Detecting A-Series Ganglioside Expression Profile Changes During Microglial Activation

Mona M. Alshaikh
The University of Western Ontario

Supervisor
Shawn Whitehead
The University of Western Ontario Co-Supervisor
Gilles Lajoie
The University of Western Ontario

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Abstract

With aging, our brains become more susceptible to disease and injury. Different regions of the brain have differing levels of vulnerability to stress and injury, and this brain region-dependent variability to vulnerability could be partly explained by the existence of glycosphingolipids within the cell’s plasma membrane called gangliosides. Gangliosides are expressed predominantly within the brain and play various roles within the central nervous system including neural repair, cell survival, and neurodegeneration. Our laboratory has demonstrated that gangliosides can shift their composition from GM1 back to GM2 and GM3 following stroke in mice and rats indicating a role for simple gangliosides in the neurodegenerative process and this shift may be part of a neuro-inflammatory cascade. However, to date, ganglioside analysis in microglia cells has not yet been done. Based on the literature and preliminary studies conducted in our laboratory, we hypothesized that GM1, GM2 and GM3 levels will change during microglia activation. BV2 cells were cultured and cells were treated with lipopolysaccharide (LPS) and interleukin 4 (IL-4) to induce M1 and M2 activation, respectively. Liquid chromatography electrospray ionization mass spectrometry LC-ESI-MS was used to quantify ganglioside levels following activation, and immunofluorescence was used to stain for GM1, GM2 and GM3 gangliosides. RT-qPCR was performed to verify what phenotypes were activated in response to observed changes in gangliosides. Our results showed a significant increase in GM1, GM2 and GM3 gangliosides in M1 BV2 cells while no change was observed in the M2 phenotype. RT-qPCR results showed a significant peak in M1 markers at 24 h following LPS while M2 markers peaked at 8 h following IL-4 exposure. Our in vitro results suggest that M1 phenotype could be selectively targeted to understand ganglioside changes following injury and that M1 microglia can be the source of the transient increase in GM1, GM2 and GM3 found in animal models of neurodegeneration.

Key Words: Gangliosides, BV2 Microglia, Neuroinflammation, Electrospray Ionization Mass Spectrometry.
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<th>Description</th>
</tr>
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<tbody>
<tr>
<td>AD</td>
<td>Alzheimer’s Disease</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
</tr>
<tr>
<td>Arg1</td>
<td>Arginase 1</td>
</tr>
<tr>
<td>CNS</td>
<td>Central Nervous System</td>
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<tr>
<td>DAPI</td>
<td>4',6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic Reticulum</td>
</tr>
<tr>
<td>ESI</td>
<td>Electrospray Ionization</td>
</tr>
<tr>
<td>GalNAc-T</td>
<td>N-acetylgalactosaminyltransferase</td>
</tr>
<tr>
<td>GAP43</td>
<td>Growth-associated Protein 43</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate Dehydrogenase</td>
</tr>
<tr>
<td>GlcCer</td>
<td>Glucosylceramide</td>
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<tr>
<td>GM1</td>
<td>monosialotetrahexosylganglioside</td>
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<tr>
<td>HD</td>
<td>Huntington’s Disease</td>
</tr>
<tr>
<td>IGF-1</td>
<td>Insulin-like Growth Factor 1</td>
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<tr>
<td>IL-1β</td>
<td>Interleukin-1 Beta</td>
</tr>
<tr>
<td>IL-4</td>
<td>Interleukin 4</td>
</tr>
<tr>
<td>LacCer</td>
<td>Lactosylceramide</td>
</tr>
<tr>
<td>LC</td>
<td>Liquid chromatography</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>LSDs</td>
<td>Lysosomal Storage Diseases</td>
</tr>
<tr>
<td>MALDI-IMS</td>
<td>Matrix-assisted Laser Desorption/ Ionization Imaging Mass Spectrometry</td>
</tr>
<tr>
<td>MCAO</td>
<td>Middle Cerebral Artery Occlusion</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>----------</td>
<td>--------------------------------------</td>
</tr>
<tr>
<td>MPP+</td>
<td>1- methyle-4-phenylpyridinium</td>
</tr>
<tr>
<td>MS</td>
<td>Mass Spectrometry</td>
</tr>
<tr>
<td>NGF</td>
<td>Nerve Growth Factor</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered Saline</td>
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<tr>
<td>PD</td>
<td>Parkinson’s Disease</td>
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<tr>
<td>PI</td>
<td>Propidium Iodide</td>
</tr>
<tr>
<td>SCI</td>
<td>Spinal Cord Injury</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard Error of the Mean</td>
</tr>
<tr>
<td>Sial-T1</td>
<td>Sialyltransferase I</td>
</tr>
<tr>
<td>Sial-T2</td>
<td>Sialyltransferase II</td>
</tr>
<tr>
<td>TNFα</td>
<td>Tumor Necrosis Alpha</td>
</tr>
<tr>
<td>WT</td>
<td>Wild-type</td>
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Section 1: INTRODUCTION
1.1 Brain Vulnerability

As we age, our brains become more vulnerable to neurodegenerative processes associated with various brain diseases and injuries. In addition, different regions of the brain have differing levels of vulnerability to stress, cell death or injury that lead to neurodegeneration. For instance, the cerebral cortex, hippocampus CA1, and amygdala are brain areas that are most severely affected by neurodegeneration throughout the progression of Alzheimer’s disease (AD) (Braak and Braak, 1991; Hyman et al., 1984; Niikura et al., 2006), whereas Parkinson’s disease (PD) is characterized by degeneration of the dopaminergic neurons of the substantia nigra (Damier et al., 1999; Dauer and Przedborski, 2003; Jenner, 1998). In addition to regional neuroanatomical differences, individual neuroanatomical regions within the brain can have variable vulnerability. For example, the CA1 region of the hippocampus is more vulnerable to hypoxia and ischemia related neurodegeneration than neurons of the dentate gyrus (Olsson et al., 2003; Schmidt-Kastner and Freund, 1991; Woodruff et al., 2011). This has also been observed with aging (Mueller et al., 2007), and chronic epileptic fit (Mathern et al., 1997). This brain region dependent variability to vulnerability could partly be explained by activity within the plasma membrane, since it is the first point of interaction with external stimuli, and harbors a variety of cellular functions, such as cell adhesion, ion conductivity, cell signaling and molecular exchange (Knight, 2013).

1.2 Plasma Membrane

The plasma membrane is a highly dynamic and heterogeneous structure that can change its physical structure, along with its density and fluidity, to meet the external demand of the external and internal cellular environment. It is composed mainly of phospholipids, proteins, cholesterol and sugar molecules (Lingwood and Simons, 2010; Singer and Nicolson, 1972). It also consists of specialized subdomains called lipid rafts that contain high levels of cholesterol and saturated lipids.
including glycosphingolipids (Pike, 2003). Lipid rafts are highly involved in signal transduction since signaling molecules such as Src family kinases, G proteins, growth factor receptors, mitogen-activated protein kinase (MAPK)3 and protein kinase C are largely found within these membrane microdomains. Lipid rafts also play critical roles within neurons by regulating intracellular trafficking and of neurotransmission. (Brown and London, 1998; Simons and Toomre, 2000). One of the main functional lipid components within lipid rafts are gangliosides (Sonnino et al., 2007)

1.3 Ganglioside Structure

Gangliosides are a diverse species of glycosphingolipids that mainly reside within the outer layer of the plasma membrane, and are present in all vertebrate cells (Sonnino et al., 2007), but are particularly enriched within the central nervous system (CNS), where they constitute 10% - 12% of the lipid mass of the brain (Kolter, 2012; Ledeen and Yu, 1982). Structurally, gangliosides contain a hydrophobic ceramide domain that is embedded within the cell membrane. This ceramide domain is made from the combination of a sphingosine backbone with variable lengths, primarily 18 and 20 carbons in length, along with a fatty acid (Fig. 1A) (Karlsson, 1970; Sonnino et al., 2007). Both d18:1 and d20:1 (18 carbon and 20 carbon sphingosine moieties) are the predominant species observed in the mammalian CNS (Sonnino and Chigorno, 2000), while published evidence of d16:1 species are found in small quantities in rat brains (Avrova et al., 1973). They also contain a hydrophilic domain consisting of variable sialyated oligosaccharide units which protrude toward the extracellular environment (Fig. 1B) (Sonnino et al., 2007). The major type of sialic acid in the mammalian brain is N-acetylneuraminic acid (NeuNAc) (Yu & Ledeen, 1970). Moreover, gangliosides can be classified either according to their sialic acid residues number or their sugar molecules. Gangliosides having 0, 1, 2 or 3 sialic acid residues are attached to other sialic acid or
galactose residues are called asialo, a-, b- and c-series gangliosides respectively. The prefix G refers to ganglio, M, D, T and Q (mono-/di-/tri-/quad) refer to the number of sialic acid residues. (Yu et al., 2011) (Fig. 1).
Figure 1. Ganglioside Structure. Gangliosides are made up of a hydrophobic ceramide base consisting of a fatty acid and sphingosine backbone of variable lengths (primarily 18 and 20 carbons), and a hydrophilic domain composed of variable number of oligosaccharide units and sialic acid residues. Figure modified from Weishaupt et al., 2015.
1.4 Ganglioside Synthesis vs. Degradation

Gangliosides maintain a homeostatic balance for each species in the healthy mammalian brain. This balance however, can be altered enzymatically via biosynthetic and catabolic pathways (Sandhoff and Harzer, 2013). Ganglioside biosynthesis starts with the formation of a hydrophobic ceramide at the cytoplasmic leaflet of the endoplasmic reticulum (ER) membrane. Ceramide is then glycosylated and converted into glucosylceramide (GlcCer) via glucosylceramide synthase (Glc-T) in the early Golgi apparatus. Glucosylceramide is then further modified into lactosylceramide (LacCer) by galactosyltransferase I (Gal-T1). LacCer is then converted into ganglioside GM3, structurally the simplest form of ganglioside, by the enzyme sialyltransferase I (Sial-T1), which adds the characteristic sialic acid residue of gangliosides to the LacCer molecule. Other ganglioside molecules are generated by sequential addition of different sugar molecules and/or sialic acid residues to GM3 (Kolter et al., 2002). For example, GM3 is modified by the addition of additional sialic acids by sialyltransferase II (Sial-T2) and sialyltransferase III (Sial-T3) to give GD3 and GT3 sequentially. GM3, GD3 and GT3 are considered the precursors for a, b, and c-series, respectively. More complex gangliosides can be produced from these precursor molecules via different glycosyltransferases enzymes including N-acetylgalactosaminyltransferase (GalNAc-T), galactosyltransferase II (Gal T-2), sialyltransferase IV (Sial-T4) and sialyltransferase V (Sial-T5), (Fig. 2) (Yu et al., 2004, 2008)
Figure 2. Schematic of ganglioside biosynthesis pathway. Ganglioside synthesis starts with the formation of hydrophobic ceramide at the cytoplasmic leaflet of the endoplasmic reticulum (ER) membrane followed by stepwise addition of oligosaccharide chains and/or sialic acids via specific enzymes. GalNAc-T: N-acetylgalactosaminyltranferase or GM2/ GD2/ GT2 synthase, Gal- T1: galactosyltransferase I, Gal- T2: galactosyltransferase II or GM1 synthase, Glc-T: glucosyltransferase, Sial-T1: sialyltransferase I or GM3 synthase, Sial-T2: sialyltransferase II or GD3 synthase, Sial-T3: sialyltransferase III or GT3 synthase, Sial-T4: sialyltransferase IV or GD1a synthase, Sial-T5: sialyltransferase V or GT1a synthase.
Ganglioside degradation takes place within endosomes and lysosomes via endocytosis (Yu et al., 2008). Similar to the biosynthesis process, catabolism is a sequential process where oligosaccharide units and/or sialic acid residues are sequentially removed by specific enzymes (glycosidases) which reside within the lumen of endosomes and lysosomes (Kolter et al., 2002; Sandhoff and Kolter, 2003). Initially, complex gangliosides containing more than one sialic acid such as GT1b and GQ1b are converted to GM1 at the plasma membrane by a membrane-bound sialidase (Miyagi and Tsuiki, 1986; Riboni et al., 1995). GM1 is then degraded in the lysosome to GM2 by β-galactosidase. GM2 is further degraded to GM3 by β-N-acetyl-hexoaminidase. Direct removal of sialic acid residues from GM1 or GM2 by sialidases produces GA1 and GA2 which degrades to LacCer. followed by degradation to ceramide by β-galactosidase and β-glucosidase. Finally, ceramide is degraded to a fatty acid and sphingosine by ceramidase (Huwiler et al., 2000; Kolter et al., 2002) (Fig. 3).

Ganglioside synthesis and degradation are strictly controlled by the activities of glycosyltransferases through glycosyltransferase gene transcription and post-translational modification (Yu et al., 2004). Perturbations in either the synthesis or degradation processes can lead to several human diseases. For example, one study showed that mutation of Sial-T1 (GM3-synthase), an enzyme responsible for the synthesis of all complex gangliosides, caused infantile-onset epilepsy syndromes in humans (Simpson et al., 2004), suggesting that complex gangliosides are critically involved in the development of the human nervous system. On the other hand, the inability to degrade gangliosides, leading to the accumulation of gangliosides in late endosomes/lysosomes is a key component in lysosomal storage diseases (LSDs) (Kolter and Sandhoff, 2010; Sandhoff and Kolter, 2003; Schuette et al., 1999). LSDs can be either due to deficiency of enzymes involved in ganglioside degradation, such as β-galactosidase or hexosaminidases which caused an accumulation of GM1 (GM1 gangliosidosis) or GM2 (GM2
gangliosidosis), or impairments in lysosomal functions (Jeyakumar et al., 2005; Kolter and Sandhoff, 2006; Vitner et al., 2010; Zervas et al., 2001).
Figure 3. **Ganglioside degradation pathway.** Ganglioside degradation is a sequential process where oligosaccharide units and/or sialic acid residues are gradually removed by specific enzymes, called glycosidases. LacCer: Lactosylceramide, β-gal: β-galactosidase, β-glu: β-glucosidase.
1.5 Ganglioside Expression in the Nervous System

Ganglioside expression patterns have been shown to change as the mammalian brain matures (Ngamukote et al., 2007). Simple gangliosides GM3 and GD3 are abundant in the embryonic mammalian brain, and this abundancy shifts to the more complex gangliosides such as GM1, GD1a, GD1b and GT1b in matured brains (Ngamukote et al., 2007; Yu et al., 2012, 1988). These complex species are also predominantly expressed in the adult human brain, accounting for 90% of the total ganglioside content (Ando et al., 1978). Work from our laboratory has shown that simple gangliosides, GM2 and GM3 are only detected at very low levels in the healthy adult rodent brain (Caughlin et al., 2015; Whitehead et al., 2011). In human brains, overall ganglioside content accumulation occurs during the gestation period until the first five postnatal years. Specifically, during this period, there is a 12-15-fold elevation in GM1a and GD1a which correlates with the most critical stage of axonal growth, dendrite arborization, and synapse formation (Svennerholm et al., 1989). In mouse brains, overall ganglioside content is 8-fold greater in the adult brain compared to the embryonic brain (Ngamukote et al., 2007). The change in ganglioside levels during brain development is mainly controlled by glycosyltransferase enzymes, involved in the ganglioside biosynthesis pathway (Sandhoff and Kolter, 2003). Conversely, human brains lose about 64% of ganglioside contents during aging (Segler-Stahl et al., 1983).

1.6 Ganglioside d18:1 and d20:1 Sphingosine Moieties

Gangliosides vary with respect to their carbon chain length within their sphingosine base. This variable sphingosine length has unique expression profiles within various regions of the brain and may play distinct roles within the CNS. For example, A key study by Whitehead et al., 2011 used matrix-assisted laser desorption/ionization imaging mass spectrometry (MALDI-IMS) to demonstrate that GM1 d18:1 was distributed throughout the healthy rat brain hippocampus, while
GM1 d20:1 was specifically expressed within the dentate gyrus of the hippocampus (Whitehead et al., 2011). More recently, our laboratory has demonstrated that the GM1 expression profile within the rat brain differs anatomically with respect to sphingosine base length (d18:1 vs. d20:1) in a number of key anatomical regions that are susceptible to disease and injury later in life, including various layers of the cortex, hippocampus and white matter tracts (Caughlin et al., 2017; Weishaupt et al., 2015). Moreover, studies have shown that d18:1 gangliosides are the predominate species within the embryonic and adult brain. d20:1 gangliosides, however, are less abundant than d:18:1 gangliosides, and dramatically decrease during brain development but conversely increase with aging (Palestini et al., 1993, 1990; Park et al., 2016; Rosenberg and Stern, 1966; Svennerholm et al., 1989). An increase in the d20:1/d18:1 ratio has been observed in mature rat cerebellum for GQ1b, GT1b, GM1, GD1b and GD1b (Valsecchi et al., 1996). Also, GM1d20:1 levels increased in healthy 4-month adult rat brain compared to embryonic cortical neurons (Park et al., 2016). These studies indicate that accumulation of d20:1 of gangliosides may be a marker of aging.

1.7 Role of Gangliosides in the Central Nervous System

The membrane localization of gangliosides allows them to interact both laterally (cis) with other signaling molecules in their own membrane plane and with other proteins on opposing cell membranes (trans) (Lopez and Schnaar, 2009; Todeschini and Hakomori, 2008). Together cis and trans ganglioside interactions play an important role in development (Yu et al., 2012) and regulating physiological processes within the CNS, such as differentiation (Jennemann et al., 2005), axon stability (Schnaar, 2010), neural regeneration (Schnaar et al., 2014; Yu et al., 2012) as well as neurodegeneration (Mlinac and Bognar, 2010; Yamashita et al., 2005). This has been inferred in studies involving genetically modified mice that lack gangliosides expressed in
embryonic and adult brain. Specifically, mice deficient in GlcCer synthase, an enzyme that convert ceramide into glucosylceramide, showed impairments in cerebellum and peripheral nerve structure and function leading to death within 3 weeks of birth (Jennemann et al., 2005). When synthesis of both b- and c-series gangliosides was inhibited in a GD3 synthase (Sial-T2) knockout mouse, nerve regeneration was inhibited following axotomy of the hypoglossal nerve (Okada et al., 2002). Another study showed an increase in both thermal and mechanical sensory responses in the GD3 synthase knockout mice with no significant difference in the sciatic nerve conduction velocity compared to the wildtype mice, suggesting the crucial role of both b- and c-series gangliosides on the maintenance and development of the nervous system (Handa et al., 2005; Niimi et al., 2011). GalNAc-T knockout mice, mainly express GM3 and GD3 and lack all complex ganglioside, displayed reduction in conduction velocity from the tibial nerve to the somatosensory cortex, sensory dysfunction, neural degeneration (Takamiya et al., 1996), anterograde degeneration and myelination defects in both CNS and peripheral nervous system (Sheikh et al., 1999). Furthermore, young GalNAc-T and Sial-T1 double knocked out mice, lack both simple and complex gangliosides, exhibited profound neurodegeneration and developmental deficits including axonal degeneration, axon-glia interaction perturbation in the cerebral cortex, and vacuolization impairment in the white-matter areas (Yamashita et al., 2005). All these studies strongly indicate the importance of gangliosides in shaping the neural structure during brain development and maintaining normal function of the CNS.

1.8 GM1 Ganglioside

Several studies have demonstrated the importance of gangliosides in neural repair and cell survival. Specifically, GM1 is one of the most abundant gangliosides in the adult human brain, and a major lipid component of lipid rafts (Gupta and Surolia, 2010). Clinical trials, along with
pre-clinical studies have shown that increased GM1 levels can improve outcome by promoting neuronal protection and repair following glutamate exposure, spinal cord injury, stroke, Huntington’s disease and Parkinson’s disease (PD) (Geisler et al., 2001; Koga et al., 1990; Lazzaro et al., 1994; Maglione et al., 2010; Park et al., 2016; Schneider et al., 2013).

A recent in vitro study using ESI-MS has shown that GM1 expression transiently increased following glutamate exposure. Interestingly, the transient increase of GM1 occurred only in viable neurons, suggesting a self-neuroprotective response (Park et al., 2016). To further examine the neuroprotective role of GM1, rat embryonic cortical neuron cultures were pre-treated with exogenous GM1 24 h prior to glutamate exposure. GM1 pre-treatment was able to significantly protect neurons following glutamate toxicity (Park et al., 2016). GM1 also can improve outcomes following spinal cord injury. When GM1 was administrated intravenously to SCI patients, recovery of spinal cord symptoms such as light touch, motor, pinprick scores, anal contraction and bladder function were all improved, although recovery was more significant with the less severe cases of SCI (Geisler et al., 2001). Furthermore, many studies suggest neuroprotective roles of GM1 in various ischemic models. Intravenous administration of GM1 reduced stroke-induced infarct size in rats following occlusion of the left cerebral artery, compared to controls (Lazzaro et al., 1994). Also, exogenous administration of GM1 significantly decreased edema ipsilateral hemisphere following stroke in the rat (Koga et al., 1990). Another study showed that GM1 treatment improved outcomes following hypoxic ischemia in fetal sheep brain. Pretreatment of GM1 and continuous infusion of GM1 for 60 hours after the insult improved primary and secondary edema, decreased neuronal loss in the cortex and hippocampus, reduced epileptiform activity and increased electrocorticographic activity (Tan et al., 1993). Clinically, however, GM1 treatment did not show any improvements when exogenously administrated to stroke patients and some patients had skin reactions and developed Guillain-Barre syndrome (Candelise and Ciccone,
Striatal neurons and fibroblasts from Huntington’s disease (HD) patients expressed low levels of GM1 and cells were more apoptotic. When Maglione et al. prevented GM1 synthesis, wild-type striatal cells were more vulnerable to apoptosis indicating that reduced levels of GM1 contribute to the progression of HD. After re-administration of GM1, HD striatal cells survived significantly longer, suggesting that GM1 could be a potential therapeutic target for HD (Maglione et al., 2010).

In PD, promising results were observed when GM1 was exogenously administered in vitro, in animal models of the disease, as well as in human clinical trials. Experimentally, it has been shown that pre-treatment of GM1 ganglioside can repair or prevent damage of dopaminergic neurons induced by 1-methyl-4-phenylpyridinium (MPP+). Dopamine neurons treated with both MPP+ and GM1 had healthier morphological appearance characterized by long and branched processes and increased levels of tyrosine hydroxylase (TH) enzyme activity compared to neurons treated with MPP+ only. However, this effect was mainly dependent on the timing of GM1 administration and the severity of neuronal damage (Schneider et al., 1995). Moreover, GM1 treatment improved PD symptoms in mice and non-human primate models by boosting dopamine levels in the striatum and increasing dopamine synthesis (Hadjiconstantinou et al., 1986; Schneider et al., 1992; Stull et al., 1994; Tilson et al., 1988). A recent study investigating the therapeutic effect of GM1 on PD rats demonstrated that an intraperitoneal injection of GM1 resulted in a significant relief in apomorphine (APO)-induced rotational behavior and blocked the inflammatory cytokine interleukin-1 beta (IL-1β) (Ba, 2016). Finally, one clinical trial showed that exogenous administration of GM1 significantly improved motor symptoms of PD human patients based on the Unified Parkinson’s Disease Rating Scale (Schneider et al., 2013).

One of the underlying neuroprotective mechanisms of GM1 is its interaction with proteins involved with development and regeneration of the nervous system. For example, one study...
showed that growth-associated protein 43 (GAP43), a protein that resides within neuron membranes and is highly expressed during the process of spinal cord regeneration (Petruska and Mendell, 2004) mRNA levels increased in spinal cord neurons treated with both GM1 and nerve growth factor (NGF) following glutamate insult. Boosting of GAP43 mRNA levels were less significant in neurons treated with either GM1 or NGF alone indicating that GM1 interdependently works with NGF to promote neuroprotection (Huang et al., 2009). Another study suggested that the neuroprotective mechanism of GM1 was due to its ability to enhance the activation of mitogen activated protein kinase (MAPK) and cAMP response element binding protein (CREB) pathways, which are essential for cell survival (Choi et al., 2003). Collectively, both in vitro, in vivo and clinical data suggest that GM1 targeted therapy has neuroprotective potential but knowledge on the timing for therapeutic intervention is still needed.

1.9 GM2 Ganglioside

GM2 ganglioside is synthesized by the addition of N-acetylgalactosamine to GM3 via GalNac-T, and is expressed only in very small quantities within the healthy brain (Kolter et al., 2002; Whitehead et al., 2011). Moreover, high levels of GM2 were detected during cortical development of ferret brain from the first post-natal day to the second post-natal day, however, GM2 was completely absent in the mature cortex of the ferret brain (Zervas and Walkley, 1999). Pathological accumulation of GM2 is a defining feature in lysosomal storage diseases including GM2 gangliosidosis, Niemann-Pick C disease, Sandhoff and Tay-Sach’s (Zervas et al., 2001).

An in vivo animal study showed a robust increase in GM2 in lysosomes and late endosomes of activated microglia in P7 mice brains 24 h following ethanol induced neurodegeneration (Saito et al., 2012). A more recent study compared GM2 accumulation in wild-type (WT) mice with GM2/GD2 synthase (GalNAc-T) KO mice following ethanol toxicity. Using immunohistochemistry,
WT mice brains showed strong GM2 staining in activated microglia which peaked 24 h after ethanol exposure. By 32 h after ethanol injections, GM2 decreased in activated microglia and were subsequently mainly expressed in reactive astrocytes. GD3 and GM3 ganglioside, conversely, accumulated in the late endosomes/lysosomes of activated microglia in GalNAc-T KO mice, while their expressions were minimal in astrocytes (Saito et al., 2015).

A previous study by Whitehead et al., 2011 using MALDI-IMS showed that GM2 levels transiently increased at 3 days within the stroke induced infarct site following middle cerebral artery occlusion (MCAO). This finding was recapitulated in a different stroke model whereby a transient increase of GM2 levels was observed at the site of stroke injury in rats 3 days after injury which returned to normal levels by 21 days (Caughlin et al., 2015).

### 1.10 GM3 Ganglioside

The simplest ganglioside, GM3 is the precursor for all complex gangliosides (Yu et al., 2008). In the CNS, GM3 expression is abundant mostly during embryonic development (Ngamukote et al., 2007) while it is present only at very low levels in the healthy adult mouse and rat brain (Caughlin et al., 2015; Whitehead et al., 2011). GM3 activates receptors that are involved in the angiogenic pathway. Those receptors include epidermal growth factor (EGF), platelet-derived growth factor (PDGF), insulin-like growth factor 1 (IGF-1) basic fibroblast growth factor (b-FGF), vascular endothelial growth factor (VEGF) and cell adhesion molecules such as integrins (Abate et al., 2006; Toledo et al., 2005). Exogenous administration of GM3 prevented the proliferation of endothelial tumor cells, which were shown to regrow after re-incubated in GM3 free media (Alessandri et al., 1997). Moreover, GM3 inhibited angiogenesis (Lopez and Schnaar, 2009) and has therefore been used as a treatment for various types of cancer (Abate et al., 2006; Seyfried and Mukherjee, 2010). Together with CD82 and CD9, metastasis-suppressing genes, GM3 prevented
metastasis by inducing apoptosis (Ono et al., 1999). Collectively, these studies indicate that GM3 has both anti-proliferative and anti-angiogenic properties.

*In vitro* studies have demonstrated neurotoxic properties of GM3. Specifically, GM3 levels increased in HT22 immortalized mouse hippocampal cells following glutamate excitotoxicity, and exogenous administration of GM3 to these cells exacerbated cell death. Also, overexpression of the gene coding for sialyltransferase I (GM3 synthase) in HT22 mouse cells resulted in additional accumulation of endogenous GM3 which exacerbated cell death (Sohn et al., 2006). An increase in GM3 was also detected *in vivo* when zebra fish embryos were microinjected with GM3 synthase mRNA leading to cell death in both neurons and neuronal precursor cells (Sohn et al., 2006). Overall, these experimental studies indicate that GM3 is neurotoxic and can cause neural cell death.

Abnormal accumulation of GM3 ganglioside is associated with the pathogenesis of both stroke and AD. Oikawa et al., 2009, examined the expression profile of amyloid-β protein deposition in mutant mice brain characterized by aberrant accumulation of GM3 due to GM2 synthase gene deficiency. These mice brains showed an elevation in amyloid-β in vascular tissues (Oikawa et al., 2009). Also, frontal and parietal cortex, regions of the brain which are associated with neurodegeneration in patients with AD, expressed high levels of GM3 and low levels of complex gangliosides (Kracun et al., 1992). Kracun et al. 1992 hypothesized that increased GM3 levels in frontal and parietal cortex might be due to accelerated lysosomal degradation of GM2 ganglioside (Kracun et al., 1992). More recently, an increase in GM3 levels within the site of the infarct was detected by MALDI-IMS in a mouse model of stroke (Whitehead et al., 2011). This finding was later confirmed by Caughlin et al., 2015, which found that GM3 increased at three days after stroke injury exacerbated with Aβ toxicity in the rat brain. By 21 days GM3 levels were increased in both the regular stroke model as well as the exacerbated stroke injury model, suggesting that the
accumulation of GM3 may play a mechanistic role in the propagation of neurodegeneration (Caughlin et al., 2015). All previously mentioned studies suggest that there is a strong relationship between the accumulation of GM3 and the induction of apoptosis, toxicity, neural death and neurodegeneration.

1.11 Microglia and Neuroinflammation

Microglia are considered the innate immune cells of the brain, constituting 20% of the glial population (Lawson et al., 1990; Moore and Thanos, 1996). They play a critical role in the development and maintenance of the brain (Hughes et al., 2004) as well as in neurodegeneration (Kim and De Vellis, 2005). In response to injury, ramified microglia exhibit functional and morphological changes, and transform into reactive microglia (Kreutzberg, 1996; Stence et al., 2001). Reactive microglia are classified into two activation states, M1 and M2. Resting microglia are activated to an M1 phenotype in response to interferon gamma (IFN-γ) or lipopolysaccharide (LPS) (Nakagawa and Chiba, 2014), which induce neuronal damage by releasing a variety of pro-inflammatory cytokines such as tumor necrosis alpha (TNFα), IL-1β, interleukin-6 (IL-6), CCL2, CXCL10, and reactive oxygen species/reactive nitrogen species (ROS/NOS). M1 microglia are thought to sustain environmental homeostasis by destroying pathogens and presenting antigens (Cherry et al., 2014; Henkel et al., 2009; Hu et al., 2012). On the contrary, microglia activated to the anti-inflammatory M2 phenotype are further divided into three sub-types including M2a, M2b, and M2c (Chhor et al., 2013; Latta et al., 2015; Sudduth et al., 2013). M2a microglia are activated by either interleukin 4 (IL-4) or interleukin 13 (IL-13) stimulation and their main function is to suppress inflammation. M2a microglia mainly express Arginase-1 (Arg1), Fizz1, CD206, Ym1 and IGF-1 (Latta et al., 2015; Mecha et al., 2015), whereas M2b activation phenotype is induced by TLRs agonists, IL-1R ligands, and mainly express CD86, IL-1RA, and suppressor of cytokine
signaling 3 (SOCS3) (Chhor et al., 2013; Mecha et al., 2015). M2c or “deactivated microglia” are activated by IL-10, TGF-β and glucocorticoids. M2c phenotype is associated with tissue regeneration following resolution of the inflammatory response (Mantovani et al., 2004). In general, M2 microglia phenotype enhances neuroprotection and blocks pro-inflammatory cytokines by releasing anti-inflammatory cytokines and neurotrophic factors (Cherry et al., 2014; Henkel et al., 2009; Ponomarev et al., 2013).

Microglia cells polarize based on a number of factors including the external stimuli, severity of insult, aging and the microenvironment (Hu et al., 2015; Norden and Godbout, 2014). Following ischemic stroke, microglia dynamically polarized around the infarct site. M2 microglia were detected around the infarct core at day 1 and temporally increased within 7 days’ post MCAO. M1 phenotype, however, were observed 3 days after MCAO and gradually elevated until 14 days (Hu et al., 2012).

1.12 Gangliosides and Microglia/ Macrophages

Most research has focused on investigating ganglioside changes in peripheral macrophages, therefore little is known about their expression changes in microglia following injury. Bobryshev et al., 1997, found that there was a five-fold increase in GM3 levels within macrophages located within human atherosclerotic lesions compared to non-inflamed areas. These GM3 positive macrophages also expressed high levels of GM3 synthase, indicating that increased GM3 levels in human atherosclerotic lesions was synthesized by macrophages (Bobryshev et al., 2006; Gracheva et al., 2009). Microglia act in parallel to their fellow immune cells in the peripheral tissues, macrophages, following injury or inflammation within the CNS (Chan et al., 2007). It has been shown that GM2 and GM3 ganglioside levels increased in lysosomes/late endosomes of activated microglia in mice brains following ethanol induced neurodegeneration (Saito et al., 2015, 2012).
1.13 Rationale

Alterations in the abundance of gangliosides after injury may play a key critical role in the neurodegenerative process. Our laboratory has demonstrated that gangliosides shift their composition from GM1 to GM2 and GM3 following stroke in mice and rats, indicating that GM2 and GM3 increase during neurodegeneration. Interestingly, the transient increase in GM2 and GM3 occurred between 3 and 7 d post stroke-reperfusion (Caughlin et al., 2015; Whitehead et al., 2011) indicating that these changes may be part of a neuroinflammatory cascade. Based on these findings, it is possible that the observed increases in GM2 and GM3 in our animal models may occur in microglia as microglial activation indicates that initial phase of the post-stroke inflammatory response, and the time of transient increase of both GM2 and GM3 (Whitehead et al., 2011) is closely consistent with microglia recruitment following brain injury (Fawcett and Asher, 1999; Iadecola and Anrather, 2011). A better understanding of how ganglioside species GM1, GM2 and GM3 change following microglial activation and how they relate to neuroinflammation may uncover a novel inflammatory mechanism, in which activated microglia increase their endogenous synthesis of the toxic GM2 and GM3 and release them to the surrounding neurons to cause further damage.

1.14 Hypothesis, Aim and Objectives:

Hypothesis: GM1, GM2 and GM3 ganglioside levels will change during microglia activation.

- Prediction 1: GM2 and GM3 ganglioside levels will increase during LPS-induced M1 microglia activation in BV2 cells.
- Prediction 2: complex GM1 levels will increase following exposure to IL-4 to induce M2
activation in BV2 cells.

Aim of the study: To investigate how ganglioside species GM1, GM2 and GM3 are altered following microglial activation, in vitro.

Objectives

1. Analyze ganglioside expression profile in BV2 microglia cell line following lipopolysaccharide (LPS) induced M1 microglial activation.

2. Analyze ganglioside expression profile in BV2 microglia cell line following interleukin 4 (IL-4) mediated anti-inflammatory M2 microglial activation.
Section 2: METHODS
2.1 M1 and M2 Activation of BV2 Microglia

2.1.1 Coating of 24-well plates

To coat the 24-well plates a 5 µg/mL of Poly-D-Lysine (EMD Millipore, Billerica, MA, USA) solution was prepared with autoclaved water. Poly-D-Lysine solution was aspirated following 3 h of incubation in the cell culture bio-hood and plates were washed once with autoclaved water. The plates were dried under ultraviolet light in the cell culture hood for 20 min.

2.1.2 Cell Culture

BV2 murine microglial cell line was donated by Dr. Tuan Trang at the University of Calgary. Cells were maintained in Dulbecco’s Modified Eagle Medium (DMEM, Wisent Bioproducts, St. Bruno, Quebec) supplemented with 10,000 units/ml penicillin, and 10,000 µg/mL of streptomycin until 80% confluency was obtained at 37 °C and 5% CO₂ for all experiments. BV2 cells were passaged every 3 days when reaching up to 75 to 80 % confluence.

2.1.3 Lipopolysaccharide (LPS) and Interleukin 4 (IL-4) Exposure

One day after seeding, BV-2 cells were stimulated with an exogenous application of 500 ng/mL LPS (E. coli serotype 055: B5, Sigma-Aldrich, St. Louis, MO, USA) for 24 h and 48 h to induce M1 or 20 ng/mL murine IL-4 (Sigma-Aldrich, St. Louis, MO, USA) for 3, 8, 12 and 24 h to induce M2 microglial activation. 10 cm plates were used for ESI-MS and 24-well plates were used for immunofluorescence analysis.
2.2 Lipid Extraction

Plates were washed with phosphate-buffered saline (PBS) on ice. Cells were then scraped with a cell scraper and collected into a glass tube. An extraction variance between samples was calculated at 7% by spiking 0.1 mM lactosyl β ceramide (d18:1/17:0) (Avanti Polar Lipids, Alabaster, AL, USA) prior to lipid extraction. Lipids were extracted by using a modified Folch method (Folch et al., 1957). Four 10 cm plates were used for every single experimental replicate (n=1). To the 0.8 volume parts of aqueous BV2 cell extract, 2 parts of methanol (MeOH) was added and homogenized using a Fisher Scientific™ laboratory homogenizer. Next, 1 part of chloroform was added to give one phase chloroform: methanol: water (C:M: W) = 1:2:0.8. This mixture was left at room temperature for 1 h with periodical vortexing every 15 min. Next, 1.2 parts of H2O was added to the sample for phase separation to achieve a final composition of C:M:W = 1:2:2. After vortexing, the sample was centrifuged at 2000 rpm for 20 min and the upper aqueous phase was collected. The lower phase in the glass tube was re-extracted with the theoretical upper phase of M:W = 2:2 and was centrifuged again at 2000 rpm for 20 min. The upper phase was added to the first extracted sample and 1 mL of KCl was added.

A solid phase extraction C18 column (Waters, Mississauga, Ontario) was used to elute the gangliosides of interest. Prior to eluting the gangliosides, the C18 column was washed with 10 mL of MeOH and 10 mL of CHCl3: MeOH (2:1). Then, the column was washed for a second time with 10 mL of MeOH, and 10 mL of MeOH:0.1KCl (1:1). The upper phase cell extract was passed through the column twice followed by a 20-mL wash of H2O. The sample was then eluted with 2 mL of MeOH and 10 mL of CHCl3: MeOH (1:1). Samples were evaporated until they were fully dry with N2 gas and stored in -80 °C until analysis. Dried samples were re-dissolved in 1 mL of CHCl3: MeOH (1:1) and were placed in a sonication bath for 15 min prior to mass spectrometry (MS) analysis.
2.3 Electrospray Ionization Mass Spectrometry Detection of Gangliosides

**Liquid Chromatography Conditions, LC− ESI-MS, and LC− ESI-MS/MS**

Samples were separated using an Agilent 1100 high-performance liquid chromatography (HPLC) system comprising a degasser, quaternary pump, autosampler, and column heater (Santa Clara, CA, U.S.A.) with a Luna 3 µm 100 Å NH2 LC column (150 mm × 1 mm; Phenomenex, Torrance, CA, U.S.A.) LC conditions were adapted from Ikeda & Taguchi, 2010. The mobile phase contained two solutions: A) acetonitrile/water (83:17) (1 mM ammonium formate) B) acetonitrile/water (1:1) (50 mM ammonium formate). The initial gradient was held for 5 min at a solution mixture (A:B) of 100:0, converted linearly to A:B=25:75 for 5 min, then converted linearly to A:B=10:90 for 5 min and held there for 10 min. The flow rate was set to 0.15 mL/min and the temperature of the column was set to 40 °C.

MS was performed using a Waters QToF Micro (Waters, Mississauga, Ontario) instrument and the spectra were analyzed using Mass Lynx v4.1 software (Waters). Flight tube voltage was set to 5630 V, MCP voltage was set to 2700 V, source tube voltage was set to 3500 V and the sample cone was set to 40 V and operated under negative ion mode. The source temperature was set to 100 °C and desolation temperature was set to 300 °C. Collision energy was set to 5 V and recorded mass ranges were (600 – 1800) m/z for MS only. The instrument was calibrated using NaCsI and GM1 standards obtained from bovine brain (Avanti Polar Lipids, Alabaster, Alabama, USA). Each experimental replicate was analyzed in triplicate.

Tandem mass spectrometry (MS/MS) was performed on each of the ganglioside species and analyzed qualitatively to ensure that the masses observed in the spectrum were indeed ganglioside species of interest. Data-dependent MS/MS analysis was performed with the collision energy set
at 70 V and recorded mass ranges were and 65 – 1800 m/z. Each of GM1, GM2 and GM3 were identified by detecting specific fragments: sialic acid, sugar molecules and ceramide base.

**Ganglioside Quantification:**

GM1, GM2 and GM3 gangliosides were identified in the chromatogram according to their mass to detect their elution time. The chromatogram was extracted to analyze the mass spectrum of the gangliosides and their peak intensities were recorded. Quantification of gangliosides was based on Park et al., 2016. To quantify these ganglioside species, the peaks were normalized in the following way. Firstly, peak intensities for all d16:1 d18:1, d20 species of GM1, GM2 and GM3 were summed for each individual MS analysis run. To account for the lipids contents per sample, a quotient factor for each run was calculated by dividing total lipid peaks by the average peak intensities of all runs. This quotient factor was divided by each of the ganglioside peaks. To further account for the total starting lipid present in the sample, a numerical factor was found between the total number of BV2 microglia present in the 10 cm plate of each time point of LPS or IL-4 exposure relative to the control 10 cm plates. DAPI cell counts were used to calculate BV2 cells of all groups, then the number of BV2 cells present in the coverslips of the control group was divided by the number of cells present in the cover slips from each time point of LPS or IL-4 exposure. This process accounted for the BV2 cells that died and detached from the plates, which resulted in reduced levels of starting lipids. Then, the numerical factors for each time point were multiplied to the peaks of each gangliosides.

**2.4 Cell Fixation and Immunofluorescence**

BV2 cells were treated with 20 µL of 1 mg/mL of propidium iodide (PI) 45 minutes prior to fixing them on coverslips. Cells were fixed with 500 µL of 4% paraformaldehyde (PFA) for two min and 500 µL of 2% PFA for 20 min, followed by PBS washes (3 x 5 min each). Cells were
incubated with primary antibodies GM1 (Abcam, Toronto, Canada), GM2 or GM3 (TCI, Oxford, UK) at a concentration of 1:100 diluted in 3% BSA in for 24 h at 4 °C. Coverslips were washed with PBS (3 x 5 min each), then incubated with FITC-conjugated secondary antibody for 2 h at room temperature. FITC-conjugated dylight 488 secondary anti-mouse (1:200, Thermo Fisher Scientific, Waltham, MA, USA) and FITC-conjugated dylight 488 secondary anti-rabbit (1:200, Thermo Fisher Scientific, Waltham, MA, USA) were used for the appropriate primary antibody. The coverslips were then washed with PBS (3 x) and double distilled H₂O (2 x), and then mounted onto microscope glass slides with Fluoroshield with DAPI (Sigma, St. Louis, MO, USA).

2.5 RNA Extraction and Quantification of Gene Expression by Real-time Quantitative Polymerase Chain Reaction (Real-Time qPCR)

Total RNA was extracted from BV2 cells following 8, 24 or 48 h of exposure to either LPS (500ng/ml) or IL-4 (20ng/ml) by spin column using a Purelink RNA Mini Kit (Thermo Scientific, Waltham, MA, USA) following the manufacturer’s protocol. All extracted RNA was used as a template to synthesize double-stranded cDNA using a high-capacity cDNA kit (Thermo Scientific, Waltham, MA, USA). Using primers designed to target known cytokines expressed under M1 and M2 phenotypes (Orihuela et al., 2015), qRT-PCR was used to analyze the expression of target cytokines in M1-activated, M2-activated and control cells. Primers are listed in Table 1 and were adopted from Orihuela et al., 2015. PCR was run using Power SYBR® Green Master Mix (Thermo Scientific, Waltham, MA, USA). CT (cycle threshold) values were determined using Bio Rad CFX Manager software and relative abundances for target genes were assessed using the delta-delta method with the internal control target of GAPDH (glyceraldehyde 3-phosphate dehydrogenase) (Chhor et al., 2013; Fleiss et al., 2015; Kang et al., 2015). Relative abundance of target genes was then determined based on increases or decreases compared to control cells.
Table 1. Quantitative Real-Time PCR Primers Sequences

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>GGGAAGCTCACTGGCATGG</td>
<td>CTTCTTGATGTCATCATACTTTGAGCAG</td>
</tr>
<tr>
<td>TNFα</td>
<td>TGGCCTCCCTCTATCAGTT</td>
<td>GCTTGTCACTCGAAATTTGAGAAG</td>
</tr>
<tr>
<td>IL-1β</td>
<td>TGGTGTGTGACGTCCCATT</td>
<td>CAGCACGAGGTTTTTTGTTG</td>
</tr>
<tr>
<td>iNOS</td>
<td>TTCACCCAGTTGTGCATCGACCTA</td>
<td>AACTCCAATCTCGGTGCCCATGTA</td>
</tr>
<tr>
<td>Arg1</td>
<td>TTGGCAAGGTGATGGAAGAGACCT</td>
<td>CGAAGCAAGCCAAGGTTAAAGCCA</td>
</tr>
</tbody>
</table>
2.6 Imaging

Images were taken using a Nikon Eclipse Ni (Nikon Instruments Inc, Melville, NY, USA) microscope. Analysis and quantification was done using ImageJ (Wayne Rasband, National Institute of Health, Bethesda, USA). A single cover slip was used to create a single experimental replicate (n=1) and three pictures from different regions of the cover slip were taken for triple technical replicates. The average pixel intensity of GM1, GM2 and GM3 per cell for both control (non-activated) and BV2 cells following LPS or IL-4 exposure were calculated using ImageJ (Wayne Rasband, National Institute of Health, Bethesda, USA). To quantify the number of viable or dead BV2 cells that expressed GM2 or GM3 at each time point of LPS exposure, the number of cells that were GM2 or GM3 positive and PI negative or positive was counted. Then, this number was divided by the total BV2 cells counted by DAPI and expressed as a percentage of total cells. The percentage of cells that express GM2 or GM3 without PI nuclei from each time point of LPS exposure was normalized to the control value.

2.7 Statistical Analysis

Comparisons of peak intensities for each ganglioside species and comparison of gene expression following M1 and M2 activation were done by one-way analysis of variance (ANOVA) and Tukey’s multiple comparisons post-hoc tests. Comparison of the intensity of different carbon chain ganglioside was done by two-way ANOVA and Tukey’s multiple comparisons test. Quantification of GM2 or GM3 positive/ PI negative or positive was done in a blind manner. Data analysis was done using Prism 6 (Graph Pad). Data is displayed as groups means ± SEM (standard error of the mean). A p-value of < 0.05 was considered statistically significant.
Section 3: RESULTS
3.1 LC-ESI-MS Identification of Gangliosides

To detect a-series gangliosides, chromatograms were extracted with Mass Lynx 4.1 to visualize the mass spectrum of GM1, GM2, GM3 and its d16:1, d18:1, and d20:1 sphingosine moieties (Fig. 4A, B, C, D). To verify if the mass that was observed in mass spectrometry was GM1, GM2, and GM3, tandem LC–ESI-MS/MS was performed. MS/MS was performed on the most abundant ganglioside d16:1 of each species. The parent molecule for each GM1, GM2 and GM3 (d16:1) was confirmed by each fragment ion and carbohydrate chain ion (Fig. 5A, B, C).
Figure 4. Mass spectra of GM1, GM2 and GM3 ganglioside from control BV2 microglia cells. Following the detection of gangliosides with a Waters QTOF ESI-MS instrument, the chromatogram was extracted using Mass Lynx 4.1 to visualize the mass spectra for GM1, GM2, GM3 and its d16:1, d18:1, and d20:1 sphingosine moieties. Peaks of GM1, GM2 and GM3 are presented relative to each species (A). Higher peaks indicate higher quantity of gangliosides. (B) represents an enlarged spectrum of GM3 d16:1, d18:1 and d20:1. (C) represents an enlarged spectrum of GM2 d16:1, d18:1 and d20:1. (D) represents an enlarged spectrum of GM1 d16:1, d18:1 and d20:1.
Figure 5. MS/MS spectrum of (A) GM1 d16:1, (B) GM2 d16:1 and (C) GM3 d16:1. Gal: galactose, Glc: glucose, GalNAc: N-acetylgalactosamine, Cer: ceramide, SA: sialic acid.
3.2 ESI-MS Analysis of GM1, GM2 and GM3 Gangliosides during M1 and M2 BV2 Cells and Analyzed Using LC-ESI-MS.

M1 Phenotype Induced by LPS

To investigate if GM1, GM2, and GM3 gangliosides levels changed during M1 microglial activation, BV2 murine microglial cells were cultured in 10 cm plates and were exposed to 500 ng/mL of LPS. Gangliosides were extracted at 24 h and 48 h following LPS exposure. LC-ESI-MS data revealed that d16:1 GM1 levels significantly increased at 24 h (p <0.01) and 48 h (p <0.01) following LPS compared to control (Fig. 6A). None of the d18:1 species of GM1 changed significantly over time compared to control levels (Fig. 6B). By 24 h and 48 h of LPS exposure, there was a significant increase in d20:1 GM1 (p <0.05, p <0.01, respectively) compared to control BV2 cells (Fig. 6C).

When analyzing GM2 levels following LPS exposure, there was a significant increase in d16:1 GM2 levels at 24 h (p <0.0001) which further increased at 48 h (p <0.0001) (Fig. 7A). Both d18:1 and d20:1 species showed a significant increase at 24 h (p <0.0001, p<0.01, respectively) which remained at the same level at 48 h (p <0.0001) compared to control (Fig. 7B, C).

At 24 h, d16:1 GM3 levels significantly increased (p <0.0001) compared to control cells, and further increased at 48 h (p <0.01) (Fig 8A). None of the d18:1 species of GM3 changed significantly over time compared to control levels (Fig. 8B). By 48 h of LPS exposure, there was a significant increase in d20:1 GM3 compared to control (p <0.01, Fig. 8C).
Figure 6. GM1 expression increases during LPS induced-M1 BV2 microglial activation. Quantified peak intensities for GM1 d16:1, GM1 d18:1 and GM1 d20:1. (A) GM1 d16:1 increased at 24 h and remained at this same level at 48 h post-exposure. (B) GM1 d18:1 levels did not change. (C) GM1 d20:1 levels significantly increased 24 h post-exposure and remained at this same level at 48 h post-exposure. Three technical replicates were analyzed from each biological sample. Data represented as group Mean +/- SEM. * p<0.05, ** p<0.01, one-way ANOVA, Tukey’s post-hoc test, biological N=6 per group.
Figure 7. GM2 expression increases during LPS induced-M1 BV2 microglial activation. Quantified peak intensities for GM2 d16:1, GM2 d18:1 and GM2 d20:1. (A) GM2 d16:1 increased at 24 h followed by a further increase at 48 h post-exposure. (B) GM2 d18:1 levels significantly increased 24 h post-exposure and remained at this same level at 48 h post-exposure. (C) GM2 d20:1 levels significantly increased 24 h post-exposure and remained at this same level at 48 h post-exposure. Three technical replicates were analyzed from each biological sample. Data represented as group Means +/- SEM. ** p<0.01, *** p<0.001, **** p<0.0001, one-way ANOVA, Tukey’s post-hoc test, biological N=6 per group.
Figure 8. GM3 expression increases during LPS induced-M1 BV2 microglial activation. Quantified peak intensities for GM3 d16:1, GM3 d18:1 and GM3 d20:1. (A) GM3 d16:1 increased at 24 h followed by a further increase at 48 h post-exposure. (B) GM3 d18:1 levels did not change post-exposure. (C) GM3 d20:1 levels significantly increased 48 h post-exposure. Three technical replicates were analyzed from each biological sample. Data represented as group Means +/- SEM. ** p<0.01, **** p<0.0001, one-way ANOVA, Tukey’s post-hoc test, biological N=6 per group.
M2 Phenotype Induced by IL-4

To investigate if GM1, GM2, and GM3 gangliosides levels changed during M2 microglial activation, BV2 murine microglial cells were cultured in 10 cm plates and were exposed to 20 ng/mL of IL-4. Gangliosides were extracted at 3, 8, 12 and 24 h following IL-4 exposure. LC-ESI-MS data showed that d16:1 GM1 levels significantly increased at 12 h (p <0.0001) and 24 h (p <0.0001) post-IL-4 exposure compared to 3 h, 8 h and control cells (Fig. 9A). At 12 and 24 h following IL-4 exposure, there was a significant increase in d18:1 GM1 levels (p <0.05, Fig 9B). Finally, d20:1 GM1 levels showed a significant increase at 24 h IL-4 treated cells compared to 8 h and control cells (p <0.05, Fig. 9C).

Levels of d16:1 GM2 showed significant increase at 12 h (p <0.01) and remained at the same level at 24 h (p <0.001) following IL-4 exposure compared to control BV2 cells (Fig. 10A). A significant increase was observed in d18:1 GM2 levels at 24 h compared 3 h, 8 h and control cells (p <0.05, Fig. 10B). d20:1 GM2 species significantly increased at 24 h compared to 3 h (p <0.05, Fig. 10C).

When analyzing GM3 levels following IL-4 exposure, there was a significant increase in d16:1 GM3 levels at 12 h (p <0.01) and 24 h (p <0.001) compared to 3 h, 8 h and control cells (Fig. 11A). However, no significant change can be seen in d18:1 and d20:1 GM3 levels following IL-4 exposure (Fig. 11B, C).
Figure 9. GM1 expression increases following IL-4 induced-M2 BV2 microglial activation. Quantified peak intensities for GM1 d16:1, GM1 d18:1 and GM1 d20:1. (A) GM1 d16:1 increased at 12h and remained at this same level at 24 h post-exposure. (B) GM1 d18:1 increased at 12h and remained at this same level at 24 h post-exposure. (C) GM1 d20:1 levels significantly increased 24 h post-exposure. Three technical replicates were analyzed from each biological sample. Data represented as group Means +/- SEM. * p<0.05, **** p<0.0001, one-way ANOVA, Tukey’s post-hoc test, biological N=6 per group.
Figure 10. GM2 expression increases following IL-4 induced-M2 BV2 microglial activation. Quantified peak intensities for GM2 d16:1, GM2 d18:1 and GM2 d20:1. (A) GM2 d16:1 increased at 12h and remained at this same level at 24 h post-exposure. (B) GM2 d18: increased at 24 h post-exposure. (C) GM2 d20:1 levels significantly increased 24 h post-exposure. Three technical replicates were analyzed from each biological sample. Data represented as group Means +/- SEM. * p <0.05, ** p <0.01, *** p <0.001, one-way ANOVA, Tukey’s post-hoc test, biological N=6 per group.
Figure 11. GM3 expression increases following IL-4 induced-M2 BV2 microglial activation. Quantified peak intensities for GM3 d16:1, GM3 d18:1 and GM3 d20:1. (A) GM3 d16:1 increased at 12h and remained at this same level at 24 h post-exposure. (B) GM3 d18:1 did not change post-exposure. (C) GM3 d20:1 levels did not change post-exposure. Three technical replicates were analyzed from each biological sample. Data represented as group Means +/- SEM. ** p<0.01, *** p <0.001, one-way ANOVA, Tukey’s post-hoc test, biological N=6 per group.
3.3 Comparison of d16:1, d18:1 and d20:1 Sphingosine Moieties

Gangliosides vary with respect to their carbon chain length within their sphingosine base. GM1, GM2 and GM3 sphingosine moieties d16:1, d18:1 and d20:1 are believed to express differently and play different roles within the CNS (Caughlin et al., 2015; Park et al., 2016; Weishaupt et al., 2015; Whitehead et al., 2011). LC-ESI-MS results showed that BV2 cells are primarily expressed the d16:1 sphingosine moieties across GM1, GM2 and GM3 gangliosides. d18:1 and d20:1 were significantly less abundant in both control and activated cells (p <0.05). However, there was no significant difference between d18:1 and d20:1 species of GM1, GM2, and GM3 (Fig. 12 & 13).
Figure 12. Comparison of peak intensities of sphingosine backbone of GM1, GM2 and GM3 ganglioside following LPS exposure. Peak intensities of (A) GM1, (B) GM2, and (C) GM3 and its d16:1, d18:1, and d20:1 moieties. * indicate that d18:1 and d20:1 is significantly lower than d16:1, p < 0.05. No significant difference between d18:1 and d:20. Data represented as Mean +/- SEM; d16:1, d18:1, d20:1 sphingosine moieties were compared by two-way ANOVA and Tukey’s post-hoc test, biological N=6 per group.
Figure 13. Comparison of peak intensities of sphingosine backbone of GM1, GM2 and GM3 ganglioside following IL-4 exposure. Peak intensities of (A) GM1, (B) GM2, and (C) GM3 and its d16:1, d18:1, and d20:1 moieties. * indicate that d18:1 and d20:1 is significantly lower than d16:1, p < 0.05. No significant difference between d18:1 and d20. Data represented as Mean +/- SEM; d16:1, d18:1, d20:1 sphingosine moieties were compared by two-way ANOVA and Tukey’s post-hoc test, biological N=6 per group.
3.4 Immunofluorescence of GM1, GM2 and GM3 Following Exposure to LPS or IL-4

Microglial activation is the initial phase of the post-stroke inflammatory response (Iadecola and Anrathner, 2012). Therefore, it is crucial to investigate if the changes in the gangliosides expression profiles noticed in *in vivo* and *in situ* animal models of neurodegeneration are related to microglia activation (Caughlin et al., 2015; Saito et al., 2015, 2012; Whitehead et al., 2011). Immunofluorescence was used to visualize gangliosides so they can be detected according to the oligosaccharide chain located on the cell surface. BV2 microglia cells were cultured in 24 well-plate and exposed to 500 ng/mL of LPS for 24 and 48 h or 20 ng/mL of IL-4 for 3, 8, 12 and 24 h. BV2 cells were fixed and incubated with GM1, GM2 and GM3 antibodies. Control BV2 cells showed small cell soma with long and fine processes. Following LPS and IL-4 exposure, they became activated and exhibited morphological changes, characterized by either hypertrophied cell bodies and short thick processes or rounded phagocytic appearance (Fig. 14A-19A).

**M1 Phenotype Induced by LPS**

First, when staining with GM1 antibody, control cells and all cells among all time points following LPS exposure were positively stained for GM1 within the cell body and the processes (Fig. 14A). When measuring optical density for GM1, there was no significant difference in GM1 levels following LPS compared to control cells (Fig. 14B).

When BV-2 cells were labelled with GM2 antibody, GM2 was either undetectable or weakly stained in control cells. By 24 h of LPS exposure, strong GM2 staining was observed in activated BV2 cells. However, GM2 staining was weaker at 48 h than 24 h post-LPS exposure (Fig. 15A). Quantification using ImageJ demonstrated that the average pixel intensity of GM2 ganglioside significantly increased at 24 h and 48 h compare to control (p <0.0001, p <0.001, respectively),
while there was a significant decline in GM2 by 48 h compared to 24 h (p < 0.0001) following LPS exposure (Fig. 15B).

Similarly, when staining for GM3, some control BV2 cells did not show GM3 staining and other displayed weak GM3. By 24 h and 48 h of LPS exposure, LPS-activated cells showed a strong positive signal of GM3 which became stronger at 48 h (Fig. 16A). Quantitation revealed a significant increase in the average pixel intensity of GM3 at 24 h and 48 h following LPS exposure compared to control (p < 0.0001, Fig. 16B).
Figure 14. Quantification of GM1 expression in BV2 cells following activation by LPS for 24 h and 48 h. (A) BV2 cells were activated by 500 ng/mL of LPS, fixed with 4% & 2% PFA, and stained with GM1 (green) and DAPI (blue). Bars indicate 50 μm for each panel. (B) Using Image J, average pixel intensity per cell of GM1 in BV2 cells was compared to control. Data represented as Mean +/- SEM; one-way ANOVA, Tukey’s post-hoc test, biological N=8 per group.
Figure 15. Quantification of GM2 expression in BV2 cells following activation by LPS for 24 h and 48 h. (A) BV2 cells were activated by 500 ng/mL of LPS, fixed with 4% & 2% PFA, and stained with GM2 (green) and DAPI (blue). Bars indicate 50 µm for each panel. (B) Using Image J, average pixel intensity per cell of GM2 in BV2 cells was compared to control. Data represented as Mean +/- SEM; one-way ANOVA, Tukey’s post-hoc test, biological N=8 per group.
Figure 16. Quantification of GM3 expression in BV2 cells following activation by LPS for 24 h and 48 h. (A) BV2 cells were activated by 500 ng/mL of LPS, fixed with 4% & 2% PFA, and stained with GM3 (green) and DAPI (blue). Bars indicate 50 µm for each panel. (B) Using Image J, average pixel intensity per cell of GM3 in BV-2 cells was compared to control. Data represented as Mean +/- SEM. **** p <0.0001, one-way ANOVA, Tukey’s post-hoc test, biological N=10 per group.
BV2 cells were also labeled with PI to determine if the observed increase of GM2 and GM3 at 24 h and 48 h following LPS (Fig 15B, 16B) resulted in cell death. By 24 h of LPS exposure, there was a significant increase in the number of BV2 cells that were GM2 or GM3 positive and PI negative (viable cells) compared to the control cells (p <0.0001). However, by 48 h of LPS exposure the number of GM2 or GM3 positive and PI-negative (viable cells) significantly decreased compared to 24 h LPS treated cells (Fig. 17A, 18A).

Similarly, the percentage of GM2 or GM3 positive and PI positive cells was detected. There was a dramatic significant increase in the number of cells that were GM2 or GM3 positive and PI positive at 48 h compared to 24 h LPS-treated cells and control cells (Fig. 17B, 18B)
Figure 17. Quantification of GM2-Positive/ PI-negative or positive BV2 cells following LPS exposure. (A) Quantification of GM2-positive/PI-negative BV2 cells normalized to control BV2 cells. (B) Quantification of GM2-positive/PI-positive BV2 cells normalized to control BV2 cells. Data represented as Mean +/- SEM. * p<0.05, ** p<0.01, **** p<0.0001, one-way ANOVA, Tukey’s post-hoc test, biological N=8 per group.
Figure 18. Quantification of GM3-Positive/ PI-negative or positive BV2 cells following LPS exposure. (A) Quantification of GM3-positive/PI-negative BV2 cells normalized to control BV2 cells. (B) Quantification of GM3-positive/PI-positive BV2 cells normalized to control BV2 cells. Data represented as Mean +/- SEM. **** p <0.0001, one-way ANOVA, Tukey’s post-hoc test, biological N=10 per group.
**M2 Phenotype Induced by IL-4**

All control cells, 3, 8, 12 and 24 h cells following IL-4 were positively stained for GM1 within the cell body and the processes (Fig. 19A). Quantitative results showed that there was a significant increase in GM1 pixel intensity per cell at 24 h compared to 3, 8, 12 h (p <0.05) and control cells following IL-4 exposure (p < 0.01) (Fig. 19B).

When staining with GM2 antibody, GM2 was undetectable in control cells. However, cells showed weak GM2 expression at 3, 8 and 12 h IL-4 treated cells until 24 h where GM2 expression increased (Fig. 20A). BV2 cells showed a marked significant increase of GM2 average pixel intensity at 24 h following IL-4 compared to control, 3, 8 and 12 h cells (p <0.001, Fig. 20B).

Finally, when staining with GM3, it was either undetectable or weakly stained in control BV2 cells, while strong positive staining was observed at 24 h following IL-4 exposure (Fig. 21A). GM3 expression was significantly higher at 24 h compared to controls and cells exposed to IL-4 at 3, 8 and 12 h (p <0.0001, Fig. 21B).
Figure 19. Quantification of GM1 expression in BV2 cells following activation by IL-4 for 3, 8, 12 and 24 h. (A) BV2 cells were activated by 20 ng/mL of IL-4, fixed with 4% & 2% PFA, and stained with GM1 (green) and DAPI (blue) and PI (red). Bars indicate 50 µm for each panel. (B) Using Image J, average pixel intensity per cell of GM1 in BV2 cells was compared to control. Data represented as Mean +/- SEM. * $p <0.05$, ** $p <0.01$, one-way ANOVA, Tukey’s post-hoc test, biological N=8 per group.
Figure 20. Quantification of GM2 expression in BV2 cells following activation by IL-4 for 3, 8, 12 and 24 h. (A) BV2 cells were activated by 20 ng/mL of IL-4, fixed with 4% & 2% PFA, and stained with GM2 (green) and DAPI (blue) and PI (red). Bars indicate 50 µm for each panel. (B) Using Image J, average pixel intensity per cell of GM2 in BV2 cells was compared to control. Data represented as Mean +/- SEM. *** p <0.001, one-way ANOVA, Tukey’s post-hoc test, biological N=8 per group.
Figure 21. Quantification of GM3 expression in BV2 cells following activation by IL-4 for 3, 8, 12 and 24 h. (A) BV2 cells were activated by 20 ng/mL of IL-4, fixed with 4% & 2% PFA, and stained with GM3 (green) and DAPI (blue) and PI (red). Bars indicate 50 µm for each panel. (B) Using Image J, average pixel intensity per cell of GM3 in BV2 cells was compared to control. Data represented as Mean +/- SEM. **** p <0.0001, one-way ANOVA, Tukey’s post-hoc test, biological N=8 per group.
BV2 cells were also labeled with PI to understand if the observed increase of GM2 and GM3 levels at 24 h following IL-4 (Fig 22B, 23B) resulted in cell death. By 24 h of IL-4 exposure, there was a significant decrease in the number of BV2 cells that were GM2-positive and PI-negative (viable cells) compared to control cells (p <0.01, Fig. 22A). However, number of GM2-positive and PI-positive significantly increased at 24 h post-IL-4 exposure (p <0.0001, Fig. 22B). Furthermore, by 24 h of IL-4 exposure, the was no significant change in the number of BV2 cells that were GM3-positive and PI-negative (viable cells) compared to the control group (Fig. 23A) while number of GM3-positive and PI-positive cells significantly increased at 24 h following IL-4 exposure (p <0.0001, Fig. 23B).
Figure 22. Quantification of GM2-Positive/PI-negative or positive BV2 cells following IL-4 exposure. (A) Quantification of GM2-positive/PI-negative BV2 cells normalized to control BV2 cells. (B) Quantification of GM2-positive/PI-positive BV2 cells normalized to control BV2 cells. Data represented as Mean +/- SEM. * p<0.05, **** p<0.0001, one-way ANOVA, Tukey’s post-hoc test, biological N=8 per group.
Figure 23. Quantification of GM3-Positive/ PI-negative or positive BV2 cells following IL-4 exposure. (A) Quantification of GM2-positive/PI-negative BV2 cells normalized to control BV2 cells. (B) Quantification of GM2-positive/PI-positive BV2 cells normalized to control BV2 cells. Data represented as Mean +/- SEM. **** p <0.0001, one-way ANOVA, Tukey’s post-hoc test, biological N=8 per group.
3.5 Detecting the time course of M1 and M2 Gene expression following exposure to LPS or IL-4

To validate that LPS and IL-4 exposure resulted in gene expression changes associated with M1 and M2 microglia activation status, RNA was extracted, and RT-qPCR was performed (Table 1). Fold change in gene expression was calculated relative to cell culture exposed to a no treatment control. All pro-inflammatory iNOS, IL-1β and TNFα markers of the M1 phenotype showed a significant peak at 24 h following LPS exposure compared to the control, no treatment, and M2 groups (Fig. 24A, B, C). Furthermore, following IL-4 induced M2 phenotype, Arg1 was significantly increased 5-fold at 8 h while no changes in Arg1 were detected following LPS exposure over time (Fig. 24D).
Figure 24. M1 and M2 gene changes in BV2 cells following LPS or IL-4 exposure. Data are shown as the average fold change (± SEM) at 0, 8, 24, and 48 h time points. Data was normalized to GAPDH and analyzed and compared to the control (no treatment) by two-way ANOVA and Tukey’s post-hoc test. * indicates statistical significance peak over all groups.
Section 4: DISCUSSION
Gangliosides maintain a homeostatic balance for each species in the healthy mammalian brain (Sandhoff and Harzer, 2013), and they play different regulatory roles within the CNS including development, differentiation (Yamashita, 2000; Yu et al., 2012), neuroprotection and cell survival (Maglione et al., 2010; Park et al., 2016; Schnaar et al., 2014; Schneider et al., 2013; Yu et al., 2012) as well as neurodegeneration (Caughlin et al., 2015; Mlinac and Bognar, 2010; Yamashita et al., 2005). However, perturbations in their homeostatic levels have been associated pre-clinical and clinical neurodegenerative diseases and injuries (Caughlin et al., 2015; Saito et al., 2015; Schneider et al., 2013; Whitehead et al., 2011). For example, shifts in ganglioside composition were observed following stroke in both rats and mice. There was a transient increase in GM2 and GM3 ganglioside within 3 and 7 d post-stroke which indicate that these changes are part of the post-stroke inflammation (Whitehead et al., 2011). Based on these findings, we hypothesized that the observed transient increase in GM2 and GM3 occurred within inflammatory cells residing the CNS; in particular, microglia as microglial activation contributes to the initial phase of the post-stroke inflammatory response (Iadecola and Anrather, 2011).

In this study, we investigated, for the first time, changes in GM1, GM2 and GM3 gangliosides and its d16:1, d18:1, and d20:1 sphingosine moieties in BV2 microglia cells following exposure to LPS induced-M1 phenotype and IL-4 induced-M2 phenotype. We predicted that GM2 and GM3 ganglioside levels would increase during M1 activation and complex GM1 gangliosides would increase during M2 activation.
4.1 Changes in Ganglioside Expression Profile following LPS Mediated-M1 Phenotype

Both animals and *in vitro* studies have strongly linked GM2 and GM3 accumulation to apoptosis, toxicity, neural death and neurodegeneration (Caughlin et al., 2015; Mlinac and Bognar, 2010; Saito et al., 2015, 2012; Sohn et al., 2006; Whitehead et al., 2011; Zervas et al., 2001). A previous study using MALDI-IMS showed that GM3 levels increased in mice brains following stroke (Whitehead et al., 2011). More recently, Caughlin et al., 2015 reported that GM3 increased at 3 days after stroke injury exacerbated with Aβ toxicity in the rat brain and at 21 days in both the regular stroke model as well as the exacerbated stroke injury model (Caughlin et al., 2015). In line with these reports, our immunofluorescence and LC-ESI-MS results showed that GM2 and GM3 levels significantly increased in BV2 cells by 24 h and 48 h following activation by LPS. These findings are also consistent with previous *in vivo* study where GM2 levels significantly peaked at 24 h in activated microglia in WT mice, and GM3 levels increased at 48 h in activated microglia in knockout mice following ethanol toxicity induced neurodegeneration (Saito et al., 2015, 2012). Although Saito et al., 2015 suggested that the observed accumulation of GM2 and GM3 gangliosides stemmed from degenerating neurons and being engulfed by activated microglia (Saito et al., 2015), the current study indicates, for the first time, that BV2 microglia can produce GM3 and GM2 following activation. Our results are also congruent with previous studies where GM3 ganglioside and GM3 synthase levels increased in macrophages within the inflamed areas of human atherosclerotic lesions which imply that increased GM3 ganglioside was derived from activated macrophages (Bobryshev et al., 2006, 1997; Gracheva et al., 2009). Collectively, the observed increase in our study of GM2 and GM3 ganglioside levels in M1-BV2 microglia following activation by LPS *in vitro* supports the hypothesis that microglia may contribute to the transient increase of GM2 and GM3 gangliosides found in animal models of neurodegeneration (Caughlin et al., 2015; Whitehead et al., 2011).
Using serum free medium in BV2 cell culture along with the toxic effect of LPS on cells as time progresses increased the susceptibility of BV2 cell death. Subsequently, their density dramatically decreased at 48 h following LPS exposure, and this was confirmed by quantification analysis using DAPI. Based on these results, BV2 cells were double stained with GM2 or GM3 antibody and PI to investigate if GM2 and GM3 levels increased at 24 h and 48 h following LPS were derived from activated or dead cells. At 24 h, the number of GM2 or GM3 positive and PI negative BV2 cells (viable cells) was higher than controls. At 48 h, however, the number of GM2 or GM3 positive and PI positive BV2 cells was higher compared to 24 h LPS-treated cells and control cells. Therefore, we hypothesized that the increase in GM2 and GM3 levels at 24 h following LPS exposure occur during BV2-M1 microglial activation while their increase at 48 h was a consequence of cell death. This was confirmed via RT-qPCR in which all M1 pro-inflammatory markers including iNOS, IL-1β and TNFα peaked at 24 h following LPS exposure compared to M2 phenotype and the control, no treatment, BV2 cells.

Unexpectedly, LC-ESI-MS showed that BV2 cells expressed significantly higher levels of GM1 ganglioside at 24 h and 48 h following LPS exposure despite the significant increase in the simple GM2 and GM3 gangliosides. Interestingly, the same phenomenon has been observed in a mouse model following stroke where a significant increase in GM1 levels was observed between 24 h and 7 d post-stroke while GM2 and GM3 levels increased between 3 and 7 d post stroke (Whitehead et al., 2011). Similarly, GM1 levels also increased significantly at 3 days in combined model of Aβ toxicity and endothelin-1 injection rat model of enhanced stroke along with an increases in both GM2 and GM3 levels (Caughlin et al., 2015). One possible explanation to the significant increase in both simple GM2 and GM3 and complex GM1 A-series gangliosides is the degradation of other complex B or C-series gangliosides. To confirm this hypothesis, future studies are required to measure the enzymatic activity that control the biosynthetic and catabolic pathways.
of gangliosides. Moreover, Moreno-Altamirano et al., 2007 found a subpopulation of peripheral blood monocyte expressed high GM1 levels and that there is a positive correlation between GM1 levels and endocytic activity. Also, the percentage of monocytes that have high GM1 increased from 2.5% at day 1 up to 50% at day 7 of culture when monocytes were differentiated to active macrophages, indicating a potential role of GM1 during monocyte macrophage differentiation (Moreno-Altamirano et al., 2007). Thus, a possible explanation to the accumulation of complex GM1 ganglioside is that BV2 microglia increase endogenous GM1 to differentiate from a resting (inactivated) to activated phenotype, possibly to increase their endocytic activity in response to injury.

4.2 Changes in Ganglioside Expression Profile following IL-4 Mediated-M2 Phenotype

GM1, GM2 and GM3 ganglioside levels were investigated in BV2 cells following IL-4 application. IL-4 was selected in our study because it has been shown that it is a successful M2 inducer in peripheral macrophages (Gordon and Martinez, 2010). We decided to investigate ganglioside changes at earlier time points than 24 h since Latta et al., 2015 found that M2 gene markers including Arg1 and MRC peaked at 8 h. In addition to 24 h, BV2 cells were exposed to IL-4 for 3, 8 and 12 h. Both LC-ESI-MS and immunofluorescence showed that GM1, as expected; and surprisingly, both GM2 and GM3 levels increased significantly at 24 h following IL-4. However, immunofluorescence images revealed that most of the GM2 and GM3 positive cells at 24 h were also PI positive, and quantification confirmed that number GM2 or GM3 positive and PI positive BV2 cells were significantly higher at 24 h. Our results therefore suggest that BV2 cells transiently activated to the M2 phenotype before 24 h since it has been shown that BV2 cells exhibited a time dependant heterogeneous morphology and phenotypes (Latta et al., 2015). This has been further supported by RT-qPCR results where Arg1 expression, M2 gene marker, peaked.
at 8 h and returned to control levels by 24 h in IL-4 treated BV2 cells. Collectively, data showed that no change in GM1, GM2 and GM3 levels were observed in M2 BV2 cells following IL-4 exposure, suggesting that M2 cells have no role in the transient gangliosides level changes observed in our previous animal studies.

4.3 BV2 cells primarily express the d16:1 sphingosine moiety

In addition to diversity based on the saccharide moiety, gangliosides also differ with respect to carbon chain length within their sphingosine moiety of the ganglioside ceramide backbone (Karlsson, 1970; Sonnino et al., 2007). Experimental studies showed that gangliosides with different sphingosine lengths are differentially expressed within various regions of the brain and play different roles within the CNS. Both d18:1 and d20:1 species are the predominantly expressed gangliosides in the mammalian brain (Caughlin et al., 2017, 2015; Sonnino and Chigorno, 2000; Weishaupt et al., 2015; Whitehead et al., 2011; Woods et al., 2013) and one study showed that the d16:1 species were found in small quantities in the rat brain (Avrova et al., 1973). Furthermore, rat embryonic cortical neurons predominantly expressed the d18:1 sphingosine moieties (Park et al., 2016). Interestingly, this is the first study to show that d16:1 is the most abundant species among GM1, GM2 and GM3 gangliosides in BV2 cells using LC-ESI-MS. Future experiment on primary microglia is needed for further confirmation.

4.4 Study Limitations:

The first limitation to this study is the use of cell line instead of using primary cells. Although several studies reported that BV2 cell line is a reliable model to study neuroinflammation associated with neurodegenerative diseases (Stansley et al., 2012; Wu et al., 2006; Yang et al., 2007), other studies showed that BV2 cells are less responsive to LPS and inflammatory
expressions were less pronounced than primary microglia (Henn et al., 2009). Another limitation of this study is that antibodies can only distinguish gangliosides according to the hydrophilic sugar chains on the cell surface (Kotani et al., 1993). Consequently, they cannot differentiate between the d16:1, d18:1 or d20:1 sphingosine moieties of GM1, GM2 and GM3 gangliosides. Also, because of this sugar-based epitope site for ganglioside, antibodies cross reactivity can occur, which is an additional potential limitation in the current study. For instance, GM1 antibody derived from Guillain-Barré syndrome patients cross reacted with GD1b (Koga et al., 2001). Thereby, it is possible that the resulted GM1 expression when staining BV2 cells with anti-GM1 cross reacted with GD1b ganglioside. This has been settled by using LC-ESI-MS which is a highly accurate, sensitive and quantitate analytical technique (Ho et al., 2003). Also, it is capable of accurately detecting ganglioside species according to their sugar chain and carbon numbers. Another limitation to this study is that LC-ESI-MS and immunofluorescence are two different techniques that have fundamental differences in sensitivity and detection methodology. Therefore, measuring GM1 levels by immunofluorescence and LC-ESI-MS did not provide consistent data in our study. LC-ESI-MS can provide both qualitative and quantitative data, and measures the signal intensity of ions of specific mass to charge ratio (Ho et al., 2003) while immunofluorescence is mostly qualitative and relies on measuring gangliosides based on their sugar moieties resulting in reduced specificity.

4.5 Future Studies:

This is the first study to investigate GM1, GM2 and GM3 ganglioside level changes in BV2 cells. We exposed BV2 cells to LPS and IL-4 to induce M1 and M2 phenotype, respectively. We hypothesized that following LPS, BV2 cells activated to the M1 phenotype and both GM2 and GM3 levels significantly increased. Then, BV2 cells will release the toxic GM2 and GM3 into the extracellular environment and exacerbate neuronal damage. To test this hypothesis, further studies
should be conducted to investigate the expression profile of GM2 and GM3 gangliosides in the isolated BV2 culture medium following activation by LPS using LC-ESI-MS to confirm that microglia release GM2 and GM3 gangliosides into the medium. Also, it would be interesting to confirm our current results by investigating ganglioside level changes in primary microglia cells.

RT-qPCR is a vital quantitative method in this study to verify what phenotypes were activated in response to the changes observed in ganglioside levels, and to confirm that LPS and IL-4 exposure resulted in gene expression changes used as markers for M1 and M2 phenotypes. Up to date, we optimized three M1 phenotype gene markers and only one M2 marker. Future studies to optimize more M1 and M2 gene markers would further validate our current RT-qPCR results.
Section 5: SUMMARY AND CONCLUSIONS
Summary of Key Findings

1. GM2 and GM3 gangliosides levels significantly increased at 24 and 48 h in M1 BV2 cells following LPS exposure compared to control cells.

2. GM1 levels significantly increased at 24 and 48 h in BV2 cells following LPS induced-M1 phenotype.

3. No significant change in GM1, GM2 and GM3 gangliosides levels were observed in M2 BV2 cells following IL-4.

4. BV2 microglia cells primarily express the d16:1 sphingosine moiety.

5. RT-qPCR results indicated a significant peak in M1 markers including iNOS, IL-1β and TNFα at 24 h following LPS while compared to the control and M2 group.

6. Following IL-4 induced M2 phenotype, Arg1 significantly peaked at 8 h compared to control and M1 group.

Conclusions:

In this study, we investigated, for the first time, GM1, GM2 and GM3 gangliosides profile changes in BV2 cells following LPS and IL-4 induced M1 and M2, respectively, using LC-ESI-MS. Our results showed a significant increase in GM1, GM2 and GM3 gangliosides levels in M1-BV2 cells following LPS exposure whereas no significant change in their levels were observed in M2 cells following exposure to IL-4. The increase in GM1, GM2 and GM3 levels following LPS suggest that M1 microglia may contribute to the transient increase of gangliosides observed in our rodent models of neurodegeneration (Caughlin et al., 2015; Whitehead et al., 2011). By understanding that gangliosides levels changed only in M1 BV2 cells following LPS as the current study suggests, we can selectively use the pro-inflammatory M1 phenotype as a target to study...
ganglioside metabolism and their relation to neurodegeneration post injury. Moreover, we suggest that following brain injury, microglia become activated into the M1 phenotype, increase their synthesis of GM2 and GM3 and release them into the extracellular environment further exacerbating neuronal damage following injury. This could open a new avenue of lipid based anti-inflammatory therapeutics targeting lipid dysregulation following neurodegenerative injury.
References


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Alzheimer’s Disease 20.


Riboni, L., Caminiti, A., Bassi, R., Tettamanti, G., 1995. The Degradative Pathway of


Curriculum Vitae
Mona Alshaikh

Education

M.Sc. Candidate, Department of Anatomy and Cell Biology, Neurobiology

King Abdulaziz University, Jeddah, KSA: 2007 – 2012
Bachelor degree in Physical Therapy

Scholarship

Fully sponsored by King Abdullah Scholarship Program for a Master’s Degree in Canada.

Researches


Training and Work Experience

March 2015
Volunteer, Organizer and Participant
International Day, University of Ottawa, Ottawa, Canada

January 2014
Volunteer, Organizer
Career Day, Saudi Arabian Cultural Bureau, Ottawa, Canada

January 2014
Volunteer, Organizer
Workshop of Academic Acceptance Keys in Canadian Universities

April – June 2013
Physical Therapist
Bagado Medical Clinics, Jeddah, Saudi Arabia

May – July 2012
Physical Therapist, Intern student
King Abdulaziz University
February – May 2013
Physical Therapist, Intern student
Abdullatif Jameel Hospital for Medical Rehabilitation

July 2011 – February 2012
Physical Therapist, Intern student
King Faisal Specialist and Research Centre

April 2011
Volunteer, Organizer in The 7th Medical Sciences Students Meeting
King Faisal Conference Centre, KAU, Jeddah, Saudi Arabia

Conferences, Presentations & Abstracts


  1. (March 9, 2017) Western Student Research Conference (WSRC), London, ON, Canada;

- Alshaikh MM, Lajoie GA & Whitehead SN. Poster Presentation: Detecting Gangliosides Expression Profile Changes During Microglial Activation.
  1. (May 30, 2016) Conference: Canadian Association for Neuroscience (CAN), Toronto, ON, Canada;
  3. (November 12, 2016) Conference: Society for Neuroscience (SFN 2016), San Diego, California, United States of America;

- The 7th Applied Medical Sciences Students Meeting, King Abdulaziz University, Jeddah, Saudi Arabia (April 2011)

- Stroke Day symposium (January 2012)

- Workshop of Rehab Technology in Neurological Rehabilitation, Jeddah, Saudi Arabia (February 2012)

- The Saudi International Rehabilitation Conference: Updates in Medical Rehabilitation of Disability Caused by Neurological Diseases, Jeddah, Saudi Arabia (March 2011):
  1. Workshop of Spider Therapy.
  3. Workshop of Rehab Technology in Neurological Rehabilitation.