF-α (250 μg/ml) (Fig. 3.6A,C,D). CytC became detectable in the supernatants from Aβ42- and TNF-α-treated BV-2 cells after eight h incubation. Increased concentrations of CytC in the supernatant of BV-2 cells exposed to

staurosporine or Aβ42 were accompanied by a significant decrease in cell viability, compared to BV-2 microglia treated with the corresponding vehicle solutions (Fig. 3.6B,E). Decrease in viability of BV-2 cells exposed to TNF-α showed only a trend towards significance (Fig. 3.6E). Incubation of BV-2 cells for 24 h in the presence of staurosporine vehicle solution did not cause release of CytC into cell supernatants (Fig. 3.6A). Similarly, CytC was not detected in the supernatants of BV-2 cells exposed to the vehicle solution of TNF-α or Aβ42 (Fig. S3.5 in Appendix).

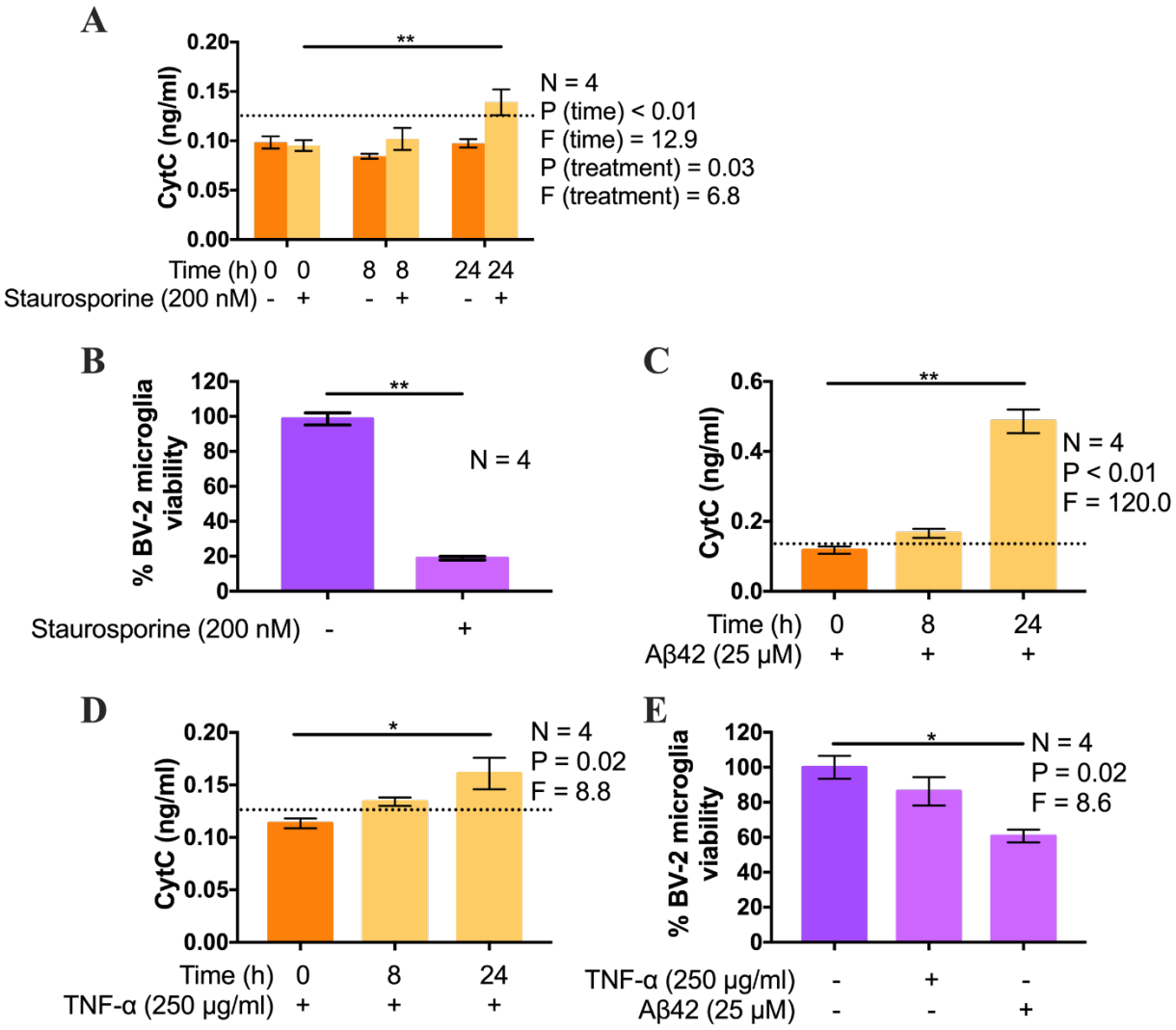


Figure 3.6. Concentrations of CytC were significantly increased in the supernatant of BV-2 microglia exposed to staurosporine (**A**), Aβ42 (**C**), or TNF-α (**D**). Viability of BV-2 cells exposed to staurosporine was decreased compared to cells exposed to the staurosporine vehicle

only (**B**). Viability of BV-2 cells exposed to Aβ42 was decreased compared to cells exposed to the vehicle of Aβ42 only (**E**). Data (means ± SEM) from four independent experiments are presented. Cell viability is expressed as a percentage of MTT absorbance from cells incubated in growth medium only (**B,E**). * $P<0.05$, ** $P<0.01$ according to the Dunnett's post-hoc test (**A,C-E**), or paired Student's t-test (**B**). P and F values for the two-way (**A**), or one-way randomized blocks ANOVA are also shown (**C-E**).

3.4 Chapter discussion

Extracellular CytC has been identified as a clinical marker for several pathologies. For example, urine or sera concentrations of CytC increase after traumatic brain injury, cancer treatment, encephalopathy, and kidney injury (Barczyk et al., 2005; Beachy and Repasky, 2008; Hosoya et al., 2006; Radhakrishnan et al., 2007; Satchell et al., 2005; Zager et al., 2004). CytC is released into the extracellular space from cells undergoing apoptosis (Ahlemeyer et al., 2002; Jemmerson et al., 2002). Previous studies have shown that extracellular CytC can modulate functions of several different cells, including splenocytes, monocytic cells and microglia (Gouveia et al., 2017; Pullerits et al., 2004). In this study, I extend the list of cell types potentially affected by extracellular CytC by demonstrating that adding CytC to tissue culture medium significantly upregulated secretion of GM-CSF, IL-1β, and IL-12 p70 by human primary astrocytes. Secretion of four other inflammatory mediators, including MCP-1, was not affected by CytC. For this experiment, I selected a subset of inflammatory mediators, which have been previously reported to be secreted by resting or activated primary human astrocytes (Choi et al., 2014; Weinberg et al., 2018). The CytC-induced increase in the secretion of GM-CSF and IL-1β by primary astrocytes was blocked by anti-TLR 4 antibodies. These data are similar to the previously reported TLR 4-mediated effects of extracellular CytC on microglia (Gouveia et al., 2017). It is interesting to note that, in this previous study, CytC also did not induce secretion of MCP-1 by microglia-like cells, while the secretion of other inflammatory mediators was not measured. Upregulated secretion of inflammatory mediators by astrocytes in response to TLR agonists has been reported before; for example, primary murine astrocytes cultures, purified with L-leucyl-L-leucine methyl ester (LLME) to remove microglia contamination, secreted five-fold higher levels of IL-1β when treated with the TLR 4 agonist LPS, TLR 2 agonist zymosan, or

TLR 3 agonist polyinosinic-polycytidylic acid (poly(I:C)) (Marinelli et al., 2015). LPS and poly(I:C) increased the gene expression of GM-CSF and IL-8 by primary human astrocytes (Bsibsi et al., 2006). Furthermore, LPS increased the secretion of IL-1β and IL-8 by primary human astrocytes (Bsibsi et al., 2007), which corresponds to our data obtained by using CytC as a stimulus.

Differential upregulation of select inflammatory mediators similar to the data obtained in this study has been reported before for a different immune stimulus. Choi *et al*. (2014) demonstrated that the combination of IL-1β and TNF-α significantly upregulated secretion of GM-CSF, IL-8 and IL-1β, but not IL-12 p70, by primary human astrocytes. Similarly, Weinberg *et al*. (2018) demonstrated that IL-1β significantly upregulated secretion of GM-CSF, IL-8, but not IL-12 p70, by primary human astrocytes. Altogether, the CytC-induced profile of inflammatory mediator secretion observed in this study is consistent with the previously reported profiles for primary human and murine astrocytes treated with TLR 4 agonists. Interestingly, anti-TLR 4 antibodies did not significantly reduce CytC-induced secretion of IL-12 p70, and their effect showed only a trend towards significance on IL-8 secretion by primary human astrocytes. This observation may indicate that CytC binds to another astrocyte receptor that initiates the secretion of IL-12 p70. For example, TLR 2 and TLR 9 have both been shown to modulate IL-12 p70 secretion by primary murine microglia and other cell types (Holley et al., 2012; Liew et al., 2005; Martin et al., 2008). However, it is important to note that the activation of TLR 4 is usually accompanied by an increased secretion of IL-12 p70 in other cell types, such as macrophages and dendritic cells (Kim and Chung, 2012; Krummen et al., 2010).

In addition to the experiments with primary human astrocytes, I confirmed the interaction of CytC with the astrocyte TLR 4 by using human U118 MG astrocytic cells, which were induced by extracellular CytC to secrete IL-1β and IL-8. Pre-incubation of these cells with anti-TLR 4 antibodies led to reduced CytC-induced secretion of IL-1β and IL-8. Isotype control antibodies recognizing SOD 1 were ineffective. I also demonstrated that CytC-induced secretion of IL-1β by U118 MG cells was inhibited by a selective inhibitor of TLR 4 signaling (Hussey et al., 2012; Wei et al., 2016). Upregulated expression of this receptor in U118 MG cells exposed to extracellular CytC, as well as a combination of CytC with IFN-γ and IL-1β, provided additional

experimental evidence indicating TLR 4 involvement. The latter two cytokines were selected since they are known to induce a reactive phenotype of astrocytes (Blasko et al., 2000; Falsig et al., 2004). Ligands that bind to specific TLRs, such as LPS, zymosan, and poly(I:C), have been shown to modulate the mRNA expression of TLRs in primary astrocytes and microglia (Bsibsi et al., 2006; El-Hage et al., 2011; Marinelli et al., 2015). Similar to our observations with extracellular CytC, poly(I:C) and LPS have been shown to upregulate the expression of TLR 3, TLR 2, and to a lesser extent, TLR 4 by primary human astrocytes (Bsibsi et al., 2006). Overall, our studies with primary human astrocytes and U118 MG astrocytic cells indicate that CytC, once released into the extracellular space from damaged cells, can induce in a TLR 4-dependent manner the secretion of a subset of inflammatory mediators by astrocytes. This interaction between extracellular CytC and astrocytes could contribute to the neuroinflammation associated with neurodegenerative disorders, such as AD.

Previous studies have shown that the supernatant from human astrocytes activated by inflammatory stimuli can cause neuronal death (Hashioka et al., 2009). Here I demonstrated that, similar to its effects on microglia (Gouveia et al., 2017), extracellular CytC induced the secretion of cytotoxins by U118 MG astrocytic cells causing death of human SH-SY5Y neuronal cells. Furthermore, exposure of neuronal cells to supernatants from CytC-stimulated primary human astrocytes showed a trend towards reduced viability with the limited number of observations made. These findings are in line with previous studies, which found that extracellular CytC causes death to nearby neurons (Ahlemeyer et al., 2002). This is consistent with the hypothesis that CytC can act similar to other DAMPs, which are known to regulate neurotoxicity and have been implicated in the pathogenesis of neurodegenerative disorders (Kato and Svensson, 2015; Land, 2015; Pointer et al., 2019). While the exact cytotoxins astrocytes release are unknown, it is likely a mixture of toxins is secreted, the composition of which depends on the nature of stimuli astrocytes encounter (Bsibsi et al., 2006; Choi et al., 2014). It is important to note that in our *in vitro* assays, measuring secretion of cytokines and cytotoxins by human astrocytes and astrocytic U118 MG cells, 5-50 µg/ml CytC was required to induce significant effects. This concentration range was selected based on a previously published study demonstrating modulation of microglia functions by 50 µg/ml CytC (Gouveia et al., 2017). This is considerably higher than the concentrations of CytC measured in supernatants of injured glial cells (up to 2 ng/ml in this

study) or neurons (~ 25 ng/ml) (Ahlemeyer et al., 2002), and cerebrospinal fluids following traumatic brain injury in children (~1 ng/ml) (Au et al., 2012). The highest level of CytC *in vivo* has been shown in human blood plasma following cardiac arrest (5 μg/ml) (Radhakrishnan et al., 2007). It is plausible that local concentration of CytC in the proximity of damaged cells could be much higher than its concentration in circulation; such a difference in concentration has been reported previously for ATP, which is another molecule released from injured cells (Beigi et al., 1999). For example, Beigi *et al.* (1999) demonstrated that ATP concentrations near the cell surface of injured cells were approximately ten-fold higher than levels detected in their cell culture supernatant. Thus, since 5 μg/ml of CytC was detected in human blood plasma following cardiac arrest by Radhakrishnan *et al.* (2007), it is plausible CytC could exist near damaged or dying cells at concentrations comparable to the effective concentration used in this study.

Our data identify TLR 4 as a possible molecular target to reduce neuroinflammation and neurotoxicity associated with neurodegenerative diseases, ischemia and traumatic brain injury (Akhter et al., 2015; Caso et al., 2007; Go et al., 2016; Walter et al., 2007). All these pathological states are characterized by excessive cell death, which could lead to increased concentrations of extracellular CytC, and consequently increased cytokine and cytotoxin secretion by glial cells. Thus, a self-perpetuating cycle of increased release of CytC, cytotoxins and associated neurotoxicity could be initiated (Gouveia et al., 2017). CytC has already been shown to be released into the cell culture medium from damaged chick neurons (Ahlemeyer et al., 2002). In this study, I demonstrated that injuring murine BV-2 microglia by three different toxic treatments, including Aβ42, induced release of CytC into the cell culture medium. CytC was also detected in supernatants from damaged human astrocytes. This is in line with a previous study which showed Aβ42 induced the release of CytC from isolated murine brain mitochondria (Kim et al., 2002). Thus, our observations identify damaged glial cells as a potential source of extracellular CytC in the CNS, especially since degenerating microglia and astrocytes have been reported in AD (Kitamura et al., 1999; Nagele et al., 2004; Navarro et al., 2018; Verkhratsky et al., 2010).

Only limited information about the cellular mechanisms mediating the extracellular release of CytC is available. Renz *et al.* (2001) suggested that unknown apoptotic processes,

rather than non-specific leakage, lead to the extracellular release of CytC from Jurkat T-cells. They based this conclusion on the observations that staurosporine, anti-CD 95 antibodies, etoposide, doxorubicin, and TNF-α plus actinomycin D induced apoptosis as well as extracellular release of CytC, while the treatments that specifically led to necrosis, such as hydrogen peroxide or a cocktail of TNF-α, actinomycin D plus carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]-fluoromethylketone (ZVAD-FMK), did not cause the release of CytC into the extracellular space (Renz et al., 2001). Furthermore, lactate dehydrogenase, an intracellular enzyme that leaks from damaged cells, was released into the extracellular space more than six hours after CytC was detected in cell culture medium (Renz et al., 2001). Interestingly, I observed that Aβ42-exposed BV-2 microglia released higher levels of CytC than the same cell type exposed to staurosporine or TNF-α even though cell viability of cells was affected less by Aβ42 compared to staurosporine treatment. It is possible that Aβ42 caused more CytC to be released from BV-2 microglia by inducing a different cell death pathway than staurosporine or TNF-α. To test this hypothesis, these experiments should be extended by measuring expression of proteins associated with a variety of cell death pathways, including caspases 1, 3, 8 and assembly of the NLRP3 (nucleotide-binding domain, leucine-rich repeat family pyrin domain containing 3) inflammasome. The extracellular release of CytC may involve an unconventional protein secretion pathway not requiring vesicular movement, which has been shown to mediate the release of IL-1β and fibroblast growth factor 2 by peripheral macrophages (Rabouille, 2017; Renz et al., 2001; Rubartelli et al., 1990). It is possible that such an unconventional protein secretion pathway used by CytC involves the ATP-binding cassette (ABC) transporter A1, which has been shown to be associated with the release of CytC from the mitochondria of primary mouse colon cells (Smith and Land, 2012). More importantly, the ABC transporter A1 is expressed by primary human monocytes and glial cells (Linder et al., 2009; Uhlén et al., 2015); therefore, these proteins may also cause the rapid extracellular release of CytC from astrocytes undergoing apoptosis, but this hypothesis requires experimental proof.

3.5 Chapter conclusion

While several different intracellular functions of CytC are well described, only limited studies have explored the effects of extracellular CytC on the functions of glia. Here I show that

extracellular CytC can modulate secretion of inflammatory mediators by astrocytes in a TLR 4-dependent manner. Additionally, I show that exposure of astrocytes to extracellular CytC induces their toxicity towards neuronal cells. I also identify damaged astrocytes and microglia as a potential source of extracellular CytC. Therefore, this study (1) highlights CytC as a novel DAMP of the CNS that could contribute to neurodegeneration after it is released into the extracellular space from damaged glial cells, in addition to neurons, and (2) provides additional evidence supporting TLR 4 as a possible therapeutic target to treat the neuroinflammation associated with neurodegeneration. Confirmation of the extracellular effects of CytC *in vivo* is the next required step.

Chapter 4: Short-chain fatty acids (SCFAs) alone or in combination regulate select immune functions of microglia-like cells

4.1 Chapter introduction

Neuroinflammation contributes to the pathogenesis of neurodegenerative disorders, including AD (McGeer and McGeer, 2013; Newcombe et al., 2018). Accumulation of pathology-associated molecules, such as Aβ, may initiate neuroinflammatory responses in AD. For example, Aβ can activate microglia, the innate immune cells of the brain, upregulating their production of cytotoxins and inflammatory mediators (Malm et al., 2015; Yu and Ye, 2015). Secreted inflammatory cytokines, such as TNF-α and IL-1β, can further activate nearby microglia, causing positive feedback leading to excessive release of inflammatory mediators and damage of surrounding brain cells, including neurons (Brown and Vilalta, 2015; Prinz et al., 2019). Identifying endogenous mechanisms that reduce the persistent activation of microglia may lead to the discovery of new therapeutic targets to treat neurodegenerative diseases, like AD.

Recent studies indicate that healthy gut microbiota play an important role in downregulating systemic and brain inflammation (Ghosh et al., 2020; Schirmer et al., 2016; Syeda et al., 2018). SCFAs are among the many metabolites released by gut microbes. Under physiological conditions of the host, SCFAs are continuously released by gut microbes (den Besten et al., 2013), but under pathological conditions there are less SCFA-producing bacteria in the gut and thus abnormal levels of circulating SCFAs (Liu et al., 2019). Once released from gut microbes, SCFAs enter the circulatory system, which allows them to act systemically. Previous studies demonstrate that select SCFAs activate FFAR 2 and 3 (Brown et al., 2003). These receptors are expressed by human and murine peripheral immune cells, including eosinophils, neutrophils, and monocytes (Alvarez-curto and Milligan, 2016; Uhlén et al., 2015). In the human CNS, FFAR 2 and 3 are expressed by both glia and neurons (Uhlén et al., 2015). The anti-inflammatory properties of certain SCFAs, notably acetate, propionate, and butyrate, have been studied extensively in the periphery (Ji et al., 2016; Nastasi et al., 2015; Tan et al., 2014), with only limited data available about their effects in the brain (Huuskonen et al., 2004). CNS effects

of SCFAs have begun to emerge since some of them can cross the BBB aided by monocarboxylate transporters and interact, for example, with microglia (Dalile et al., 2019).

Effects of SCFAs on microglia have been studied before with inconsistent outcomes. For example, butyrate reduced the secretion of IL-6 and TNF-α by LPS-stimulated primary murine microglia, but increased the secretion of IL-6 by LPS-stimulated N9 microglia (Huuskonen et al., 2004). The same study demonstrated that propionate and butyrate, but not valerate, upregulated the secretion of nitric oxide by LPS-stimulated N9 microglia. SCFAs have also been shown to inhibit inflammatory cytokine secretion by other types of human monocytic cells. For example, acetate, propionate, and butyrate, but not formate, decreased secretion of TNF-α and IFN-γ by LPS-stimulated human peripheral blood mononuclear cells (Cox et al., 2009). All four of these SCFAs decreased the secretion of MCP-1 by LPS-stimulated primary human monocytes, but only acetate, propionate, and butyrate decreased their secretion of IL-10 (Cox et al., 2009). Effects of SCFAs on neutrophils have also been reported. High concentrations of butyrate, acetate, and propionate decreased the phagocytic activity, as well as production of ROS, by bovine polymorphonucleocytes treated with opsonized zymosan or phorbol 12-myristate 13-acetate, respectively (Mills et al., 2006).

To date, the majority of studies on the anti-inflammatory properties of SCFAs have used them at concentrations that are much higher than those measured in human sera. Therefore, the results of these studies may not reflect the physiological effects of SCFAs in the brain, where the concentration of SCFAs would likely be lower than serum levels. I hypothesized that SCFAs act synergistically at physiological serum concentrations as anti-inflammatory mediators. Even though the majority of previous studies only use the most abundant SCFAs, namely acetate, butyrate, and propionate, I chose to also include formate and valerate as additional key SCFA metabolites of gut microbes (Macfarlane and Macfarlane, 2003; Morrison and Preston, 2016). Individual SCFAs were added to cell culture medium on their own or as a mixture consisting of the above five SCFAs in a 16:8:8:1:1 ratio. This ratio, as well as the 5 – 500 μM initial concentration range used in our study, were selected based on previously published physiological serum concentrations of SCFAs (Hanzlik et al., 2005; Ktsoyan et al., 2016; Zhao et al., 2007). Due to the previously-reported interspecies differences in microglia responses (Bhattacherjee et

al., 2019; Davis et al., 2018; Du et al., 2017), I used two different human cell lines to model select immune functions of human microglia (Klegeris et al., 2005; Pointer et al., 2019). I demonstrate that the SCFA mixture as well as individual SCFAs decreased the release of IL-1β, TNF-α, MCP-1, and cytotoxins by stimulated microglia-like cells.

4.2 Materials and methods

4.2.1 Reagents

MTT, N,N-dimethylformamide, fMLP, bisBenzimide (Hoechst 33258), luminol sodium salt, LPS, PBS, phosphatase substrate tablets, and PI were obtained from Sigma Aldrich (Oakville, ON, Canada). Human recombinant IFN-γ as well as ELISA development kits for MCP-1, IL-1β and TNF-α were purchased from PeproTech (Embrun, ON, Canada). Sodium butyrate and sodium acetate were purchased from Cayman Chemicals (Ann Arbor, MI, USA). Sodium propionate and sodium formate were purchased from Alfa Aesar (Ward Hill, MA, USA). Sodium valerate was purchased from Bide Pharmatech (Yangpu, Shanghai, China). GLPG 0974, a human FFAR 2 and 3 antagonist, was purchased from Tocris (Oakville, ON, Canada). FITC externally-labelled one μm fluorescent polystyrene latex beads were purchased from Bangs Laboratories (Fishers, IN, USA). Texas red fluorophore-conjugated MemBrite Fix membrane stain was purchased from Biotium (Fremont, CA, USA). All other reagents were from Thermofisher Scientific (Ottawa, ON, Canada).

4.2.2 Cell culture models

Human THP-1 monocytic and HL-60 myelomonocytic cell lines, which express FFAR 2 (Uhlén et al., 2015), were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). They were used to model select functions of human microglia (Klegeris et al., 2005; Pointer et al., 2019). The human neuroblastoma SH-SY5Y cell line was donated by Dr. R. Ross (Fordham University, Bronx, NY, USA). Cells were cultured in DMEM-F12 supplemented with 10% CBS, penicillin (100 U/ml) and streptomycin (100 μg/ml) in T-75 flasks incubated at 37 °C in humidified 5% CO_2 and 95% air atmosphere.

4.2.3 Secretion of inflammatory mediators and cytotoxins by human THP-1 microglia-like cells

The experiments were performed as described in a previously published study (Pointer et al., 2019), with minor modifications. THP-1 microglia-like cells were plated in 24-well plates at a concentration of 2 x 10^5 cells/ml in one ml DMEM-F12 containing 5% CBS, penicillin (100 U/ml) and streptomycin (100 µg/ml). One well was used for each experimental condition. SCFA mixture, individual SCFAs on their own, or their vehicle solution (H_2O) were added to culture media for 15 min. In select experiments, cells were incubated for 30 min with GLPG 0974 (0.1 µM) or an equivalent volume of solvent (ethanol) before SCFAs were added. Cells were stimulated with LPS (20 ng/ml) plus IFN-γ (100 U/ml) and cell-free supernatants were collected 48 h later. The following SCFAs were used: acetate, propionate, butyrate, valerate, and formate; they were added to cells individually or as a mixture in a 16:8:8:1:1 ratio.

Concentrations of IL-1β, MCP-1, and TNF-α in THP-1 cell supernatants were measured using PeproTech ELISA development kits according to the manufacturer's instructions. To study the effect of SCFAs on secretion of cytotoxins by microglia, 0.4 ml of the collected THP-1 supernatants were transferred to each well containing SH-SY5Y neuronal cells that had been plated 24 h earlier at a concentration of 3 x 10^5 cells/ml in 0.4 ml of DMEM-F12 containing 5% CBS, penicillin (100 U/ml) and streptomycin (100 µg/ml). After 72 h incubation, neuronal cell viability was assessed by the MTT assay and cell death was assessed by the PI assay. Viability of THP-1 cells at the end of the 48 h incubation period with SCFAs and stimuli was also assessed by the MTT and PI assays.

When neuronal cells are exposed to supernatants from SCFA-treated THP-1 monocytic cells, some of the SCFAs could be transferred onto SH-SY5Y cells possibly affecting their viability; therefore, a control experiment was performed where SH-SY5Y cells were plated at 3 x 10^5 cells/ml in 0.4 ml DMEM-F12 medium containing 5% CBS, penicillin (100 U/ml) and streptomycin (100 µg/ml). One well was used for each experimental condition. After 24 h incubation to allow their adherence, SH-SY5Y cells were exposed to varying concentrations SCFAs or the SCFA mixture. Neuronal cell viability was assessed by the MTT and PI assays 72 h later.

4.2.4 The respiratory burst activity of human HL-60 cells

Differentiated human HL-60 cells were used as model cells to assess the effect of SCFAs on the respiratory burst of phagocytes as described previously (Pointer et al., 2019). Cells were primed with LPS for 24 h and then stimulated with bacterial peptide fMLP to induce the release of ROS, which reacted with luminol added to the cell culture medium to produce chemiluminescence (CHL). HL-60 cells were differentiated by incubation with 1.3% dimethyl sulfoxide for five days. Differentiated HL-60 cells were seeded in 24-well plates at a concentration of 1 x 10^6 cells/ml in one ml phenol red-free DMEM-F12 supplemented with 2% CBS. One well was used for each experimental condition. Cells were treated with formate (0.015-1 mM), valerate (0.015-1 mM) or their vehicle solution, followed by the priming agent (500 ng/ml LPS). After 24 h incubation, cells were washed, transferred to 96-well plates, luminol (0.85 mg/ml) and fMLP (1.2 µM) added, and FLUOstar Omega plater reader and the accompanying Mars Analysis software (BMG Labtech, Guelph, ON, Canada) were used to measure CHL of each well over a nine min period.

4.2.5 Phagocytic activity of human THP-1 microglia-like cells

Phagocytosis was assessed as described in a previously published study (Ohgidani et al., 2014), with some modifications. THP-1 microglia-like cells were plated in poly-D-lysine-coated, 4-chambered petri dishes at 1 x 10^6 cells/ml in 0.5 ml DMEM-F12 medium containing 5% CBS, penicillin (100 U/ml) and streptomycin (100 µg/ml). One chamber was used for each experimental condition. Cells were incubated for 24 h with valerate (1 mM) or formate (1 mM) in the presence or absence of LPS (200 ng/ml) plus IFN-γ (150 U/ml). One µm diameter externally FITC-labelled latex beads were added in a 10:1 bead:cell ratio. After one h incubation, light from non-internalized beads was quenched by adding 0.05% trypan blue to the culture medium. Supernatant, trypan blue, and beads were removed from all wells by washing two times with one ml PBS. 400 µl of 70% ethanol were added to all wells for five min, and the wells were washed two times with one ml PBS. 300 µl of one µg/ml Texas red membrane stain were added to all wells for five min followed by a single washing step with one ml PBS. Prior to imaging, bisBenzimide (2 µg/ml) nuclear stain was added to all wells in 0.5 ml PBS.

Cells were visualized using a Zeiss AxioObserver.Z1 widefield epifluorescence microscope with Zen image acquisition software at an excitation/emission of 350/470 nm for bisBenzimide, 470/520 nm for the fluorescent beads, and 594/615 nm for Texas red membrane stain. Images of 30-50 cells per well were acquired and analyzed by another researcher, and thus data were collected in a blinded manner. MFI was measured using ImageJ software (National Institute of Health, USA). Only viable cells, identified by trypan blue exclusion, were examined. Additionally, THP-1 cells were visualized using an Olympus FluoView confocal microscope (model #FV1000) and imaged using FV10-ASW software to confirm that fluorescing beads were engulfed by cells.

4.2.6 Assessing cell viability/death

Cell viability was monitored by the MTT assay as described in previously published studies (Hansen et al., 1989; Wenzel et al., 2019). This assay measures the reduction of water-soluble tetrazolium dye MTT to an insoluble purple formazan product by viable cells. MTT (0.5 mg/ml) was added to the cell cultures and the plates were incubated for one h at 37 °C in a 5% CO_2 incubator. The resulting formazan crystals were dissolved by adding a volume of sodium dodecyl sulfate (20% w/v)/ N,N-dimethylformamide (50% v/v) solution equal to that of the culture medium present in the well, then incubating the plates for three h. Optical densities were measured at 570 nm using the FLUOstar Omega microplate reader. Cell viability data are presented as percent compared to values obtained from cells incubated in growth medium only.

PI, a fluorescent DNA dye which cannot penetrate membranes of live cells, was used to assess cell death (Nieminen et al., 1992; Wenzel et al., 2019). Cells were incubated with 20 µg/ml PI at 37 °C for 20 min followed by fluorescence intensity measurements using 485 nm excitation and 625 nm emission filters. Cell death data are presented as percent compared to values obtained from cells lysed in growth medium only.

4.2.7 Data analysis

Data were analyzed using the randomized block design one-way or two-way ANOVA, followed by Dunnett's or Tukey's post-hoc test. Independent experiments are defined herein as assays performed on different days. Detection limits were calculated using the following formula:

$$\textbf{Detection limit} = \frac{[(\text{ODmean } of\ six\ sample\ blanks)+3*(standard\ deviation\ of\ ODmean\ comprising\ six\ sample\ blanks)]-b}{m}$$

Where:

ODmean = mean optical density

m = slope value obtained from the linear standard curve

b = intercept value with the optical density axis obtained from the linear standard curve

4.3 Results

4.3.1 SCFAs alone or in mixture reduce secretion of IL-1β, MCP-1, and TNF-α by stimulated human THP-1 microglia-like cells

Five different SCFAs, widely recognized as key metabolites of gut microbes, were chosen for this study (Macfarlane and Macfarlane, 2003; Morrison and Preston, 2016). In the first series of experiments, the five SCFAs were mixed at an approximate physiological ratio and used at 5-500 μM total concentration. Figure 4.1 illustrates that stimulating THP-1 cells with LPS plus IFN-γ induced their secretion of IL-1β, MCP-1, and TNF-α. SCFA mixture at the highest total concentration tested (500 μM), when added before stimulation, significantly inhibited secretion of all three cytokines, compared to stimulated cells treated with the vehicle solution only (Fig. 4.1A,C,E). In addition, the SCFA mixture at lower concentrations (5-250 μM) significantly inhibited secretion of IL-1β but not the other two cytokines. The SCFA mixture at the concentrations used did not significantly affect the viability of THP-1 cells according to the MTT and PI assays (see fig. 4.3C,D).

In the second series of experiments, the same assays were used to confirm the significant inhibitory effect of the SCFA mixture at the highest total concentration, and to test the effects of individual SCFAs, at the maximum concentration used within the SCFA mixture, on cytokine secretion by THP-1 cells (Fig. 4.1 B,D,F). All five individual SCFAs reduced the secretion of MCP-1, compared to cells treated with vehicle solution only (Fig. 4.1D). All individual SCFAs tested, except propionate, significantly reduced the secretion of IL-1β (Fig. 4.1B), while three out of five SCFAs tested (propionate, formate and valerate) reduced the secretion of TNF-α (Fig. 4.1F). Individual SCFAs at the concentrations used did not significantly affect the viability of THP-1 cells according to the MTT and PI assays (see fig. 4.4C,D).

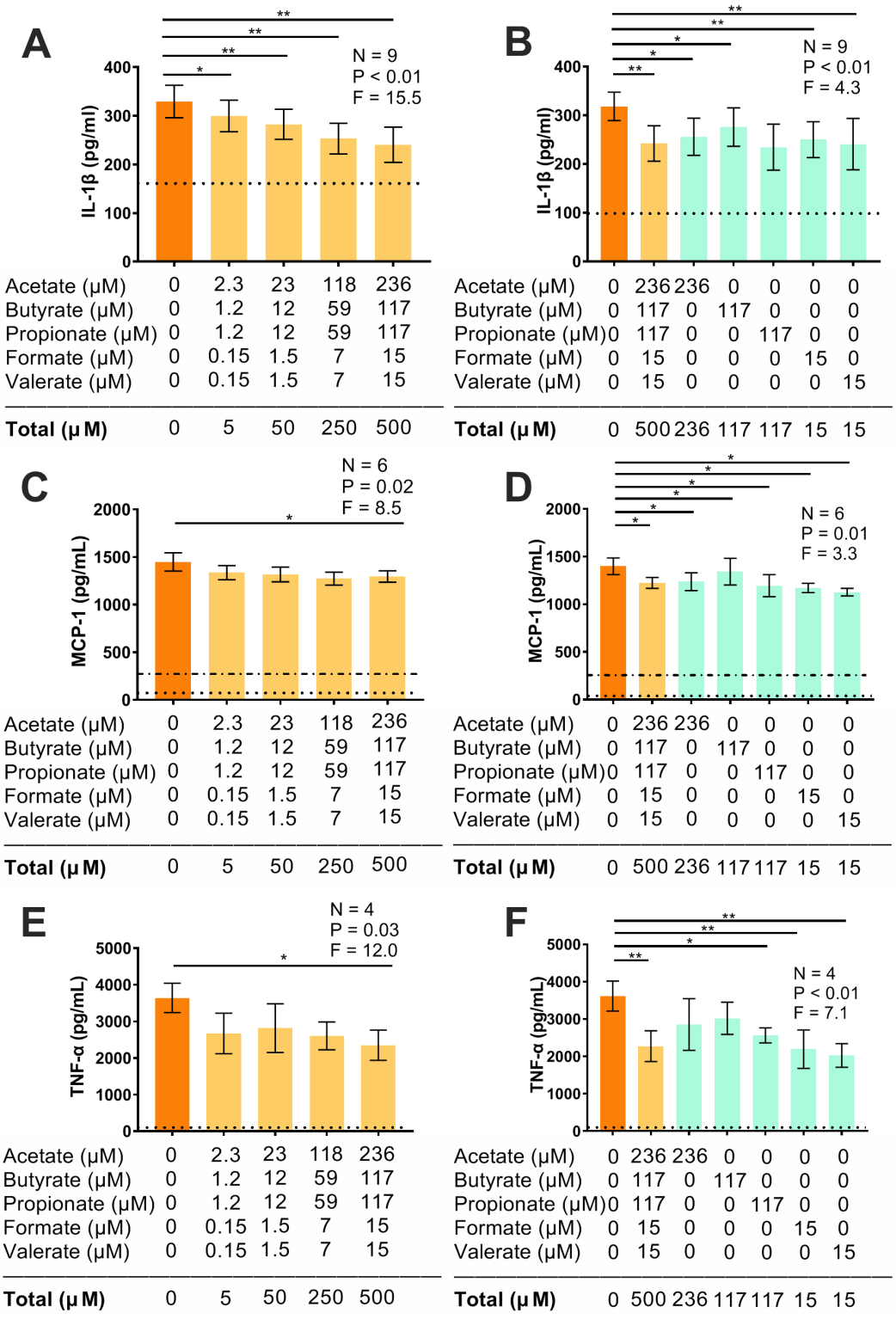


Figure 4.1. Effects of the SCFA mixture and individual SCFAs on cytokine secretion by stimulated human THP-1 monocytic cells. SCFA mixtures at different concentrations (**A,C,E**) or

individual SCFAs at their respective maximum concentrations used in the mixture (**B,D,F**) were added to THP-1 cells 15 min before their stimulation with LPS (20 ng/ml) plus IFN-γ (100 U/ml). After a 48 h incubation period, concentrations of IL-1β (**A,B**), MCP-1 **(C,D)**, and TNF-α (**E,F**) in cell-free supernatants were measured by ELISAs. Data (means ± SEM) from four to nine independent experiments performed on different days are presented. * $P<0.05$, ** $P<0.01$ compared to stimulated THP-1 cells treated with SCFA vehicle only, according to the Dunnett's post-hoc test. P and F values for the one-way randomized blocks ANOVA are shown, as well as the detection limit of the ELISAs as a dotted line. Dotted and dashed lines represent the concentration of MCP-1 in the supernatant of unstimulated THP-1 cells (**C,D**). IL-1β and TNF-α were not detected in the supernatant of unstimulated THP-1 cells.

4.3.2 The inhibitory effect of the SCFA mixture on IL-1β secretion by stimulated THP-1 microglia-like cells is not mediated by FFAR 2 or FFAR 3

Since FFAR 2 and FFAR 3 have been implicated as the molecular targets of certain SCFAs (Ang et al., 2016; Li et al., 2018), and since activation of both these receptors has been shown to induce cytokine secretion by immune cells (Ang et al., 2016), I tested a hypothesis that the inhibitory effects of SCFA mixture on THP-1 cell cytokine secretion are mediated by these receptors. The FFAR 2 and 3 antagonist (GLPG 0974, 0.1 μM), or its solvent, was added to THP-1 cell cultures, followed by their treatment with the SCFA mixture (500 μM, Fig. 4.2A), the SCFA formate alone (500 μM, Fig. 4.2B), or their corresponding solvents. Subsequently, THP-1 cells were stimulated with LPS plus IFN-γ for 48 h to induce IL-1β secretion. Figure 4.2 illustrates that GLPG 0974 on its own did not affect IL-1β secretion by stimulated THP-1 cells. GLPG 0974 did not block the inhibitory effect of the SCFA mixture on IL-1β secretion (Fig. 4.2A), but it blocked the inhibitory effect of formate alone on IL-1β secretion by stimulated THP-1 cells (Fig. 4.2B). GLPG 0974, SCFA mixture, or formate on its own at the concentrations used did not significantly affect the cell viability according to the MTT assay (Fig. S4.1 in Appendix).

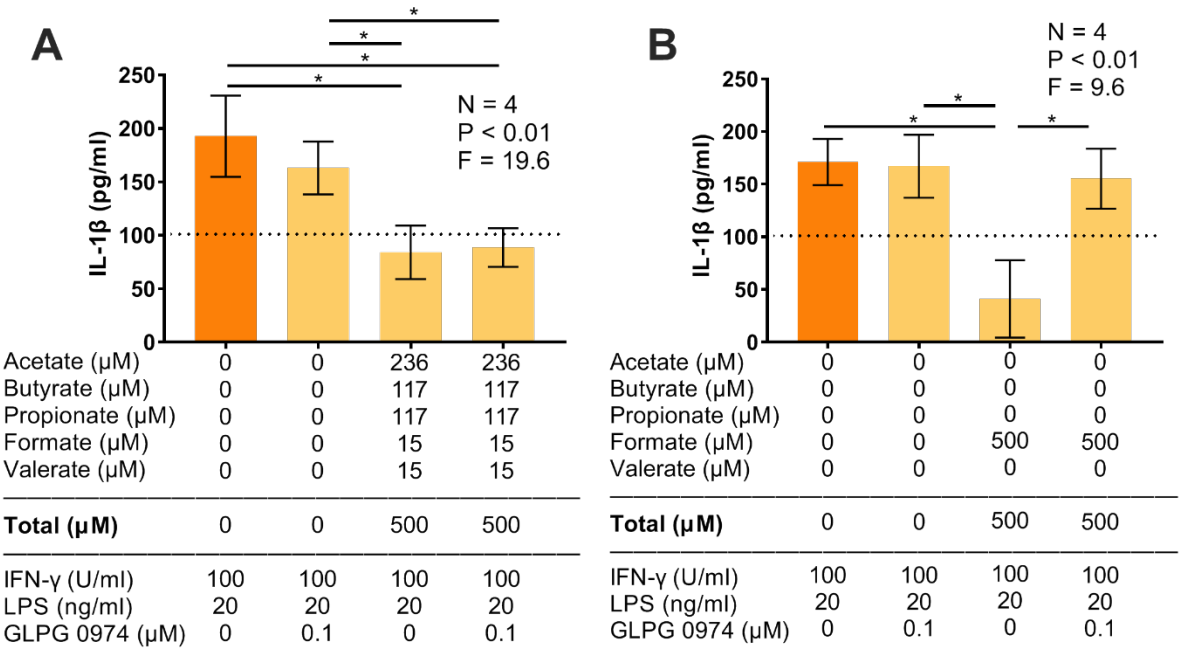


Figure 4.2. Effect of the SCFA mixture (**A**) and the SCFA formate on its own (**B**) in the presence or absence of the FFAR 2 and 3 antagonist on IL-1β secretion by stimulated THP-1 cells. 0.1 μM GLPG 0974 was added to THP-1 cells first. After 30 min incubation, 500 μM SCFA mixture (**A**) or formate alone (**B**) were added to THP-1 cells, followed by their stimulation with LPS plus IFN-γ 15 min later. After a 48 h incubation period, concentrations of IL-1β in cell-free supernatants were measured by ELISA. Data (means ± SEM) from four independent experiments performed on different days are presented. * P<0.05 according to the Tukey's post-hoc test. P and F values for the one-way randomized blocks ANOVA are shown, as well as the detection limit of the IL-1β ELISA as a dotted line. IL-1β was not detected in the supernatant of unstimulated THP-1 cells.

4.3.3 SCFA mixture reduces secretion of cytotoxins by stimulated THP-1 microglia-like cells

Since the SCFAs inhibited secretion of inflammatory cytokines by THP-1 cells, I studied the effect of the SCFA mixture on secretion of cytotoxins by these microglia-like cells. Increasing concentrations of the SCFA mixture (5-500 μM total concentration, Fig. 4.3) were added to THP-1 cell cultures prior to their stimulation with LPS plus IFN-γ. Figures 4.3A,B

illustrate that LPS plus IFN-γ stimulation increased the cytotoxicity of THP-1 supernatants towards SH-SY5Y neuronal cells, which could be observed as reduced viability of SH-SY5Y recorded by two different assays. The SCFA mixture in a concentration-dependent manner reduced the toxicity of THP-1 supernatants towards SH-SY5Y neuronal cells, compared to supernatants from THP-1 cells stimulated in the absence of SCFAs (Fig. 4.3A,B). At the concentrations tested, the SCFA mixture did not significantly affect viability of THP-1 cells according to the MTT and PI assays (Fig. 4.3C,D).

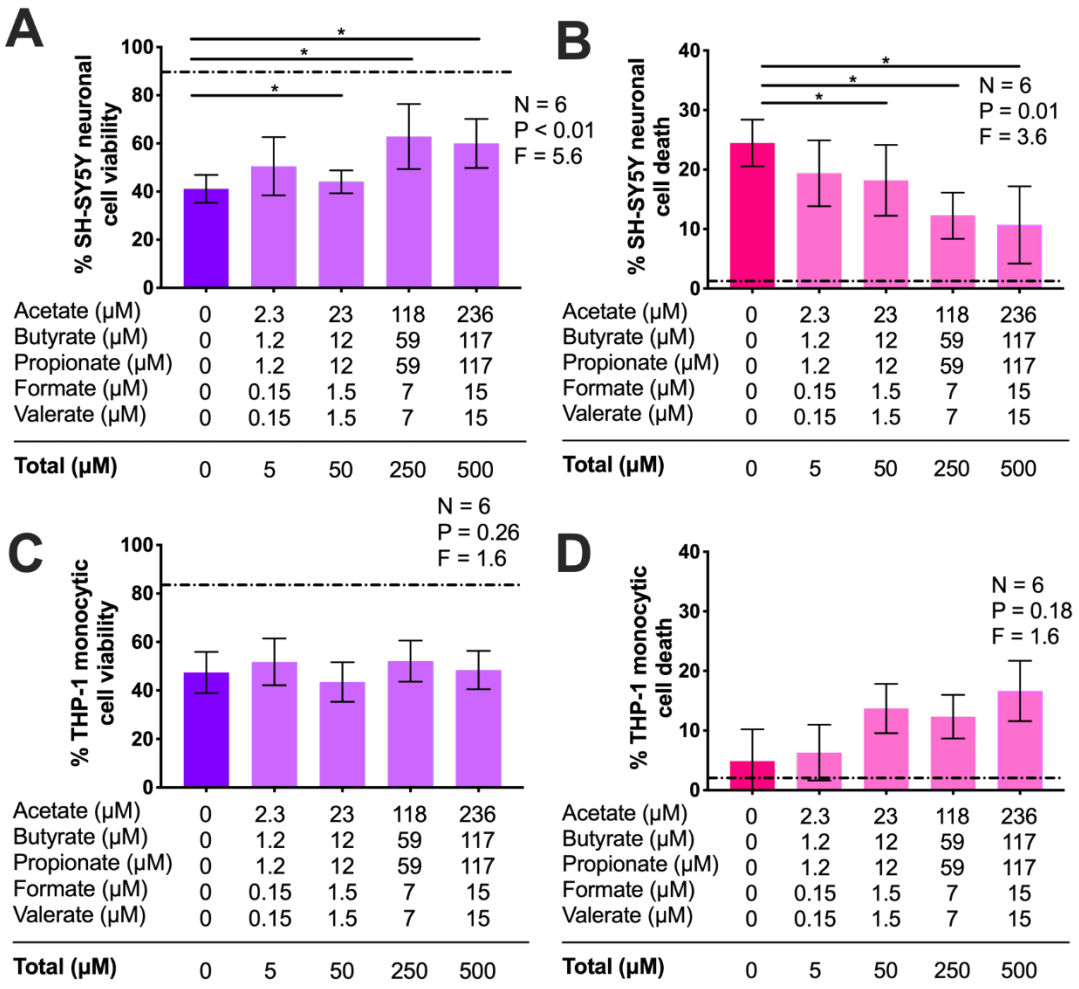


Figure 4.3. Effect of the SCFA mixture on cytotoxicity of stimulated human THP-1 monocytic cells towards human SH-SY5Y neuronal cells. Viability (**A**) and death (**B**) of human SH-SY5Y cells was measured by the MTT and PI assays respectively after 72 h incubation with supernatants from THP-1 cells that had been stimulated for 48 h with LPS (20 ng/ml) plus IFN-γ (100 U/ml) in the presence or absence of different concentration of the SFCA mixture. Effects of

the SCFA mixture on viability (**C**) and death (**D**) of THP-1 cells at the end of the 48 h incubation period with the stimuli were also assessed. Data (means ± SEM) from six independent experiments performed on different days are presented. * $P<0.05$, compared to stimulated and SCFA vehicle-treated samples, according to the Dunnett's post-hoc test. P and F values for the one-way randomized blocks ANOVA are shown. Dotted and dashed lines represent the mean signal from SH-SY5Y cells incubated in tissue culture medium only and not exposed to THP-1 supernatants (**A,B**), or the mean signal from unstimulated THP-1 cells (**C,D**).

The same assays were repeated to confirm the significant inhibition of THP-1 cell cytotoxicity by the SCFA mixture at the highest concentration, and to test the effects of individual SCFAs at the maximum concentration used within SCFA mixture on THP-1 cell-mediated toxicity (Fig. 4.4A,B). The supernatants from stimulated THP-1 cells exposed to formate (15 μM) or valerate (15 μM) alone, but not acetate (236 μM), butyrate (117 μM) or propionate (117 μM), increased the viability of SH-SY5Y cells, compared to cells exposed to supernatants from stimulated THP-1 cells treated with vehicle solution (Fig. 4.4A,B). SCFA mixtures or individual SCFAs at concentration used did not significantly affect the viability of stimulated THP-1 cells according to two independent assays (Fig. 4.4C,D).

Since the SCFAs could be transferred onto SH-SY5Y cells with the THP-1 cell supernatants, I tested the possible direct effects of the SCFA mixture and individual SCFAs on SH-SY5Y viability. SCFA mixture (5-500 μM) or individual SCFAs at the maximum concentration used had no significant effect on viability of SH-SY5Y cells when added to neuronal cells for 72 h (Fig. S4.2 in Appendix).

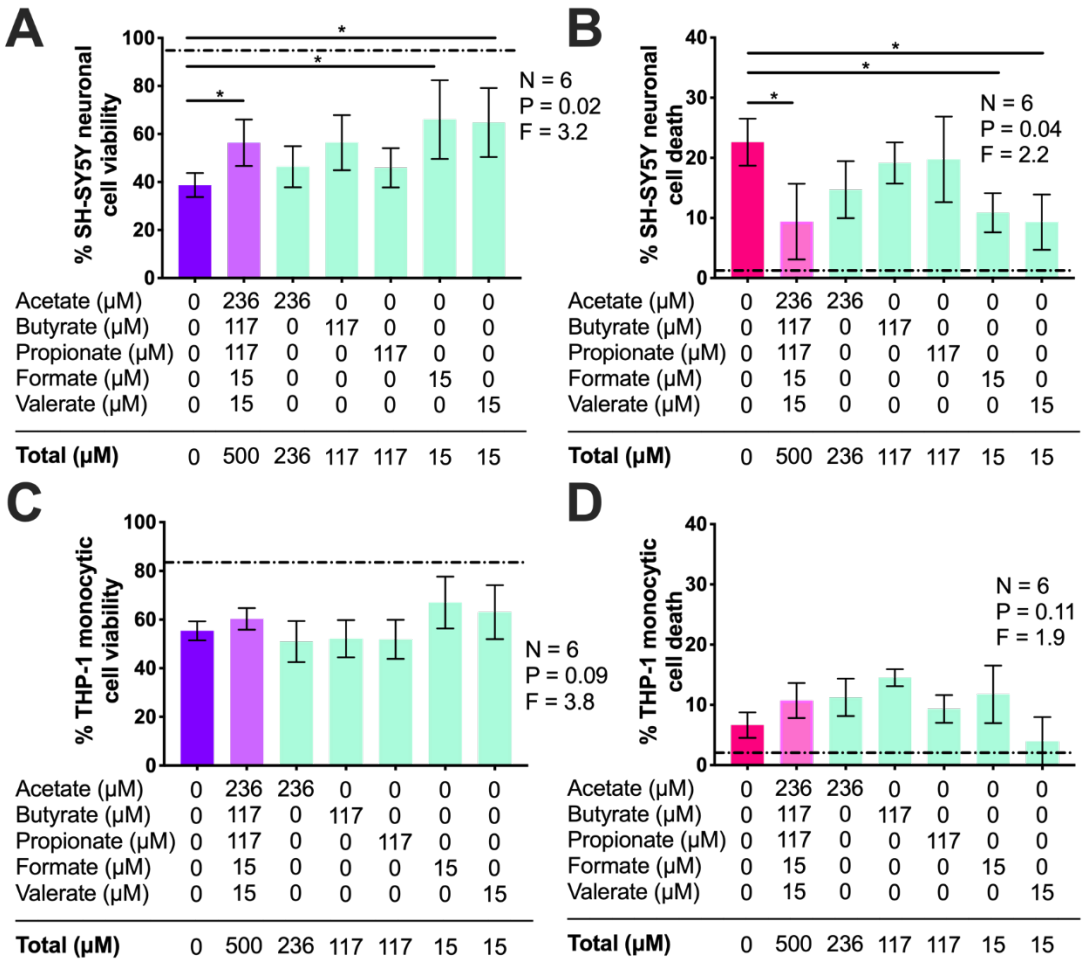


Figure 4.4. Effect of the individual SCFAs on cytotoxicity of stimulated human THP-1 monocytic cells towards human SH-SY5Y neuronal cells. Viability (**A**) and death (**B**) of human SH-SY5Y cells was measured by the MTT and PI assays respectively after 72 h incubation with supernatants from THP-1 cells that had been stimulated for 48 h with LPS (20 ng/ml) plus IFN-γ (100 U/ml) in the presence or absence of the SCFA mixture or individual SCFAs at respective maximum concentrations used in the mixture. Effects of the mixture and individual SCFAs on viability (**C**) and death (**D**) of THP-1 cells at the end of the 48 h incubation period with the stimuli were also assessed. Data (means ± SEM) from six independent experiments performed on different days are presented. * $P<0.05$, compared to stimulated and SCFA vehicle-treated samples, according to the Dunnett's post-hoc test. P and F values for the one-way randomized blocks ANOVA are shown. Dotted and dashed lines represent the mean signal from SH-SY5Y cells incubated in tissue culture medium only and not exposed to THP-1 supernatants (**A,B**), or the mean signal from unstimulated THP-1 cells (**C,D**).

4.3.4 Formate, but not valerate, reduces the respiratory burst response of stimulated HL-60 microglia-like cells

Since formate and valerate on their own inhibited secretion of select cytokines and cytotoxins by THP-1 cells, further studies were performed to assess their effects on respiratory burst and phagocytic activity of microglia-like cells. First, I evaluated whether increasing concentrations of formate (15-1000 μM) and valerate (15-1000 μM) reduced the fMLP-induced production of ROS by differentiated HL-60 cells primed with LPS (Pointer et al., 2019). Figure 4.5 demonstrates that fMLP-induced respiratory burst response was higher in LPS-primed compared to unprimed differentiated HL-60 cells. When added during the LPS priming, formate at the highest concentration studied significantly lowered the levels of ROS after fMLP stimulation (Fig. 4.5A). Figure 4.5C shows that valerate did not affect the fMLP-induced respiratory burst response of LPS-primed, differentiated HL-60 cells. At the concentrations studied, neither formate nor valerate affected the viability of LPS-primed, differentiated HL-60 cells, as measured by the MTT assay (Fig. 4.5B,D).

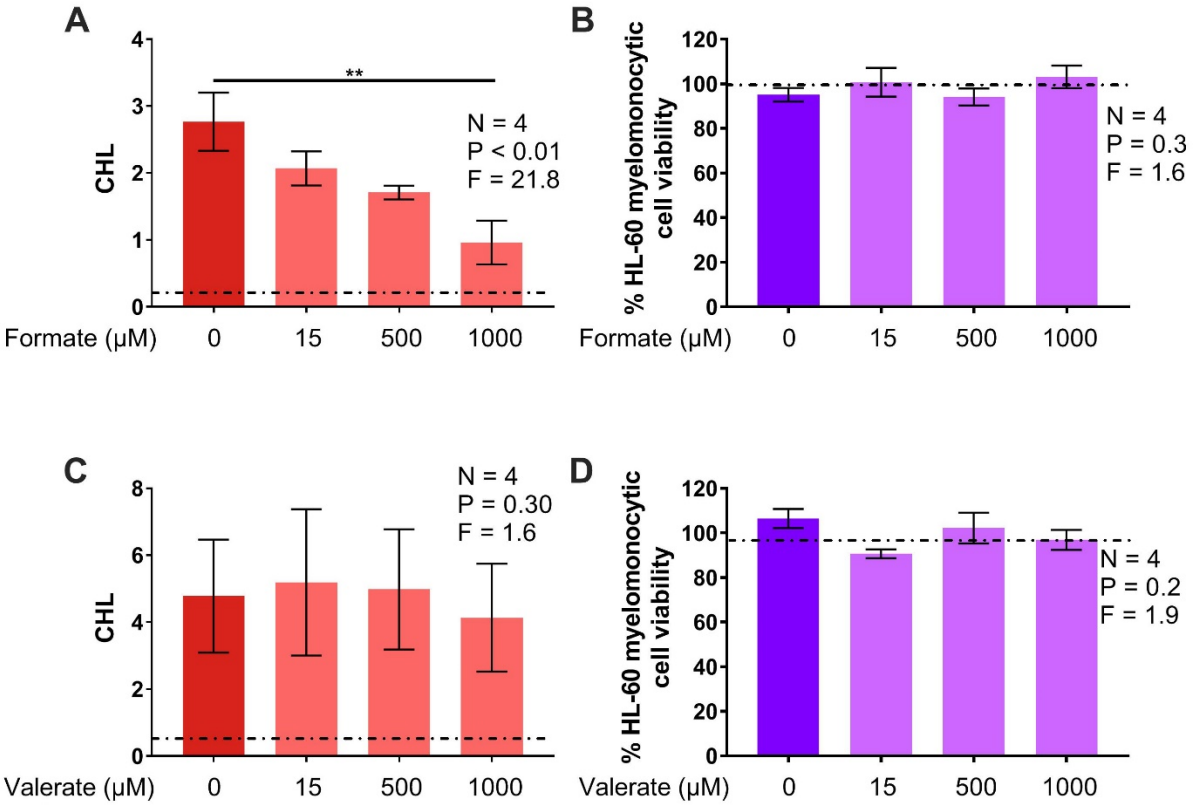


Figure 4.5. Effect of formate and valerate on the respiratory burst of differentiated human HL-60 myelomonocytic cells. Dimethyl sulfoxide-differentiated human HL-60 cells were primed with LPS for 24 h in the presence or absence of increasing concentrations of formate (**A,B**) or valerate (**C,D**). The respiratory burst response was then induced by adding fMLP, and luminol-dependent CHL signal was measured (**A,C**); cell viability was assessed in parallel wells by the MTT assay (**B,D**). Data (means ± SEM) from four independent experiments performed on different days are presented. ** P<0.01 compared to LPS-primed and fMLP stimulated HL-60 cells not exposed to SCFAs, according to the Dunnett's post-hoc test. P and F values for the one-way randomized blocks ANOVA are shown. Dotted and dashed lines represent CHL signal from differentiated HL-60 cells that had not been primed with LPS, but were stimulated with fMLP (**A,C**), or viability data from differentiated HL-60 cells that had not been primed with LPS or stimulated with fMLP (**B,D**).

4.3.5 Formate and valerate decrease the phagocytic activity of stimulated THP-1 microglia-like cells

Since formate reduced the respiratory burst response of HL-60 cells at the highest concentration tested (1 mM), I used the same concentration to evaluate the effects of formate and valerate on phagocytic activity of THP-1 cells. Either of the two SCFAs was added to THP-1 cell cultures in the presence or absence of LPS plus IFN-γ, a combination of stimuli which is known to upregulate phagocytosis (Voss et al., 2017). THP-1 cell phagocytosis of fluorescent latex beads was studied 24 h later. Figure 4.6 illustrates that LPS plus IFN-γ significantly upregulated the phagocytic activity of THP-1 cells compared to cells exposed to vehicle solution. Formate and valerate on their own did not modulate phagocytic activity of THP-1 cells, but both these SCFAs significantly inhibited the LPS plus IFN-γ-upregulated phagocytosis of latex beads.

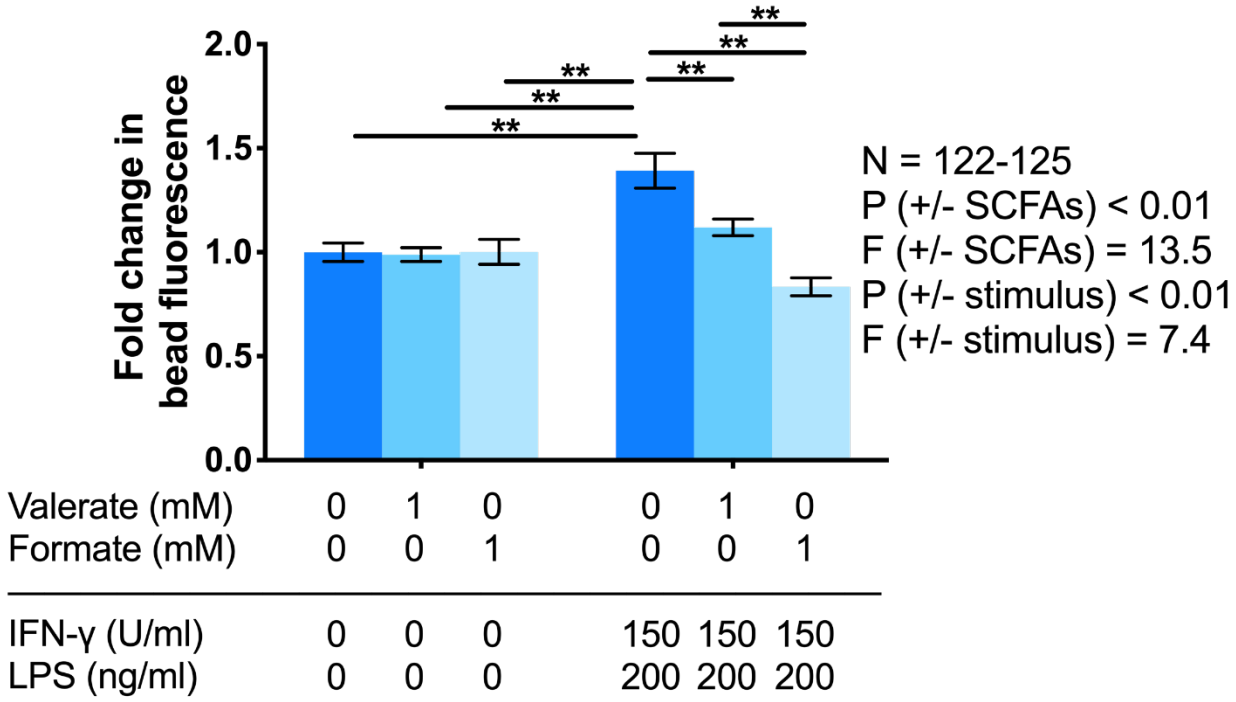


Figure 4.6. Effects of formate and valerate on phagocytosis of latex beads by unstimulated and stimulated THP-1 cells. Data (means ± SEM) from four independent experiments performed on different days (N=122-125 cells per experimental condition) are presented as a fold-change of phagocytosed bead fluorescence in live cells compared to the signal obtained from THP-1 cells exposed to vehicle solutions only. ** P<0.01 according to Tukey's post-hoc test. P and F values for a two-way randomized blocks ANOVA are shown.

4.4 Chapter discussion

Anti-inflammatory properties of SCFAs have been studied by using animal models of peripheral inflammatory diseases (Maslowski et al., 2009; Masui et al., 2013). For example, colons of the colitis model mice receiving 150 mM acetate in their drinking water had significantly higher levels of the anti-inflammatory cytokine IL-10, as well as lower levels of the pro-inflammatory TNF-α, compared to colitis-model mice not treated with acetate (Masui et al., 2013). The anti-inflammatory properties of SCFAs have also been demonstrated by studying *in vitro* immune activation of different types of mononuclear phagocytes, including microglia (Ang et al., 2016; Huuskonen et al., 2004). In most cases, effects of individual SCFAs have been investigated, with limited numbers of studies using mixtures (Canfora et al., 2017; McLoughlin et al., 2017). Clinical relevance of using SCFA mixtures was demonstrated by Canfora *et al*. (2017) who showed that fasting, overweight or obese males rectally administered with 24 mmol acetate, 8 mmol propionate, and 8 mmol butyrate had significantly lower plasma levels of the pro-inflammatory IL-1β, compared to control subjects treated with saline solution. The same SCFAs mixed at two other ratios (18:14:8 mmol or 18:8:14 mmol acetate:propionate:butyrate) had no effect on the plasma IL-1β levels.

We investigated the neuroimmune effects of acetate, propionate, butyrate, formate, and valerate on their own or mixed at approximate physiological ratios found in human peripheral blood (Hanzlik et al., 2005; Ktsoyan et al., 2016; Zhao et al., 2007). The SCFA mixture I used had acetate at the highest concentration compared to other fatty acids, which makes it similar to the mixture found to have anti-inflammatory activity in clinical research (Canfora et al., 2017). Given the prominent role microglia play in neuroimmune reactions, I studied the effects of SCFAs on secretion of cytokines and cytotoxins by human microglia-like cells. Additionally, I studied the effects of individual SCFAs on the respiratory burst and phagocytic activities of microglia-like cells. Clinical and experimental evidence indicates that these select microglia functions become dysregulated in AD (Brown and Vilalta, 2015; Fu et al., 2014; Meraz-Ríos et al., 2013); therefore, identification of molecules and mechanism that can be used to modulate these functions may reveal novel therapeutic strategies for slowing the progression of AD. Human cell lines were used instead of primary mouse or rat microglia due to the previously

reported interspecies differences in microglia responses to immune stimulation; for example, human CD 33, a microglial receptor, regulates the phagocytic activity of microglia, unlike its murine homologue (Bhattacherjee et al., 2019). Unlike their murine counterparts, human microglia are activated by IL-1β (Davis et al., 2018), which may differently affect cytotoxic secretions of microglia from these two species. Furthermore, after oxygen–glucose deprivation and reoxygenation, human, mouse, and rat microglia express different baseline levels of chemokines and cytokines, including IL-1β, MCP-1, and TNF-α (Du et al., 2017).

Reactive microglia release a mixture of inflammatory cytokines and neurotoxins, possibly contributing to the progression of neurodegenerative diseases (Brown and Vilalta, 2015; Brabazon et al., 2018; Klegeris et al., 2008). In the first series of experiments, I used human microglia-like THP-1 cells to investigate secretion of three inflammatory cytokines, which have been reported to be regulated by individual SCFAs in different cell types (Ang et al., 2016; Huang et al., 2017; Russo et al., 2012; Zhou et al., 2014). For example, Huang *et al.* (2017) showed that acetate and butyrate alone inhibited secretion of IL-1β and MCP-1 by SV-40 MES 13 murine glomerular mesangial cells stimulated with LPS. However, it is important to note that butyrate also reduced cell viability, which could be responsible for the decreased cytokine secretion (Huang et al., 2017). Another study showed butyrate inhibited the secretion of TNF-α by LPS-stimulated human Caco-2 epithelial cells (Russo et al., 2012). Furthermore, by using LPS-stimulated primary human peripheral blood monocytes, Ang *et al.* (2016) demonstrated that acetate decreased the secretion of IL-1β and MCP-1. Even though different cell types were used, some of these previous observations are consistent with the data obtained herein by using LPS plus IFN-γ-stimulated THP-1 cells; for example, acetate and butyrate alone downregulating the secretion of IL-1β and MCP-1.

Previous studies have shown that all the SCFAs used in our study are FFAR 2 and FFAR 3 agonists. Thus, HEK293T cells were shown to respond to treatment with acetate, propionate, butyrate, formate, or valerate alone only after being transfected with FFAR 2 or FFAR 3 (Brown et al., 2003). Other studies revealed that individual SCFAs and FFAR 2 and 3 agonists induced secretion of similar set of cytokines. For example, Ang *et al.* (2016) showed that acetate alone, as well as the FFAR 2 agonist (2S)-2-(4-chlorophenyl)-3,3-dimethyl-N-(5-phenylthiazol-2-

yl)butanamide, and the FFAR 3 agonist AR420626 at different time points, decreased the secretion of IL-1β and MCP-1 by primary human peripheral blood monocytes stimulated with LPS.

Very few studies have tested the capacity of FFAR 2 and 3 antagonists to block the inhibitory effects of SCFAs on cytokine secretion. GLPG 0974 reduced the inhibitory effects of acetate, propionate, and butyrate on IL-6 secretion by LPS- or TNF-α-stimulated primary human umbilical vein endothelial cells, but this FFAR 2 and 3 antagonist only reduced the effect of acetate on IL-8 secretion by the same cells (Li et al., 2018). In our study, GLPG 0974 blocked the inhibitory effect of high concentration formate on IL-1β secretion by LPS plus IFN-γ-stimulated THP-1 cells, but it did not modify the inhibitory effect of the SCFA mixture in the same experimental model. Therefore, it is possible that SCFAs at low concentrations act through other SCFA receptors of monocytic cells, such as the niacin receptor 1 (Gpr109a) (Wannick et al., 2018), which regulates the secretion of cytokines including IL-1β (Rahman et al., 2014; Singh et al., 2014).

Individual SCFAs can engage several different inhibitory mechanisms that reduce the secretion of cytokines by immune cells. As previously mentioned in this thesis, acetate, propionate, butyrate, formate and valerate activate FFAR 2 and 3 (Brown et al., 2003), while butyrate can also activate Gpr109a (Singh et al., 2014). Earlier, Cousens *et al.* (1979) showed that propionate, butyrate, valerate, and, to a much lesser degree, acetate inhibited histone deacetylases. Prevention of the activation of NF-κB has been established as the common mechanisms used by FFAR 2 and 3 and Gpr109a agonists, as well as histone deacetylase inhibitors (Lee et al., 2013; Place et al., 2005; Zandi-nejad et al., 2013). It is important to note that activation of NF-κB commonly leads to upregulated secretion of cytokines by immune cells (Liu et al., 2017).

The respiratory burst response of phagocytes, including macrophages, microglia, and neutrophils, is characterized by NADPH oxidase-dependent rapid release of large quantities of ROS (Thomas, 2017). Even though this cellular response is aimed at neutralizing foreign pathogens, the cytotoxic ROS can be harmful to the cells of the host. Similar to our observations

showing inhibitory effect of formate on the respiratory burst of macrophages, previous studies have demonstrated individual SCFAs regulate the levels of ROS, or ROS-related proteins. For example, sodium butyrate decreased the intracellular levels of ROS in LPS-stimulated human Caco-2 epithelial cells (Russo et al., 2012), while acetate and butyrate upregulated SOD activity and decreased intracellular ROS levels in SV-40 MES 13 cells stimulated by LPS (Huang et al., 2017). Interestingly, sodium butyrate supplementation of feed upregulated the gene expression of SOD 1, 2, and 3, as well as glutathione peroxidase 3 in the mammary tissue of goats (Memon et al., 2019).

Inflammatory mediators and ROS released by reactive microglia could have toxic effects on surrounding cells, including neurons (Kim and Joh, 2006; Takeuchi, 2010; Uttara et al., 2009). For example, previous studies have demonstrated that incubating primary human neurons with the subset of inflammatory mediators I studied, including IL-1β and TNF-α, causes neurons to die (Annis et al., 2013). In another study, Yang *et al.* (2011) showed that adding MCP-1 to the primary murine microglia-neuron co-cultures, but not pure cultures of primary murine neurons, induced neurotoxicity. In our study, SCFA mixtures, as well as formate and valerate on their own, reduced the cytotoxicity of THP-1 cell supernatants towards neuronal cells. These data are consistent with our observation that SCFA mixtures, as well as formate and valerate alone, decrease the release of potentially cytotoxic molecules IL-1β, TNF-α, MCP-1 and ROS. Interestingly, it has been demonstrated that suberoylanilide hydroxamic acid, a histone deacetylase inhibitor, reduces the toxicity of IFN-γ-activated primary human astrocytes towards SH-SY5Y neuronal cells (Hashioka et al., 2012).

It is also important to highlight that SCFAs ability to reduce the secretion of IL-1β, TNF-α and MCP-1 by THP-1 cells – a peripheral immune cell that is being used herein as a microglial model - would plausibly decrease microglial activation and neuron death. In mice, IL-1β and TNF-α cross the BBB from blood-to-brain using a saturable transport mechanism (Banks, 2005) and MCP-1 reduces the integrity of the BBB (Yao and Tsirka, 2014). It is unknown whether IL-1β, TNF-α and MCP-1 engage the same mechanisms in humans. If the human cytokines act in the same manner as their murine counterparts, decreasing the levels of these inflammatory mediators in the periphery could prevent adverse glial activation and neuronal death (Annis et

al., 2013; Lively and Schlichter, 2018; Yang et al., 2011). Therefore, our observation that SCFAs alone or in mixture reduce the secretion of IL-1β, TNF-α and MCP-1 by THP-1 monocytic cells could be relevant to immune signaling between the periphery and CNS.

We studied effects of formate and valerate on phagocytosis, which is commonly viewed as a protective function since it ensures removal of tissue debris and is used to eliminate foreign pathogens. Recent studies indicate that adversely activated microglial can phagocytose live neurons, causing their death (phagoptosis); therefore, inhibiting their phagocytic activity could be neuroprotective under certain pathological conditions (Brown and Neher, 2014; Fu et al., 2014; Hornik et al., 2016). I demonstrated that formate and valerate decreased the phagocytic activity of THP-1 cells stimulated with LPS plus IFN-γ. These data are similar to previously reported effects of butyrate on the phagocytic activity of LPS-stimulated primary chicken macrophages (Zhou et al., 2014). However, in another study, butyrate increased the phagocytic activity of IL-4-stimulated primary murine bone marrow-derived macrophages (Fernando et al., 2016). It has been suggested that SCFAs activate different signaling pathways in human and murine macrophages (Ang et al., 2016), which may explain the opposite effects of butyrate on human and murine macrophage phagocytosis.

4.5 Chapter conclusion

It is important to point out that most *in vitro* studies on biological effects of SCFAs have used them at millimolar concentrations, which may be well above the physiological SCFA concentration in human serum and brain tissue. This study highlights the potential of SCFAs alone and in combinations to regulate microglial functions that are disrupted in AD. Further investigation is required to determine the primary mechanisms engaged by the SCFA mixture since the inhibitory effect of SCFAs appears to be only partially dependent on the FFAR 2 or 3. It is also important to note that only future *in vivo* experiments will be able to establish conclusively that SCFAs produced by gut microbes enter the CNS at sufficiently high concentrations to regulate microglial activation and beneficially modulate the progression of neuroinflammatory processes in AD.

5. Conclusion

5.1 Discussion

Chapters 2-4 demonstrate the ability of CL, CytC and SCFAs to modulate select functions of glia. Based on the data I collected, as well as previously published literature, figure 5.1 illustrates a possible mechanism engaged by these three endogenous molecules in the human body, and how these mechanisms contribute to the pathogenesis of AD.

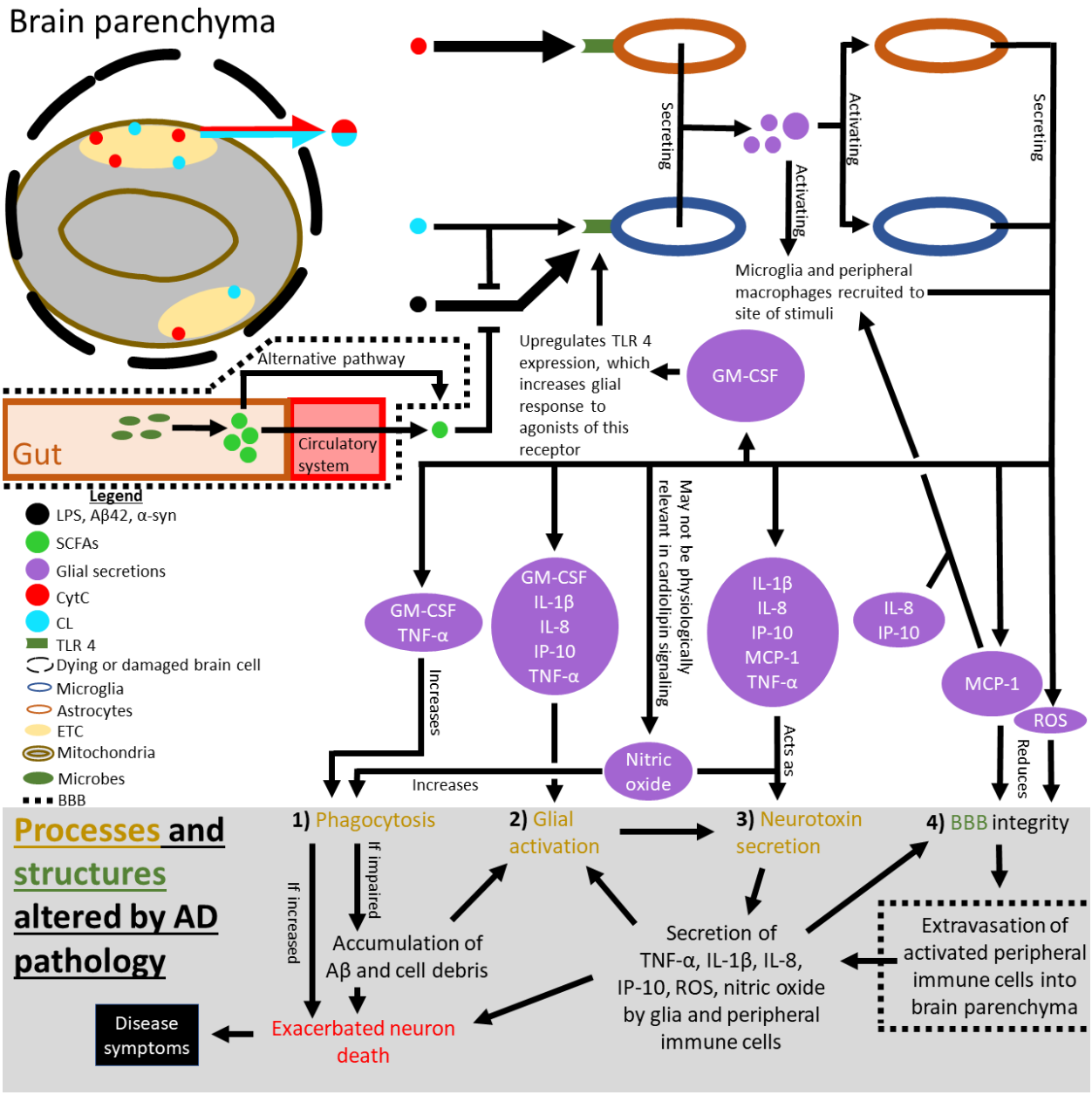


Figure 5.1. A proposed schematic for the signaling events described in this thesis, and how they may contribute to the pathogenesis of AD. Extracellular CL, CytC and SCFAs modulate the secretion of inflammatory mediators that **1**) alter phagocytic activity (Giulian and Ingeman, 1988; Neniskyte et al., 2014), **2**) activate glia (Clarner et al., 2015; Lively and Schlichter, 2018; McLarnon, 2016; Parajuli et al., 2012), **3**) cause neurons to die (Annis et al., 2013; Demaurex and Scorrano, 2009; McLarnon, 2016; Sui et al., 2004; Yang et al., 2011), and **4**) reduce BBB integrity (Ding et al., 2017; Song et al., 2020; Yao and Tsirka, 2014). A subset of inflammatory mediators, namely IL-8, IP-10, and MCP-1, recruit microglia and peripheral macrophages

towards the site of injury, facilitating the activation of more cells (Clarner et al., 2015; McLarnon, 2016; Sheehan et al., 2007). GM-CSF also upregulates the expression of TLR 4 (Parajuli et al., 2012), which sensitizes glia to TLR 4 agonists such as Aβ. Each of these events can contribute to the pathogenesis of neurological disorders such as AD (Leitner et al., 2019; Thériault et al., 2015). CytC and CL are constitutively present in mitochondria of brain cells, and can be released into extracellular space under pathological conditions, whereas SCFAs are produced in the gut by microbes. Thus, SCFAs need to move from the gut to the brain parenchyma, or modify peripheral signaling pathways, to modulate glial immune responses. For example, SCFAs may **i**) cross the BBB through monocarboxylate transporters and act directly on microglia, or **ii**) modulate the secretion of peripheral inflammatory mediators that can cross the BBB and regulate glia functions (see chapter 4 for expanded commentary).

As figure 5.1 illustrates, the effects of CL, CytC and SCFAs may be more pronounced in the brain parenchyma where multiple cell types exist simultaneously. For example, while the experiments described in this thesis show CytC induces the secretion of cytotoxins by astrocytic cells, these experiments were conducted in the absence of microglia. Since CytC induces astrocytes to secrete GM-CSF, IL-1β and IL-8, which activate microglia (Lively and Schlichter, 2018; McLarnon, 2016; Parajuli et al., 2012), I would expect substantially more cytotoxins to be secreted in the human brain parenchyma where microglia and astrocytes co-exist, inducing neuron death. Furthermore, GM-CSF upregulates the expression of TLR 4 by microglia, and primes microglia for an enhanced immune response when subsequently exposed to TLR 4 agonists (Parajuli et al., 2012). Thus, it is plausible that CytC may adversely sensitize microglia causing their excessive response to subsequent challenge with TLR 4 agonists, including the disease-related proteins Aβ and α-syn. It is also important to point out that GM-CSF promotes the proliferation of microglia, but not astrocytes (Giulian and Ingeman, 1988; Liva et al., 1999), which may lead to increased numbers of activated microglia in pathological conditions.

Extracellular MCP-1 and ROS reduce BBB integrity, thus increasing the permeability of this structure. As extensively reviewed by Thériault *et al.* (2015), reduced BBB integrity enables the entry of peripheral immune cells into the brain parenchyma, where they can contribute to the

removal of Aβ deposits. While studies have demonstrated peripheral macrophages clear Aβ deposits more readily than microglia (Gate et al., 2010), it is important to state that the presence of peripheral cells in the brain can have adverse consequences. For example, a previous study demonstrates T cells that infiltrate the CNS reduce the spatial memory of AD-model mice (Laurent et al., 2017). Upregulated ROS production in the brain has been associated with reduced BBB integrity (Song et al., 2020). From a mechanistic perspective, it is possible that ROS directly injure the BBB; for example, excessive ROS produced by microglia induces apoptosis of pericytes (Ding et al., 2017; Song et al., 2020), which, as described in section 1.2, are the principal cell type forming the BBB.

Several different inflammatory mediators, including GM-CSF, TNF-α and nitric oxide, directly modulate microglial phagocytosis (Giulian and Ingeman, 1988; Kopec and Carroll, 2000; Neniskyte et al., 2014). For example, GM-CSF increased the phagocytic activity of microglia 3.2 fold (Giulian and Ingeman, 1988). Therefore, CytC-induced secretion of GM-CSF by astrocytes could lead to upregulated phagocytic activity of microglia . While the data in Chapter 2 demonstrate that CL induces the secretion of nitric oxide by human THP-1 cells, this may not translate to a physiologically-relevant observation in murine cells. For instance, even though nitric oxide upregulates the phagocytic activity of murine microglia (Kopec and Carroll, 2000), CL does not induce the secretion of nitric oxide by these cells (Pointer et al., 2019). Therefore, CL-induced upregulation of murine microglia phagocytosis is likely not mediated by nitric oxide. As demonstrated by Kopec and Carroll (2000), molecules such as Aβ can increase the phagocytic activity of murine microglia without inducing their secretion of nitric oxide. While upregulated phagocytic activity of immune cells has been suggested to be neuroprotective in AD (Gate et al., 2010; Leitner et al., 2019), it is critical to highlight that it can directly cause neurons to die. For example, Neniskyte *et al.* (2014) demonstrate that TNF-α upregulates rat microglial phagocytosis and causes neurons to die. This study illustrates that it is essential to regulate phagocytosis to prevent excessive neuronal death contributing to the onset of AD symptoms.

5.2 Study considerations and limitations

Immortalized cell lines were utilized in most of the experiments described in this thesis. These cells were derived from cancerous tissues, or were experimentally created through viral transfection (Maqsood et al., 2013). Due to these origins, immortalized cells are representative of the subpopulation of cells they were derived from. Their characteristics, such as morphology, gene expression or secretome, may not be representative of the whole population of primary cells. To overcome this limitation, several key experiments in this thesis were repeated using primary cells.

Primary cells also have limitations compared to immortalized cell lines. While primary cells may express more native characteristics, their morphology, gene expression and secretome may drastically change when removed from their native environment (Gosselin et al., 2017). In an attempt to overcome this limitation, scientists have historically conducted *in vivo* studies. However, animal models have their own unique sets of drawbacks compared to primary cells, which were described in sections 1.5 and 2.4. In brief, structural and expression differences exist between murine and human receptors, including TLR 4 (Lizundia et al., 2008; Vaure and Liu, 2014). These differences can contribute to a drug failing clinical trials in humans, despite the success of the drug during a preclinical trial using animal models of corresponding diseases (Oura et al., 2017; Shepherd and Sridhar, 2003). Furthermore, primary murine microglia used in experiments described in this thesis were obtained from eight-week old mice. While six to 20 weeks of age have been consistently described as adult mice (Jackson et al., 2017), it is known that microglia extracted from older animals secrete higher levels of cytokines and exhibit decreased phagocytic activity compared to microglia derived from younger animals (Rawji et al., 2016). Astrocytes extracted from aged animals also secrete cytokines at higher concentrations compared to cells obtained from young animals (Palmer and Ousman, 2018). Therefore, it is possible that the effects of CL, CytC and SCFAs may differ if primary cells were derived from aged animals. Recent research indicates that the optimal time of therapeutic intervention for AD precedes symptoms, and thus tissues from younger animals and patients should be used in experimental and clinical studies (Crous-Bou et al., 2017). The experiments in chapter 3 utilizing primary human astrocytes were conducted using cells derived from three patients only due to the

very limited supply of surgically resected human brain tissue. If additional human tissue samples become available, these experiments should be repeated. Larger sample size could increase statistical power to a level sufficient to delineate additional effects of CytC. For example, with more samples, the experiment described in Table 3.1 may demonstrate that CytC significantly increases the secretion of MCP-1 by primary human astrocytes.

Recently, new brain organoid technologies have been developed in an attempt to overcome the limitations of two-dimensional cell cultures and animal models. Brain organoids are self-assembling three-dimensional cell cultures that resemble a fetal human brain (Velasco et al., 2019), and have been used to study a variety of diseases, including AD (Lancaster and Huch, 2019; Papaspyropoulos et al., 2020). Cells in these three-dimensional cultures can receive and send signals to a variety of cell types native to their normal tissues, unlike in two-dimensional cultures where some cell types are often excluded. For example, two-dimensional cell cultures do not often simultaneously consist of neurons, oligodendrocytes, astrocytes, microglia and progenitor cells. Cells that make up brain organoids (e.g. neurons, oligodendrocytes, astrocytes and microglia) are derived from inducible pluripotent stem cells (iPSC), which can be generated by reprogramming somatic cells in blood, such as CD 34-positive or erythroid progenitor cells. Importantly, brain organoids are generated with a genetic background identical to the human the iPSCs are derived from. Therefore, this technology allows the generation of cell cultures that have disease-relevant genetic backgrounds.

The experiments described in this thesis should be extended to organoid models. Not only do these three-dimensional cultures better encapsulate the brain than two-dimensional cultures, the cells of brain organoids express receptors and respond to stimuli similar to their primary human counterpart. Thus, this technology overcomes some limitations of *in vivo* models, such as the interspecies differences in immune responses and receptor expression of microglia described in sections 1.5 and 2.4. This technology would have been particularly beneficial to chapter 4, where a non-glial human cell line was used because (1) murine brain cells do not express the hypothesized receptor targets FFAR 2 and 3, and (2) there is no human microglial cell line that is widely available and well characterized. While the reproducibility of organoid model systems have been questioned, recent studies demonstrate that brain organoids have sample-to-sample

variation similar to fetal human and adult mice brains (Nascimento et al., 2019; Velasco et al., 2019). Altogether, organoids enable researchers to study a system that better represents the human brains, compared to traditional two-dimensional cell cultures.

Experiments conducted in chapters 2-4 measured a limited number of cytokines at only one timepoint. Cytokines secreted by microglia and astrocytes reach peak concentrations at distinct timepoints after glial stimulation (Lee et al., 1993; Norden et al., 2016). For example, TNF-α is maximally secreted by microglia eight h after their exposure to LPS (Lee et al., 1993). The same study also demonstrates that TNF-α activate microglia and astrocytes. Since the experiments described throughout this thesis measured cytokines in glial supernatant 24 h and 48 h after exposure to stimuli, the observed effects may not be caused directly by CL, CytC and SCFAs, but indirectly by the cytokines that are released from stimulated glia. Furthermore, only the classical pro-inflammatory cytokines were measured in chapters 2-4. As previously described in section 1.4 and 1.6, TLR 4 agonists induce the secretion of a broad range of pro- and anti-inflammatory cytokines. In an attempt to better profile the direct effects of CL, CytC and SCFAs on the glia secretome, experiments described herein should be repeated to measure a wider range of cytokines. In addition, these repeated experiments should use shortened simulation periods.

Identifying ligand targets using receptor antagonists has limitations (Lin et al., 2019). For example, it is possible that the antagonists used in this thesis could have unexpected off-target effects, leading to the incorrect identification of TLR 4. In an attempt to overcome this limitation, the experiments described in chapter 3 used TLR 4-blocking antibodies, as well as a synthetic receptor antagonist. The experiments described in this thesis should be repeated using cells with the implicated target receptor knocked out using clustered regularly interspaced short palindromic repeats (CRISPR)-CRISPR associated protein (Cas) 9 technology. This has been recently described as the gold standard to identify ligand-protein target interactions, as antagonists and RNA-interfering molecules are often less specific than expected (Lin et al., 2019).

It is important to highlight that the experiments described in chapter 3 exposed human astrocytes to bovine CytC. Many species are tolerant to antigens produced by the host-organism

(self-tolerance); therefore, immune cells do not respond to normal constituents of the host (Weigle, 1980). However, small differences in amino acid residues can cause a molecule to induce an immune response, even if a protein is highly conserved between species. For example, subcutaneous injections of human CytC, but not mouse CytC, into mice induced an immune response in the animals (Mamula et al., 1992). Therefore, it is possible that human CytC may have no effect on human astrocytes. It is paramount to test human-derived CytC on human astrocytes to enhance the translatability of this research. In addition, the experiments in chapter 2 should be repeated using human CL derived from brain tissue, where there exist unique CL species with a variety of different acyl chain lengths and degrees of unsaturation (Pointer and Klegeris, 2017).

5.3 Significance of research

In chapter 2, I discovered previously unknown molecular mechanisms engaged by extracellular CL, and novel neuroinflammation-resolving properties of this phospholipid. I demonstrated for the first time that CL inhibits the secretion of MCP-1 and TNF-α by α-syn and Aβ42-stimulated microglia, suggesting this phospholipid may have disease-modifying properties. I established that CL alone induces the secretion of inflammatory mediators IP-10, MCP-1, and nitric oxide, but not at concentrations that are cytotoxic towards neuronal cells. Previous studies demonstrated CL inhibits secretion of cytokines by LPS-stimulated immune cells, as well as upregulates microglial phagocytosis (Balasubramanian et al., 2015; Pizzuto et al., 2019; Pointer et al., 2019). Yet, I am the first to experimentally describe TLR 4 as the receptor that mediates the effects of CL on microglia and microglia-like cells. Altogether, the data reported in chapter 2 supports my hypothesis that CL modulates the secretion of cytokines and cytotoxins by microglia, as well as their phagocytic activity, in a TLR 4-dependent manner. Experimental results described in chapter 2 justify developing CL or TLR 4 inhibitors as possible therapeutic agents to reduce the adverse neuroinflammation observed in AD patients.

In chapter 3, I discovered CytC is released from dying glial cells and subsequently induces the secretion of cytokines and cytotoxins from astrocytes in a TLR 4-dependent manner, which supports my chapter 3 hypothesis. Previous studies demonstrated CytC modulates immune

responses of non-astrocytic cells. For example, CytC primed the respiratory burst response of differentiated HL-60 myelomonocytic cells, and induced the secretion of nitric oxide by BV-2 microglia (Gouveia et al., 2017). Yet, the effects of CytC on astrocyte functions were unknown. I demonstrated for the first time that CytC alone induces the secretion of cytokines from primary human astrocytes in a TLR 4-dependent manner. In addition, I established that CytC induces the secretion of cytotoxins by astrocytic cells at sufficiently high concentrations to kill neurons. Importantly, I discovered that CytC is released from damaged glial cells, including microglia exposed to Aβ42 or TNF-α. With the data reported herein showing CytC release from glia, and previous studies showing its release from neurons (Ahlemeyer et al., 2002), there is accumulating evidence that CytC sufficiently contributes to the increased neuroinflammation exhibited in conditions of exacerbated cell death, including neurodegenerative diseases.

In chapter 4, I discovered that SCFAs at physiologically relevant concentrations modulate the secretion of cytokines and cytotoxins by microglia-like cells, as well as their phagocytic activity. Previous studies demonstrated that individual SCFAs at concentrations well-above levels measured in human sera modulate the secretion of cytokines by microglia (Huuskonen et al., 2004). Yet, only limited studies described effects of SCFAs alone or in mixture at physiological serum concentrations. I discovered that SCFAs mixed at concentrations reported in human sera reduced the secretion of IL-1β, MCP-1, TNF-α and cytotoxins by microglia-like cells. I demonstrated for the first time that FFAR 2 or 3 mediated the ability of formate alone, but not the SCFA mixture, to reduce the secretion of IL-1β by microglia-like cells. I also discovered that formate and valerate at high concentrations reduced the phagocytic activity of microglia-like cells. Therefore, it is possible that formate and valerate may reduce phagoptosis (phagocytosis of live neurons), slowing the death of neurons in AD (Clayton et al., 2017). I demonstrated that formate inhibits the respiratory burst response of macrophages. Since there are less SCFA-producing microbes in the gut of AD patients (Liu et al., 2019), it is possible that therapeutic agents that lead to the recolonization by and proliferation of these microbes may have AD-modifying properties .

Overall, I discovered previously unknown molecular mechanisms and functional consequences of microglia and astrocyte activation by extracellular CL, CytC and SCFAs. In

addition, I discovered receptors that partially regulate effects of these endogenous molecules. The novel knowledge described in this thesis about the regulation of glial functions by endogenous molecules offers insight into possible therapeutic agents that could be developed to prevent or treat neurological disorders, such as AD. For example, since TLR 4 modulates glial functions that become dysregulated in AD and blocking this receptor reduces the secretion of cytotoxic secretions of glia, therapeutic agents that inhibit TLR 4 may slow the progression of AD. Experiments described in chapter 4 also justify future studies to determine whether therapies that promote the colonization and proliferation of SCFA-producing bacteria reduce neuroinflammation, and subsequently slow the onset of AD symptoms.

References

Ahlemeyer, B., Klumpp, S., Krieglstein, J., 2002. Release of cytochrome c into the extracellular space contributes to neuronal apoptosis induced by staurosporine. Brain Res. 934, 107–116.

Ajmone-Cat, M.A., Nicolini, A., Minghetti, L., 2003. Prolonged exposure of microglia to lipopolysaccharide modifies the intracellular signaling pathways and selectively promotes prostaglandin E2 synthesis. J. Neurochem. 87, 1193–1203.

Akhter, R., Sanphui, P., Das, H., Saha, P., Biswas, S.C., 2015. The regulation of p53 up-regulated modulator of apoptosis by JNK/c-Jun pathway in β-amyloid-induced neuron death. J. Neurochem. 134, 1091–1103.

Alvarez-curto, E., Milligan, G., 2016. Metabolism meets immunity: the role of free fatty acid receptors in the immune system. Biochem. Pharmacol. 114, 3–13.

Amor, S., Peferoen, L.A., Vogel, D.Y., Breur, M., Valk, P., Baker, D., 2014. Innate and adaptive immune responses in neurodegeneration and repair. Immunology 141, 287–291.

Ang, Z., Er, J.Z., Tan, N.S., Lu, J., Liou, Y.C., Grosse, J., Ding, J.L., 2016. Human and mouse monocytes display distinct signalling and cytokine profiles upon stimulation with FFAR2/FFAR3 short-chain fatty acid receptor agonists. Sci. Rep. 6, 34145.

Annis, D.S., Mosher, D.F., Roberts, D.D., 2013. IL-1β and TNF-α induce neurotoxicity through glutamate production: a potential role for neuronal glutaminase. J. Neurochem. 125, 897–908.

Atlante, A., Calissano, P., Bobba, A., Azzariti, A., Marra, E., Passarella, S., 2000. Cytochrome c is released from mitochondria in a reactive oxygen species (ROS)-dependent fashion and can operate as a ROS scavenger and as a respiratory substrate in cerebellar neurons undergoing excitotoxic death. J. Biol. Chem. 275, 37159–37166.

Au, A.K., Aneja, R.K., Bell, M.J., Bayir, H., Feldman, K., Adelson, P.D., Fink, E.L., Kochanek, P.M., Clark, R.S.B., 2012. Cerebrospinal fluid levels of high-mobility group box 1 and cytochrome c predict outcome after pediatric traumatic brain injury. J. Neurotrauma 29, 2013–2021.

Baeten, K.M., Akassoglou, K., 2011. Extracellular matrix and matrix receptors in blood-brain barrier formation and stroke. Dev. Neurobiol. 71, 1018–1039.

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.

Balasubramanian, K., Maeda, A., Lee, J.S., Mohammadyani, D., Dar, H.H., Jiang, J.F., St Croix, C.M., Watkins, S., Tyurin, V.A., Tyurina, Y.Y., Klöditz, K., Polimova, A., Kapralova, V.I., Xiong, Z., Ray, P., Klein-Seetharaman, J., Mallampalli, R.K., Bayir, H., Fadeel, B., Kagan, V.E., 2015. Dichotomous roles for externalized cardiolipin in extracellular signaling: promotion of phagocytosis and attenuation of innate immunity. Sci. Signal. 8, 1–33.

Balestrino, R., Schapira, A.H. V, 2020. Parkinson disease. Eur. J. Neurol. 27, 27–42.

Banks, W.A., 2009. Characteristics of compounds that cross the blood-brain barrier. BMC Neurol. 9, S3–S3.

Banks, W.A., 2005. Blood-brain barrier transport of cytokines: a mechanism for neuropathology. Curr. Pharm. Des. 11, 973–984.

Barczyk, K., Kreuter, M., Pryjma, J., Booy, E.P., Maddika, S., Ghavami, S., Berdel, W.E., Roth, J., Los, M., 2005. Serum cytochrome c indicates in vivo apoptosis and can serve as a prognostic marker during cancer therapy. Int. J. Cancer 116, 167–173.

Beachy, S.H., Repasky, E.A., 2008. Using extracellular biomarkers for monitoring efficacy of therapeutics in cancer patients: an update. Cancer Immunol. Immunother. 57, 759–775.

Beigi, R., Kobatake, E., Aizawa, M., Dubyak, G.R., 1999. Detection of local ATP release from activated platelets using cell surface-attached firefly luciferase. Am. J. Physiol. 276, C267–C278.

Berer, K., Mues, M., Koutrolos, M., Rasbi, Z. Al, Boziki, M., Johner, C., Wekerle, H., Krishnamoorthy, G., 2011. Commensal microbiota and myelin autoantigen cooperate to trigger autoimmune demyelination. Nature 479, 538–541.

Bhattacherjee, A., Rodrigues, E., Jung, J., Luzentales-Simpson, M., Enterina, J.R., Galleguillos, D., St. Laurent, C.D., Nakhaei-Nejad, M., Fuchsberger, F.F., Streith, L., Wang, Q., Kawasaki, N., Duan, S., Bains, A., Paulson, J.C., Rademacher, C., Giuliani, F., Sipione, S., Macauley, M.S., 2019. Repression of phagocytosis by human CD33 is not conserved with mouse CD33. Commun. Biol. 2, 450.

Bianchi, R., Kastrisianaki, E., Giambanco, I., Donato, R., 2011. S100B protein stimulates microglia migration via RAGE-dependent upregulation of chemokine expression and release. J. Biol. Chem. 286, 7214–7226.

Blasko, I., Veerhuis, R., Stampfer-Kountchev, M., Saurwein-Teissl, M., Eikelenboom, P., Grubeck-Loebenstein, B., 2000. Costimulatory effects of interferon-γ and interleukin-1β or tumor necrosis factor α on the synthesis of Aβ1-40 and Aβ1-42 by human astrocytes. Neurobiol. Dis. 7, 682–689.

Blázquez, E., Velázquez, E., Hurtado-Carneiro, V., Ruiz-Albusac, J.M., 2014. Insulin in the brain: its pathophysiological implications for states related with central insulin resistance, type 2 diabetes and Alzheimer's disease. Front. Endocrinol. (Lausanne). 5, 161–182.

Bohatschek, M., Werner, A., Raivich, G., 2001. Systemic LPS injection leads to granulocyte influx into normal and injured brain: effects of ICAM-1 deficiency. Exp. Neurol. 172, 137–152.

Brabazon, F., Bermudez, S., Shaughness, M., Khayrullina, G., Byrnes, K.R., 2018. The effects of insulin on the inflammatory activity of BV2 microglia. PLoS One 13, e0201878.

Brown, A.J., Goldsworthy, S.M., Barnes, A.A., Eilert, M.M., Tcheang, L., Daniels, D., Muir, A.I., Wigglesworth, M.J., Kinghorn, I., Fraser, N.J., Pike, N.B., Strum, J.C., Steplewski, K.M., Murdock, P.R., Holder, J.C., Marshall, F.H., Szekeres, P.G., Wilson, S., Ignar, D.M., Foord, S.M., Wise, A., Dowell, S.J., 2003. The orphan G protein-coupled receptors GPR41 and GPR43 are activated by propionate and other short chain carboxylic acids. J. Biol. Chem. 278, 11312–11319.

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

Brown, G.C., Vilalta, A., 2015. How microglia kill neurons. Brain Res. 1628, 288–297.

Brown, R.C., Davis, T.P., 2002. Calcium modulation of adherens and tight junction function. Stroke 33, 1706–1711.

Bsibsi, M., Bajramovic, J.J., Van Duijvenvoorden, E., Persoon, C., Ravid, R., Van Noort, J.M., Vogt, M.H.J., 2007. Identification of soluble CD14 as an endogenous agonist for toll-like receptor 2 on human astrocytes by genome-scale functional screening of glial cell derived proteins. Glia 55, 473–482.

Bsibsi, M., Persoon-Deen, C., Verwer, R.W.H., Meeuwsen, S., Ravid, R., Van Noort, J.M., 2006. Toll-like receptor 3 on adult human astrocytes triggers production of neuroprotective mediators. Glia 53, 688–695.

Bushnell, G.W., Louie, G. V., Brayer, G.D., 1990. High-resolution three-dimensional structure

of horse heart cytochrome c. J. Mol. Biol. 214, 585–595.

Canfora, E.E., Van Der Beek, C.M., Jocken, J.W.E., Goossens, G.H., Holst, J.J., Olde Damink, S.W.M., Lenaerts, K., Dejong, C.H.C., Blaak, E.E., 2017. Colonic infusions of short-chain fatty acid mixtures promote energy metabolism in overweight/obese men: a randomized crossover trial. Sci. Rep. 7, 1–12.

Cao, W., Zheng, H., 2018. Peripheral immune system in aging and Alzheimer's disease. Mol. Neurodegener. 13, 51–68.

Caso, J.R., Pradillo, J.M., Hurtado, O., Lorenzo, P., Moro, M.A., Lizasoain, I., 2007. Toll-like receptor 4 is involved in brain damage and inflammation after experimental stroke. Circulation 115, 1599–1608.

Causevic, M., Ramoz, N., Haroutunian, V., Davis, K.L., Buxbaum, J.D., 2003. Lack of association between the levels of the low-density lipoprotein receptor-related protein (LRP) and either Alzheimer dementia or LRP exon 3 genotype. J. Neuropathol. Exp. Neurol. 62, 999–1005.

Chakraborty, K., Raundhal, M., Chen, B.B., Morse, C., Tyurina, Y.Y., Khare, A., Oriss, T.B., Huff, R., Lee, J.S., Croix, S.C.M., Watkins, S., Mallampalli, R.K., Kagan, V.E., Ray, A., Ray, P., 2017. The mito-DAMP cardiolipin blocks IL-10 production causing persistent inflammation during bacterial pneumonia. Nat. Commun. 8, 1–15.

Chen, F., Kuhn, D.C., Gaydos, L.J., Demers, L.M., 1996. Induction of nitric oxide and nitric oxide synthase mRNA by silica and lipopolysaccharide in PMA-primed THP-1 cells. APMIS 104, 176–182.

Chhor, V., Le Charpentier, T., Lebon, S., Oré, M.V., Celador, I.L., Josserand, J., Degos, V., Jacotot, E., Hagberg, H., Sävman, K., Mallard, C., Gressens, P., Fleiss, B., 2013. Characterization of phenotype markers and neuronotoxic potential of polarised primary microglia in vitro. Brain. Behav. Immun. 32, 70–85.

Cho, H., Hashimoto, T., Wong, E., Hori, Y., Wood, L.B., Zhao, L., Haigis, K.M., Hyman, B.T., Irimia, D., 2013. Microfluidic chemotaxis platform for differentiating the roles of soluble and bound amyloid-β on microglial accumulation. Sci. Rep. 3, 1823.

Choi, S.S., Lee, H.J., Lim, I., Satoh, J., Kim, S.U., 2014. Human astrocytes: secretome profiles of cytokines and chemokines. PLoS One 9, 1–8.

Clarner, T., Janssen, K., Nellessen, L., Stangel, M., Skripuletz, T., Krauspe, B., Hess, F.-M.,

Denecke, B., Beutner, C., Linnartz-Gerlach, B., Neumann, H., Vallières, L., Amor, S., Ohl, K., Tenbrock, K., Beyer, C., Kipp, M., 2015. CXCL10 triggers early microglial activation in the cuprizone model. J. Immunol. 194, 3400–3413.

Clayton, K.A., Van Enoo, A.A., Ikezu, T., 2017. Alzheimer's disease: the role of microglia in brain homeostasis and proteopathy. Front. Neurosci. 11, 680.

Cloonan, S.M., Choi, A.M.K., 2013. Mitochondria: sensors and mediators of innate immune receptor signaling. Curr. Opin. Microbiol. 16, 327–338.

Cox, M.A., Jackson, J., Stanton, M., Rojas-Triana, A., Bober, L., Laverty, M., Yang, X., Zhu, F., Liu, J., Wang, S., Monsma, F., Vassileva, G., Maguire, M., Gustafson, E., Bayne, M., Chou, C.C., Lundell, D., Jenh, C.H., 2009. Short-chain fatty acids act as anti-inflammatory mediators by regulating prostaglandin E2 and cytokines. World J. Gastroenterol. 15, 5549–5557.

Crous-Bou, M., Minguillón, C., Gramunt, N., Molinuevo, J.L., 2017. Alzheimer's disease prevention: from risk factors to early intervention. Alzheimers Res. Ther. 9, 71.

Culmsee, C., Michels, S., Scheu, S., Arolt, V., Dannlowski, U., Alferink, J., 2019. Mitochondria, microglia, and the immune system—how are they linked in affective disorders? Front. Psychiatry 9, 739.

Cunningham, C., Dunne, A., Lopez-Rodriguez, A.B., 2019. Astrocytes: heterogeneous and dynamic phenotypes in neurodegeneration and innate immunity. Neuroscientist 25, 455–474.

Dalile, B., Van Oudenhove, L., Vervliet, B., Verbeke, K., 2019. The role of short-chain fatty acids in microbiota–gut–brain communication. Nat. Rev. Gastroenterol. Hepatol. 16, 461–478.

Daneman, R., Prat, A., 2015. The blood-brain barrier. Cold Spring Harb. Perspect. Biol. 7, a020412–a020435.

Daneman, R., Zhou, L., Kebede, A.A., Barres, B.A., 2010. Pericytes are required for blood–brain barrier integrity during embryogenesis. Nature 468, 562–566.

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, 1–18.

Davis, R.L., Buck, D.J., McCracken, K., Cox, G.W., Das, S., 2018. Interleukin-1β-induced inflammatory signaling in C20 human microglial cells. Neuroimmunol. Neuroinflamm. 5, 1–11.

Deane, R., Sagare, A., Zlokovic, B. V, 2008. The role of the cell surface LRP and soluble LRP in blood-brain barrier Aβ clearance in Alzheimer's disease. Curr. Pharm. Des. 14, 1601–1605.

Deguchi, H., Fernández, J.A., Hackeng, T.M., Banka, C.L., Griffin, J.H., 2000. Cardiolipin is a normal component of human plasma lipoproteins. Proc. Natl. Acad. Sci. U. S. A. 97, 1743–1748.

Demaurex, N., Scorrano, L., 2009. Reactive oxygen species are NOXious for neurons. Nat. Neurosci. 12, 819–820.

den Besten, G., van Eunen, K., Groen, A.K., Venema, K., Reijngoud, D.-J., Bakker, B.M., 2013. The role of short-chain fatty acids in the interplay between diet, gut microbiota, and host energy metabolism. J. Lipid Res. 54, 2325–2340.

Deshpande, M., Zheng, J., Borgmann, K., Persidsky, R., Wu, L., Schellpeper, C., Ghorpade, A., 2005. Role of activated astrocytes in neuronal damage: potential links to HIV-1-associated dementia. Neurotox. Res. 7, 183–192.

Ding, X., Zhang, M., Gu, R., Xu, G., Wu, H., 2017. Activated microglia induce the production of reactive oxygen species and promote apoptosis of co-cultured retinal microvascular pericytes. Graefe's Arch. Clin. Exp. Ophthalmol. 255, 777–788.

Du, Y., Deng, W., Wang, Z., Ning, M., Zhang, W., Zhou, Y., Lo, E.H., Xing, C., 2017. Differential subnetwork of chemokines/cytokines in human, mouse, and rat brain cells after oxygen–glucose deprivation. J. Cereb. Blood Flow Metab. 37, 1425–1434.

Dukay, B., Csoboz, B., Tóth, M.E., 2019. Heat-shock proteins in neuroinflammation. Front. Pharmacol. 10, 1–22.

El-Hage, N., Podhaizer, E.M., Sturgill, J., Hauser, K.F., 2011. Toll-like receptor expression and activation in astroglia: differential regulation by HIV-1 Tat, gp120, and morphine. Immunol. Invest. 40, 498–522.

Erny, D., Hrabě de Angelis, A.L., Jaitin, D., Wieghofer, P., Staszewski, O., David, E., 2015. Host microbiota constantly control maturation and function of microglia in the CNS. Nat. Neurosci. 18, 965–977.

Esen, N., Kielian, T., 2014. Central role for MyD88 in the responses of microglia to pathogen-

associated molecular patterns. J. Immunol. 176, 6802–6811.

Falsig, J., Latta, M., Leist, M., 2004. Defined inflammatory states in astrocyte cultures: correlation with susceptibility towards CD95-driven apoptosis. J. Neurochem. 88, 181–193.

Fan, Y., Xie, L., Chung, C.Y., 2017. Signaling pathways controlling microglia chemotaxis. Mol. Cells 40, 163–168.

Faraco, G., Fossati, S., Bianchi, M.E., Patrone, M., Pedrazzi, M., Sparatore, B., Moroni, F., Chiarugi, A., 2007. High mobility group box 1 protein is released by neural cells upon different stresses and worsens ischemic neurodegeneration in vitro and in vivo. J. Neurochem. 103, 590–603.

Fellay, J., Marzolini, C., Meaden, E.R., Back, D.J., Buclin, T., Chave, J.-P., Decosterd, L.A., Furrer, H., Opravil, M., Pantaleo, G., Retelska, D., Ruiz, L., Schinkel, A.H., Vernazza, P., Eap, C.B., Telenti, A., 2002. Response to antiretroviral treatment in HIV-1-infected individuals with allelic variants of the multidrug resistance transporter 1: a pharmacogenetics study. Lancet 359, 30–36.

Fellner, L., Irschick, R., Schanda, K., Reindl, M., Klimaschewski, L., Poewe, W., Wenning, G.K., Stefanova, N., 2013. Toll-like receptor 4 is required for α-synuclein dependent activation of microglia and astroglia. Glia 61, 349–360.

Fernando, M.R., Saxena, A., Reyes, J.L., McKay, D.M., 2016. Butyrate enhances antibacterial effects while suppressing other features of alternative activation in IL-4-induced macrophages. Am. J. Physiol. - Gastrointest. Liver Physiol. 310, G822–G831.

Forsyth, R., Fray, A., Boutelle, M., Fillenz, M., Middleditch, C., Burchell, A., 1996. A role for astrocytes in glucose delivery to neurons? Dev. Neurosci. 18, 360–370.

Frohman, E.M., Frohman, T.C., Gupta, S., de Fougerolles, A., van den Noort, S., 1991. Expression of intercellular adhesion molecule 1 (ICAM-1) in Alzheimer's disease. J. Neurol. Sci. 106, 105–111.

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.

Galloway, D.A., Phillips, A.E.M., Owen, D.R.J., Moore, C.S., 2019. Phagocytosis in the brain: homeostasis and disease. Front. Immunol. 10, 790.

Gandy, K.A.O., Zhang, J., Nagarkatti, P., Nagarkatti, M., 2019. The role of gut microbiota in shaping the relapse-remitting and chronic-progressive forms of multiple sclerosis in mouse

models. Sci. Rep. 9, 6923–6940.

Garry, P.S., Ezra, M., Rowland, M.J., Westbrook, J., Pattinson, K.T.S., 2015. The role of the nitric oxide pathway in brain injury and its treatment - From bench to bedside. Exp. Neurol. 263, 235–243.

Gate, D., Rezai-Zadeh, K., Jodry, D., Rentsendorj, A., Town, T., 2010. Macrophages in Alzheimer's disease: the blood-borne identity. J. Neural Transm. 117, 961–970.

Gengler, S., Hamilton, A., Hölscher, C., 2010. Synaptic plasticity in the hippocampus of a APP/PS1 mouse model of Alzheimer's disease is impaired in old but not young mice. PLoS One 5, e9764–e9764.

Ghosh, T.S., Rampelli, S., Jeffery, I.B., Santoro, A., Neto, M., Capri, M., Giampieri, E., Jennings, A., Candela, M., Turroni, S., Zoetendal, E.G., Hermes, G.D.A., Elodie, C., Meunier, N., Brugere, C.M., Pujos-Guillot, E., Berendsen, A.M., De Groot, L.C.P.G.M., Feskins, E.J.M., Kaluza, J., Pietruszka, B., Bielak, M.J., Comte, B., Maijo-Ferre, M., Nicoletti, C., De Vos, W.M., Fairweather-Tait, S., Cassidy, A., Brigidi, P., Franceschi, C., O'Toole, P.W., 2020. Mediterranean diet intervention alters the gut microbiome in older people reducing frailty and improving health status: the NU-AGE 1-year dietary intervention across five European countries. Gut 69, 1218–1228.

Giulian, D., Ingeman, J.F., 1988. Colony-stimulating factors as promoters of ameboid microglia. J. Neurosci. 8, 4707–4717.

Go, M., Kou, J., Lim, J.E., Yang, J., Fukuchi, K.I., 2016. Microglial response to LPS increases in wild-type mice during aging but diminishes in an Alzheimer's mouse model: implication of TLR4 signaling in disease progression. Biochem. Biophys. Res. Commun. 479, 331–337.

Gong, T., Liu, L., Jiang, W., Zhou, R., 2020. DAMP-sensing receptors in sterile inflammation and inflammatory diseases. Nat. Rev. Immunol. 20, 95–112.

Goodsell, D.S., 2004. The molecular perspective: cytochrome c and apoptosis. Oncologist 9, 226–227.

Gosselin, D., Skola, D., Coufal, N.G., Holtman, I.R., Schlachetzki, J.C.M., Sajti, E., Jaeger, B.N., O'Connor, C., Fitzpatrick, C., Pasillas, M.P., Pena, M., Adair, A., Gonda, D.D., Levy, M.L., Ransohoff, R.M., Gage, F.H., Glass, C.K., 2017. An environment-dependent transcriptional network specifies human microglia identity. Science 356, 1248–1259.

Gouveia, A., Bajwa, E., Klegeris, A., 2017. Extracellular cytochrome c as an intercellular

signaling molecule regulating microglial functions. Biochim. Biophys. Acta - Gen. Subj. 1861, 2274–2281.

Grazioli, S., Pugin, J., 2018. Mitochondrial damage-associated molecular patterns: from inflammatory signaling to human diseases. Front. Immunol. 9, 832.

Greene, C., Hanley, N., Campbell, M., 2019. Claudin-5: gatekeeper of neurological function. Fluids Barriers C.N.S. 16, 3–18.

Hamilton, N., Attwell, D., Hall, C., 2010. Pericyte-mediated regulation of capillary diameter: a component of neurovascular coupling in health and disease. Front. Neuroenergetics 2, 5–19.

Hampl, R., Marie, B., Lucie, S., 2015. Hormones and the blood-brain barrier. Horm. Mol. Biol. Clin. Investig. 21, 159–164.

Han, X.J., Hu, Y.Y., Yang, Z.J., Jiang, L.P., Shi, S.L., Li, Y.R., Guo, M.Y., Wu, H.L., Wan, Y.Y., 2017. Amyloid β-42 induces neuronal apoptosis by targeting mitochondria. Mol. Med. Rep. 16, 4521–4528.

Han, Z., Tian, R., Ren, P., Zhou, W., Wang, P., Luo, M., Jin, S., Jiang, Q., 2018. Parkinson's disease and Alzheimer's disease: a Mendelian randomization study. BMC Med. Genet. 19, 215.

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.

Hanzlik, R.P., Fowler, S.C., Eells, J.T., 2005. Absorption and elimination of formate following oral administration of calcium formate in female human subjects. Drug Metab. Dispos. 33, 282–286.

Harach, T., Marungru, N., Duthilleul, N., Cheatham, V., Coy, K.D.M., Frisoni, G., 2017. Reduction of Abeta amyloid pathology in APPPS1 transgenic mice in the absence of gut microbiota. Sci. Rep. 7, 1–13.

Haran, J.P., Bhattarai, S.K., Foley, S.E., Dutta, P., Ward, D.V., Bucci, V., McCormick, B.A., 2019. Alzheimer's disease microbiome is associated with dysregulation of the anti-inflammatory p-glycoprotein pathway. MBio 10, 1–14.

Haruwaka, K., Ikegami, A., Tachibana, Y., Ohno, N., Konishi, H., Hashimoto, A., Matsumoto, M., Kato, D., Ono, R., Kiyama, H., Moorhouse, A.J., Nabekura, J., Wake, H., 2019. Dual microglia effects on blood brain barrier permeability induced by systemic inflammation.

Nat. Commun. 10, 5816–5833.

Hashioka, S., Han, Y., Fujii, S., Kato, T., Monji, A., 2007. Phosphatidylserine and phosphatidylcholine-containing liposomes inhibit amyloid β and interferon-γ-induced microglial activation. Free Radic. Biol. Med. 42, 945–954.

Hashioka, S., Klegeris, A., McGeer, P.L., 2012. The histone deacetylase inhibitor suberoylanilide hydroxamic acid attenuates human astrocyte neurotoxicity induced by interferon-γ. J. Neuroinflammation 9, 1–8.

Hashioka, S., Klegeris, A., Schwab, C., McGeer, P.L., 2009. Interferon-γ-dependent cytotoxic activation of human astrocytes and astrocytoma cells. Neurobiol. Aging 30, 1924–1935.

Hashioka, S., McLarnon, J.G., Ryu, J.K., Youssef, A.M., Abd-El-Aziz, A.S., Neeland, E.G., Klegeris, A., 2011. Pyrazole compound 2-MBAPA as a novel inhibitor of microglial activation and neurotoxicity in vitro and in vivo. J. Alzheimers Dis. 27, 531–541.

Hayashi, T., Crain, B., Yao, S., Caneda, C.D., Cottam, H.B., Chan, M., Corr, M., Carson, D.A., 2014. Novel synthetic toll-like receptor 4/MD2 ligands attenuate sterile inflammation. J. Pharmacol. Exp. Ther. 350, 330–340.

Heusinkveld, H.J., Westerink, R.H.S., 2017. Comparison of different in vitro cell models for the assessment of pesticide-induced dopaminergic neurotoxicity. Toxicol. In Vitro 45, 81–88.

Holley, M.M., Zhang, Y., Lehrmann, E., Wood, W.H., Becker, K.G., Kielian, T., 2012. Toll-like receptor 2 (TLR2)-TLR9 crosstalk dictates IL-12 family cytokine production in microglia. Glia 60, 29–42.

Hornik, T.C., Vilalta, A., Brown, G.C., 2016. Activated microglia cause reversible apoptosis of pheochromocytoma cells, inducing their cell death by phagocytosis. J. Cell Sci. 129, 65–79.

Hosoya, M., Kawasaki, Y., Katayose, M., Sakuma, H., Watanabe, M., Igarashi, E., Aoyama, M., Nunoi, H., Suzuki, H., 2006. Prognostic predictive values of serum cytochrome c, cytokines, and other laboratory measurements in acute encephalopathy with multiple organ failure. Arch. Dis. Child. 91, 469–472.

Hovens, I.B., Nyakas, C., Schoemaker, R.G., 2014. A novel method for evaluating microglial activation using ionized calcium-binding adaptor protein-1 staining: cell body to cell size ratio. Neuroimmunol. Neuroinflamm. 1, 82–88.

Huang, W., Guo, H.L., Deng, X., Zhu, T.T., Xiong, J.F., Xu, Y.H., Xu, Y., 2017. Short-chain fatty acids inhibit oxidative stress and inflammation in mesangial cells induced by high

glucose and lipopolysaccharide. Exp. Clin. Endocrinol. Diabetes 125, 98–105.

Hussey, S.E., Liang, H., Costford, S.R., Klip, A., DeFronzo, R.A., Sanchez-Avila, A., Ely, B., Musi, N., 2012. TAK-242, a small-molecule inhibitor of toll-like receptor 4 signalling, unveils similarities and differences in lipopolysaccharide- and lipid-induced inflammation and insulin resistance in muscle cells. Biosci. Rep. 33, 37–47.

Hüttemann, M., Pecina, P., Rainbolt, M., Sanderson, T.H., Kagan, V.E., Samavati, L., Doan, J.W., Lee, I., 2011. The multiple functions of cytochrome c and their regulation in life and death decisions of the mammalian cell: from respiration to apoptosis. Mitochondrion 11, 369–381.

Huuskonen, J., Suuronen, T., Nuutinen, T., Kyrylenko, S., Salminen, A., 2004. Regulation of microglial inflammatory response by sodium butyrate and short-chain fatty acids. Br. J. Pharmacol. 141, 874–880.

Ingelsson, M., 2016. Alpha-synuclein oligomers-neurotoxic molecules in Parkinson's disease and other Lewy body disorders. Front. Neurosci. 10, 408.

Iyer, S.S., He, Q., Janczy, J.R., Elliott, E.I., Zhong, Z., Olivier, A.K., Sadler, J.J., Knepper-Adrian, V., Han, R., Qiao, L., Eisenbarth, S.C., Nauseef, W.M., Cassel, S.L., Sutterwala, F.S., 2013. Mitochondrial cardiolipin is required for NLRP3 inflammasome activation. Immunity 39, 311–323.

Jackson, S.J., Andrews, N., Ball, D., Bellantuono, I., Gray, J., Hachoumi, L., Holmes, A., Latcham, J., Petrie, A., Potter, P., Rice, A., Ritchie, A., Stewart, M., Strepka, C., Yeoman, M., Chapman, K., 2017. Does age matter? The impact of rodent age on study outcomes. Lab. Anim. 51, 160–169.

Jemmerson, R., LaPlante, B., Treeful, A., 2002. Release of intact, monomeric cytochrome c from apoptotic and necrotic cells. Cell Death Differ. 9, 538–548.

Jensen, C.J., Massie, A., Keyser, J. De, 2013. Immune players in the CNS: the astrocyte. J. Neuroimmune Pharmacol. 8, 824–839.

Ji, J., Shu, D., Zheng, M., Wang, J., Luo, C., Wang, Y., Guo, F., Zou, X., Lv, X., Li, Y., Liu, T., Qu, H., 2016. Microbial metabolite butyrate facilitates M2 macrophage polarization and function. Sci. Rep. 6, 1–10.

Johnstone, M., Gearing, A.J.H., Miller, K.M., 1999. A central role for astrocytes in the inflammatory response to β-amyloid; chemokines, cytokines and reactive oxygen species

are produced. J. Neuroimmunol. 93, 182–193.

Joshi, A.U., Minhas, P.S., Liddelow, S.A., Haileselassie, B., Andreasson, K.I., Dorn, G.W., Mochly-Rosen, D., 2019. Fragmented mitochondria released from microglia trigger A1 astrocytic response and propagate inflammatory neurodegeneration. Nat. Neurosci. 22, 1635–1648.

Józefowski, S., Czerkies, M., Sobota, A., Kwiatkowska, K., 2011. Determination of cell surface expression of toll-like receptor 4 by cellular enzyme-linked immunosorbent assay and radiolabeling. Anal. Biochem. 413, 185–191.

Kagan, V.E., Tyurin, V.A., Jiang, J., Tyurina, Y.Y., Ritov, V.B., Amoscato, A.A., Osipov, A.N., Belikova, N.A., Kapralov, A.A., Kini, V., Vlasova, I.I., Zhao, Q., Zou, M., Di, P., Svistunenko, D.A., Kurnikov, I. V, Borisenko, G.G., 2005. Cytochrome c acts as a cardiolipin oxygenase required for release of proapoptotic factors. Nat. Chem. Biol. 1, 223–232.

Kang, D.E., Pietrzik, C.U., Baum, L., Chevallier, N., Merriam, D.E., Kounnas, M.Z., Wagner, S.L., Troncoso, J.C., Kawas, C.H., Katzman, R., Koo, E.H., 2000. Modulation of amyloid β-protein clearance and Alzheimer's disease susceptibility by the LDL receptor-related protein pathway. J. Clin. Invest. 106, 1159–1166.

Kapellos, T.S., Taylor, L., Lee, H., Cowley, S.A., James, W.S., Iqbal, A.J., Greaves, D.R., 2016. A novel real time imaging platform to quantify macrophage phagocytosis. Biochem. Pharmacol. 116, 107–119.

Kato, J., Svensson, C.I., 2015. Role of extracellular damage-associated molecular pattern molecules (DAMPs) as mediators of persistent pain. Prog. Mol. Biol. Transl. Sci. 131, 251–279.

Kertesz, Z., Yu, B.B., Steinkasserer, A., Haupt, H., Benham, A., Sim, R.B., 1995. Characterization of binding at human β2-glycoprotein I to cardiolipin. Biochem. J. 310, 315–321.

Kierdorf, K., Erny, D., Goldmann, T., Sander, V., Schulz, C., Perdiguero, E.G., Wieghofer, P., Heinrich, A., Riemke, P., Hölscher, C., Müller, D.N., Luckow, B., Brocker, T., Debowski, K., Fritz, G., 2013. Microglia emerge from erythromyeloid precursors via Pu.1- and Irf8-dependent pathways. Nat. Neurosci. 16, 273–280.

Kim, H.S., Chung, D.H., 2012. TLR4-mediated IL-12 production enhances IFN-γ and IL-1β

production, which inhibits TGF-β production and promotes antibody-induced joint inflammation. Arthritis Res. Ther. 14, R210.

Kim, H.S., Lee, J.H., Lee, J.P., Kim, E.M., Chang, K.A., Park, C.H., Jeong, S.J., Wittendorp, M.C., Seo, J.H., Choi, S.H., Suh, Y.H., 2002. Amyloid β peptide induces cytochrome c release from isolated mitochondria. Neuroreport 13, 1989–1993.

Kim, Y.S., Joh, T.H., 2006. Microglia, major player in the brain inflammation: their roles in the pathogenesis of Parkinson's disease. Exp. Mol. Med. 38, 333–347.

Kitamura, Y., Taniguchi, T., Shimohama, S., 1999. Apoptotic cell death in neurons and glial cells: implicated for Alzheimer's disease. Jpn. J. Pharmacol. 79, 1–5.

Klegeris, A., 2020. Regulation of neuroimmune processes by damage- and resolution-associated molecular patterns. Neural Regen. Res. 16, 423–429.

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.

Klegeris, A., Bissonnette, C.J., McGeer, P.L., 2003. Reduction of human monocytic cell neurotoxicity and cytokine secretion by ligands of the cannabinoid-type CB2 receptor. Br. J. Pharmacol. 139, 775–786.

Klegeris, A., Giasson, B.I., Zhang, H., Maguire, J., Pelech, S., McGeer, P.L., 2006. Alpha-synuclein and its disease-causing mutants induce ICAM-1 and IL-6 in human astrocytes and astrocytoma cells. FASEB J. 20, 2000–2008.

Klegeris, A., Li, J., Bammler, T.K., Jin, J., Zhu, D., Kashima, D.T., Pan, S., Hashioka, S., Maguire, J., McGeer, P.L., Zhang, J., 2008. Prolyl endopeptidase is revealed following SILAC analysis to be a novel mediator of human microglial and THP-1 cell neurotoxicity. Glia 56, 675–685.

Klegeris, A., Walker, D.G., McGeer, P.L., 1999. Toxicity of human THP-1 monocytic cells towards neuron-like cells is reduced by non-steroidal anti-inflammatory drugs (NSAIDs). Neuropharmacology 38, 1017–1025.

Komarova, Y.A., Kruse, K., Mehta, D., Malik, A.B., 2017. Protein interactions at endothelial junctions and signaling mechanisms regulating endothelial permeability. Circ. Res. 120, 179–206.

Kopec, K.K., Carroll, R.T., 2000. Phagocytosis is regulated by nitric oxide in murine microglia.

Nitric Oxide 4, 103–111.

Krummen, M., Balkow, S., Shen, L., Heinz, S., Loquai, C., Probst, H.C., Grabbe, S., 2010. Release of IL-12 by dendritic cells activated by TLR ligation is dependent on MyD88 signaling, whereas TRIF signaling is indispensable for TLR synergy. J. Leukoc. Biol. 88, 189–199.

Ktsoyan, Z.A., Mkrtchyan, M.S., Zakharyan, M.K., Mnatsakanyan, A.A., Arakelova, K.A., Gevorgyan, Z.U., Sedrakyan, A.M., Hovhannisyan, A.I., Arakelyan, A.A., Aminov, R.I., 2016. Systemic concentrations of short chain fatty acids are elevated in salmonellosis and exacerbation of familial mediterranean fever. Front. Microbiol. 7, 1–10.

Kuno, R., Wang, J., Kawanokuchi, J., Takeuchi, H., Mizuno, T., Suzumura, A., 2005. Autocrine activation of microglia by tumor necrosis factor-α. J. Neuroimmunol. 162, 89–96.

Lancaster, M.A., Huch, M., 2019. Disease modelling in human organoids. Dis. Model. Mech. 12, 1–14.

Land, W.G., 2015. The role of damage-associated molecular patterns (DAMPs) in human disease. Sultan Qaboos Univ. Med. J. 15, 157–170.

Lannes, N., Eppler, E., Etemad, S., Yotovski, P., Filgueira, L., 2017. Microglia at center stage: a comprehensive review about the versatile and unique residential macrophages of the central nervous system. Oncotarget 8, 114393–114413.

Laurent, C., Dorothée, G., Hunot, S., Martin, E., Monnet, Y., Duchamp, M., Dong, Y., Légeron, F.-P., Leboucher, A., Burnouf, S., Faivre, E., Carvalho, K., Caillierez, R., Zommer, N., Demeyer, D., Jouy, N., Sazdovitch, V., Schraen-Maschke, S., Delarasse, C., Buée, L., Blum, D., 2017. Hippocampal T cell infiltration promotes neuroinflammation and cognitive decline in a mouse model of tauopathy. Brain 140, 184–200.

Lee, J.K., Tansey, M.G., 2013. Microglia isolation from adult mouse brain. Methods Mol. Biol. 1041, 17–23.

Lee, S.C., Liu, W., Dickson, D.W., Brosnan, C.F., Berman, J.W., 1993. Cytokine production by human fetal microglia and astrocytes. Differential induction by lipopolysaccharide and IL-1 beta. J. Immunol. 150, 2659–2667.

Lee, S.U., In, H.J., Kwon, M.S., Park, B., Jo, M., Kim, M., Cho, S., Lee, S., Lee, H., Kwak, S., Kim, S., 2013. β-Arrestin 2 mediates G protein-coupled receptor 43 signals to muclear factor-κB. Biol. Pharm. Bull. 36, 1754–1759.

Leitner, G.R., Wenzel, T.J., Marshall, N., Gates, E.J., Klegeris, A., 2019. Targeting toll-like receptor 4 to modulate neuroinflammation in central nervous system disorders. Expert Opin. Ther. Targets 23, 865–882.

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, C., Zhao, R., Gao, K., Wei, Z., Yin, M.Y., Lau, L.T., Chui, D., Hoi Yu, A.C., 2011. Astrocytes: implications for neuroinflammatory pathogenesis of Alzheimer's disease. Curr. Alzheimer Res. 8, 67–80.

Li, M., van Esch, B.C.A.M., Henricks, P.A.J., Folkerts, G., Garssen, J., 2018. The anti-inflammatory effects of short chain fatty acids on lipopolysaccharide- or tumor necrosis factor α-stimulated endothelial cells via activation of GPR41/43 and inhibition of HDACs. Front. Pharmacol. 9, 1–12.

Li, T., Chen, X., Zhang, C., Zhang, Y., Yao, W., 2019. An update on reactive astrocytes in chronic pain. J. Neuroinflammation 16, 140.

Liew, F.Y., Patel, M., Xu, D., 2005. Toll-like receptor 2 signalling and inflammation. Ann. Rheum. Dis. 64, iv104–iv105.

Lin, A., Giuliano, C.J., Palladino, A., John, K.M., Abramowicz, C., Yuan, M. Lou, Sausville, E.L., Lukow, D.A., Liu, L., Chait, A.R., Galluzzo, Z.C., Tucker, C., Sheltzer, J.M., 2019. Off-target toxicity is a common mechanism of action of cancer drugs undergoing clinical trials. Sci. Transl. Med. 11, eaaw8412.

Linder, M., Mäyränpää, M., Peränen, J., Pietilä, T., Pietiäinen, V., Uronen, R., Olkkonen, V., Kovanen, P., Ikonen, E., 2009. Rab8 regulates ABCA1 cell surface expression and facilitates cholesterol efflux in primary human macrophages. Arterioscler. Thromb. Vasc. Biol. 29, 883–888.

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.

Liu, J., Konermann, L., 2009. Irreversible thermal denaturation of cytochrome c studied by electrospray mass spectrometry. J. Am. Soc. Mass Spectrom. 20, 819–828.

Liu, P., Wu, L., Peng, G., Han, Y., Tang, R., Ge, J., Zhang, L., Jia, L., Yue, S., Zhou, K., Li, L., Luo, B., Wang, B., 2019. Altered microbiomes distinguish Alzheimer's disease from amnestic mild cognitive impairment and health in a Chinese cohort. Brain. Behav. Immun. 80, 633–643.

Liu, T., Zhang, L., Joo, D., Sun, S.C., 2017. NF-κB signaling in inflammation. Signal Transduct. Target. Ther. 2, 1–9.

Liva, S.M., Kahn, M.A., Dopp, J.M., de Vellis, J., 1999. Signal transduction pathways induced by GM-CSF in microglia: significance in the control of proliferation. Glia 26, 344–352.

Lively, S., Schlichter, L.C., 2018. Microglia responses to pro-inflammatory stimuli (LPS, IFNγ+TNFα) and reprogramming by resolving cytokines (IL-4, IL-10). Front. Cell. Neurosci. 12, 1–19.

Lizundia, R., Sauter, K.S., Taylor, G., Werling, D., 2008. Host species-specific usage of the TLR4-LPS receptor complex. Innate Immun. 14, 223–231.

Löscher, W., Potschka, H., 2002. Role of multidrug transporters in pharmacoresistance to antiepileptic drugs. J. Pharmacol. Exp. Ther. 301, 7–14.

Lotze, M.T., Tracey, K.J., 2005. High-mobility group box 1 protein (HMGB1): nuclear weapon in the immune arsenal. Nat. Rev. Immunol. 5, 331–342.

Lv, J., Hu, W., Yang, Z., Li, T., Jiang, S., Ma, Z., Chen, F., Yang, Y., 2018. Focusing on claudin-5: a promising candidate in the regulation of BBB to treat ischemic stroke. Prog. Neurobiol. 161, 79–96.

Lyck, R., Reiss, Y., Gerwin, N., Greenwood, J., Adamson, P., Engelhardt, B., 2003. T-cell interaction with ICAM-1/ICAM-2 double-deficient brain endothelium in vitro: the cytoplasmic tail of endothelial ICAM-1 is necessary for transendothelial migration of T cells. Blood 102, 3675–3683.

Macfarlane, S., Macfarlane, G.T., 2003. Regulation of short-chain fatty acid production. Proc. Nutr. Soc. 62, 67–72.

Madeira, J.M., Little, J.P., Klegeris, A., 2012. Microglia secretome: from neurotoxins to neurotrophins, in: Kaur, C., Ling, E. (Eds.), Microglia: Biology, Functions and Roles in Disease. Nova Publishers, New York, NY, USA, pp. 73–92.

Malm, T.M., Jay, T.R., Landreth, G.E., 2015. The evolving biology of microglia in Alzheimer's disease. Neurotherapeutics 12, 81–93.

Mamula, M.J., Lin, R., Janeway, C.A., Hardin, J.A., 1992. Breaking T cell tolerance with foreign and self co-immunogens: a study of autoimmune B and T cell epitopes of cytochrome c. J. Immunol. 149, 789–795.

Maqsood, M.I., Matin, M.M., Bahrami, A.R., Ghasroldasht, M.M., 2013. Immortality of cell lines: challenges and advantages of establishment. Cell Biol. Int. 37, 1038–1045.

Marinelli, C., Di Liddo, R., Facci, L., Bertalot, T., Conconi, M.T., Zusso, M., Skaper, S.D., Giusti, P., 2015. Ligand engagement of toll-like receptors regulates their expression in cortical microglia and astrocytes. J. Neuroinflammation 12, 244.

Martin, S.F., Dudda, J.C., Bachtanian, E., Lembo, A., Liller, S., Dürr, C., Heimesaat, M.M., Bereswill, S., Fejer, G., Vassileva, R., Jakob, T., Freudenberg, N., Termeer, C.C., Johner, C., Galanos, C., Freudenberg, M.A., 2008. Toll-like receptor and IL-12 signaling control susceptibility to contact hypersensitivity. J. Exp. Med. 205, 2151–2162.

Maslowski, K.M., Vieira, A.T., Ng, A., Kranich, J., Sierro, F., Yu, D., Schilter, H.C., Rolph, M.S., Mackay, F., Artis, D., Xavier, R.J., Teixeira, M.M., Mackay, C.R., 2009. Regulation of inflammatory responses by gut microbiota and chemoattractant receptor GPR43. Nature 461, 1282–1286.

Masui, R., Sasaki, M., Funaki, Y., Ogasawara, N., Mizuno, M., Iida, A., Izawa, S., Kondo, Y., Ito, Y., Tamura, Y., Yanamoto, K., Noda, H., Tanabe, A., Okaniwa, N., Yamaguchi, Y., Iwamoto, T., Kasugai, K., 2013. G protein-coupled receptor 43 moderates gut inflammation through cytokine regulation from mononuclear cells. Inflamm. Bowel Dis. 19, 2848–2856.

McAllister, M.S., Krizanac-Bengez, L., Macchia, F., Naftalin, R.J., Pedley, K.C., Mayberg, M.R., Marroni, M., Leaman, S., Stanness, K.A., Janigro, D., 2001. Mechanisms of glucose transport at the blood–brain barrier: an in vitro study. Brain Res. 904, 20–30.

McGeer, P.L., McGeer, E.G., 2013. The amyloid cascade-inflammatory hypothesis of Alzheimer disease: implications for therapy. Acta Neuropathol. 126, 479–497.

McLarnon, J.G., 2016. Chemokine interleukin-8 (IL-8) in Alzheimer's and other neurodegenerative diseases. J. Alzheimer's Dis. Park. 6, 6–9.

McLoughlin, R.F., Berthon, B.S., Jensen, M.E., Baines, K.J., Wood, L.G., 2017. Short-chain fatty acids, prebiotics, synbiotics, and systemic inflammation: a systematic review and meta-analysis. Am. J. Clin. Nutr. 106, 930–945.

McManus, C.M., Brosnan, C.F., Berman, J.W., 1998. Cytokine induction of MIP-1α and MIP-1β

in human fetal microglia. J. Immunol. 160, 1449–1455.

Memon, M.A., Dai, H., Wang, Y., Xu, T., Aabdin, Z. ul, Bilal, M.S., Chandra, R.A., Shen, X., 2019. Efficacy of sodium butyrate in alleviating mammary oxidative stress induced by sub-acute ruminal acidosis in lactating goats. Microb. Pathog. 137, 103781.

Meraz-Ríos, M.A., Toral-Rios, D., Franco-Bocanegra, D., Villeda-Hernández, J., Campos-Peña, V., Gomez-Gonzalez, B., 2013. Inflammatory process in Alzheimer's Disease. Front. Integr. Neurosci. 7, 1–15.

Mestas, J., Hughes, C.C.W., 2004. Of mice and not men: differences between mouse and human immunology. J. Immunol. 172, 2731–2738.

Michaud, J.P., Hallé, M., Lampron, A., Thériault, P., Préfontaine, P., Filali, M., Tribout-Jover, P., Lanteigne, A.M., Jodoin, R., Cluff, C., Brichard, V., Palmantier, R., Pilorget, A., Larocque, D., Rivest, S., 2013. Toll-like receptor 4 stimulation with the detoxified ligand monophosphoryl lipid A improves Alzheimer's disease-related pathology. Proc. Natl. Acad. Sci. U. S. A. 110, 1941–1946.

Mills, S.W., Montgomery, S.H., Morck, D.W., 2006. Evaluation of the effects of short-chain fatty acids and extracellular pH on bovine neutrophil function in vitro. Am. J. Vet. Res. 67, 1901–1907.

Molinaro, A., Caesar, R., Holm, L.M., Tremaroli, V., Cani, P.D., Bäckhed, F., 2017. Host–microbiota interaction induces bi-phasic inflammation and glucose intolerance in mice. Mol. Metab. 6, 1371–1380.

Morris, M.E., Rodriguez-Cruz, V., Felmlee, M.A., 2017. SLC and ABC transporters: expression, localization, and species differences at the blood-brain and the blood-cerebrospinal fluid barriers. AAPS J. 19, 1317–1331.

Morrison, D.J., Preston, T., 2016. Formation of short chain fatty acids by the gut microbiota and their impact on human metabolism. Gut Microbes 7, 189–200.

Moser, J., Heeringa, P., Jongman, R.M., Zwiers, P.J., Niemarkt, A.E., Yan, R., de Graaf, I.A., Li, R., Ravasz Regan, E., Kümpers, P., Aird, W.C., van Nieuw Amerongen, G.P., Zijlstra, J.G., Molema, G., van Meurs, M., 2016. Intracellular RIG-I signaling regulates TLR4-independent endothelial inflammatory responses to endotoxin. J. Immunol. 196, 4681–4691.

Mosmann, T., 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J. Immunol. Methods 65, 55–63.

Nagele, R.G., Wegiel, Jerzy, Venkataraman, V., Imaki, H., Wang, K.C., Wegiel, Jarek, 2004. Contribution of glial cells to the development of amyloid plaques in Alzheimer's disease. Neurobiol. Aging 25, 663–674.

Nascimento, J.M., Saia-Cereda, V.M., Sartore, R.C., da Costa, R.M., Schitine, C.S., Freitas, H.R., Murgu, M., de Melo Reis, R.A., Rehen, S.K., Martins-de-Souza, D., 2019. Human cerebral organoids and fetal brain tissue share proteomic similarities. Front. Cell Dev. Biol. 7, 1–16.

Nastasi, C., Candela, M., Bonefeld, C.M., Geisler, C., Hansen, M., Krejsgaard, T., Biagi, E., Andersen, M.H., Brigidi, P., Ødum, N., Litman, T., Woetmann, A., 2015. The effect of short-chain fatty acids on human monocyte-derived dendritic cells. Sci. Rep. 5, 1–10.

Nau, R., Sörgel, F., Eiffert, H., 2010. Penetration of drugs through the blood-cerebrospinal fluid/blood-brain barrier for treatment of central nervous system infections. Clin. Microbiol. Rev. 23, 858–883.

Navarro, V., Sanchez-Mejias, E., Jimenez, S., Muñoz-Castro, C., Sanchez-Varo, R., Davila, J.C., Vizuete, M., Gutierrez, A., Vitorica, J., 2018. Microglia in Alzheimer's disease: activated, dysfunctional or degenerative. Front. Aging Neurosci. 10, 140.

Neniskyte, U., Vilalta, A., Brown, G.C., 2014. Tumour necrosis factor alpha-induced neuronal loss is mediated by microglial phagocytosis. FEBS Lett. 588, 2952–2956.

Newcombe, E.A., Camats-Perna, J., Silva, M.L., Valmas, N., Huat, T.J., Medeiros, R., 2018. Inflammation: the link between comorbidities, genetics, and Alzheimer's disease. J. Neuroinflammation 15, 276.

Nguyen, T.L.A., Vieira-Silva, S., Liston, A., Raes, J., 2015. How informative is the mouse for human gut microbiota research? Dis. Model. Mech. 8, 1–16.

Nieminen, A.L., Gores, G.J., Bond, J.M., Imberti, R., Herman, B., Lemasters, J.J., 1992. A novel cytotoxicity screening assay using a multiwell fluorescence scanner. Toxicol. Appl. Pharmacol. 115, 147–155.

Nitta, T., Hata, M., Gotoh, S., Seo, Y., Sasaki, H., Hashimoto, N., Furuse, M., Tsukita, S., 2003. Size-selective loosening of the blood-brain barrier in claudin-5-deficient mice. J. Cell Biol. 161, 653–660.

Norden, D.M., Trojanowski, P.J., Villanueva, E., Navarro, E., Godbout, J.P., 2016. Sequential activation of microglia and astrocyte cytokine expression precedes increased Iba-1 or GFAP

immunoreactivity following systemic immune challenge. Glia 64, 300–316.

Oblak, A., Jerala, R., 2015. The molecular mechanism of species-specific recognition of lipopolysaccharides by the MD-2/TLR4 receptor complex. Mol. Immunol. 63, 134–142.

Ohgidani, M., Kato, T.A., Setoyama, D., Sagata, N., Hashimoto, R., Shigenobu, K., Yoshida, T., Hayakawa, K., Shimokawa, N., Miura, D., Utsumi, H., Kanba, S., 2014. Direct induction of ramified microglia-like cells from human monocytes: dynamic microglial dysfunction in Nasu-Hakola disease. Sci. Rep. 4, 1–7.

Orihuela, R., McPherson, C.A., Harry, G.J., 2016. Microglial M1/M2 polarization and metabolic states. Br. J. Pharmacol. 173, 649–665.

Ottum, P.A., Arellano, G., Reyes, L.I., Iruretagoyena, M., Naves, R., 2015. Opposing roles of interferon-gamma on cells of the central nervous system in autoimmune neuroinflammation. Front. Immunol. 6, 539.

Oura, T., Cosimi, A.B., Kawai, T., 2017. Chimerism-based tolerance in organ transplantation: preclinical and clinical studies. Clin. Exp. Immunol. 189, 190–196.

Palmer, A.L., Ousman, S.S., 2018. Astrocytes and aging. Front. Aging Neurosci. 10, 337.

Papaspyropoulos, A., Tsolaki, M., Foroglou, N., Pantazaki, A.A., 2020. Modeling and targeting Alzheimer's disease with organoids. Front. Pharmacol. 11, 1–8.

Parajuli, B., Sonobe, Y., Kawanokuchi, J., Doi, Y., Noda, M., Takeuchi, H., Mizuno, T., Suzumura, A., 2012. GM-CSF increases LPS-induced production of proinflammatory mediators via upregulation of TLR4 and CD14 in murine microglia. J. Neuroinflammation 9, 268.

Patching, S.G., 2017. Glucose transporters at the blood-brain barrier: function, regulation and gateways for drug delivery. Mol. Neurobiol. 54, 1046–1077.

Paudel, Y.N., Shaikh, M.F., Chakraborti, A., Kumari, Y., Aledo-Serrano, Á., Aleksovska, K., Alvim, M.K.M.H., Othman, I., 2018. HMGB1: a common biomarker and potential target for TBI, neuroinflammation, epilepsy, and cognitive dysfunction. Front. Neurosci. 12, 628.

Paul, D., Ge, S., Lemire, Y., Jellison, E.R., Serwanski, D.R., Ruddle, N.H., Pachter, J.S., 2014. Cell-selective knockout and 3D confocal image analysis reveals separate roles for astrocyte- and endothelial-derived CCL2 in neuroinflammation. J. Neuroinflammation 11, 10.

Perea, G., Sur, M., Araque, A., 2014. Neuron-glia networks: integral gear of brain function. Front. Cell. Neurosci. 8, 378.

Pizzuto, M., Lonez, C., Baroja-Mazo, A., Martínez-Banaclocha, H., Tourlomousis, P., Gangloff, M., Pelegrin, P., Ruysschaert, J.M., Gay, N.J., Bryant, C.E., 2019. Saturation of acyl chains converts cardiolipin from an antagonist to an activator of toll-like receptor-4. Cell. Mol. Life Sci. 76, 3667–3678.

Place, R.F., Noonan, E.J., Giardina, C., 2005. HDAC inhibition prevents NF-κB activation by suppressing proteasome activity: down-regulation of proteasome subunit expression stabilizes IκBα. Biochem. Pharmacol. 70, 394–406.

Pointer, C.B., Klegeris, A., 2017. Cardiolipin in central nervous system physiology and pathology. Cell. Mol. Neurobiol. 37, 1161–1172.

Pointer, C.B., Wenzel, T.J., Klegeris, A., 2019. Extracellular cardiolipin regulates select immune functions of microglia and microglia-like cells. Brain Res. Bull. 146, 153–163.

Potokar, M., Jorgačevski, J., Zorec, R., 2016. Astrocyte aquaporin dynamics in health and disease. Int. J. Mol. Sci. 17, 1–18.

Prinz, M., Jung, S., Priller, J., 2019. Microglia biology: one century of evolving concepts. Cell 179, 292–311.

Prinz, M., Priller, J., 2017. The role of peripheral immune cells in the CNS in steady state and disease. Nat. Neurosci. 20, 136–144.

Pulgar, V.M., 2019. Transcytosis to cross the blood-brain barrier, new advancements and challenges. Front. Neurosci. 12, 1019.

Pullerits, R., Bokarewa, M., Jonsson, I.M., Verdrengh, M., Tarkowski, A., 2004. Extracellular cytochrome c, a mitochondrial apoptosis-related protein, induces arthritis. Rheumatology 44, 32–39.

Qin, H., Niyongere, S.A., Lee, S.J., Baker, B.J., Benveniste, E.N., 2008. Expression and functional significance of SOCS-1 and SOCS-3 in astrocytes. J. Immunol. 181, 3167–3176.

Qiu, Z., Strickland, D.K., Hyman, B.T., Rebeck, G.W., 2001. Elevation of LDL receptor-related protein levels via ligand interactions in Alzheimer disease and in vitro. J. Neuropathol. Exp. Neurol. 60, 430–440.

Rabouille, C., 2017. Pathways of unconventional protein secretion. Trends Cell Biol. 27, 230–240.

Radde, R., Bolmont, T., Kaeser, S.A., Coomaraswamy, J., Lindau, D., Stoltze, L., Calhoun, M.E., Jäggi, F., Wolburg, H., Gengler, S., Haass, C., Ghetti, B., Czech, C., Hölscher, C.,

Mathews, P.M., Jucker, M., 2006. Abeta42-driven cerebral amyloidosis in transgenic mice reveals early and robust pathology. EMBO Rep. 7, 940–946.

Radhakrishnan, J., Wang, S., Ayoub, I.M., Kolarova, J.D., Levine, R.F., Gazmuri, R.J., 2007. Circulating levels of cytochrome c after resuscitation from cardiac arrest: a marker of mitochondrial injury and predictor of survival. Am. J. Physiol. Circ. Physiol. 292, 767–775.

Rahman, M., Muhammad, S., Khan, M.A., Chen, H., Ridder, D.A., Müller-Fielitz, H., Pokorná, B., Vollbrandt, T., Stölting, I., Nadrowitz, R., Okun, J.G., Offermanns, S., Schwaninger, M., 2014. The β-hydroxybutyrate receptor HCA2 activates a neuroprotective subset of macrophages. Nat. Commun. 5, 1–11.

Ransohoff, R.M., 2016. A polarizing question: do M1 and M2 microglia exist. Nat. Neurosci. 19, 987–991.

Rawji, K.S., Mishra, M.K., Michaels, N.J., Rivest, S., Stys, P.K., Yong, V.W., 2016. Immunosenescence of microglia and macrophages: impact on the ageing central nervous system. Brain 139, 653–661.

Renz, A., Berdel, W.E., Kreuter, M., Belka, C., Schulze-osthoff, K., Los, M., 2001. Rapid extracellular release of cytochrome c is specific for apoptosis and marks cell death in vivo. Blood 98, 1542–1549.

Restrepo, A., Smith, C.A., Agnihotri, S., Shekarforoush, M., Kongkham, P.N., Seol, H.J., Northcott, P., Rutka, J.T., 2011. Epigenetic regulation of glial fibrillary acidic protein by DNA methylation in human malignant gliomas. Neuro. Oncol. 13, 42–50.

Rhea, E.M., Banks, W.A., 2019. Role of the blood-brain barrier in central nervous system insulin resistance. Front. Neurosci. 13, 521–535.

Roager, H.M., Licht, T.R., 2018. Microbial tryptophan catabolites in health and disease. Nat. Commun. 9, 1–10.

Roberfroid, M.B., Bornet, F., Bouley, C., Cummings, J.H., 1995. Colonic microflora: nutrition and health. Nutr. Rev. 53, 127–130.

Rock, R.B., Hu, S., Deshpande, A., Munir, S., May, B.J., Baker, C.A., Peterson, P.K., Kapur, V., 2005. Transcriptional response of human microglial cells to interferon-γ. Genes Immun. 6, 712–719.

Roh, J.S., Sohn, D.H., 2018. Damage-associated molecular patterns in inflammatory diseases. Immune Netw. 18, e27.

Roodveldt, C., Labrador-Garrido, A., Gonzalez-Rey, E., Lachaud, C.C., Guilliams, T., Fernandez-Montesinos, R., Benitez-Rondan, A., Robledo, G., Hmadcha, A., Delgado, M., Dobson, C.M., Pozo, D., 2013. Preconditioning of microglia by α-synuclein strongly affects the response induced by toll-like receptor (TLR) stimulation. PLoS One 8, 1–17.

Rothhammer, V., Mascanfroni, I.D., Bunse, L., Takenaka, M.C., Kenison, J.E., Mayo, L., Chao, C., Patel, B., Yan, R., Blain, M., Alvarez, J.I., Kébir, H., Anandasabapathy, N., Izquierdo, G., Jung, S., Obholzer, N., Pochet, N., Clish, C.B., Prinz, M., Prat, A., Antel, J., Quintana, F.J., 2016. Type I interferons and microbial metabolites of tryptophan modulate astrocyte activity and central nervous system inflammation via the aryl hydrocarbon receptor. Nat. Med. 22, 586–597.

Rubartelli, A., Cozzolino, F., Talio, M., Sitia, R., 1990. A novel secretory pathway for interleukin-1 beta, a protein lacking a signal sequence. EMBO J. 9, 1503–1510.

Rupp, N.J., Wegenast-Braun, B.M., Radde, R., Calhoun, M.E., Jucker, M., 2011. Early onset amyloid lesions lead to severe neuritic abnormalities and local, but not global neuron loss in APPPS1 transgenic mice. Neurobiol. Aging 32, 2324.e1-2324.e6.

Russo, I., Luciani, A., de Cicco, P., Troncone, E., Ciacci, C., 2012. Butyrate attenuates lipopolysaccharide-induced inflammation in intestinal cells and Crohn's mucosa through modulation of antioxidant defense machinery. PLoS One 7, 1–10.

Rustenhoven, J., Park, T.I.H., Schweder, P., Scotter, J., Correia, J., Smith, A.M., Gibbons, H.M., Oldfield, R.L., Bergin, P.S., Mee, E.W., Faull, R.L.M., Curtis, M.A., Scott Graham, E., Dragunow, M., 2016. Isolation of highly enriched primary human microglia for functional studies. Sci. Rep. 6, 1–11.

Saitou, M., Furuse, M., Sasaki, H., Schulzke, J.D., Fromm, M., Takano, H., Noda, T., Tsukita, S., 2000. Complex phenotype of mice lacking occludin, a component of tight junction strands. Mol. Biol. Cell 11, 4131–4142.

Sampson, T.R., Debelius, J.W., Thron, T., Wittung-stafshede, P., Knight, R., Mazmanian, S.K., Sampson, T.R., Debelius, J.W., Thron, T., Janssen, S., Shastri, G.G., Ilhan, Z.E., Challis, C., Schretter, C.E., Rocha, S., Gradinaru, V., Chesselet, M., 2016. Gut microbiota regulate motor deficits and neuroinflammation in a model of parkinson's disease. Cell 167, 1469–1480.

Satchell, M.A., Lai, Y., Kochanek, P.M., Wisniewski, S.R., Fink, E.L., Siedberg, N.A., Berger,

R.P., DeKosky, S.T., Adelson, P.D., Clark, R.S.B., 2005. Cytochrome c, a biomarker of apoptosis, is increased in cerebrospinal fluid from infants with inflicted brain injury from child abuse. J. Cereb. Blood Flow Metab. 25, 919–927.

Sato, S., Nomura, F., Kawai, T., Takeuchi, O., Mühlradt, P.F., Takeda, K., Akira, S., 2000. Synergy and cross-tolerance between toll-like receptor (TLR) 2- and TLR4-mediated signaling pathways. J. Immunol. 165, 7096–7101.

Savettieri, G., Liegro, I. Di, Catania, C., Licata, L., Pitarresi, G.L., D'Agostino, S., Schiera, G., De Caro, V., Giandalia, G., Giannola, L.I., Cestelli, A., 2000. Neurons and ECM regulate occludin localization in brain endothelial cells. Neuroreport 11, 1081–1084.

Scheperjans, F., Aho, V., Pereira, P.A.B., Koskinen, K., Paulin, L., Pekkonen, E., Haapaniemi, E., Kaakkola, S., Eerola-Rautio, J., Pohja, M., Kinnunen, E., Murros, K., Auvinen, P., 2015. Gut microbiota are related to Parkinson's disease and clinical phenotype. Mov. Disord. 30, 350–358.

Schindler, S.M., Frank, M.G., Annis, J.L., Maier, S.F., Klegeris, A., 2018. Pattern recognition receptors mediate pro-inflammatory effects of extracellular mitochondrial transcription factor A (TFAM). Mol. Cell. Neurosci. 89, 71–79.

Schirmer, M., Smeekens, S.P., Vlamakis, H., Jaeger, M., Oosting, M., Franzosa, E.A., Ter Horst, R., Jansen, T., Jacobs, L., Bonder, M.J., Kurilshikov, A., Fu, J., Joosten, L.A.B., Zhernakova, A., Huttenhower, C., Wijmenga, C., Netea, M.G., Xavier, R.J., 2016. Linking the human gut microbiome to inflammatory cytokine production capacity. Cell 167, 1125–1136.

Schroder, K., Irvine, K.M., Taylor, M.S., Bokil, N.J., Le Cao, K.A., Masterman, K.A., Labzin, L.I., Semple, C.A., Kapetanovic, R., Fairbairn, L., Akalin, A., Faulkner, G.J., Baillie, J.K., Gongora, M., Daub, C.O., Kawaji, H., McLachlan, G.J., Goldman, N., Grimmond, S.M., Carninci, P., Suzuki, H., Hayashizaki, Y., Lenhard, B., Hume, D.A., Sweet, M.J., 2012. Conservation and divergence in toll-like receptor 4-regulated gene expression in primary human versus mouse macrophages. Proc. Natl. Acad. Sci. U. S. A. 109, 944–953.

Seeley, J.J., Ghosh, S., 2017. Molecular mechanisms of innate memory and tolerance to LPS. J. Leukoc. Biol. 101, 107–119.

Serneels, L., Van Biervliet, J., Craessaerts, K., Dejaegere, T., Horré, K., Van Houtvin, T., Esselmann, H., Paul, S., Schäfer, M.K., Berezovska, O., Hyman, B.T., Sprangers, B., Sciot,

R., Moons, L., Jucker, M., Yang, Z., May, P.C., Karran, E., Wiltfang, J., D'Hooge, R., De Strooper, B., 2009. γ-Secretase heterogeneity in the Aph1 subunit: relevance for Alzheimer's disease. Science 324, 639–642.

Sheehan, J.J., Zhou, C., Gravanis, I., Rogove, A.D., Wu, Y.P., Bogenhagen, D.F., Tsirka, S.E., 2007. Proteolytic activation of monocyte chemoattractant protein-1 by plasmin underlies excitotoxic neurodegeneration in mice. J. Neurosci. 27, 1738–1745.

Shepherd, F.A., Sridhar, S.S., 2003. Angiogenesis inhibitors under study for the treatment of lung cancer. Lung Cancer 41, 63–72.

Shi, S., Liang, D., Chen, Y., Xie, Y., Wang, Y., Wang, L., Wang, Z., Qiao, Z., 2016. Gx-50 reduces β-amyloid-induced TNF-α, IL-1β, NO, and PGE2 expression and inhibits NF-κB signaling in a mouse model of Alzheimer's disease. Eur. J. Immunol. 46, 665–676.

Shields, A.M., Panayi, G.S., Corrigall, V.M., 2011. Resolution-associated molecular patterns (RAMP): RAMParts defending immunological homeostasis? Clin. Exp. Immunol. 165, 292–300.

Shinohara, M., Fujioka, S., Murray, M.E., Wojtas, A., Baker, M., Rovelet-Lecrux, A., Rademakers, R., Das, P., Parisi, J.E., Graff-Radford, N.R., Petersen, R.C., Dickson, D.W., Bu, G., 2014. Regional distribution of synaptic markers and APP correlate with distinct clinicopathological features in sporadic and familial Alzheimer's disease. Brain 137, 1533–1549.

Simard, M., Nedergaard, M., 2004. The neurobiology of glia in the context of water and ion homeostasis. Neuroscience 129, 877–896.

Singh, N., Gurav, A., Sivaprakasam, S., Brady, E., Padia, R., Shi, H., Thangaraju, M., Prasad, P.D., Manicassamy, S., Munn, D.H., Lee, J.R., Offermanns, S., Ganapathy, V., 2014. Activation of Gpr109a, receptor for niacin and the commensal metabolite butyrate, suppresses colonic inflammation and carcinogenesis. Immunity 40, 128–139.

Smith, B., Land, H., 2012. Anticancer activity of the cholesterol exporter ABCA1 gene. Cell Rep. 2, 580–590.

Smith, Q.R., 2000. Transport of glutamate and other amino acids at the blood-brain barrier. J. Nutr. 130, 1016S-1022S.

Song, K., Li, Y., Zhang, H., An, N., Wei, Y., Wang, L., Tian, C., Yuan, M., Sun, Y., Xing, Y., Gao, Y., 2020. Oxidative stress-mediated blood-brain barrier (BBB) disruption in

neurological diseases. Oxid. Med. Cell. Longev. 2020, 4356386.

Sorice, M., Circella, A., Cristea, I.M., Garofalo, T., Renzo, L. Di, Alessandri, C., Valesini, G., Esposti, M.D., 2004. Cardiolipin and its metabolites move from mitochondria to other cellular membranes during death receptor-mediated apoptosis. Cell Death Differ. 11, 1133–1145.

Spielman, L.J., Gibson, D.L., Klegeris, A., 2018. Unhealthy gut, unhealthy brain: the role of the intestinal microbiota in neurodegenerative diseases. Neurochem. Int. 120, 149–163.

Stratoulias, V., Venero, J.L., Tremblay, M.-È., Joseph, B., 2019. Microglial subtypes: diversity within the microglial community. EMBO J. 38, e101997.

Štros, M., 2010. HMGB proteins: interactions with DNA and chromatin. Biochim. Biophys. Acta - Gene Regul. Mech. 1799, 101–113.

Subedi, L., Lee, J.H., Yumnam, S., Ji, E., Kim, S.Y., 2019. Anti-inflammatory effect of sulforaphane on LPS-activated microglia potentially through JNK/AP-1/NF-κB inhibition and Nrf2/HO-1 activation. Cells 8, 194.

Sui, Y., Potula, R., Dhillon, N., Pinson, D., Li, S., Nath, A., Anderson, C., Turchan, J., Kolson, D., Narayan, O., Buch, S., 2004. Neuronal apoptosis is mediated by CXCL10 overexpression in simian human immunodeficiency virus encephalitis. Am. J. Pathol. 164, 1557–1566.

Sweeney, M.D., Zhao, Z., Montagne, A., Nelson, A.R., Zlokovic, B. V, 2018. Blood-brain barrier: from physiology to disease and back. Physiol. Rev. 99, 21–78.

Syeda, T., Sanchez-Tapia, M., Pinedo-Vargas, L., Granados, O., Cuervo-Zanatta, D., Rojas-Santiago, E., Díaz-Cintra, S., Torres, N., Perez-Cruz, C., 2018. Bioactive food abates metabolic and synaptic alterations by modulation of gut microbiota in a mouse model of Alzheimer's disease. J. Alzheimers Dis. 66, 1657–1682.

Takeuchi, H., 2010. Neurotoxicity by microglia: mechanisms and potential therapeutic strategy. Clin. Exp. Neuroimmunol. 1, 12–21.

Tan, J., McKenzie, C., Potamitis, M., Thorburn, A.N., Mackay, C.R., Macia, L., 2014. The role of short-chain fatty acids in health and disease. Adv. Immunol. 121, 91–119.

Tanaka, A., Nakamura, S., Seki, M., Fukudome, K., Iwanaga, N., Imamura, Y., Miyazaki, T., Izumikawa, K., Kakeya, H., Yanagihara, K., Kohno, S., 2013. Toll-like receptor 4 agonistic antibody promotes innate immunity against severe pneumonia induced by coinfection with

influenza virus and Streptococcus pneumoniae. Clin. Vaccine Immunol. 20, 977–985.

Tang, Y., Le, W., 2016. Differential roles of M1 and M2 microglia in neurodegenerative diseases. Mol. Neurobiol. 53, 1181–1194.

Tanokashira, D., Mamada, N., Yamamoto, F., Taniguchi, K., Tamaoka, A., Lakshmana, M.K., Araki, W., 2017. The neurotoxicity of amyloid β-protein oligomers is reversible in a primary neuron model. Mol. Brain 10, 4.

Tărlungeanu, D.C., Deliu, E., Dotter, C.P., Kara, M., Janiesch, P.C., Scalise, M., Galluccio, M., Tesulov, M., Morelli, E., Sonmez, F.M., Bilguvar, K., Ohgaki, R., Kanai, Y., Johansen, A., Esharif, S., Ben-Omran, T., Topcu, M., Schlessinger, A., Indiveri, C., Duncan, K.E., Caglayan, A.O., Gunel, M., Gleeson, J.G., Novarino, G., 2016. Impaired amino acid transport at the blood-brain barrier is a cause of autism spectrum disorder. Cell 167, 1481-1494.e18.

Thériault, P., ElAli, A., Rivest, S., 2015. The dynamics of monocytes and microglia in Alzheimer's disease. Alzheimers Res. Ther. 7, 41.

Thomas, D.C., 2017. The phagocyte respiratory burst: historical perspectives and recent advances. Immunol. Lett. 192, 88–96.

Thompson, K.K., Tsirka, S.E., 2017. The diverse roles of microglia in the neurodegenerative aspects of central nervous system (CNS) autoimmunity. Int. J. Mol. Sci. 18, 1–15.

Uhlén, M., Fagerberg, L., Hallström, B.M., Lindskog, C., Oksvold, P., Mardinoglu, A., Sivertsson, Å., Kampf, C., Sjöstedt, E., Asplund, A., Olsson, I., Edlund, K., Lundberg, E., Navani, S., Szigyarto, C.A., Odeberg, J., Djureinovic, D., Takanen, J.O., Hober, S., Alm, T., Edqvist, P., Berling, H., Tegel, H., Mulder, J., Rockberg, J., Nilsson, P., Schwenk, J.M., Hamsten, M., Feilitzen, K. Von, Forsberg, M., Persson, L., Johansson, F., Zwahlen, M., Heijne, G. Von, Nielsen, J., Pontén, F., 2015. Tissue-based map of the human proteome. Science 347, 12604191–12604199.

Ullian, E.M., Harris, B.T., Wu, A., Chan, J.R., Barres, B.A., 2004. Schwann cells and astrocytes induce synapse formation by spinal motor neurons in culture. Mol. Cell. Neurosci. 25, 241–251.

Uttara, B., Singh, A.V., Zamboni, P., Mahajan, R.T., 2009. Oxidative stress and neurodegenerative diseases: a review of upstream and downstream antioxidant therapeutic options. Curr. Neuropharmacol. 7, 65–74.

Vaure, C., Liu, Y., 2014. A comparative review of toll-like receptor 4 expression and functionality in different animal species. Front. Immunol. 5, 1–15.

Velasco, S., Kedaigle, A.J., Simmons, S.K., Nash, A., Rocha, M., Quadrato, G., Paulsen, B., Nguyen, L., Adiconis, X., Regev, A., Levin, J.Z., Arlotta, P., 2019. Individual brain organoids reproducibly form cell diversity of the human cerebral cortex. Nature 570, 523–527.

Venegas, C., Heneka, M.T., 2017. Danger-associated molecular patterns in Alzheimer's disease. J. Leukoc. Biol. 101, 87–98.

Verkhratsky, A., Olabarria, M., Noristani, H.N., Yeh, C.Y., Rodriguez, J.J., 2010. Astrocytes in Alzheimer's disease. Neurotherapeutics 7, 399–412.

Vijay, N., Morris, M.E., 2014. Role of monocarboxylate transporters in drug delivery to the brain. Curr. Pharm. Des. 20, 1487–1498.

Voss, J.J.L.P., Ford, C.A., Petrova, S., Melville, L., Paterson, M., Pound, J.D., Holland, P., Giotti, B., Freeman, T.C., Gregory, C.D., 2017. Modulation of macrophage antitumor potential by apoptotic lymphoma cells. Cell Death Differ. 24, 971–983.

Wakida, N.M., Cruz, G.M.S., Ro, C.C., Moncada, E.G., Khatibzadeh, N., Flanagan, L.A., Berns, M.W., 2018. Phagocytic response of astrocytes to damaged neighboring cells. PLoS One 13, 1–25.

Walter, S., Letiembre, M., Liu, Y., Heine, H., Penke, B., Hao, W., Bode, B., Manietta, N., Walter, J., Schulz-Schüffer, W., Fassbender, K., 2007. Role of the toll-like receptor 4 in neuroinflammation in Alzheimer's disease. Cell. Physiol. Biochem. 20, 947–956.

Wang, D., Bordey, A., 2009. The astrocyte odyssey. Neuroscience 86, 342–367.

Wang, Siwen, Song, R., Wang, Z., Jing, Z., Wang, Shaoxiong, Ma, J., 2018. S100A8/A9 in inflammation. Front. Immunol. 9, 1–14.

Wannick, M., Assmann, J.C., Vielhauer, J.F., Offermanns, S., Zillikens, D., Sadik, C.D., Schwaninger, M., 2018. The immunometabolomic interface receptor hydroxycarboxylic acid receptor 2 mediates the therapeutic effects of dimethyl fumarate in autoantibody-induced skin inflammation. Front. Immunol. 9, 1–10.

Wei, Z., Sun, X., Xu, Q., Zhang, Y., Tian, F., Sun, H., Zheng, H., Dai, J., 2016. TAK-242 suppresses lipopolysaccharide-induced inflammation in human coronary artery endothelial cells. Pharmazie 71, 583–587.

Weigle, W.O., 1980. Analysis of autoimmunity through experimental models of thyroiditis and allergic encephalomyelitis. Adv. Immunol. 30, 159–273.

Weinberg, R.P., Koledova, V. V, Schneider, K., Sambandan, T.G., Grayson, A., Zeidman, G., Artamonova, A., Sambanthamurthi, R., Fairus, S., Sinskey, A.J., Rha, C., 2018. Palm fruit bioactives modulate human astrocyte activity in vitro altering the cytokine secretome reducing levels of TNF-α, RANTES and IP-10. Sci. Rep. 8, 16423.

Welser-Alves, J. V, Milner, R., 2013. Microglia are the major source of TNF-α and TGF-β1 in postnatal glial cultures; regulation by cytokines, lipopolysaccharide, and vitronectin. Neurochem. Int. 63, 47–53.

Wenzel, T.J., Bajwa, E., Klegeris, A., 2019. Cytochrome c can be released into extracellular space and modulate functions of human astrocytes in a toll-like receptor 4-dependent manner. Biochim. Biophys. Acta - Gen. Subj. 1863, 129400.

Wenzel, T.J., Gates, E.J., Ranger, A.L., Klegeris, A., 2020a. Short-chain fatty acids (SCFAs) alone or in combination regulate select immune functions of microglia-like cells. Mol. Cell. Neurosci. 105, 103493.

Wenzel, T.J., Kwong, E., Bajwa, E., Klegeris, A., 2020b. Resolution-associated molecular patterns (RAMPs) as endogenous regulators of glia functions in neuroinflammatory disease. C.N.S. Neurol. Disord. Drug Targets 10, 483–494.

White, H., Venkatesh, B., 2011. Clinical review: ketones and brain injury. Crit. Care 15, 219.

Yang, G., Meng, Y., Li, W., Yong, Y., Fan, Z., Ding, H., Wei, Y., Luo, J., Ke, Z.J., 2011. Neuronal MCP-1 mediates microglia recruitment and neurodegeneration induced by the mild impairment of oxidative metabolism. Brain Pathol. 21, 279–297.

Yao, Y., Tsirka, S.E., 2014. Monocyte chemoattractant protein-1 and the blood-brain barrier. Cell. Mol. Life Sci. 71, 683–697.

Yu, Y., Ye, R.D., 2015. Microglial Aβ receptors in Alzheimer's disease. Cell. Mol. Neurobiol. 35, 71–83.

Zager, R.A., Johnson, A.C.M., Hanson, S.Y., 2004. Proximal tubular cytochrome c efflux: determinant, and potential marker, of mitochondrial injury. Kidney Int. 65, 2123–2134.

Zandi-nejad, K., Takakura, A., Jurewicz, M., Chandraker, A.K., Offermanns, S., Mount, D., Abdi, R., 2013. The role of HCA2 (GPR109A) in regulating macrophage function. FASEB J. 27, 4366–4374.

Zanoni, I., Ostuni, R., Marek, L.R., Barresi, S., Barbalat, R., Barton, G.M., Granucci, F., Kagan, J.C., 2011. CD14 controls the LPS-induced endocytosis of toll-like receptor 4. Cell 147, 868–880.

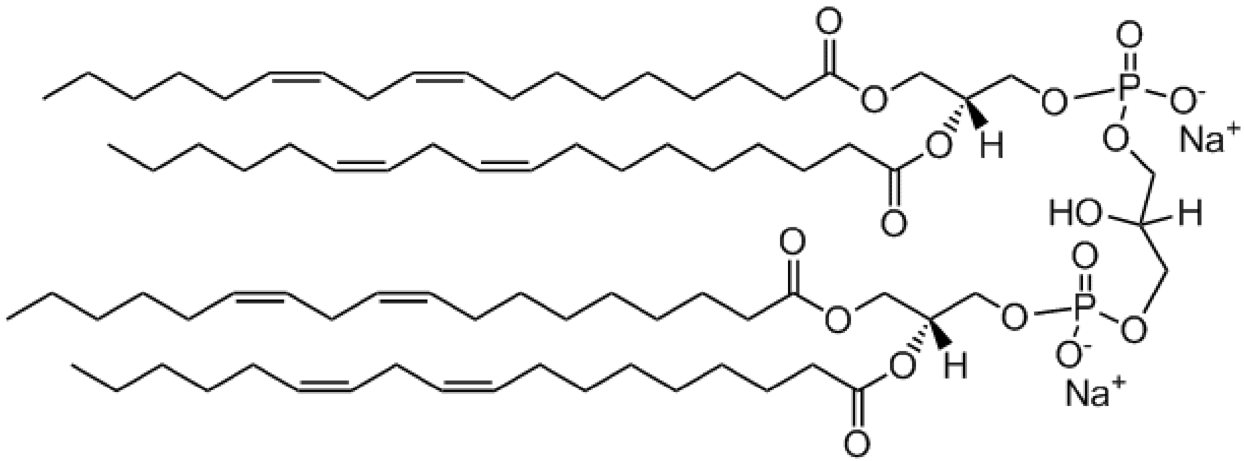
Zhang, L., Bell, R.J.A., Kiebish, M.A., Seyfried, T.N., Han, X., Gross, R.W., Chuang, J.H., 2011. A mathematical model for the determination of steady-state cardiolipin remodeling mechanisms using lipidomic data. PLoS One 6, e21170.

Zhang, L., Mizumoto, K., Sato, N., Ogawa, T., Kusumoto, M., Niiyama, H., Tanaka, M., 1999. Quantitative determination of apoptotic death in cultured human pancreatic cancer cells by propidium iodide and digitonin. Cancer Lett. 142, 129–137.

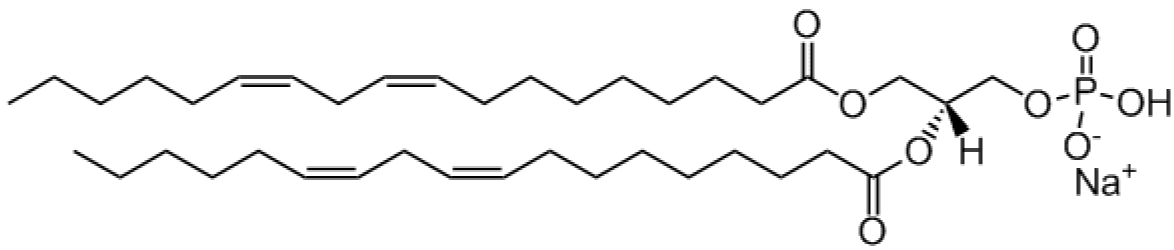
Zhao, G., Liu, J. fu, Nyman, M., Jönsson, J.Å., 2007. Determination of short-chain fatty acids in serum by hollow fiber supported liquid membrane extraction coupled with gas chromatography. J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 846, 202–208.

Zhou, Z.Y., Packialakshmi, B., Makkar, S.K., Dridi, S., Rath, N.C., 2014. Effect of butyrate on immune response of a chicken macrophage cell line. Vet. Immunol. Immunopathol. 162, 24–32.

Appendix



B



C

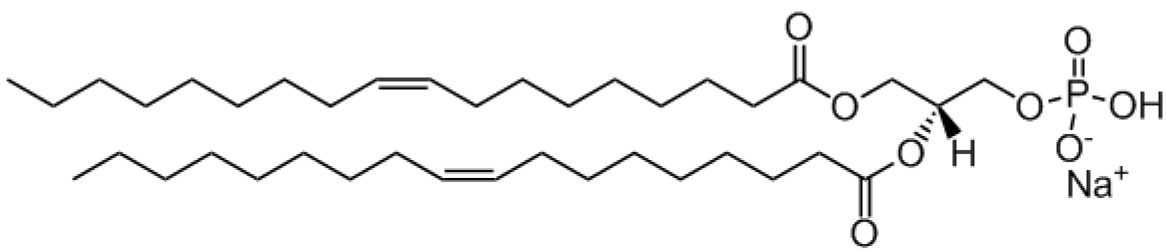


Figure S2.1. The chemical structure of the most abundant form of CL (**A)**, PA182 (**B**), and PA181 (**C**).

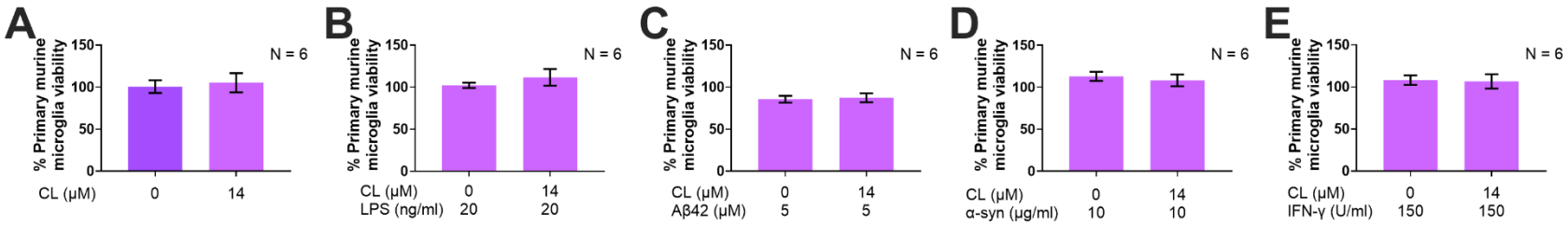


Figure S2.2. Effects of CL on the viability of unstimulated and stimulated primary murine microglia. CL was added to cell cultures to reach a final concentration of 14 μM. Cells were left unstimulated (**A**) or were stimulated with 20 ng/ml LPS (**B**), 5 μM Aβ42 (**C**), 10 μg/ml α-syn (**D**), or 150 U/ml IFN-γ (**E**) 15 min later. After 48 h incubation, viability of primary murine microglia was measured by the MTT assay. Data (means ± SEM) from six independent experiments performed on separate days are presented. No significance was detected according to the paired Student's *t*-test with Holm-Bonferroni correction for multiple comparisons.

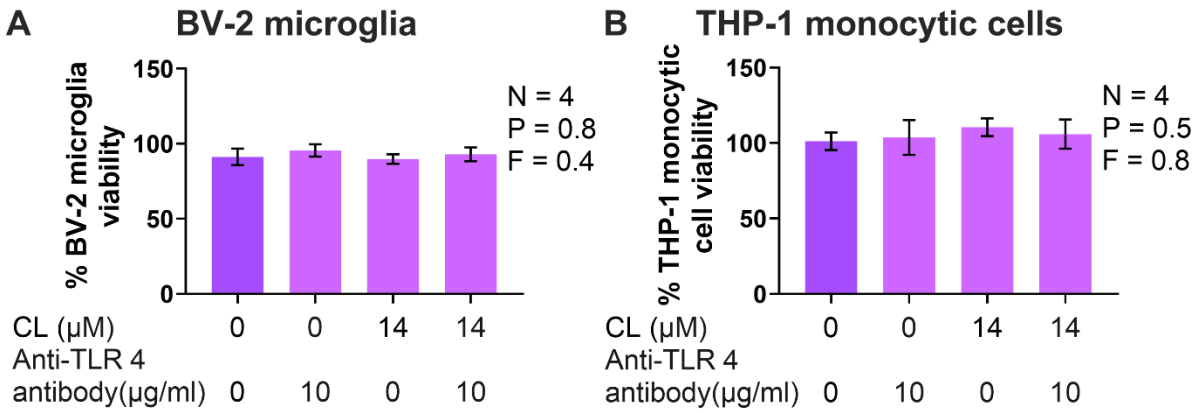


Figure S2.3. Effects of CL the viability of murine BV-2 microglia (**A**) and human THP-1 monocytic cells (**B**) in the presence or absence of TLR 4-blocking antibodies. 10 μg/ml of anti-TLR 4 antibodies were added to cell cultures 30 min before stimulation with CL (**A,B**). After 48 h incubation, viability of BV-2 microglia (**A**) and THP-1 cells (**B**) was measured by the MTT assay. Data (means ± SEM) from four independent experiments performed on separate days are presented. P and F values calculated by the one-way randomized blocks ANOVA are shown.

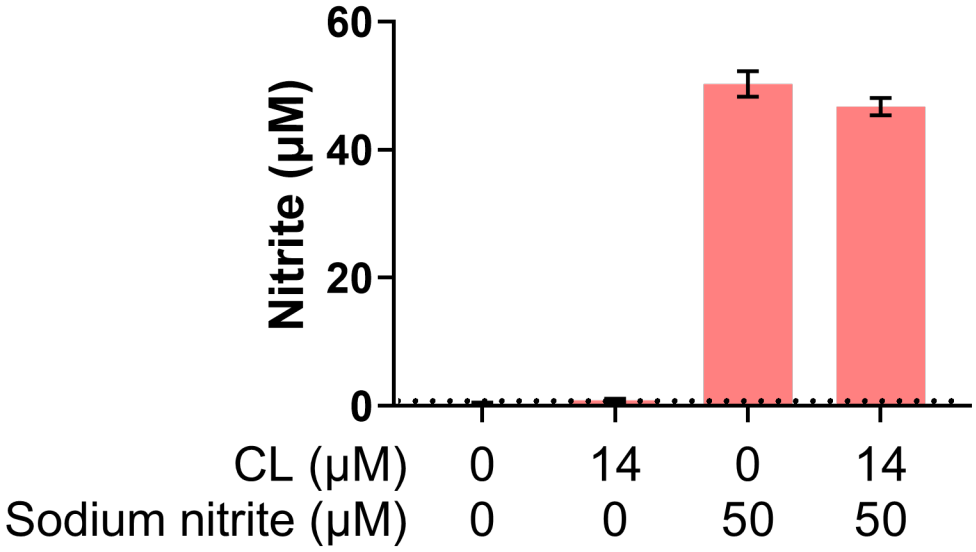


Figure S2.4. Chemical interaction between CL or its solvent and the Griess reagents. Solutions of cardiolipin or its solvent was mixed with Griess reagents in the presence or absence of sodium nitrite. Data (means ± SEM) from three experiments are presented.

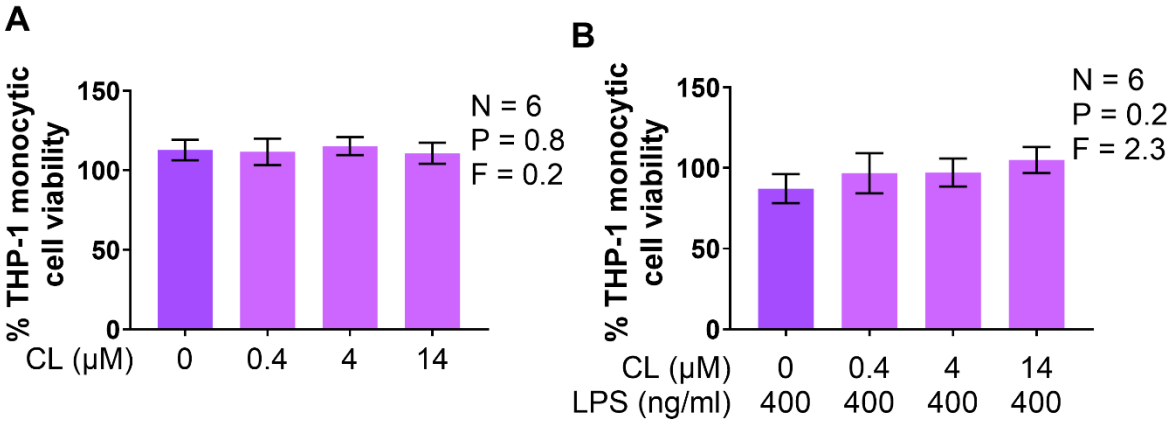


Figure S2.5. Effects of CL on the viability of human THP-1 monocytic cells in the presence or absence of LPS. Increasing concentrations of CL (0.4-14 μM) were added to cell cultures. Cells were left unstimulated (**A**) or were stimulated with 400 ng/ml (**B**) LPS 15 min later. After 48 h incubation, the viability of THP-1 cells were measured with the MTT assay. Data (means ± SEM) from six independent experiments performed on separate days are presented. P and F values calculated by the one-way randomized blocks ANOVA are shown.

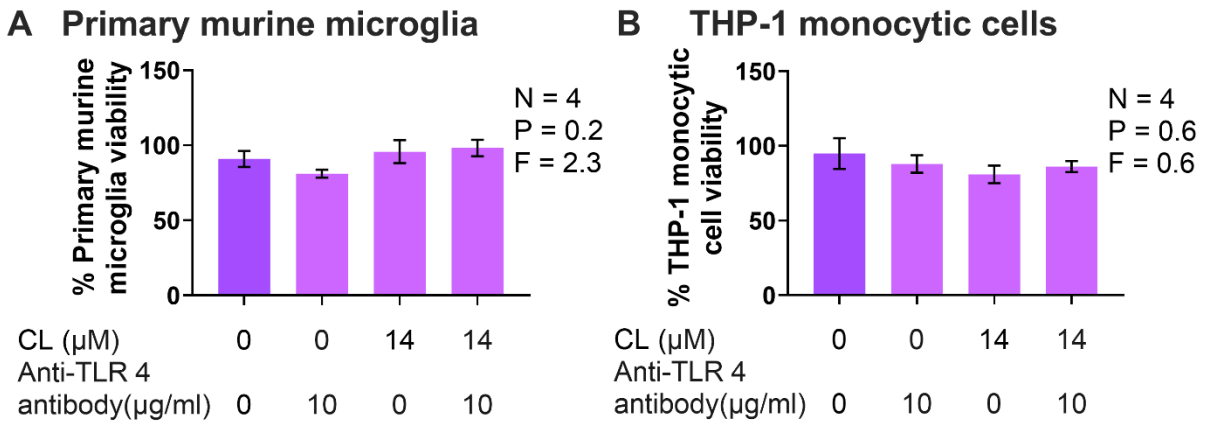


Figure S2.6. Effects of CL on the viability of primary murine microglia (**A**) or human THP-1 monocytic cells (**B**) in the presence or absence of TLR 4-blocking antibodies. 10 μg/ml of anti-TLR 4 antibodies were added to cell cultures first. After 30 min incubation, CL was added to reach the final concentrations of 14 μM. After a 24 h incubation period, viability of primary murine microglia (**A**) or THP-1 cells (**B**) was measured with the MTT assay. Data (means ± SEM) from four independent experiments performed on separate days are presented. P and F values calculated by the one-way randomized blocks ANOVA are shown.

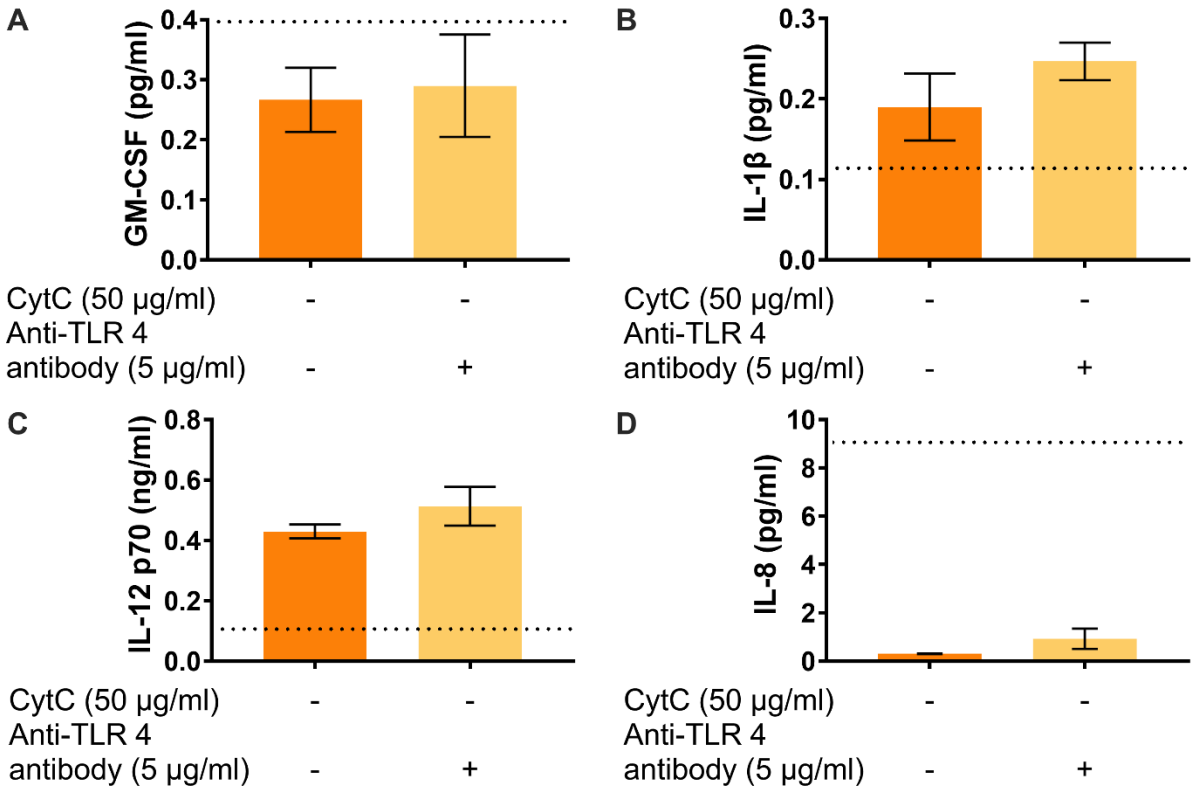


Figure S3.1. Anti-human TLR 4 antibodies alone did not affect the secretion of GM-CSF (**A**), IL-1β (**B**), IL-12 p70 (**C**) or IL-8 (**D**) by human primary astrocytes. 10 µg/ml of anti-TLR 4 antibodies were added to cell cultures first. 48 h later, concentrations of of GM-CSF (**A**), IL-1β (**B**), IL-12 p70 (**C**) or IL-8 (**D**) in cell-free supernatants were measured by the ELISA. Data (means ± SEM) from three independent experiments performed on separate days, using cells extracted from three different subjects, are presented. No significance was detected according to the paired Student's t-test with the Holm-Bonferroni correction for multiple comparisons. The detection limits of the ELISAs are shown as dotted lines.

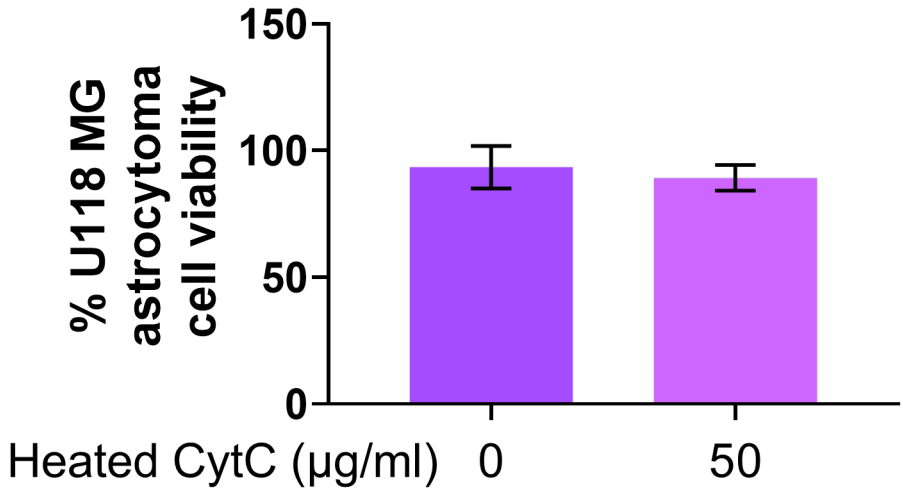


Figure S3.2. Effects of heated CytC on the viability of U118 MG astrocytic cells. Heated CytC was added to reach the final concentrations of 50 µg/ml. After a 48 h incubation period, viability of U118 MG astrocytic cells was measured with the MTT assay. Data (means ± SEM) from five independent experiments performed on separate days are presented. No significance was detected according to the paired Student's t-test.

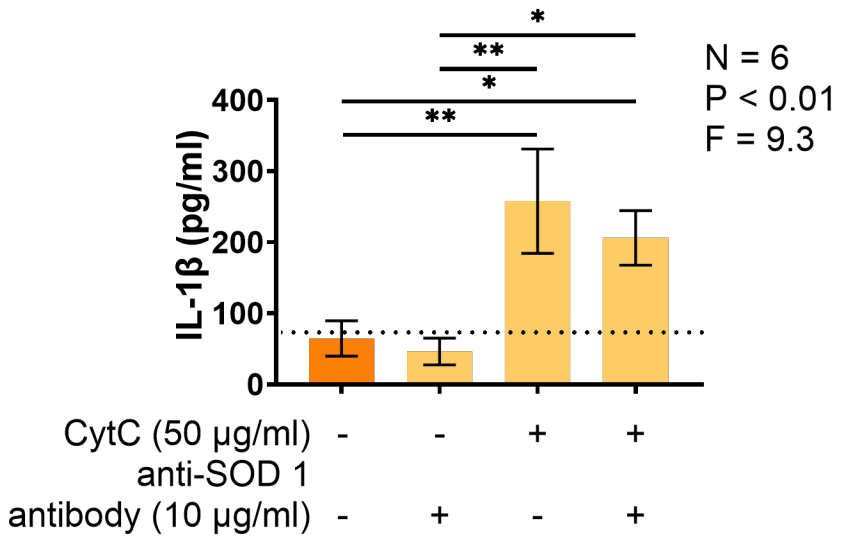


Figure S3.3. Anti-human SOD 1 antibodies had no effect on the CytC-induced secretion of IL-1β by U118 MG astrocytic cells. 10 µg/ml of anti-SOD 1 antibodies were added to cell cultures first. After 30 min incubation, CytC was added to reach the final concentrations of 50 µg/ml. 48 h later, concentrations of IL-1β in cell-free supernatants were measured by the ELISA. Data (means ± SEM) from six independent experiments performed on separate days are presented. * $P<0.05$, ** $P<0.01$ according to the Tukey's post-hoc test. P and F values for the one-way randomized blocks ANOVA are also shown, as well as the detection limit of the ELISAs as a dotted line.

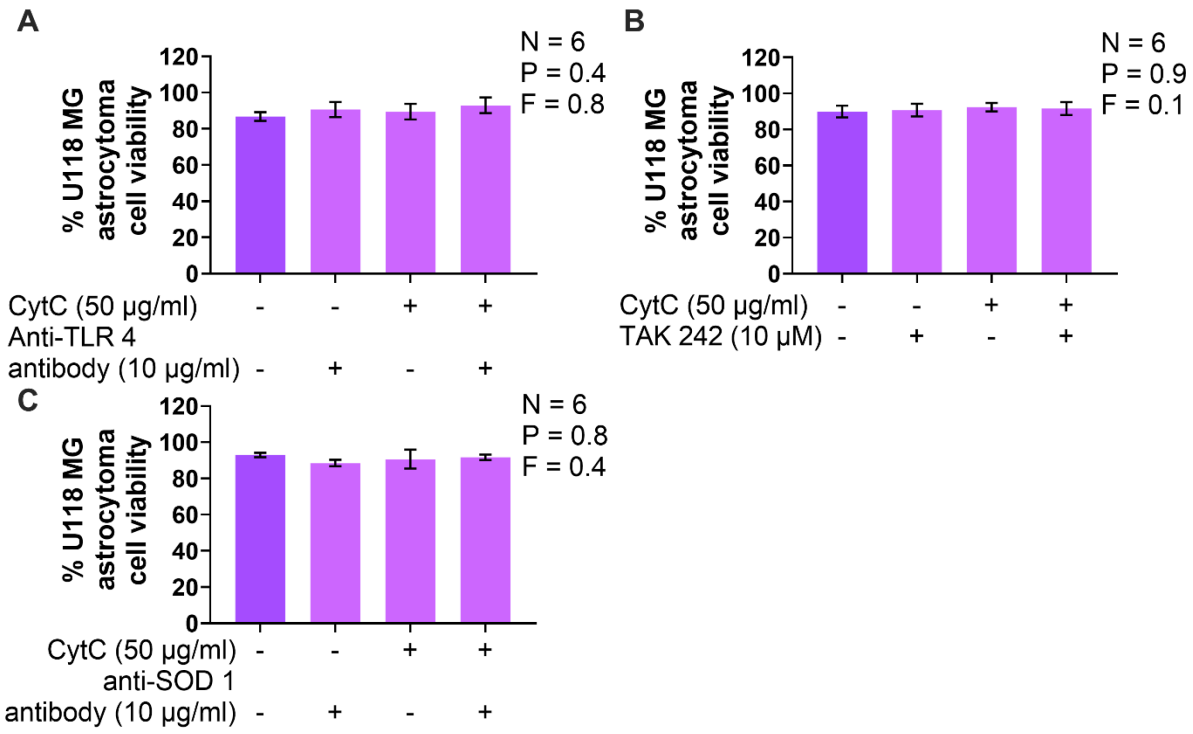


Figure S3.4. Anti-TLR 4 antibodies (**A**), TAK 242 (**B**), and anti-human SOD 1 antibodies (**C**) did not affect the viability of U118 MG astrocytic cells. 10 µg/ml of antibodies (**A,C**) or 10 µM TAK 242 (**B**) was added to cell cultures first. Cells were left untreated or were treated with CytC (50 µg/ml) 30 min later. After a 48 h incubation period, viability of U118 MG astrocytic cells was measured by the MTT assay. Data (means ± SEM) from six independent experiments performed on separate days are presented. P and F values for the one-way randomized blocks ANOVA are also shown.

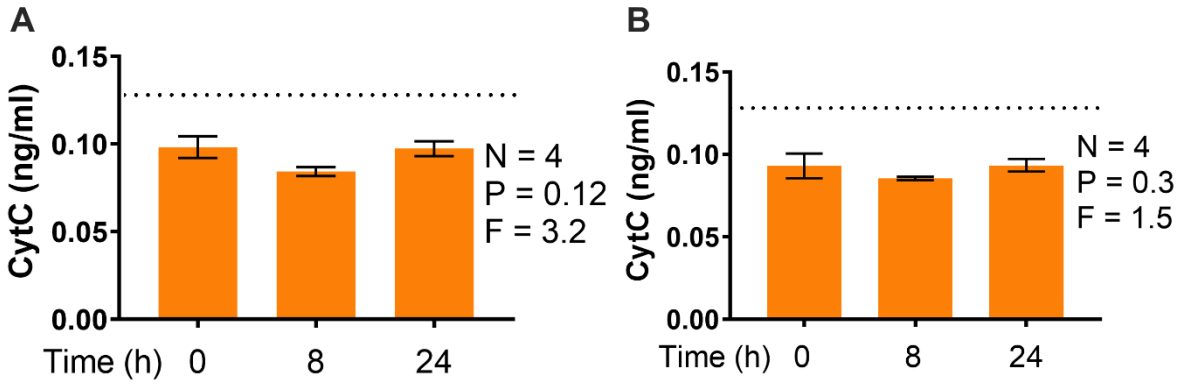


Figure S3.5. CytC was not detected in culture medium from BV-2 microglia exposed to the vehicle solutions of TNF-α (**A**) or Aβ42 (**B**). Data (means ± SEM) from four independent experiments performed on different days are presented. P and F values for the one-way randomized blocks ANOVA are shown, as well as the detection limit of the ELISA as a dotted line.

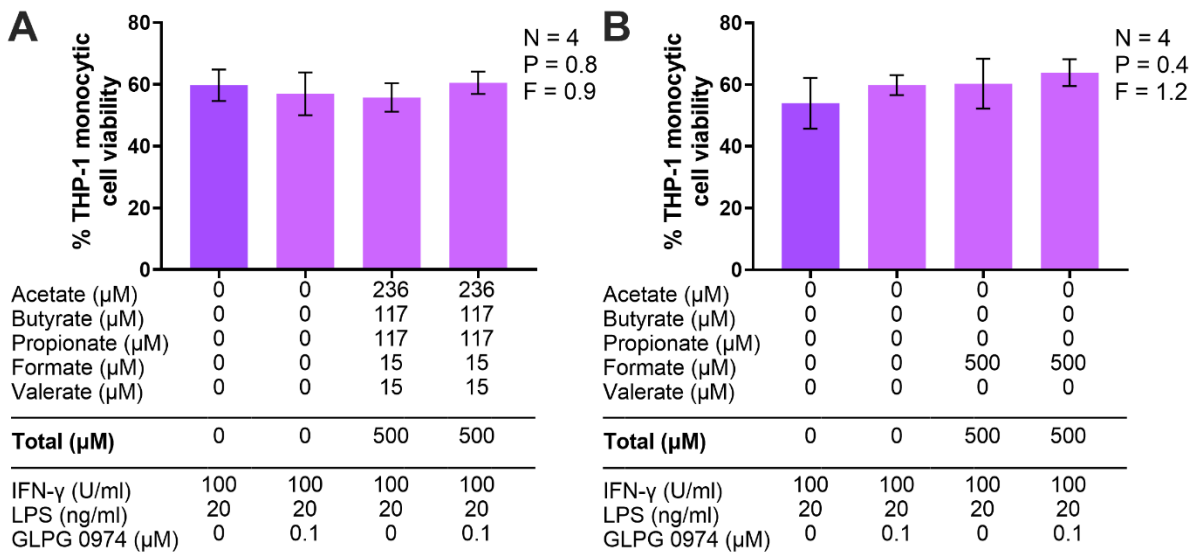


Figure S4.1. Effect of the SCFA mixture (**A**) and the SCFA formate on its own (**B**) in the presence or absence of the FFAR 2 and 3 antagonist on the viability of THP-1 cells. 0.1 µM GLPG 0974 was added to THP-1 cells first. After 30 min incubation, 500 µM SCFA mixture (**A**) or formate alone (**B**) were added to THP-1 cells, followed by their stimulation with LPS plus IFN-γ 15 min later. After a 48 h incubation period, viability of THP-1 cells was measured by the MTT assay. Data (means ± SEM) from four independent experiments performed on different days are presented. P and F values for the one-way randomized blocks ANOVA are shown.

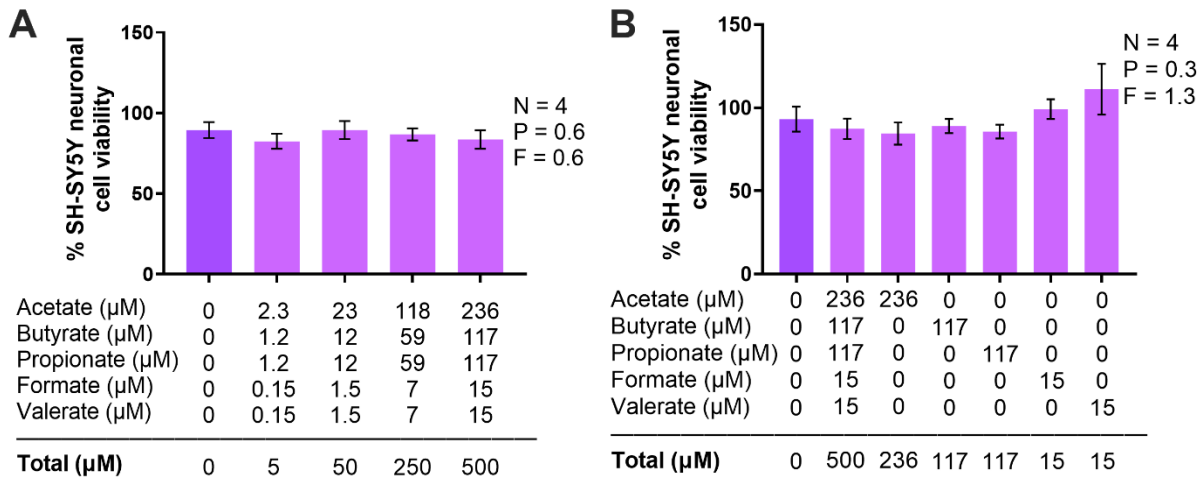


Figure S4.2. Effect of the SCFA mixture (**A**) and individual SCFAs (**B**) on viability of human SH-SY5Y neuronal cells. Viability human SH-SY5Y cells was measured by the MTT assay respectively after 72 h incubation with SCFAs (**A**), or individual SCFAs at their maximum concentration in mixture (**B**). Data (means ± SEM) from four independent experiments performed on different days are presented. P and F values for the one-way randomized blocks ANOVA are shown.