THE ROLE OF ASTROCYTES IN RESPONSE TO
STIMULATION WITH THE MICROBIAL ENDOTOXIN LIPOPOLYSACCHARIDE

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ABSTRACT

Esteban Adrian Oyarzabal: The Role Of Astrocytes In Response To
Stimulation With The Microbial Endotoxin Lipopolysaccharide
(Under the direction of Michael D. Aitken)

Though the cause of Parkinson's disease (PD) remains unknown, many suspect its etiology includes both genetic mutations and exposures to environmental factors (e.g., microbial infections, heavy metals, pesticides, air pollution). This work attempted to understand the immunological roles of astrocytes and microglia, two brain cell types with innate immune properties, when exposed to the Gram-negative bacterial endotoxin lipopolysaccharide (LPS) in a mouse model of PD. This study found the immunoreactivity of astrocytes to LPS was attributed to the presence of microglia in 'enriched' astrocyte cultures. Reconstituting microglia back into a novel highly-enriched astrocyte culture (with <0.001% microglia) showed less than 0.5-1% microglia can account for the erroneous detection of proinflammatory factors by LPS-treated 'enriched' astrocytes. LPS was found to activate astrocytes via TNF-α produced by microglia, stimulating cytotoxic and neurotrophic factors release. Nonetheless, activated astrocytes protected neurons from microglial-derived bystander damage in this neuroinflammation-induced model of PD.

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LIST OF ABBREVIATIONS AND SYMBOLS

AD   Alzheimer's disease

ATP   Adenosine triphosphate

BDNF   Brain derived neurotrophic factor

CDNF   derived neurotrophic factor

CNS   Central nervous system

BBB   Blood-brain barrier

BCSFB   Blood-cerebral spinal fluid barrier

BMEC   Brain microvascular endothelial cells

BPI   Bactericidal/permeability increasing protein

BSA   Bovine serum albumin

C6   Cell line 6 (rat astroglioma)

CSF   Cerebral spinal fluid

DAnergic   Dopaminergic

DAMPs   Danger associated molecular patterns

ECM   Extracellular matrix

EGFP   Enhanced green flourecent protein

ELISA   Enzyme-linked immunosorbent assay

FBS   Fetal bovine serum

FGF   Fibroblast growth factor

GADPH   Glyceraldehyde 3-phosphate dehydrogenase

GDNF   Glia derived neurotrophic factor

GFAP   Glial fibrillary acidic protein

HDL   High-density lipoproteins

Iba	Ionized calcium-binding adapter molecule
iNOS	Inducible nitric oxide synthase
ISF	Interstidal fluid
LBP	Lipopolysaccharide-binding protein
LDL	Low-density lipoproteins
LME	L-leucine methyl ester
MCEC	Modified cuboidal epithelial cell
mCD14	membrane-bound CD14
MCM	Microglia conditioned media
MCP	Monocyte chemoattractant protein
M/G	Mixed glial culture
MIP	Macrophage inflammatory protein
mRNA	Messenger ribonucleic acid
NGF	Neuronal growth factor
NO	Nitric Oxide Radical
NO_2^-	Nitrite
N_2O_3	Dinitrogen Trioxide
NOX	Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase
NT	Neutrophin
ONOO−	Peroxynitrite
PAMPs	Pathogen associated molecular patterns
PBS	Phosphate buffered saline
PET	Position emission tomography
PD	Parkinson's disease
PRR	Pattern recognition receptors

PSA-NCAM   Polysialylated neuronal cell adhesion molecule

rBPI23   Recombinant N-terminal fragment of bacterial/permeability increasing protein

SAHA   Suberoylanilide hydroxamic acid

sCD14   Soluble CD14

SN   Substantia nigra

SNpc   Substantia nigra pars compacta

TLR   Toll-like receptors

TSE   Transmittable spongiform encephalopathies

CHAPTER 1: BACKGROUND AND LITERATURE REVIEW

"We sit on the threshold of important new advances in neuroscience that will yield increased understanding of how the brain functions and of more effective treatments to heal brain disorders and diseases. How the brain behaves in health and disease may well be the most important question in our lifetime."

- Dr. Richard D. Broadwell
Professor of Pathology at UMSM

Serving as the control center for the entire body, the brain is perhaps our most important organ. Adverse changes to the structure or biochemistry of the brain can result in decreased muscle rigidity; loss of sensation, movement, coordination or function to peripheral organs and tissue; cognitive deficits (e.g., IQ, memory); behavioral changes (e.g., addiction; changes in activity, mood, eating and sleep patterns); seizures; chronic pain; loss of consciousness; and death. Although many adverse changes to the brain arise from genetic polymorphisms and complications during fetal development, the role of environmental exposures, both during development and adulthood, have begun to receive more attention in the study of central nervous system (CNS) dysfunction.

In the late 1970s a group of environmental toxicologists began formalizing the study of CNS toxicity into today's mainstream field of neurotoxicology. Neurotoxicology is the study of adverse changes in the structure or function of the central and/or peripheral nervous system following an exposure to a chemical, physical, or biological agent. These exposures may include nutritional imbalance, stress, hypoxia, physical injury (e.g. head trauma, neurosurgery), pharmaceutical compounds, air pollution, industrial toxicants and infectious agents and their toxins. Unlike toxicological effects that occur in peripheral systems, the CNS has very limited capacities for biotransformation and efflux; anti-oxidant generation; resident immune response; energetic reserves; and ability to regenerate lost cells—making the brain susceptible to long-term damage from even the most minor perturbations.

This chapter serves as a primer of the immune system in the CNS and the pathological consequences that may arise from neuroinflammation derived from direct and indirect exposures to the microbial endotoxin lipopolysaccharide. The goal of this chapter is to provide sufficient background information for readers to understand the original research described in Chapter Two.

Infectious Agents and CNS Dysfunction: A Brief History

The effects of environmental agents on the CNS have been recognized for thousands of years, but the first recorded accounts of infectious agents resulting in neurological complications come from influenza outbreaks in Europe during the 12th Century.   Similar accounts were described at the turn of the last century among individuals that developed viral encephalopathy [1-4]. In 1961 Poskanzer and Schwab postulated a link between patients who had been infected with the Spanish Influenza and/or Encephalitis Lethargica during 1914-17 and who later developed Parkinson's disease (PD) [5].   Since then, the association between viral infections and the development of neurological disorders has been investigated extensively [6].

The association between bacterial infections and the development of neurological disorders has only recently started to gain more attention. Beginning in the late 18th Century with several cases of neuromuscular paralysis from the consumption of tainted blood sausage, Kerner described these bacterial toxin poisonings—now identified as botulism—as having "[t]he capacity [to interrupt] nerve conduction…in the same way as…an electrical conductor by rust" [7]. In 1933 Rich and McCordock were the first to demonstrate that the bacterium that causes Tuberculosis could infiltrate the subarachnoid space of the brain and induce inflammation to cause neurological morbidity [8]. Furthermore, several case reports of patients that developed bacterial vaginosis during pregnancy yielded children with brain damage sparked a series of epidemiological studies that linked bacterial infections during pregnancy with higher risk of their offspring developing certain neurological disorders and being born with activated immune systems [9, 10]—an association that had already been made with maternal viral infections in 1956 [11].

The role of bacterial infections in neurodegenerative disease originated in 1906 when Alzheimer suggested that "microorganisms" could be responsible for the formation of senile plaques observed in the brain parenchyma and around blood vessels of patients with Alzheimer's disease (AD)—later being compared to Prion-like infectiousness of transmissible spongiform encephalopathies (TSEs) in the late 1970s. Alzheimer's theory was strengthened after several epidemiological studies—some using biological samples to confirm past or present infections among study participants—found loose associations between bacterial infections and neurodegenerative disorders (**Table 1.1**). Interestingly, among the several bacterial strains

that were screened, only bacterial species that have been established as causative agents in human cases of bacterial encephalitis showed associations.

Table 1.1 Bacteria associated with the development of neurodegenerative disorders.

Neurodegenerative Disorder	Bacteria with Probable Associations	Sources
Amyotrophic lateral sclerosis	*Borrelia burgdorferi* (Lyme disease), *Chlamydia pneumoniae*, *Mycoplasma spp.*	[12-16]
Alzheimer's disease	*Borrelia burgdorferi*, *Chlamydia pneumoniae*, *Mycoplasma spp.*, *Helicobacter pylori*	[17-41]
Multiple Sclerosis	*Borrelia burgdorferi*, *Chlamydia pneumoniae*, *Mycoplasma spp.*	[42-49]
Parkinson's disease	*Chlamydia pneumoniae*, *Helicobacter pylori*, *Nocardia asteroides, Bordetella pertussis*	[50-64]

Bacterial Toxins: Understanding the Role of Lipopolysaccharides

Beyond direct infection, some bacteria are capable of generating non-infectious, bioactive toxins that are independently capable of inducing an immune response without the presence of living bacteria. In the late 19th Century, Pfeiffer discovered that heat-inactivated *Vibrio cholerae* could still induce fever when administered to guinea pigs [65]. Since then these bacterial toxins have been classified as either exotoxins or endotoxins. Exotoxins are usually highly toxic secreted proteins generated by a living bacterium, while endotoxins are usually lipid components released from the outer membrane of a dead or phagocytized bacterium. Exotoxins such as botulism and tetanus have long been recognized as direct neurotoxins (pathogenic regardless of the host's immune response). On the other hand, endotoxins are only pathogenic through a severe immune response—a condition known as endotoxemia. Although endotoxins are produced by both Gram-positive and Gram-negative bacteria, varying greatly among bacterial species and strains, human cases of endotoxemia are primarily derived from the lipopolysaccharide (LPS) on membrane fragments from Gram-negative bacterial infections [66-68].

Nearly seven decades of research establishing our current understanding of the immune system and cytokine signaling has enabled us to understand that the pathological effects of LPS are indirect. LPS is thought to mediate its indirect toxicity by initiating a cascade of host-generated factors such as prostaglandins, cytokines (e.g. IL-1, IL-6 and TNF -α), nitric oxide, superoxide, vasoactive amines, proteases (e.g. matrix metalloproteinases) and products of the complement and coagulation cascades that are

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themselves directly or indirectly cytotoxic [69]. Animal models and occupational exposure cases in humans have shown that LPS is bioactive *in vivo* and dose-dependently correlates with measures of immune activation and the display of symptoms such as inflammation, fever, fatigue, headache, leukopenia, nausea, joint and muscle pain, diarrhea, and in some cases mild amnesia [69, 70]. High-dose exposures to LPS are capable of triggering sepsis including hemorrhaging, disseminated intravascular coagulation, shock, multiple organ failure, and death—all without a detectable infection [69, 71, 72]. In fact, the mortality rate of patients with septic shock ranges from 20 to 80% [71].   Due to its clinical relevance in sepsis and septic shock, as well as its replicability in certain animal species, LPS has become the archetypical immunological stimulant for immunological research.

LPS and Parkinson's Disease

PD is a neurodegenerative disorder characterized by the chronic progressive loss of both the neurotransmitter dopamine and dopaminergic (DAnergic) neurons in the substantia nigra (SN) that innervate the corpus striatum—the axis responsible for smooth, controlled body movements [73]. Although the etiology of PD remains unclear, the majority of researchers suspect idiopathic PD is "environmentally-driven" and most likely stems from a combination of risk factors such as age, genetic predisposition, and xenobiotic exposures [74, 75].   Beyond containing an element of environmental risk, all human neurodegenerative diseases also share an age-dependent onset, progressive degeneration of distinct neuronal populations, formation of abnormal protein aggregates and neurofibrillary tangles, regional increase of heavy metal accumulation and localized neuroinflammation and oxidative stress in regions of degeneration.

Among the various environmental factors associated with higher incidences of PD, the associative link between the endotoxin LPS and PD originated in 1998 when a single intranigral injection of LPS in rats was reported to induce neuroinflammation resulting in a subsequent loss of dopamine in the nigrostriatal projection within 2 to 4 days [76]. In 2000, the same group conducted a follow-up study establishing that the

LPS-induced neurodegeneration selectively destroyed only DAnergic neurons[1] [77] and our group showed

that the DAnergic neurodegeneration was a direct result of the neuroinflammatory response [78]—which

was further supported by using an anti-inflammatory drug naloxone to prevent the inflammation-mediated

neurodegeneration [79]. Moreover, a similar account was described in humans in a 2001 case report of an

occupational exposure to LPS by a laboratory researcher who developed Parkinsonian Syndrome three weeks

after being exposed to 10 μg of *Salmonella minnesota* LPS [80]. Position Emission Tomography (PET) scans

confirmed that the patient's motor deficits were a result of severe structural damage to her substantia nigra

pars compacta (SNpc) [80]. Several years later a 2006 case report of an elderly Nigerian male who developed

acute Parkinsonian Syndrome within days after having developed severe sepsis from a Gram-negative

bacterial infection [58], further suggests an associative link. Although results from epidemiological studies

identifying infections as a risk factor for PD have primarily found associations with viral infections such as

influenza and herpes simplex virus [6, 63], studies have also identified an increased risk for developing PD

among individuals previously infected with the bacteria that causes whooping cough [59, 60, 64] or with

Helicobacter pylori [50-55, 57]—both LPS-containing gram-negative bacteria. Additionally, the involvement

of an existing chronic infection before an intranigral injection of LPS was recently shown to exacerbate

DAnergic neuronal loss, suggesting heterogeneous toxicities with regards to multiple environmental 'hits'

[81, 82].

In 2007, our group pursued the hypothesis that endotoxemia—commonly associated with sepsis and

severe infections—could result in the neurodegeneration of DAnergic neurons. Interestingly, unlike the

aforementioned studies and case reports that induced acute Parkinsonian Syndrome, a single systemic

intraperitoneal injection of LPS (5 mg/kg) in mice induced a delayed and progressive loss of DAnergic

neurons in the SNpc similar to that observed in idiopathic PD—whereby DAnergic neuronal losses of ~20%

and ~50% where observed at 7 and 10 months after LPS treatment, respectively [83]. Beyond recapitulating

the delayed, progressive degeneration of nigrostriatal DAnergic neurons that coincided with striatal

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[1] *DAnergic neurons are thought to be inherently more susceptible to oxidative stress than other neurons as a result of lower reserves of the anti-oxidant glutathione (on account of the quinones formed during DA(MDA: define DA) metabolism) and limited ability to synthesize neurotrophic factors. This deficit is exacerbated in the SN during inflammation due to its disproportionally high density of microglia (i.e., 4-5 times higher).*

dopamine loss and motor deficits in rotarod or open field activities [83, 84], this model also displays temporary reversal of motor deficits in response to levodopa treatment [84], and showed a marked sex difference in DAnergic neuronal loss [84] that echoes the sex differences observed in the prevalence and incidence of PD [85-90].   Some of these findings have been replicated and confirmed by other groups and the model has even recently been implemented in Wistar rats successfully [91]. In addition, this chronic neuroinflammation model of PD has been independently reviewed by several groups [92-95].

Interestingly, a single intraperitoneal injection of LPS administered to pregnant rodent dams also resulted in offspring with sustained, unbalanced inflammation (low levels of pro-resolution factors); fewer DAnergic neurons in the SNpc; reduced levels of brain dopamine; aggregation of 'Lewy body-like' formations; decreased antioxidant levels in brain; and greater susceptibilities for subsequent environmental 'hits' (i.e. infections, neurotoxicants, etc.)[96-98]. This finding suggests that the environmental hit associated with either the development or 'priming' of PD may occur as early as fetal development. Furthermore, transgenic mice that are deficient in Parkin, a gene whereby functional mutations have been associated with early onset familial PD, showed a selective loss of DA neurons in the SNpc with fine motor deficits upon chronic, systemic exposure to low dose LPS [99]. Similar findings were shown in transgenic mice overexpressing a mutated human SNCA gene (A57T), another familial PD mutation associated with increased risk of developing synucleinopathy and PD [100]. Although both gene mutations are themselves insufficient to cause PD in these rodent models, an environmental 'hit' may be required to propel pathogenesis of PD. Taken together these studies support a potential association between the bacterial endotoxin LPS and the neurodegeneration of DAnergic neurons associated with PD.

Environmental Exposures to LPS

LPS is ubiquitous in the environment where it has been detected in organic dust [101-103], cigarette smoke [104, 105], environmental pollution [106, 107] and occupation settings [108-110] ranging anywhere from rural to urban settings [111]. LPS is considered a persistent toxin because of its capacity to withstand severe environmental conditions such as desiccation, autoclaving and distillation [112]. Furthermore, human exposures are highly prevalent—whereby LPS and LPS containing Gram-negative bacteria have been

detected in the normal flora of the skin, respiratory airway and lower intestinal tract [113]. Interestingly, in organs that get continuous exposures to LPS, the body is capable of suppressing the immune system through a homeostatic mechanism that results in an endotoxin-tolerance effect [113]. For instance, LPS is considered to be a common component of normal gut flora—a region of the body that does not typically experience chronic inflammation—whereby small amounts of LPS are regularly absorbed into the hepatoportal blood supply where it is rapidly detoxified by the Kupffer cells of the liver without major incidence. In pathological conditions, such as those experienced by severe burn patients with severely damaged gut mucosa integrity, large amounts of LPS are thought to enter the systemic blood circulation resulting in a potent and life-threatening systemic inflammatory response [114, 115].

Does LPS Enter the Brain?

In 1885 Ehrlich observed that upon injecting a water soluble dye into the peripheral circulation, neither the brain nor the spinal cord were stained—implicating the existence of a barrier system between the CNS and the systemic circulation [116]. This defensive barrier refers to two ensheathing structures termed the blood-brain barrier (BBB) and the blood-cerebral spinal fluid barrier (BCSFB). The BBB is comprised of brain microvascular endothelial cells (BMEC; connect by tight junctions that restrict paracellular diffusion), pericytes, astrocytes and a basement membrane that together form the cerebral capillaries of the brain [117, 118]. In contrast the BCSFB is composed of a layer of modified cuboidal epithelial cells (MCEC; connected by less restrictive tight junctions that allow the passage of blood components necessary for the formation of CSF) that contain the CSF in the choroid plexus of each of the four cerebral ventricles [117, 118]. The term 'barrier' is actually a misnomer because although these enveloping structures can restrict the entry of most environmental factors in healthy individuals, they also play a major role in the influx of essential nutrients and ions and the efflux of metabolic waste products, drugs and other toxicants [119-122].

Because bacteria lack the advanced neuroinvasion mechanisms adapted by neurotropic viruses [123], thus excluding cases of neuronal tube defects or trauma that result in damage to the integrity of the BBB, most bacteria and their toxins are restricted from entry into the CNS. Nonetheless bacterial toxins have been reported to enter the CNS from the peripheral system via three modes of entry [124, 125]. The first

mechanism is retrograde axoplasmic transport mode that occurs only for bacterial exotoxins (e.g. tetanus toxin and botulinum toxin), whereby the neurotoxin enters a peripheral sensory-nerve ending or neuromuscular junction and travels along the retrograde axoplasmic transport system to the neuronal soma in the CNS. The second mechanism is paracellular penetration, whereby toxins can infiltrate the CNS, usually at the choroid plexus or at a dysfunctional region of the BBB, by passing between the tight junctions of BMECs or MCECs. The third mechanism occurs when bacterial toxins are released from bacterial infections of the CNS (e.g. bacterial meningitis, bacterial encephalitis and brain abscesses). Although the incidences are extremely rare, direct exposure of bacterial toxin to the CNS can result in long-term neurological sequelae or often result in a lethal neuroimmune response.

Among bacterial toxins, the ability of LPS to enter the CNS has been studied extensively [83, 126, 127]. The CNS is a lipid-rich system that is vulnerable to exposures by small, lipophilic toxicants that can readily diffuse passively through BMEC membranes. LPS is hydrophobic due to its lipid tails and has a net negative charge at physiological pH, which limits its ability to pass through the BBB easily. Furthermore, the luminal membrane of BMEC is more negative charged, thus it is thermodynamically unfavorable for LPS to diffuse across into the CNS due to mutual repulsion of ions—even through paracellular penetration. In 2010, Banks and Robinson confirmed that LPS radiolabeled with the isotope [125]iodine administered peripherally did not readily enter the CNS (~0.025% of the administered dose), even when observed in a Multiple Sclerosis model where the BBB was severely disrupted [127]. Similar findings were confirmed in postmortem examination of patients who died from complications with Gram-negative sepsis with encephalopathy that did not display disseminated cerebral micro-abcsesses usually associated with direct CNS infections [128]. Although sepsis-associated encephalopathy typically results in the breakdown of the BBB, these encephalopathies are thought to occur from systemic inflammation rather than by directly infecting bacteria and/or their toxins in the CNS [129]. It is, therefore, believed that in the majority of cases, peripherally derived LPS does not directly stimulate the CNS—even in cases of BBB breakdown.

LPS-Induced Neuroinflammation

Neuroinflammation is a self-defense reaction against a pathogenic stimulus or injury in the CNS. Under normal circumstances, neuroinflammation is a highly regulated process whereby coordinated networks of immune cells sequester and eliminate the source of the injurious stimulant, restoring homeostasis in the tissue. In the CNS this transient process is sequentially characterized by coagulation, edema (whereby the swelling induces pain), hypoxia-induced increase in blood supply, vascular permeability at the afflicted site allowing the infiltration of leukocytes, phagocytosis of the injurious stimulant, formation of granulation tissue (glial scar), and tissue repair which terminates the inflammation through the release of pro-resolution factors; this process of overlapping events is termed an 'acute neuroinflammatory response'. If the injurious stimulant is persistent, the neuroinflammation may continue unresolved, becoming a 'chronic neuroinflammatory response', which may result in more severe damage than that caused by the original insult [130]. Chronic inflammation has been identified to be a hallmark of not only infectious CNS diseases but also of brain trauma, ischemia, and autoimmune, neurophsyciatric, and neurodegenerative disorders of the CNS [131, 132]. Understanding the distinction between the neurotoxic and neuroprotective effects of neuroinflammation in non-infectious CNS diseases is a contentious topic that is currently being investigated.

LPS can induce neuroinflammation either indirectly through peripheral stimulation or, in rare cases, through direct stimulation. When LPS is administered through an intraperitoneal injection, it accumulates at the lining of the abdominal organs, recruiting neutrophils and inducing inflammation-mediated hyperpermeability of tissue membrane allowing the endotoxin translocation into the bloodstream where it binds to plasma components [133] and accumulates primarily in the liver and to a lesser extent in the spleen, kidneys and lungs [69]. Kupffer cells, the resident macrophages of the liver sinusoids, represent 80% to 90% of the total fixed-tissue macrophages of the body and are thought to be, in coordination with hepatocytes, primarily responsible for 80% of the clearance of LPS into the bile and crucial in LPS-mediated neuroinflammation through their inflammatory response [69]. Upon *in vitro* stimulation with LPS, Kupffer cells rapidly release pro-inflammatory factors such as TNF-α, IL-1β, IL-6, IL-18 and can induce the production of nitric oxide, prostaglandins, and reactive oxygen and nitrogen radicals which have also been detected in the circulating blood supply *in vivo* and in *ex vivo* liver tissue cultures after LPS stimulation [69]. TNF-α and

IL-1β generated by Kupffer cells are believed to play a pivotal role in peripherally induced neuroinflammation either by direct neural stimulation with cytokine receptors present on afferent vagus nerve endings [134] or through a humoral pathway where the cytokines travel through the bloodstream passing into the CNS through vesicular transcytosis to activate resident immune cells of the CNS [135].

Although it has been reported that inflammatory signals transmitted through the vagus nerve to the CNS can induce neuroinflammation [134], this route is primarily thought to contribute to pain, fever, and changes in corticosteroid and norepinephrine levels. This is verified *in vivo* through studies of intraperitoneal injections of either IL-1β, TNF-α, or LPS in intact and vagotomized rodents [136] and has been implicated in immune suppression through the release of acetylcholine from descending vagal afferent fibers that bind to α7- nicotinic acetylcholine receptors on Kupffer cells—attenuating the release of TNF-α, IL-1β, IL-6, and IL-18—in an LPS-induced sepsis model [137]. Interestingly, fever is generally induced within 10 minutes after an intraperitoneal injection of LPS and TNF-α—the first of the cytokines to be transcribed, translated and secreted into the bloodstream—is undetectable until 30 minutes after treatment. These observations suggest the existence of either another inflammatory signal capable of stimulating the vagus nerve or of an LPS receptor in the afferent nerve endings that can innervate the vagus nerve.

Due in part to the timetable that inflammatory factors are detected in the CNS, neuroinflammation is primarily thought to be induced through humoral cytokines. Qin *et al.* (2007) reported that when mice were treated intraperitoneally with LPS, TNF-α protein peaked in the blood and in liver and brain tissues 60 minutes after treatment [83]. Liver tissue expressed TNF-α mRNA within minutes of stimulation (peaking at 30 minutes) whereas TNF-α mRNA was detected in brain tissue 30 minutes after treatment (peaking at 1 hour) [83]. To test whether liver-derived TNF-α initiated neuroinflammation in the brain, Qin *et al.* administered LPS intraperitoneally into wild-type mice and mice with a genetic ablation of the TNF-α receptors TNF-R1 and TNF-R2 [83]. As expected, LPS was capable of generating TNF-α mRNA and protein in liver tissue in both mice, but TNF-α mRNA and protein was detected in brain tissue only in wild-type mice [83]—indicating that TNF-α receptors are necessary for peripheral LPS to result in the generation of brain TNF-α mRNA and protein. This model was further validated by treating both types of mice with a systemic injection of TNF-α that induced brain TNF-α mRNA and protein in wild-type mice, but not in mice that lacked

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the TNF-α receptors [83]. Moreover, treatment with TNF-α failed to induce the production of monocyte

chemoattractant protein-1 (MCP-1) and IL-1β in brain tissue of TNF-α receptor knock-out mice [83],

confirming the role of humoral TNF-α as the primary factor that drives neuroinflammation in LPS-induced

endotoxemia.

The most important finding of Qin *et al.* was that even though TNF-α protein in liver tissue returned

to baseline concentrations 9 hours after LPS treatment, it remained elevated in brain tissue throughout the 10

month duration of the study (now confirmed up to 30 months) [83]. In other words, LPS-induced

endotoxemia produced an acute inflammatory response in the peripheral system that triggered a chronic

inflammatory response in the CNS. Interestingly, this chronic neuroinflammation was shown to be directly

responsible for the selective delayed and progressive loss of DAnergic neurons in SNpc, similar to that

observed in idiopathic PD.   Block *et al.* (2007) theorized that the neuroinflammatory response induced from

the LPS endotoxemia becomes chronic through a positive feedback loop wherein the resident immune cells of

the brain become activated by humoral TNF-α to release inflammatory factors that result in their autocrine

re-stimulation and paracrine stimulation of other resident immune cells (amplifying the signal). Some of

these factors are also thought to be cytotoxic and are thought to injure DAnergic neurons to release

endogenous stimulatory paracrine signals—taken together resulting in a vicious stimulatory cycle that

produces chronic neuroinflammation [138].

Many have argued that concentrations of humoral cytokines that enter the brain are insufficient to

generate a significant acute neuroinflammatory response in the brain—let alone a chronic inflammatory

response. Instead some have hypothesized that LPS and released pro-inflammatory factors travel through

the bloodstream and bind directly to their receptors that are expressed on luminal surface BMECs or MCECs

of either the choroid plexus or the circumventricular organs, inducing an inflammatory response in these

cells resulting in either a direct release of pro-inflammatory factors into the adjacent brain tissue or a

breakdown of the BBB allowing cytokines as well as other serum-derived immunoactivators to enter the

brain parenchyma to activate the resident immune cells of the CNS. *In vitro* cultures have supported that both

endothelial and epithelial cells of the CNS are capable of generating an immune response when treated with

either IL-1β, TNF-α, or LPS [139-141]—but these findings are not so clear *in vivo* because it is difficult to

differentiate the attributable effects of these cells from astrocytes, pericytes and microglia. Interestingly, although Sumi *et al.* (2010) did not evaluate the ability of LPS-treated cultured primary rat MBECs to produce cytokines, they did find that the presence of microglia were required to induce permeability in the MBECs monolayer as detected in culture by decreased transendothelial electrical resistance and increased permeability to sodium-fluorescein. Furthermore, they demonstrated that the changes in MBEC integrity were due to microglia-induced oxidative damage resulting in the fragmentation of the tight-junction proteins zonula occludens-1, claudin-5, and occludin.

LPS has been shown to be sequestered by circulating factors and reversibly bind to BMECs in very low concentrations [127], but whether it can directly stimulate endothelial cells *in vitro* has yet to be definitively established. Interestingly, bi-directional treatment with either LPS or TNF-α has been observed to result in an acute breakdown of the BBB in rodents [142-144] occurring between 6 and 24 hours after treatment—a phenomenon that has also been observed in patients with Gram-negative bacteremia-induced sepsis-associated encephalopathy through the detection of large serum proteins in their CSF [145]. Taken together, all the proposed modes of action for LPS-induced neuroinflammation have been experimentally confirmed to be indirect. The role of direct LPS stimulation in the CNS and the detection mechanisms that are required to generate a neuroinflammatory response, they are addressed in a later section (*see* **Detecting LPS in the CNS**).

The Resident Immune System of the CNS

The immune system in humans is comprised of both adaptive and innate immunity. Innate immunity occurs when innate immune cells, expressing pattern recognition receptors (PRRs) such as pathogen-associated molecular pattern (PAMP) receptors, detect and immediately produce a response to stimulatory signals such as infectious agents. Adaptive immunity is generated after an innate immune response, when innate immune cells produce a certain threshold of 'non-self' antigens recognized by specialized adaptive immune cells that are recruited to the site of infection and specifically target and destroy infectious agents— whereby novel antigen-receptors can be synthesized quickly through gene rearrangement and passed down generationally through mitosis resulting in immunological memory. Regardless of their different response

mechanisms, innate and adaptive immune cells all stem from the same hematepoeitc lineage making them difficult to study in their non-activated states due to the similarity among their markers.

Until recently, the CNS was widely regarded as an 'immune-privileged' organ system that was isolated by its 'barriers' from the peripheral immune system, lacked a draining lymphatic system and was populated with 'immunoincompetant' resident innate immune cells. In 1991, Hickey *et al.* were the first to report that activated CD4 T lymphocytes could cross the BBB and host an adaptive immune response in the CNS. In 1991, Yamada *et al.* showed that cervical lymph nodes are involved in the drainage of interstitial fluid (ISF) and CSF from the brain. Today, we recognize the 'immunoincompetence' of the resident innate immune cells of the CNS as a unique adaptation to tightly control immunological responses to limit damage to this highly sensitive organ system with limited re-generative abilities.

Simplistically, the CNS is comprised of neurons, endothelial cells, pericytes and glial cells. Glial cells are comprised of several cell types including astrocytes, microglia, oligodendrocytes and ependymal cells. It is thought that these cells, along with pericytes and endothelial cells, form the resident innate immune network of the CNS—due to their *in vitro* ability to express PAMP receptors and present surface antigens, two features commonly associated with innate immune cells. Technically, innate immune cells also possess the ability to phagocytize microbes and cellular debris; undergo morphological and epigenetic changes during activation (also known as polarization); produce and release cytokines, chemokines and other soluble factors to recruit additional immune cells and to induce complement receptors to facilitate pathogen clearance— which are functions that have only been observed *in vivo* in microglia and astrocytes. It should be noted that during neuroinflammatory episodes, perivascular macrophages, fibroblasts and leukocytes of both the innate and adaptive immune systems are recruited into the CNS to assist in the inflammatory response. In general, these cells are thought to be recruited after an initial innate immune response by the resident immune cells to assist in the clearance of the injurious stimulant; thus for the sake of this primer, I will focus primarily on microglia and astrocytes as the resident innate immune cells of the CNS.
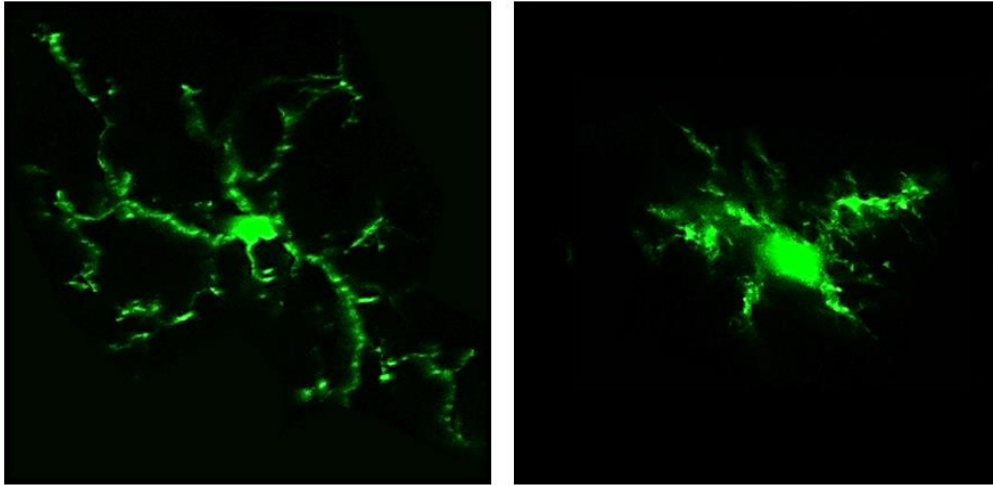
1.   Microglia

Microglia are commonly referred to as the resident 'macrophages' of the CNS. First described in detail in 1919 by Río Hortega, these cells are thought to be derived from yolk-sac macrophage precursors and migrate through the cephalic mesenchyme before vascularization to the brain pial surface where they invade the neuroepithelium of the CNS at the roof of the 4th ventricle [146]. Occurring during early embryogenesis, microglial progenitors are thought to be recruited by the early neuronal expression of the chemotactic signals MCP-1 and macrophage inflammatory protein-1 (MIP-1) [147]. Once in the brain, these yolk-sac macrophage precursors transform into the microglia and their progenitor cells, making up 10-15% of the cells of the adult CNS, where they are thought to proliferate until a few months after birth and turnover without any significant contribution from bone marrow derived monocytes [148]. During embryogenesis, microglia play a fundamental role in CNS vascularization [149] as well as neural progenitor differentiation and clearance, synaptic pruning and neuroapoptotic debris clearance [150, 151]. In a healthy brain, microglia are ubiquitously distributed throughout the brain where they remain in a quiescent state through constitutive stimulation with the immunosuppressive cytokine TGF-β [152, 153] and through cell-to-cell contact with CD200 [154], CX3CL1 [155] and polysialylated neuronal cell adhesion molecule (PSA-NCAM; [156])—proteins that are thought to degrade over time with aging and are thought to be associated with the age-dependent shift towards more activated profiles [157].

We now know that this 'quiescent state' is a misnomer because *in vivo* two-photon imaging of EGFP-expressing microglia in rodents show that inactivated microglia are actively surveying cells through cell-to-cell contact and through a series of receptors that screen the ISF for endogenous danger-associated molecular patterns (DAMPs) and PAMPs. Upon detecting a stimulatory signal, microglia swiftly undergo a hypertrophic morphological change that increases the size of their soma while retracting their processes (**Figure 1.1**), shift metabolic priorities and migrate to the site of the distress. Epigenetic reprogramming is thought to mediate this process, controlling the up- and down-regulation of a variety of genes associated with this polarization state—which in turn modulates the release of cytokines and chemokines and the induction of phagocytosis

and antigen presentation. Interestingly, the response by microglia after being activated is unique to the stimulant and is, for the most part, dose-dependent.

Figure 1.1 Two-photon 3D reconstruction of quiescent (left) and LPS-activated (right) microglia in the SNpc as imaged in CX3CR1-EGFP$^{+/-}$ mice.

The acute polarization of microglia into an activated state is commonly referred to as microgliosis. Similar to other myeloid-derived innate immune cells, activated microglia are primarily responsible for detecting and phagocytizing stimulatory xenobiotics/endogenous debris and generating a variety of toxic substances such as reactive oxygen species, reactive nitrogen species, proinflammatory cytokines (including death signals) and prostaglandins that are meant to kill infectious agents but are also known to injure by-standard cells such as neurons. Once the injurious stimuli are cleared, microglia release a series of pro-resolution factors that either induce their de-activation or apoptosis—thus resulting in an acute neuroinflammatory response. Microgliosis is thought to protect the CNS in acute neuroinflammatory events that are associated with mechanical injuries, ischemia, and infections of the CNS. On the other hand, unresolved microglial activation referred to reactive microgliosis produces chronic neuroinflammation resulting in the continuous release of cytotoxic factors that are associated with progressive neurodegeneration [130].

2.   **Astrocytes**

Astrocytes are the most abundant cell type in the CNS. First described over 150 years ago, astrocytes were thought to strictly play a role in the structural scaffolding of the brain and providing essential energetic support for neurons due to their physical interactions in the neurovascular unit. Since then, researchers have uncovered that astrocytes have many more functions including modulating synaptic transmission through neurotransmitter uptake, transport and catabolism and by releasing ATP to inhibit synaptic transmission; regulating ion concentration in the ICF; providing energetic support to ischemic neurons through energetic reserves; maintaining the blood-brain barrier and modulating blood flow at the artery and arteriole level; coordinating neural development, synaptic cleft formation and axonal myelination; forming glial scars; regulating long-term potentiation through calcium waves; and participating in innate immune functions [158].

The innate immune functions of astrocytes are still relatively controversial, as are described later in this chapter. Unlike microglia that share the same mesodermic lineage as 'professional' innate and adaptive immune cells, astrocytes are derived from radial glia that are themselves derived from neuroepithelial cells of the ectoderm. Regardless of this, astrocytes have been reported to share many similarities to microglia with regard to innate immune functions including the expression of cell surface DAMPs and PAMPs receptors [159]; the ability to shift into a hypertrophied morphology upon activation (i.e. reactive astrogliosis) [160]; the generation of cytokines and free radicals upon activation [161]; the capacity for phagocytic clearance of cellular debris [162] and insoluble fibrous protein aggregates [163]; and the ability to recruit and activate leukocytes through the release of chemokines and through antigen presentation [164].

Detecting LPS in the CNS

In the rare cases that LPS is capable of entering the CNS, it is thought that both microglia and astrocytes possess innate immune receptors to detect and sequester LPS—resulting in their activation and a subsequent neuroinflammatory response. It should be cautioned that although some of the work has been confirmed *in vivo*, most reports derived their findings using *in vitro* studies with either primary/secondary cell cultures or immortalized cell lines derived from transformed tumor cells. Although cell cultures are a necessary tool that allows researchers to differentiate the effect of a treatment on individual or multiple cell

types, all findings from cell-culture studies should be cautiously interpreted because even though the growing conditions of these cultures try to mimic those found *in vivo*, it is nearly impossible to replicate the dynamic intricacy of a living organism.

1.   Structural Description of LPS

LPSs are large amphipathic glycolipids that range in molecular weight from a few thousand to over 10,000 Daltons and are essential for structural and functional integrity of Gram-negative bacteria [165]. LPSs are thought to represent 75% of the total outer membrane of each bacterium, whereby a single *Escherichia coli* bacterium is estimated to contain ~3.5 x 10^6 monomers of LPS [166]. The structure of LPSs consists of three covalently linked domains (**Figure 1.2**): the variable O-polysaccharide extracellular moiety, the inner and outer transmembrane core oligosaccharide moiety and the intercellular lipid A moiety [165]. LPSs vary greatly in the composition of the O-polysaccharide moiety among different bacterial species and strains, ranging in length anywhere from 0 to 40 repeating units—composed of three sugars with an additional sugar connected to the first and third sugar units—resulting in two subclasses of LPSs [165]. One class of LPSs, known as rough LPSs, is derived from bacterial strains that have atypical O-polysaccharide moieties that are either absent or reduced in length [165]. LPSs derived from bacterial strains with full-length O-polysaccharide moieties, usually ranging from 20 to 40 repeating units in length, are known as smooth LPSs [165]. Interestingly, rough LPSs are more hydrophobic and tend to be cleared from the blood quicker than smooth LPS [166]. Unlike the O-polysaccharide moieties, the Lipid A moiety is a highly-conserved phosphoglycolipid that contains a head composed of two phosphorylated glucosamine sugars with four to seven ester- and amide-linked acetylated fatty acid tails that have been shown through studies using similar synthetic fragments to be the essential component necessary for the recognition and subsequent stimulation of LPSs by the innate immune system [165].

One particular method deployed by macrophages and neutrophils to detoxify phagocytized LPSs in lysozomes are to release acyloxyacyl hydrolases to degrade the lipid tails of the Lipid A moiety resulting in a deacylated LPS metabolite with decreased biological activity and in some cases an antagonistic profile to LPS receptors [167] . Kupffer cells are also capable of digesting the O-antigen portion of LPSs resulting in greater

hydrophobicity and improving their clearance [168, 169]. The final method Kupffer cells deploy for LPS

clearance utilizes scavenger receptors and released alkaline phosphatases to dephosphorylate the Lipid A

moiety, resulting in neutralizing its biological activity and increased binding to hepatocytes for clearance

[170]. Enzymatic modifications of the structure of LPSs are important in the detoxification and clearance of

LPSs from the body.
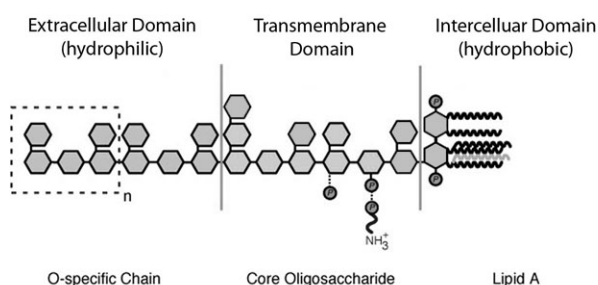
Figure 1.2 The basic structure of lipopolysaccharides and their domains.

Although Lipid A is the most conserved domain in LPSs, variable structures that differ in the head-group

substituents and in the number, distribution, and composition of the fatty acid tails have been detected

among bacterial species. These atypical LPSs differ from the archetypal structure found in *Escherichia coli*

and S*almonella* and have been shown to have lower binding affinities than typical LPS receptors in LPSs

derived from *Helicobacter pylori*, *Porphyromonas gingivalis* and *Francisella tularensis*; activate an atypical

signaling pathway with LPSs derived from *Porphyromonas gingivalis*; show greater levels of activity in

smooth LPS from *Salmonella minnesota* and *Rhodocyclus gelatinosus*; and result in a complete inhibition of the

typical LPS receptor in rough LPSs derived from *Rhodobacter sphaeroides* [166]. These differences are

thought to occur because the Lipid A structures of some LPS monomers, usually depending on the number of

acyl chains, are cylindrical (tilt angle of the lipids to the backbone of less than 15°) forming micelles whereas

other Lipid A structures are conical (tilt angles greater than 45°) and are thought to form inverted cubic

aggregates [171]. The type of aggregates formed, as well as other structural features (i.e. ratio of

hydrophobic /hydrophilic regions) among LPSs and environmental conditions (pH, salt concentration,

temperature, presence of divalent cations and hydration) may play important roles in the kinetics of LPS-

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mediated signaling and whether the LPSs will act as agonist or antagonist at the binding site of PRRs —thus modulating biological activity.

The outer membrane component of most Gram-negative bacteria also contains lipoproteins, which are themselves activators of the innate immune system; thus most commercially available LPSs has been shown to be contaminated with other bacterial components. For the remainder of this thesis, only LPS studies that used ultra-purified smooth LPS extracted from *Escherichia coli* or *Salmonella* will be considered to avoid additional confounding variables with regards to the response to LPS stimulation.

2.   Pathogen-Associated Molecular Pattern (PAMP) Receptors

The innate immune system has the ability to recognize both xenobiotics and endogenous DAMPs through a limited number of highly promiscuous PRRs. Originating in single celled organisms, these receptors have evolved into multi-cellular organisms, where they are expressed in a variety of different cellular compartments depending on where they are most likely to detect their respective stimulatory ligands. PAMP receptors are a sub-class of PRRs that are capable of detecting highly conserved molecular components of microorganisms. These receptors not only detect living microbes and their toxins, but partake in their degradation through direct phagocytosis, lyses via an oxidative burst or by tagging them with complement or antibodies to mediate their targeted destruction by the adaptive immune system (i.e., opsonization). Although other bacterial and viral components are also recognized by PAMPs, this section focuses on the receptors that are present in the CNS and that have been identified to recognize LPS.

Over the past three decades, researchers have focused on elucidating how LPS binds to cells to generate an innate immune response—much of the work on LPS stems from the field of endotoxin-induced septicemia in the peripheral system and has been validated experimentally to also include cells of the CNS. In 1986 LPS was recognized to bind to both the β2-integrin receptor CD11b/CD18 and soluble CD14 (formerly known as MY-4 antigen) and later in 1991 LPS was recognized to bind to class A scavenger receptors; all of which were incapable of mediating LPS-induced cellular activation. In 1990, membrane-bound CD14 (formerly known as monocyte-specific antigen) was implicated in LPS-induced cellular activation, but structural analysis of

membrane-bound CD14 showed that the PRR was tethered on the cell surface with a glycosylphosphatidylinositol linker and lacked a necessary transmembrane domain to mediate the intercellular signaling observed during LPS stimulation. Thus, both forms of CD14 were hypothesized to be accessory proteins to a putative LPS receptor. In 1998, toll-like receptor-4 (TLR-4) was identified as the putative receptor known to interact with CD14 and LPS to induce the bulk of the cellular activation induced by LPS. The ability for LPS to induce a weak activation in TLR-4 deficient animals suggests that additional putative receptors may exist but have not yet been identified.

a.   Plasma Septins

Studies have shown that once in the bloodstream LPS can bind to erythrocytes, mononuclear cells, platelets, neutrophils and plasma macromolecules. Plasma macromolecules including albumin, low-density lipoprotein (LDL), high-density lipoprotein (HDL), LPS-binding protein (LBP) and soluble CD14 (sCD14), collectively referred to as 'septins', strongly associate with the Lipid A moiety of LPS. One study showed that 96% of all administered LPS was bound to septins within 20 minutes of being introduced into the bloodstream [133]. LBP has been identified to not only play an important role in the functional neutralization and clearance of LPS in the blood [172], but it has also been identified to enhance the LPS-mediated immune response signals by PRRs [173]. LPS, due to its amphipathic nature, forms large micellar structures in the aqueous environment of the blood where LBP is thought to selectively capture LPS monomers (**Figure 1.3**) at a positively charged region at the N-terminal and transfer them to lipoproteins [174]. LPS-lipoprotein complexes have been shown to be biologically inactive *in vitro* [175] and are thought to be excreted in the bile [176] and thus are considered a major route of LPS clearance in the body. The role of LBP in inflammation is thought to be mediated by the transfer of LPS to either sCD14 or membrane-bound CD14 (mCD14)—integral components required for the bulk of the PRR-mediated immune activation that are described in detail in subsequent sections.

Figure 1.3 Schematic of how LPS-binding protein (LBP) removes an LPS monomer from LPS micelles.
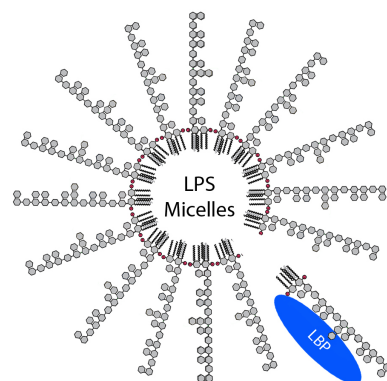
Although LPS is known to bind to CD14 independently of LBP-mediated transfer through the slow spontaneous diffusion of LPS monomers from their aggregate structures, the presence of LBP has been shown to increase the binding kinetics by 100 to 1,000-fold, thereby enhancing the sensitivity of immune cells to LPS [177]. Serum LBP and sCD14 are acute-phase proteins—whose serum concentrations increase significantly during inflammation—generated primarily by hepatocytes. Two isoforms of sCD14 have been isolated in the blood; one isoform (48kDa) is thought to be derived from proteolytic cleavage of the glycosylphosphatidyl inisotol-anchor that tethers mCD14 to extracellular membrane, and the other isoform (55kDa) is thought to be directly secreted by cell that express mCD14 [178]—the characterization of these isoforms and their respective binding kinetics to LPS have yet to be investigated.   Interestingly, although sCD14 is known to mediate the activation of PRRs in mCD14-negative cells, sCD14 is also thought to play a dual-role by competing for LPS in a protective manner with mCD14 and stripping LPS from ligated PRRs—therefore reducing the capacity LPS to induce PRR-mediated signaling. The mechanisms that govern these contrasting roles of sCD14 are thought to be mediated by differing binding sites that either shield or expose the Lipid A portion of LPS (**Figure 1.4**). Supraphysiological concentrations of sCD14—25-fold greater than the measured endogenous levels—can attenuate LPS-induced release of TNF-α *in vitro* and *in vivo* and has even been shown to be protective in murine models of lethal endotoxemia [179].
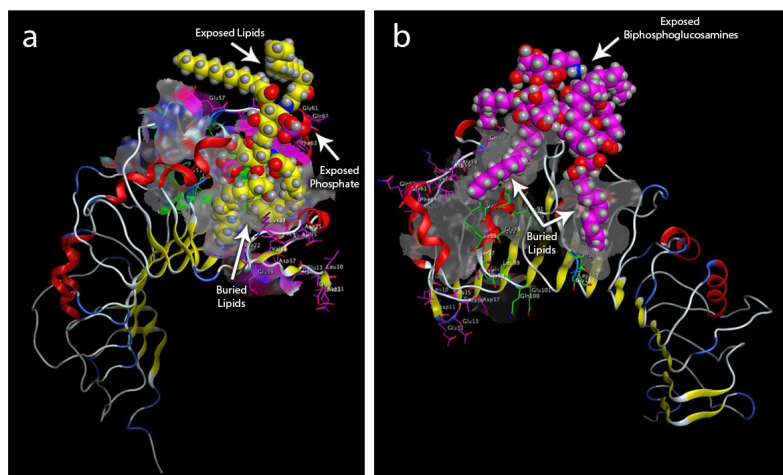
Figure 1.4 Structure-based pharmacophore predictions of low-energy binding interaction between LPS and sCD14. Rendering (a) predicts that some of the lipid A region gets buried in a hydrophobic pocket leaving behind a few exposed fatty acid tails that allow for its transfer to lipoproteins or LPS receptors. Rendering (b) predicts that LPS is capable of binding to another region of sCD14 that completely conceals the lipid A region preventing its transfer and signaling.

Studies using transgenic knockout mice that lack the ability to produce LBP have shown

hyporesponsiveness to LPS challenge *in vitro* [180] but had no difference *in vivo* [181]. Whereas, CD14

ablated transgenic rodents are hyporesponsive to low-dose LPS challenge [182] and sCD14 was

demonstrated to play a role in LBP-mediated transfer of LPS to serum lipoproteins [183]. Although a great

deal of work has been done to study LBP and sCD14 in the blood, their presence in the CNS has not been well

characterized. As previously described both the ICF and CSF are isolated from the systemic blood by the

BBB—which serves as a barrier for most circulating proteins including septins. In fact, plasma proteins such

as fibrinogen [184] and albumin [185] are considered endogenous activators of the innate immune system

and can result in an inflammatory response upon entry into the parenchymal tissue of the CNS. Nevertheless,

both LBP and sCD14 have been identified and quantified in the CSF in both healthy and diseased individuals.

LBP and sCD14 were found to be present in low concentration in the CSF of healthy individuals and are

considered to be biomarkers of BBB breakdown and neuroinflammation due to their high levels in the CSF of

individuals with head and spine trauma, bacterial meningitis, sepsis and neurodegenerative disease [186].

ELISAs conducted on the CSF of murine sepsis models have shown that sCD14 is detected in the CSF starting

24 h after an intracardiac injection of LPS, peaking around 48 h [187]. *In situ* hybridization detecting

intracellular production of CD14 mRNA identified that microglia and not astrocytes are capable of generating

CD14 mRNA—further supporting previous findings that microglia express surface mCD14 whereas

astrocytes do not and thus require sCD14 for LPS-induced PRR signaling [187]. Interestingly, the levels of

CD14 generated by microglia are negligible compared to those generated by infiltrating intrathecal

leukocytes—which are now thought to generate the bulk of the sCD14 detected in the CSF during

inflammation [187].

Even though sCD14 and mCD14 cannot be distinguished by measuring CD14 mRNA, another study

looked at CD14 mRNA in the parenchymal tissue and found that systemic administration of LPS in rats caused

a rapid expression of CD14 mRNA in the cirumventricular organs and later in the brain parenchyma [188].

Interestingly, pretreatment of mice with anti-TNF-α antibody to neutralize the cytokine TNF-α significantly

reduced the levels of LPS-induced CD14 mRNA transcription [189]. This study was further confirmed by

intracerebroventricular administration of recombinant rat TNF-α that stimulated the transcription of CD14

mRNA as early as 1h (peaking at 6h) after administration.   To date, no studies have quantified the amount of

LBP or sCD14 in the parenchymal tissue; thus it is difficult to speculate the role of either LBP or sCD14 as

PRRs in the brain or whether they share the same functions as in the peripheral system.

A lesser studied septin that is known to bind to LPS is a 50kDa protein known as

bactericidal/permeability-increasing (BPI) protein that is generated and stored in the azurophilic granules of

polymorphonuclear leukocytes [173]. BPI is thought to play an important role by neutralizing the bioactive

effects of LPS by binding the anionic phosphate groups of the Lipid A moiety to the cationic structure of BPI

[173].   BPI has a high affinity for LPS monomers, but unlike LBP, BPI lacks the ability to disperse micellar LPS

aggregates—thus implying that the protective effects of BPI may require LBP for optimal binding. The

presence of BPI in brain tissue or CSF has not been reported with the exception of one study that used a

synthetic form of BPI known as recombinant N-terminal fragment of BPI (rBPI23) in an endotoxemia-induced

model of meningitis in rabbits [190]. Intracisternal injection of meningococcal endotoxin resulted in high

levels of TNF-α and serum proteins in the CSF—which was significantly reduced following an administration

of rBPI23 to the subarachnoid cistern [190]. Yet, when rBPI23 was administered intravenously it showed no

protection [190]. Animals treated intervenously with rBPI23 showed no detectable levels of rBPI23 in the

CSF [190]—suggesting that BPI may not be capable of traversing the BBB even when it is damaged and may

not play a role in neuroinflammation.

b.   Toll-like Receptors (TLR)

The toll-like family of receptors is a class of PRRs first identified in mammals in the late 1990s. As of

now 13 mammalian receptors have been identified—with nine of the receptors being functionally conserved

among human and murine species. Capable of identifying molecular motifs from both PAMPs and DAMPs, the

discovery of the toll-like receptor-4 (TLR-4) was awarded the Nobel Prize in Physiology or Medicine in 2011.

In 1998, two independent publications confirmed TLR4 as the putative receptor that detects LPS and

participates in intracellular signaling [191, 192].   Speculation that mCD14 most likely interacts with, at the

time, a putative PRR that could detect LPS originated from two inbred strains of mice that expressed wild-

type levels of mCD14 yet were incapable of generating an innate immune response during endotoxemia. The

mutations thought to be responsible for this variation were identified and the defective genes were cloned,

confirming that hyporesponsiveness to LPS on C3H/HeJ and C57BL/10ScCr mice were a result of a functional missense mutation and a null mutation, respectively, in the gene that encodes the TLR-4 receptors [193]. Although the initial hypothesis regarding the LPS PRR was incorrect, today we know that CD14 plays an integral role as an associated protein that interacts with TLR-4 and the adapter protein MD-2 to induce LPS signaling [194].

TLR-4 is usually constitutively expressed on the surface of innate immune cells where it is complexed with a small cysteine-rich glycoprotein called MD-2 that gets bound to the ectodomain of TLR-4 in the golgi apparatus—where they are both stored for easy shuttling to and from the cell surface membrane. MD-2 has been shown to be capable of binding directly to LPS in a CD14-dependent manner to induce LPS signaling [195]. *In vitro* and *in vivo* studies using MD-2 ablated mice have shown that MD-2 is required for TLR-4-mediated LPS binding and signaling [194, 196]. Interestingly, MD-2 is also released in the serum by dendritic cells in a soluble form (sMD-2) which is also thought to bind to LPS and associate with cells that express TLR-4 but lack MD-2 [197]—but no additional studies have been done to investigate the relevance of this phenomenon. Endothelial cells, epithelial cells, vascular smooth muscle cells, fibroblasts and astrocytes are thought to express TLR-4 and MD-2 but are devoid of mCD14, suggesting that these cells only undergo LPS-induced activation through interaction with LPS bound to sCD14.

The TLR-4 receptor is a transmembrane protein that has leucine-rich repeats in the extracellular domain responsible for ligation with the assistance of associated proteins and a highly conserved Ig-like cytoplasmic toll/interleukin-1 receptor (TIR) domain responsible for downstream intercellular signaling [198]. Upon ligation with LPS the TLR-4/MD-2/CD-14 complex is thought to undergo dimerization with another ligated TLR-4/MD-2/CD-14 complex through lipid raft shuttling to form a homodimer that undergoes a conformation change that triggers the LPS-induced cell signaling transduction at the TIR domain [198]. The conformational activation results in the recruitment of the adaptor proteins MyD88 by PIP_2 and TIRAP, allowing MyD88 to binds to the TIR domain of TLR-4 receptor [198]. Once bound, IRAK-4, IRAK-1, and TRAF6 are recruited to the receptor where IRAK-1 binds to the death domain of MyD88 [198]. IRAK-4 then phosphorylates IRAK-1 where it complexes with TRAF6 and dissociates from the TLR-4 receptor resulting in associations with TAK1, TAB1, TAB2, Ubc13 and Uev1A, which together activate TAK1 to phosphorylate the IKK complex and MAP

kinases [198]. Once phosphorylated, the IKK complex, which is made up of IKKα, IKKβ, and NEMO/IKKγ, interacts with the inactive NFκB and IκBα complex to phosphorylate the IκBα inhibitor, which results in its ubiquitination and subsequent proteasomal degradation [198]. Upon IκBα degradation the nuclear receptor NFκB translocates to the nucleus and begins transcribing target genes until IκBα is re-synthesized to re-inhibit NFκB—acting as a negative-feedback loop [198].

Phosphorylation of the MAP kinases Jun N-terminal kinase (JNK), extracellular signal-regulated protein kinases 1 and 2 (ERK1/2) and P38 results in an NFκB-independent signaling pathway mediated by the transcription factor AP-1 [198]. For instance, JNK activation results in the phosphorylation of c-Jun and ATF2, which together are recruited assembly components of AP-1 that once formed translocates to nucleus and begins transcribing target genes. Together both NFκB and AP-1transcription factors are responsible for the transcription of all early released pro-inflammatory cytokines during LPS-induced TLR-4 signaling [198]. Interestingly, a MyD88-independent pathway has been identified to be responsible for late released pro-inflammatory cytokines that occur 90 minutes after LPS stimulation [198]. Several laboratories have identified that upon ligation with LPS, TLR-4 receptors are internalized to either the golgi apparatus or an early endosome within 60 minutes after stimulation [198]. In fact, TLR-4 is thought to be ubiquitinated and bound to the endosomal sorting protein Hrs transported into a clathrin-coated early endosome by Rab11a whereby Dynamin is thought to mediate the budding-in process of endocytosis [199]. The use of a Dynamin antagonist was shown to block the endocytosis of TLR-4 and showed enhanced My-D88-dependent signaling while completely abolishing MyD88-independent signaling [199]. These findings, along with the kinetic profile for the MyD88-dependent signaling pathway, which is thought to terminate well before the internalization of the TLR-4 receptors, suggest that the MyD88-indenpendent signaling pathway is dependent on TLR-4 internalization [199].

Briefly, the MyD88-independent pathway is thought to occur in response to MyD88-dependent signaling where TRAM is thought to bind to the TIR domain of the ligated TLR-4 receptor complex and recruit TRIF once internalized in the early endosome [198]. The activation of TRIF leads to association of TBK1 through TRAF3 and NAP1 mediating the phosphorylation and activation of the transcription factor IRF-3 [198]. Once phosphorylated IRF3 forms a homodimer and translocates to the nucleus, where it induces IFN-β and Cxcl10

genes expression [198]. The release of interferons, which is unique to signaling on the MyD88-independent pathway of TLR-4, can result in autocrine signaling that activates the STAT1 transcription factor for the induction of iNOS and NO and is also thought to promote the production of proinflammatory cytokines such as IL-1β and IL-6 [198]. Beyond having impaired interferon and IL-6 production, TRAM-deficient mice also expressed reduced, delayed NFκB signaling and TNF-α production after LPS stimulation—suggesting some cross talk between the My-D88-dependent and My-D88-independent pathways [200].

Numerous *in vivo* studies in rodents and humans have reported that microglia express the TLR-4 receptor—albeit with heterogeneity in the level of expression in a variety CNS compartments [201] and, in one case, with regards to the cellular compartment localization [202]. Furthermore, *in vitro* studies with enriched microglial cultures or microglioma cell lines have also been reported to express the TLR-4 receptor [203-212]. In contrast, the expression of the TLR-4 receptor in astrocytes has been much more controversial. Although several groups using both *in vitro* and *in vivo* approaches have published that TLR-4 is undetectable in astrocytes [205, 206, 213-216], a handful of studies have been able to detect constitutive low-levels of TLR-4 expression that increases following activation [202, 217-220]. In 2005, using *in situ* hybridization, Chakravarty and Herkenham demonstrated that TLR-4 mRNA was expressed in both microglia and astrocytes yet the surface expression of TLR-4 was not validated [207].

c.   **β2-integrin receptors**

The family of β2-integrin receptors is involved in cell-to-cell attachment by binding and adhering to extra-cellular matrix (ECM) components on the outer surface of cells. β2-integrin receptors are transmembrane glycoproteins that form obligate heterodimers between a conserved CD18 β-chain and variable CD11 α-chain. The variable CD11 subunits have allowed the β2-integrin receptors to be classified into three types of receptors: CD11a/CD18 (i.e., α1β2-integrin, LFA-1 or ITGAL); CD11b/CD18 (i.e., α2β2-integrin, CR3, MAC-1 or ITGAM); and CD11c/CD18 (i.e., α3β2-integrin, CR4, p150,95 or ITGAX). The CD18 subunit of all three receptors is known to bind to the phosphorylated glucosamines of the lipid A and O-antigen domain of LPS [221], suggesting that CD18 may not only play a role in detecting and sequestering endotoxin but may also bind to LPS aggregates that form micelles and to living Gram-negative bacteria. Interestingly, a high-dose LPS treatment in serum-free media (i.e. not containing LBP and thus preventing

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TLR-4 signaling) was shown to still act on CD11b/CD18, suggesting that LPS polymers in micelles are in fact capable of inducing signaling [222]. Furthermore, the role of the β2-integrin receptors to bind to living bacteria has been dually confirmed through the use of antibodies against CD18, which prevents monocyte surface binding of the Gram-negative bacterium *Escherichia coli* [223], and additionally in monocytes extracted from patients with leukocyte adhesion deficiency type I [224] syndrome (CD18-deficiency), which are unable attach to LPS-bound to erythrocytes or unopsonized Gram-negative bacteria [225]. As previously mentioned, the β2-integrin receptor CD11b/CD18 was the first described LPS receptor [223], but further investigation into the LPS response of monocytes obtained from patients with LADI syndrome showed that CD18 was not required for the generation of the cytokines TNF-α and IL-1β, leading to the discovery of CD14 and later TLR-4 [226].

The cellular attachment of immune cells to bacteria is thought to be a key component of phagocytosis and bacterial clearance. For this reason the β2-integrin receptors were hypothesized to be involved in phagocytosis of live bacteria and bacterial debris, yet unlike true phagocytic receptors expressed constitutively by professional phagocytes, phagocytosis through β2-integrin receptors either requires complement-opsonization of the target or extracellular stimulatory signals such as chemokines, cytokines and PAMPs to activate Rap1, a Ras-family small GTP-binding protein, that accumulates after polarization to activate the phagocytic properties of β2-integrin receptors in macrophages [227]. CD11b/CD18 is known to play an essential role in the uptake and catabolism of complement-opsonised bacteria. Yet upon examining the internalization of Alexa488-labeled LPS by microglia under confocal microscopy, our group found that pre-treatment with inhibitors of phagocytosis (cytochalasin D or fucoidan) showed no difference in the internalization of LPS in CD11b/CD18 deficient mouse cultures [222]—suggesting that CD11b/CD18 does not play a role in the phagocytosis of LPS. In contrast, when cells were treated with inhibitors of phagocytosis and broad scavenger receptor antagonist, internalization of LPS was prevented and clearly demonstrated that scavenger receptors and not β2-integrin receptors are involved in the phagocytosis of LPS [222].

Beyond just attaching to ECM components and LPS, β2-integrin receptors are also thought to stimulate signal transduction by phosphorylation of p38, JNK-1, JNK-2, ERK-1 and ERK-2 and inducing the translocation of NF-κB to the nucleus [228]. The existence of another PRR that was capable of inducing an

immune response and releasing an oxidative burst of superoxide in TLR-4 deficient mice when treated with high concentrations of LPS sparked the research that led to the discovery of β2-integrin receptor-mediated signaling. LPS-treated monocytes that were pre-treated with antibodies against CD18, CD11b or CD11c had partially attenuated activation [229, 230]—suggesting the involvement of β2-integrin receptors in mounting an innate immune response. Perera *et al.* (2001) noted that CD11b/CD18, CD14 and TLR-4 were all required to generate optimal induction of COX-2 and both IL-12 p70, whereas the induction of TNF-α (at low dose LPS) and ICSBP and IP-10 (at high dose LPS) required only CD14 and TLR4 and are CD11b/CD18-independent [228]. Our laboratory published that LPS-treated neuron-glia cultures from CD11b/CD18 deficient mice showed attenuated TNF-α production, diminished DAnergic neuronal loss and a complete absence of extracellular superoxide even at high doses of LPS (100 to 1000ng/ml) [222]. A later study confirmed this intercellular signaling pathway by measuring a CD11b/CD18-dependent and TLR-4-independent increased level of PI3K that in turn phosphorylates p47^{phox} and PIP$_3$ triggering their translocation from the cytosol to membrane-bound gp91^{phox} to generate superoxide [231]. Although the intercellular signaling capacity of β2-integrin receptors has been described, the signaling of LPS by the cytoplasmic domain of CD18 that leads to downstream phosphorylation of PI3K and the MAPK family have yet to be fully resolved. Although microglia are known to express all three β2-integrin receptors, they, like most macrophages, predominantly express CD11b/CD18, whereas astrocytes are thought to lack these receptors. In fact, the antibody OX-42 that shares an epitope in both CD11b and CD11c is commonly used as a selective cellular marker to identify microglia in brain tissue.

There are indications that the β2-integrin receptors can also associate with the TLR-4 receptor in the absence of CD14 to induce a significantly weaker LPS-mediated activation—a mechanism thought to be unfavorable due to activation kinetics [228, 232]. Transient associations between CD14 and CD11b/CD18 have also been reported in response to LPS stimulation in neutrophils [233]. Although these associations are speculative, the observation that NF-κB translocation and MAPK activation are depressed in both CD14- and CD11b/CD18-deficient mice and completely abolished in TLR-4-deficient mice implicate that all three PRRs are necessary to elicit an optimal and complete inflammatory response to LPS [228].

d.   Scavenger Receptors

Scavenger receptors are membrane-bound or soluble proteins that bind to modified low-density lipoproteins to induce endocytosis and phagocytosis. Six different classes of receptors have been assigned according to differences in their multi-domain structures. Class A scavenger receptors (SR-As) are constitutively expressed transmembrane glycoproteins with three extracellular C-terminal cysteine-rich domains expressed on the surface of phagocytic cells thought to be responsible for binding polyanionic ligands such as LPS [234]. Two functional alternatively spliced variants of SR-As have been identified that vary in structure but share the conserved cysteine-rich binding regions: SR-AI and SR-AII.

Lipid IVA, a precursor to Lipid A, and Lipid A can bind to SR-As resulting in their internalization without inducing cellular polarization or cytokine production [234]—thus SR-As are considered to be true phagocytic receptors similar to mannose and β glucan receptors. LPS binding by macrophages are significantly reduced in SR-A deficient mice compared to wild-type mice and are more susceptible to LPS-induced endotoxemia with enhanced pro-inflammatory cytokine secretion [235]—supporting the hypothesis that internalization of LPS by SR-As may function to protect the body by removing LPS that could otherwise stimulate an innate immune response. Microglia are known to express both SR-AI and SR-AII, whereas astrocytes lack these receptors [236]. As previously mentioned, the internalization of Alexa488-labeled LPS by microglia was shown to be exclusively dependent on SR-As and was further confirmed to only occur in microglia—when compared to neurons [206], astrocytes [205, 237] and oligodendrocytes [205].

MARCO is another SR-A transcribed from a different gene than SR-AI and SR-AII. MARCO contains a longer collagenous domain and completely lacks the α-helical coiled coil domain shared by SR-AI and SR-AII. Although MARCO still expresses the conserved cysteine-rich binding regions thought to be involved in LPS binding, studies suggest that MARCO cannot bind to LPS but rather to unopsonized Gram-negative bacteria such as *Escherichia coli* and *Neisseria meningitides*—suggesting the existence of another unidentified Gram-negative extracellular component involved in binding. LPS is known to induce MARCO transcription by downstream TLR-4 signaling, providing a reliable marker for macrophage polarization. In the CNS, MARCO is solely expressed on microglia and not astrocytes and commonly used as a measure of M2 polarization in

microglia—an activation profile that results in depressed pro-inflammatory factor release and increased phagocytosis.

A recent publication suggests that SR-A is required for proper LPS-mediated TLR4 signaling by macrophages [238]. In the study, macrophages were treated with fucoidan (an SR-A ligand) prior to LPS and the investigators found greater association between SR-A and TLR-4 (as evaluated with immunoprecipitation), enhanced phosphorylated IκBα, higher activities of NF-κB, and overall higher production of TNF-α and IL-1β [238]. These changes were not evident in LPS-treated macrophages derived from TLR-4 and SR-A-deficient macrophages when fucoidan was present [238]—suggesting that both TLR-4 and SR-A must be present for proper induction of NF-κB in LPS signaling. More work must be done to validate these findings with regards to its role in mediating a proper innate immune response to LPS.

3.   **The Controversy**

As described in the previous section, microglia are well equipped to detect and engulf LPS by constitutively expressing surface TLR-4, β2-integrin and scavenger receptors. In contrast, astrocytes may possibly express TLR-4, albeit at much lower expression levels than microglia and in an environment that normally does not contain sCD14 required for their signaling, and do not express either β2-integrin or scavenger receptors.   For this reason, the role of astrocytes as innate immune cells of the CNS, and not as *accessary cells that participate during a neuroimmune response*, has been a topic of debate in the field of neuroimmunology.   As described in Saura (2007), the controversy arises primarily from published studies that relied on stimulating primary enriched 'astrocyte' cell cultures known to contain contaminating microglial cells with LPS, confounding their findings and either solely or predominantly attributing the PRR-mediated immune responses to astrocytes [239].

a.   **Primary Astrocyte Cultures**

The greatest challenge for determining the innate immune functions of astrocytes has been culture-limited. Although *in vivo, ex vivo* (e.g. slice cultures), and mixed cell type *in vitro* studies produce more physiologically relevant data, it is also important to understand the independent roles of each cell type of the CNS. In order to accomplish this, astrocytes must be isolated and enriched to reduce confounding variables from other cell types. For this reason, primary astrocyte cultures are widely used to study the role of

astrocytes to innate immune stimulants. Moreover, primary astrocytes are relatively easy to culture since

they acclimate to a wide-range of *in vitro* conditions, transdifferentiation with high turnover to produce high

yields, and generate highly reproducible results.

Although several protocols exist to date, the fundamental methods and maintenance conditions have not

changed much since they were first established in 1972 [240]. Briefly, primary murine astrocyte cultures are

prepared by initially generating mixed glial cultures from either late stage embryos (embryonic day 19-23,

depending if derived from rat or mouse) or young neonates (postnatal day 0-3), as this is when radial glia

rapidly transdifferentiate into the various sub-populations of astrocytes (i.e. astrogenesis) that populate the

CNS [241]. Most protocols harvest glial cells from cortical tissue because it harbors high density of astrocytes

and relatively low density of microglia, thus resulting in more purified cultures after enrichment [78]. This

tissue is usually dissociated mechanically or by enzymatic digestion into a single cell suspension composed of

69% astrocytes and radial glia, 9% microglia and 9% oligodendrocyte precursor cells [242]. These cells are

plated and grown in more or less the same serum-containing growth medium at 37°C in a humidified

incubator with 5% CO_2 in air. Mixed glial cultures are generally grown until they become confluent ($\sim$ 7 to 14

days, depending on the initial seeding density), usually ending with a composition of 85% astrocytes and

radial glia, 9% microglia (and fibroblasts) and 4% differentiated oligodendrocytes and their precursors

(unpublished). Astrocyte enrichment is usually achieved shortly after single cell suspension or after

confluence using one of several techniques presented in **Table 1.2**.

Table 1.2 Established astrocyte enrichment protocols and their purities.

Astrocyte 'Enrichment' Method Details	Purity	Source
Shake-off method	90-96%	[243]
Shake-off method with 2-4 passages	95-98%	[244]
Shake-off method with AraC after confluence	90-98%	[245]
Shake-off method with LME	96-98%	[246]
Shake-off method with AraC and LME	>99%	[247]
Shake-off method with liposomal clodronate	>99%	[248]
Vigorous wash method	>99%	[249]
Discontinous percoll gradient method	98%	[250]
FAC Sorting	98%	[251]
MAC Sorting	95%	[242, 252]
Shake-off method with saporin	>99%	[253]
Neural stem cell differentiation	>99%	[254]
Genetic Microglial Ablation	~100%	[237]
Immunopanning method	>99%	[255]
Genetic Conditional Microglial Ablation	>99%	[220]

Although each method goes to relatively great lengths to minimize microglial contamination, no protocol to date is capable of generating pure astrocytes cultures. In part, one of the greatest design flaws of most protocols is that they do not initiate astrocyte enrichment methods until the astrocyte monolayer reaches confluence. One particular issue with waiting so long is that a small number of microglia become trapped under this monolayer (**Figure 1.5**) as astrocytes rapidly reach confluence, getting shielded from shaking and microglial-specific toxicants [239], and are eventually involved in rapidly repopulating surface microglia [254]. It is for this reason that attempting to evaluate the true immunological role of astrocytes is nearly always confounded and would require a completely microglia-free condition to adequately assess.
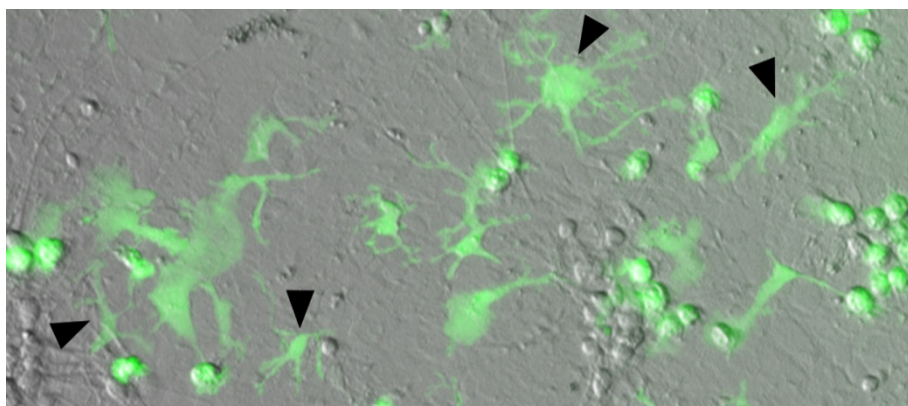
Figure 1.5 This image clearly shows that although the majority of microglia assemble on the surface of the astrocyte monolayer, the microglia highlighted with black arrows appear to be below this monolayer. These cells were visualized by using CX3CR1-EGFP$^{+/-}$ mice.

Another issue also arises in the variable subpopulations of astrocytes found in each culture. Cultured astrocytes are categorized as either type-1 astrocytes that form a tight monolayer at the bed or as type-2 astrocytes which grow on the top of the monolayer. The commonly used 'shake-off method' selectively generates a type-1 enriched astrocyte culture, which could unknowingly confound the results of a study. For these reasons, as well as the cost of live animals, many have attempted to circumvent this issue by using immortal astroglioma cell lines—which bring their own issues with their usage including ethical questions regarding their origins [256], differential quiescent and active profiles [257], and potential impurities due to mycoplamsic contaminations or mixtures with other cell lines [258].

b.   Detecting 'Cytotoxic' Pro-inflammatory Factors

To assess whether astrocytes respond to LPS, many studies have focused on assessing their ability to release inflammatory factors such as the cytokines and free radicals. These markers are not only chosen due

to their unequivocal expression in LPS-stimulated 'professional' innate immune cells, such as microglia, but also due to their cytotoxicity and assumed role in inflammation-mediated neurotoxicity. For this reason, I will focus on two heavily studied inflammatory factors thought to be released from 'enriched astrocytes' after LPS stimulation: the cytokine TNF-α and the free radical nitric oxide (•NO).

TNF-α is a pro-inflammatory cytokine that was originally described as a pyrogen (fever inducer) released during sepsis and could induce systemic apoptosis. Today, TNF-α is appreciated as a cytokine that can not only generate and maintain inflammation, but also regulate it. It is mainly produced by 'professional' innate immune cells, such as microglia, and to a lesser extent astrocytes, and is thought to be neurotoxic by acting on neuronal TNF receptor 1 to silence NF-κB cell survival signals and promote TRADD-FADD binding to recruit cysteine protease caspase-8, that at high concentration acts by autoproteolyticly cleaving itself into caspase-3 to signal cellular apoptosis [259]. Although this signal is relatively weak, compared to other pro-apoptotic signals such as Fas, the chronic low-grade production of TNF-α associated with chronic neuroinflammation may result in progressive neurodegeneration. New studies suggest that autocrine signaling of TNF-α by microglia may up-regulate glutaminase-stimulating microglia to release excessive glutamate, causing excitoneurotoxicity [260]. Interestingly, although it was shown that TNF-α is insufficient to account for all of the neurotoxicity during inflammation, its combination with pyrogallol, a superoxide generating compound, was sufficient [261]—suggesting that TNF-α is necessary but not sufficient to induce inflammation-mediated neurotoxicity.

Extensive search of the literature on the ability of primary 'enriched astrocytes' to release TNF-α after LPS stimulation was not conclusive (**Table 1.3**). Although there is no doubt that astrocytes possess the ability to generate and release TNF-α, the main question is whether astrocytes are capable of generating TNF-α upon LPS stimulation and not as a result of a secondary signal—such as paracrine signaling by TNF-α from microglia that can act on TNF-α receptors 1 and 2 located on astrocytes to induce their own TNF-α generation. Only two studies used *in situ* hybridization and verified that astrocytes either lack the capacity of directly synthesizing TNF-α mRNA upon LPS stimulation or only express TNF-α mRNA in a small subset of cells, which may potentially be a result of its proximity to a microglial contaminant.

Table 1.6 Studies investigating if astrocytes treated with LPS generate TNF-α.

Type of Culture	Known Microglial Presence	Detectable TNF-α after LPS	Astrocyte-Colocalization	Source
Primary Rat Cortical Astrocytes	+	+	NA	[262]
Primary Rat Cortical Astrocytes	(-)	+	NA	[263]
Primary Mouse Whole Brain Astrocytes	+	+	NA	[264]
Secondary Rat Cortical Astrocytes	(-)	+	NA	[265]
Primary Mouse Cortical Astrocytes	+	-	-	[266]
Secondary Rat Cortical Astrocytes	(-)	+	NA	[267]
Secondary Human Fetal Whole Brain Astrocytes	(-)	-	NA	[268]
Primary Rat Cortical Astrocytes	+	+	NA	[269]
Secondary Human Fetal and Adult Whole Brain Astrocytes	+	+	+/-	[270]
Primary Rat Cortical Astrocytes	+	+	NA	[217]
Primary Mouse Cortical Astrocyte	+	+	NA	[218]
Primary Mouse Cortical Astrocytes	NA	+	NA	[271]

(-): *although these papers claim to have no 'detectable microglia', this author is not aware of any protocol to date that generates pure astrocyte cultures completely absent of microglia.* NA: not applicable.

•NO is generated by activated macrophages, endothelial cells, and hepatocytes whereby it is rapidly transformed into various reactive nitrogen intermediates or reactive nitrogen/oxygen intermediates that are thought to have direct microbicidal/tumoricidal effects. •NO is also known to induce localized vasodilation, vascular damage and increase leukocyte adhesion and infiltration during inflammatory processes. Upon their activation, both microglia and astrocytes generate the soluble enzyme inducible nitric oxide synthase (iNOS) which catalyzes L-arginine to form •NO in the extracellular milieu. Once secreted, gaseous •NO is quickly oxidized primarily into nitrite (NO_2^-) at physiological pH, where it can remain stable for several hours—and thus can be measured using Greiss reagent as an accurate proxy for the generation of •NO. The co-administration of L-N(G)-nitroarginine, a selective inhibitor of NOS, was capable of rescuing DAnergic neurons from LPS *in vitro* and *in vivo*—implicated that •NO or its intermediates are responsible for inflammation-mediated neurodegeneration [272, 273]. Interestingly, our laboratory has published similar findings with regards to the generation of the reactive oxygen species superoxide ($O_2^{•-}$) by NAD(P)H oxidase (NOX). The co-administration of selective NOX inhibitors and the use of NOX2-deficient mice were also able to protect DAnergic neurons from LPS *in vitro* and *in vivo*—suggesting that although $O_2^{•-}$ and •NO may be themselves cytotoxic, their fused reactive nitrogen intermediate products may significantly contribute to inflammation-mediated neurodegeneration. Products such as peroxynitrite (ONOO−), nitrite(•NO_2), and

dinitrogen trioxide (N_2O_3) can peroxidize the lipid bilayer or pass through anion channels into a cell to peroxidize thiols, amino acid residues, DNA bases, and antioxidants, causing excessive cellular damage.

It is not surprising that the literature on the ability for astrocytes to synthesize iNOS or release NO_2^- after LPS stimulation was, similar to TNF-α, unclear for primary 'enriched astrocyte' cultures (**Table 4**). Among the studies that have examined this question, only a select few took the additional steps to validate that iNOS was co-localized on astrocytes using either double-immunofluorescent staining of iNOS and GFAP or *in situ* hybridization of iNOS mRNA on GFAP-positive immunocytochemistry stained astrocytes. Even fewer studies confirmed that the iNOS was due to contaminating microglial cells by labeling microglial markers. To further emphasize this point, we discovered that even our own laboratory misinterpreted its findings in a 1996 publication with regards to this very issue [274]. The publication acknowledges that LPS-treated 'enriched astrocyte' cultures rarely double-stained for iNOS and GFAP during immunocytochemistry, but our group did not take the additional step to determine if the iNOS-positive, GFAP-negative cells were in fact microglia— which can, with near certainty, be identified as microglia strictly from their unmistakable morphology [274]. In 2010, our group published a paper entitled *Astrogliosis in CNS pathologies: is there a role for Microglia?* where we speculated that the activation of astrocytes may be initiated and sustained by microglial activation—implicating that astrocytes may not undergo activation by direct stimulation to their innate immune receptor, but instead serve as accessory cells that participate during a neuroimmune response.

Table 1.3 Studies investigating if astrocytes treated with LPS generate NO2- or iNOS.

Type of Culture	Known Microglial Presence	Detectable NO_2^-/iNOS after LPS	Astrocyte-Colocalization	Source
Primary Rat Cortical Astrocyte	+	-	NA	[275]
Primary Rat Cortical Astrocyte	NA	+	NA	[276]
Primary Rat Cortical Astrocyte	+	+	NA	[277]
Primary Rat Cortical Astrocyte	+	+	+	[278]
Primary Whole Brain Astrocyte	+	+	+/-	[274]
Primary Rat Cortical Mixed Glia	+	-	-	[279]
Primary Rat Cortical Astrocyte	(-)	-	NA	[279]
Primary Rat Cortical Mixed Glia	+	-	-	[280]
Primary Rat Spinal Cord Astrocytes	(-)	+	+	[281]
Primary Mouse Cortical Mixed Glia	+	-	-	[216]
Primary Mouse Cortical Astrocyte	+	+	NA	[218]
Primary Mouse Cortical Astrocyte	+	+	-	[282]
Primary Mouse Cortical Mixed Glia	+	-	-	[239]
Primary Rat Cortical Astrocyte	+	+	NA	[271]

(-): *although these papers claim to have no 'detectable microglia', this author is not aware of any protocol to date that generates pure astrocyte cultures completely absent of microglia.* NA: not applicable.

c.   Defining the True Role of Astrocytes in Neuroinflammation

The true role of reactive astrogliosis during chronic neuroinflammation remains a mystery. Many still believe that astrocytes, along with microglia, play a predominantly harmful role during chronic neuroinflammation, due to their ability to generate and release inflammatory factors that are themselves either neurotoxic or partake in a dysfunctional modulation of vascular tone, leading to neurovascular uncoupling by restricting blood flow and influx of required metabolites [283]. Additionally, LPS-activated astrocytes show inhibited abilities to uptake excess glutamate and, therefore, may indirectly perpetuate glutamate neuroexcitotoxicity.

In contrast, many have argued that astrocytes serve a protective role during inflammation. The first line of evidence supporting this theory came in 1999, when a group developed a conditional astrocyte ablation model that could selectively destroy activated astrocytes [164]. These mice endured a forebrain stab injury and were shown to have delayed wound healing, much greater and more persistent leukocyte infiltration, sustained leaky BBB, and neuronal degeneration from neuroexcitotoxicity (attenuated by glutamate receptor inhibition) [164]. Additional studies further supported these findings, suggesting that activated astrcoytes actively assist in protecting the CNS during injury. In addition, it is known that LPS-stimulated astrocytes are capable of generating a variety of neurotrophins that can help mitigate their neurotoxicity and promote neuroprotection.

Neurotrophins are a family of secreted growth factors that regulate processes such as neuronal survival and differentiation; neurite outgrowth; synaptic formation and plasticity; and are acute modulators of neuronal function through receptor-mediated intercellular signaling [284]. In the healthy CNS, neurotrophins are predominantly produced by neurons (both intracellularly and secreted) and to a lesser extent by glial cells. This role is thought to shift during pathological events, whereby neurons suppress their neurotrophin production and astrocytes aid their survival by secreting neurotrophins. In midbrain, astrocytes are thought to generate fibroblast growth factor-2 (FGF-2), cerebral dopamine neurotrophic factor (CDNF), brain-derived neurotrophic factor (BDNF), glial cell line-derived neurotrophic factor (GDNF), nerve growth factor (NGF), and neurotrophin-3 (NT-3) through their activation, mediated through monoaminergic neurotransmitter and cytokine receptors.

Among these factors, GDNF and BDNF stand out as the factors known to induce the most significant

neuroprotection in the nigrostriatal system—even at miniscule doses [285]. Upon secretion, GDNF and BDNF

bind to either GFRα1 or TrkB and p75, respectively—receptors expressed on neurons and glial cells.

Although the neuroprotective effects of GDNF and BDNF are in part associated with their direct interaction

with neurons, recent findings also show that they may indirectly protect neurons by modulating microglial

activation and suppressing neuroinflammation [286]. For instance, the administration of exogenous GDNF to

midbrain organotypic culture inhibited LPS-induced microglial activation and •NO production [287].

Furthermore, our laboratory has shown that some widely prescribed anticonvulsant and histone deacetylase

inhibitor drugs have 'off-label' functions that can protect dopaminergic neurons during LPS-induce

neuroinflammation by increasing the expression of both GDNF and BDNF in astrocytes and reducing

microglial activation [288]. Together these studies suggest that upon activation, astrocytes most likely play a

far more important role regulating inflammation and reducing neuronal injury than partaking in their

collateral damage.

RESEARCH AIMS

The overall goal of this research was to determine if astrocytes can independently generate an innate immune response to LPS stimulation. An investigation of LPS exposure on highly-enriched astrocyte cultures greatly reduces the confounding factors associated with microglial contamination, allowing for more accurate discrimination of the attributable roles of astrocytes in the innate immune response. My thesis research was to investigate the effects of contaminating microglia in primary astrocyte cultures on the hypothesis that ***astrocytes require the presence of microglia to produce an innate immune response to LPS and that, upon activation, generate a response that is more beneficial than hurtful***.

This hypothesis was addressed through the following research aims:

Aim I   Investigate the innate immune response to LPS on highly enriched astrocyte cultures by measuring their capacity to release TNF-α and NO_2^- or synthesize iNOS

Aim II   Investigate the interactive role of contaminating microglia in the response of astrocytes to generate the aforementioned factors in response to LPS stimulation

Aim III Investigate the neurotrophic response of astrocytes in response to LPS and their potential interactive roles with microglia

CHAPTER 2: RESULTS AND DISCUSSION

Note: This Chapter was prepared in manuscript format and submitted and accepted as a peer-reviewed publication in the journal <u>Glia</u>.

Deciphering the Roles of Astrocytes and Microglia in LPS-mediated Neuroinflammation

Overview

Contamination of microglia in primary enriched astroglial cultures is usually discounted, but accumulating evidence suggests that even a small percent of microglia are sufficient to misinterpret the results of a study. By developing a modified highly-enriched astroglial culture protocol, we demonstrate that LPS failed to induce the synthesis and release of TNF-α and iNOS/NO in astroglia. Reconstituting microglia back into highly-enriched astroglial cultures show that as few as 0.5% and 1% microglial contamination can produce significantly detectable expression of TNF-α and iNOS, respectively, upon stimulation with LPS. Surprisingly, microglial-astroglial interactions are required for LPS to induce the expression of proinflammatory and neurotrophic factors from astroglia. Specifically, we found that microglia-derived soluble factors, particularly TNF-α, play a pivotal role as a paracrine signal to regulate the release of GDNF by astroglia. Although astroglia are capable of releasing both cytotoxic and neurotrophic factors, our findings show that astroglia tend to serve a neuroprotective role during neuroinflammation. Taken together, these findings suggest that astroglia may not possess the ability to directly recognize innate immune stimuli, but rather depend on glial cross-talk to elicit their role as a counterbalance over microglial activation and support neuronal survival for a complete immune response. These results caution that even a minor contamination of microglia may account for the detectable proinflammatory factors in astroglial cultures.

Introduction

In the central nervous system (CNS), the detection and subsequent inflammatory response to perturbations from cellular stress, damage or death, abnormal protein aggregates, and xenobiotic infiltration are thought to be initiated by both microglia and astroglia [132, 159, 289-293]. Although microglia and astroglia are commonly described as the resident innate immune cells of the CNS, only microglia are considered "professional" immune cells due to their myeloid lineage. The evidence that astroglia play a key role during neuroinflammation, adaptive immune cell recruitment, and tissue repair is inarguable; however, recent findings question whether astroglia are cable of independently detecting a variety of inflammogens to stimulate their activation [220].

The controversy, as addressed in a review by Saura, stems from numerous publications reporting an inflammatory response in primary enriched astroglia cultures—contaminated with anywhere from 0.5% to 10% microglial cells—using the archetypical bacterial inflammogen lipopolysaccharide (LPS) [239, 276, 294, 295]. The working theory is that a few microglia are capable of generating a detectable inflammatory response that serve as secondary messengers to mediate the activation of astroglia, which, if left unaddressed, can confound the conclusions of a study. This paracrine signaling and, to a lesser extent, the cell-to-cell contact between microglia and astroglia may play an important role not only in reciprocal activation state regulation but in mediating a proper inflammatory response to a wide-variety of insults [294, 296, 297]. Although still controversial, mounting evidence now suggests that pro-inflammatory factors, such as TNF-α and nitric oxide radical (NO), once thought to be released by astroglia upon stimulation with LPS, are now thought to be predominantly attributed to microglial contamination and may serve as the aforementioned paracrine signals that activate astroglia [294, 298]. This misattribution challenges a potential misconception that reactive astrogliosis generates high levels of cytotoxic factors, resulting in their deleterious role towards neurons during chronic neuroinflammation rather than a beneficial one [293].

The main purpose of this study was to accurately discriminate the attributable roles of astroglia in the innate immune response to LPS and to further understand the role of astrogliosis in neuroinflammation. To reduce the confounding factors associated with microglial contamination, we developed a highly-enriched astroglial culture protocol containing less than 0.005% microglial contamination. Here, we report that LPS

failed to induce the expression of TNF-α and nitrite (NO_2^-) in highly-enriched astroglial cultures, but instead required soluble factors from activated microglia to generate a weak pro-inflammatory response. In assessing the role of microglia-mediated astrogliosis, we found that the neuroprotective effect of glial cell line-derived neurotrophic factor (GDNF) released by astroglia outweigh their capacity to release cytotoxic factors, implicating a beneficial role for astrogliosis. Most importantly, we observed that the release of GDNF from astroglia was dependent on the paracrine signaling of TNF-α released by activated microglia suggesting the importance of glial cross-talk during LPS-induced neuroinflammation.

Materials and Methods

Animals: Timed-pregnant (gestational day 14) female Fisher 344 rats were purchased from Charles River Laboratories (Raleigh, NC, USA). Timed-pregnant C57BL/6J and B6.129S-Tnftm1Gkl/J (TNF-α deficient) mice were bred by the animal husbandry staff at NIEHS using breeders obtained from Jackson Laboratories (Ben Harbor, ME, USA). TNF R1/R2 knockout mice were kindly gifted from Dr. Perry Blackshear at the National Institute of Environmental Health Sciences. Rat and mouse dams were housed in polycarbonate cages in animal facilities with controlled environment conditions with a 12 h artificial light-dark cycle and provided fresh deionized water and NIH 31 chow ad libitum. All animal procedures were approved by the Institutional Animal Care and Use Committee and conducted in strict accordance with the National Institutes of Health animal care and use guidelines.

Reagents: Poly-D-lysine, cytosine β-D-arabinofuranoside (Ara-c), L-leucine methyl ester (LME) and 3,3´-diaminobenzidine and urea-hydrogen peroxide tablets were purchased from Sigma-Aldrich (St. Louis, MO, USA). Lipopolysaccharide (LPS; *E. coli* strain O111:B4) was purchased from Calbiochem (San Diego, CA, USA). Cell culture ingredients were obtained from Life Technologies (Grand Island, NY, USA). Suberoylanilide hydroxamic acid was purchased from Cayman Chemical (Ann Arbor, MI, USA). Anti-GFAP and antibody diluent were purchased from DAKO (Carpinteria, CA, USA). Anti-Iba1 antibody was purchased from Wako Pure Chemicals (Richmond, VA, USA). Anti-iNOS antibody was purchased from Abcam (Cambridge, MA, USA). TNF-α ELISA kit and Anti-GDNF and isotype control antibodies were purchased from R&D Systems (Minneapolis, MN, USA). Alexa Fluor 488 goat anti-rabbit IgG and Alexa Fluor 594 goat anti-rabbit IgG were

purchase from Invitrogen (Carlsbad, CA, USA). Goat anti-rabbit biotinylated secondary antibody was purchased from Vector Laboratory (Burlingame, CA, USA).

Primary cortical mixed glia and highly-enriched astroglial cultures: Primary cortical mixed glia (M/G) cultures were prepared from rat pup brains at postnatal day 1-3, as previously described [299]. Briefly, the cortices were isolated, the meninges and blood vessels removed, the tissue gently dissociated through trituration and the single cell suspension plated on either 6-well plates or 24-well plates pre-coated in poly-D-lysine (20 µg/ml) at 1×10^6 cells/well or 1×10^5 cells/well, respectively. Cells were maintained in DMEM-F12 (1:1) media supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 1 mM sodium pyruvate, 100 µM non-essential amino acids, 50 U/ml penicillin, and 50 µg/ml streptomycin. Media was refreshed every 3 days until they were experimentally treated 7 days after seeding. Highly-enriched primary cortical astroglial cultures, were prepared using a modified protocol [300]. Highly-enriched astroglial cultures were derived from M/G cultures by supplementing 1 mM of L-leucine methyl ester (LME) to the DMEM-F12 media 72 hours after seeding. Highly-enriched astroglial cultures were experimentally treated at 5-7 days after LME treatment. Immunocytochemistry revealed M/G cultures contained ~15% microglia (Iba-1-immunoreactive cells), whereas highly-enriched astroglia cultures contained less than 0.005% microglia.

Microglia-enriched cultures: Microglia-enriched cultures were prepared from primary M/G cultures as previously described [299]. Briefly, M/G cultures were plated on 150 cm³ flasks pre-coated in poly-D-lysine (20 µg/ml) at 5×10^7 cells/flask. Microglia-enriched cultures were maintained in DMEM-F12 media changed every three days for two weeks. At two weeks, microglia were shaken off with an Incubator Orbital Shaker at 180 rpm for 40 minutes and re-plated either on 24-well plates pre-coated in poly-D-lysine (20 µg/ml) at 5×10^5 cells/well for the collection of microglia conditioned media or added to highly-enriched astroglia cultures for reconstitution studies. Immunocytochemistry revealed microglia-enriched cultures contained less than 1% contamination of astroglia (GFAP-immunoreactive cells).

Neuron-glia and reconstituted neuron-microglia cultures: Rat mesencephalic neuron-glia cultures were prepared following the protocol as described previously [299]. Briefly, midbrain tissues were dissected from day 14 embryos, and then gently triturated into single cell suspension. Cells were then seeded (5×10^5

cells/well) in poly D-lysine (20 µg/ml) (Sigma-Aldrich, St. Louis, MO) precoated 24-well plates. Forty-eight hours post-seeding, 8-10 µM of cytosine β-D-arabinofuranoside was added to cultures. After 3 days, media containing Ara-c were removed and replaced with fresh media. The neuron-enriched cultures were > 98% pure. Microglia cells were prepared from the brains of 1-day-old rat pups using the protocol described previously [299]. Briefly, meninges and blood vessels were removed, and brain tissues were triturated and seeded (5×10^7 cells) in 150 cm^3 flasks. Media were changed every three days for two weeks. After two weeks, microglia cells were shaken off from the confluent cell monolayer and plated on top of the neuron-enriched culture described above (80% neurons and 20% microglia). The neuron-microglia cultures were treated 24 hours after microglia were added.

Cell-line cultures: Mycoplasma-free rat C6 astrocytoma cells were maintained in DMEM supplemented with 10% FBS, 50 U/ml penicillin, and 50 µg/ml streptomycin. Cells were split on T75 flasks and used for experiments at passages 3–5, where they were seeded on 24-well plates at $1x10^5$ cells/well and treated 24 hours later.

Immunofluorescence and immunocytochemical staining: For immunofluorescence, M/G and highly-enriched astroglia cultures were fixed with 3.7% formaldehyde in phosphate-buffered saline (PBS) for 20 minutes and incubated for 20 minutes with blocking solution (BSA 1%/Triton X-100 0.4%/Normal Goat Serum 4% in PBS). Cells were immunostained overnight at 4°C with either rabbit polyclonal antibody against Iba-1 (1:750) or with rabbit polyclonal antibody against GFAP (1:1000) diluted in Antibody Diluent. Cells were washed for 10 minutes in PBS (three times) and incubated overnight at 4°C with mouse monoclonal antibody against iNOS (1: 250). Cells were washed for 10 minutes in PBS (three times) and incubated for 2 hours with the secondary antibodies Alexa Fluor 488 goat anti-rabbit IgG (1:750), Alexa Fluor 594 goat anti-rabbit IgG (1:750) or Alexa Fluor 594 goat anti-mouse IgG (1:750). Cells were washed for 10 minutes in PBS (three times) and visualized under microscope (Nikon, model ECLIPSE TE3000, Garden City, NY, USA) connected to AxioCam MRm camera operated with AxioVision software (Carl Zeiss, Thornwood, NY, USA).

For microglial quantification using immunohistochemistry, fixed cultures were treated with 1 % hydrogen peroxide for 10 minutes, immunostained overnight with Iba-1 antibody and incubated for 1 hour with PBS containing 0.3% Triton X-100 and a biotinylated secondary antibody (goat anti-rabbit antibody, 1:227; Vector Laboratory, Burlingame, CA). After washing (three times) with PBS, the cultures were incubated for 1 hour with the Vectastain ABC reagents (Vector Laboratory, Burlingame, CA) diluted in PBS containing 0.3% Triton X-100. To visualize the signal, the cultures were incubated with 3,3´-diaminobenzidine and urea-hydrogen peroxide tablets dissolved in water. Iba-1 positive cells were manually counted under a microscope (Nikon, model DIAPHOT, Garden City, NY, USA) by at least two investigators and the results were averaged.

RNA analysis: Total RNA was extracted from cultures with RNeasy Minikit (Qiagen, Valencia, CA) and reverse transcribed with an oligo dT primer. Real-time PCR amplification was performed using SYBR Green PCR Master Mix and an ABI 7900 HT Sequence Detection System according to manufacturer's protocols (Applied Biosystems, Foster City, CA, USA). The primers were designed using Vector NTI software (v.11, Invitrogen, Carlsbad, CA, USA) and validated for efficacy through melting curve analyses. **Table 2.1** contains the primer sequences used to amplify the mRNA of each target genes.

Table 2.1 Target genes and their respective mRNA primer sequences.

Primer	GeneBank ID	Sequence (5'→3')
Rat Iba-1	NM_017196	TTT CTG AGT TGC CCT AAT TGG AG (forward)
		GCT GTC ATT AGA AGG TCC TCG G (reverse)
Rat iNOS	NM_012611	GAG TGA GGA GCA GGT TGA GGA TTA C (forward)
		AGG AAA AGA CCG CAC CGA AG (reverse)
Rat TNFα	NM_012675	CCA GAC CCT CAC ACT CAG ATC ATC (forward)
		CCT CCG CTT GGT GGT TTG CT (reverse)
Rat GDNF	NM_019139	CAG AGG GAA AGG TCG CAG AGG (forward)
		TAG CCC AAA CCC AAG TCA GTG (reverse)
Rat BDNF	NM_012513	CGA TGC CAG TTG CTT TGT CTT C (forward)
		AAG TTC GGC TTT GCT CAG TGG (reverse)
Mouse GDNF	NM_010275	GGC TGA CCT TGA ACT TAC TGC TTG (forward)
		CCT GTG GAT ACG GTG TGA TTG AT (reverse)
Mouse/Rat GAPDH (internal control)	NM_008084 NM_017008	TTC AAC GGC ACA GTC AAG GC (forward) GAC TCC ACG ACA TAC TCA GCA CC (reverse)

Amplification conditions were 95°C for 10 seconds, 55°C for 30 seconds, and 72°C for 30 seconds for 40 cycles. All samples were tested in triplicate from at least three independent experiments and normalized with GAPDH using the $2^{-\Delta\Delta Ct}$ method.

Measuring the release of Nitrite and TNF-α: The release of •NO was assessed by measuring the accumulated levels of nitrite (NO_2^-) using 50 μl/well of culture supernatants through an assay performed with the addition of Griess reagent, as described previously [301]. The release of TNF-α was measured from culture supernatant with a commercial enzyme-linked immunosorbent assay (ELISA) kit from R&D Systems, in accordance to the manufacturer's instructions. Culture supernatant was collected at 3 and 24 hours after treatment for TNF-α and nitrite assays, respectively. Both colorimetric assays were quantified using a SPECTAmax PLUS 384 spectrophotometer (Molcular Devices, Sunnyvale, CA, USA).

Functional Assessment of DA Neurons using [³H]-DA Uptake Assay: The ability of dopaminergic neurons to uptake dopamine has served as a good indicator of the health and viability of these neurons—correlating nicely with immunocytochemical counts of TH-immonoreactive neurons. Briefly, this functional assessment (detailed in [301]) measures the rate of uptake of radiolabeled dopamine for 21 minutes at 37°C. Nonspecific dopamine uptake was also observed by competitively inhibiting dopamine receptor uptake with mazindol. Cells were washed and lysed to release internalized radiolabeled dopamine that was quantified with a liquid scintillation counter (Tri-Carb 4000; Packard, Meriden, CT). Specific dopamine uptake by dopaminergic neurons was calculated by subtracting the amount of radioactivity observed in the presence of mazindol from that observed in the absence of mazindol.

Statistics: Data are presented as the mean ± SEM. Comparison of more than two groups were performed using one-way ANOVA followed by Bonferroni's post-hoc multiple comparison test. Comparisons of more than two parameters were performed by two-way ANOVA analysis followed by Bonferroni's post-hoc multiple comparison test. Data were analyzed using Prism (v6.00, GraphPad, San Diego, CA). P-values less than or equal to 0.05 were considered statistically significant.

Results

Highly-enriched astroglia cultures were prepared with L-Leu methyl ester (LME)

Traditional methods for enriching astroglia have attempted to remove contaminating microglia through prolonged orbital shaking coupled with multiple generations of passaging or the use of antimitotic drugs and/or selective microglial toxicants resulting in cultures with 1-5% microglial contaminations [300, 302, 303]. Since as little as 1% of contaminating microglia have been shown to confound the results of a study [304], our group developed highly-enriched astroglial cultures by initiating LME treatment (0.6-1 mM) three days after seeding—prior to the formation of a confluent monolayer in M/G cultures. Upon reaching confluence, cultures were examined for contaminating microglia. Immunofluorescent staining for the astroglial marker GFAP and microglial marker Iba-1 showed a significant reduction in Iba-1-immunoreactive cells with increasing concentrations of LME and the morphology of astroglia was not altered after LME treatment (**Figure 2.1A**). Contaminating microglia were more sensitively quantified by examining Iba-1 mRNA levels of M/G cells treated with 1 mM of LME, which only expressed 0.36 ± 0.1 % of the transcripts expressed by M/G cultures ($P < 0.001$; **Figure 2.1B**). Moreover, using immunocytochemical staining, Iba-1-immunoreactive cells were directly counted as a percent of the total cells, whereby M/G and M/G with 0.6, 0.8, and 1 mM of LME cultures have 13.46 ± 0.98, 2.69 ± 0.58, 0.08 ± 0.01, and 0.004 ± 0.001% microglia, respectively. These results indicated that M/G cultures treated with 1 mM of LME were nearly entirely absent of contaminating microglia and did not significantly recover their microglial populations until 7 days after LME was removed (**Figure 2.1C**).
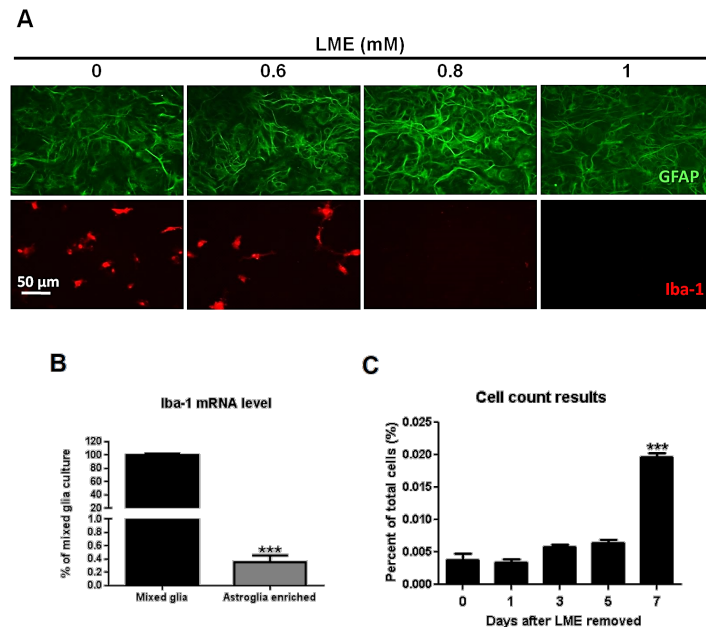
Figure 2.1 Assessment of contaminating microglia in highly-enriched astroglial cultures. (A) Three days after seeding, primary rat M/G cultures were treated with various concentrations of LME (0.6 to 1 mM) for 3-4 days. Cultures underwent immunofluorescence staining using antibodies against GFAP (green, for astroglia) and Iba-1(red, for microglia). Scale bar: 50 µm. (B) Real-time PCR assay for Iba-1 mRNA in M/G and M/G with 1 mM LME. (C) LME (initially at 1 mM) was removed from highly-enriched astroglial cultures and counted several days after removal to assess the repopulation of contaminating microglia using immunocytochemical staining for Iba-1-immunoreactive cells. Values represent mean ± SEM from three independent experiments, with duplicates in each experiment. *** $P < 0.0001$, Bonferroni's t-test compared to Day 0.

Highly-enriched astroglial cultures maintained functional integrity in response to suberoylanilide hydroxamic acid (SAHA)

To insure that treatment with 1 mM LME did not functionally damage astroglia, we assessed their ability to produce neurotrophic factors. Previously we reported that histone deacetylase (HDAC) inhibitors can up-regulate the expression of neurotrophic factors in astroglia [305-307]. Therefore, highly-enriched astroglial cultures were treated with an HDAC inhibitor, suberoylanilide hydroxamic acid (SAHA) [308], and the mRNA expressions of glial cell line-derived neurotrophic factor (GDNF) and brain-derived neurotrophic factor (BDNF) were measured. The basal mRNA expression levels in highly-enriched astroglial cultures showed no difference when compared to their M/G culture counterparts. Treatment with SAHA increased the expression of GDNF mRNA to 150 and 200% and BDNF mRNA to 480 and 680%, in M/G and highly-enriched

astroglial cultures, respectively, when compared to basal M/G expression (**Figure 2.2**). These results

indicated that astroglia treated with 1 mM LME retained their physiological function.
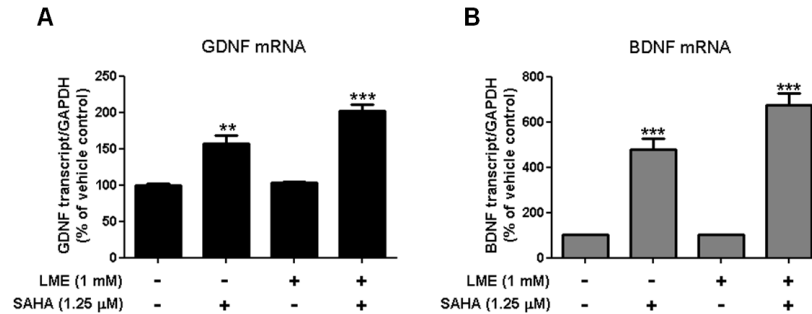
Figure 2.2 LME-treated astroglia express similar basal expression levels of neurotrophic factors and retain the ability to increase this expression upon treatment with the HDAC inhibitor SAHA. M/G and highly-enriched astroglial cultures were treated with 1.25 μM of SAHA for 12 hours and assessed for changes in mRNA expression levels of (A) GDNF and (B) BDNF. Values were present as mean ± SEM from three independent experiments, with duplicates. ** P <0.01, *** P < 0.0001, Bonferroni's *t*-test compared to basal expression levels of vehicle-treated M/G cultures.

LPS failed to elicit the release of NO and TNF-α from highly-enriched astroglial and C6 cultures

M/G and highly-enriched astroglial cultures stimulated with 1, 10, 100 and 1,000 ng/ml of LPS

induced a significant release of NO_2^- and TNF-α in M/G cultures but not highly-enriched astroglial cultures. To

corroborate these findings, pure rat astroglioma (C6) cell lines were treated with LPS and, like highly-

enriched astroglial cultures, failed to induce a significant release of NO_2^- and TNF-α. NO_2^- and TNF-α were also

measured in LPS-treated enriched astroglial cultures derived from the traditional shake-off method [302],

whereby 4% Iba-1-immunoreactive cells generated significantly higher levels of TNF-α and NO_2^- (data shown

only for 100 ng/ml of LPS in **Figures 2.3A and 2.3B**).

Highly-enriched astroglial cultures generated extremely low levels of NO_2^- and TNF-α, thus due to the

detection limitation of their assays of ~0.5 μM and 30 pg/ml, respectively, we also performed qRT-PCR to

detect TNF-α and iNOS mRNA at various time points after LPS stimulation in M/G, enriched microglia and

highly-enriched astroglial cultures. Messenger RNA levels for TNF-α were significantly increased in both M/G

and enriched microglia cultures after LPS treatment (P < 0.0001). The *post hoc* test revealed that there are

significant differences of TNF-α level in M/G and enriched microglia cultures compared to highly-enriched

astroglial cultures at 3 and 6 hours after LPS treatment (M/G culture: 3h, P<0.0001; 6h, P<0.001; enriched

microglia culture: 3h, *P*<0.001; 6h, *P*<0.05; **Figure 2.3C**). A great disparity was detected in iNOS expression of

LPS-stimulated enriched microglia and M/G cultures compared to highly enriched astroglial cultures (*P* <

0.0001). The *post hoc* test revealed that there are significant differences of iNOS level in M/G and enriched

microglia cultures compared to highly-enriched astroglial cultures at 3 and 6 hours after LPS treatment (M/G

culture: 3h, *P*<0.001; 6h, *P*<0.001; enriched microglia culture: 3h, *P*<0.001; 6h, *P*<0.001; **Figure 2.3D**). Taken

together, these results indicated that LPS cannot induce detectable levels of NO_2^- and TNF-α from highly-

enriched astroglial cultures.
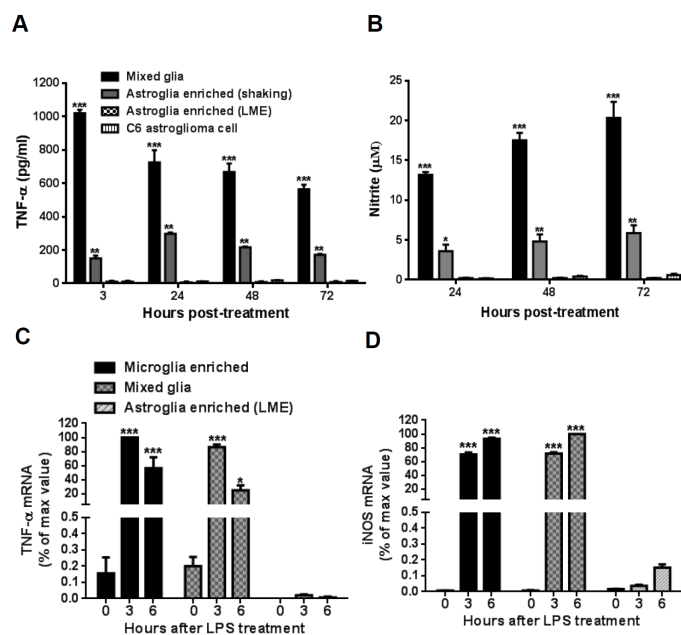
Figure 2.3 LPS treatment did not elicit the release of TNF-α or NO_2^- from highly-enriched astroglial and C6 astroglioma cultures. M/G, enriched astroglia (shake-off method), highly-enriched astroglia (LME-treated method) and C6 astroglioma cell cultures were treated with 100 ng/ml of LPS and the supernatant from each was examined for NO_2^- (A) and TNF-α (B) at different time points after treatment. M/G, enriched microglia and highly-enriched astroglial cultures were treated with 100 ng/ml of LPS for 0, 3 and 6 hours and pelleted for mRNA analysis. The levels of TNF- α and iNOS mRNA in M/G and highly-enriched astroglial cultures were normalized to the percentage of microglia in their respective cultures. Expression of TNF-α (C) and iNOS (D) mRNAs were determined for each culture as a percent of the maximum value of microglia enriched culture. Data show mean ± SEM from three independent experiments, each with triplicates. * *P* < 0.05, ** *P* < 0.01, *** *P* < 0.0001, Bonferroni's *t*-test compared to highly-enriched astroglial cultures.

Microglia were the major source of Nitrite and TNF-α in 'enriched astroglial' cultures

The degree of microglial contamination varies greatly among laboratories that perform primary

astroglial cultures (see supplemental attachment to [239]). Although most studies appropriately caution the

potential confounding role of residual microglia in their cultures, only one study to date reported that as little

as 1% contaminating microglia was sufficient to affect the results of their most sensitive assay (qPCR) [304]. However, the number of microglia required to confound the results of a study are still unknown. To address this issue, we reconstituted highly-enriched astroglial cultures with various amounts of enriched microglia ranging from 0.5 to 20% of the total cells of the cultures. We found that even when contaminating microglia accounted for approximately 0.5% of the total cells, they produced significantly detectable levels of TNF-α ($P < 0.0001$) as compared to highly-enriched astroglial cultures 3 hours after LPS stimulation (**Figure 2.4A**). NO_2^- production 24 hours after LPS treatment was significantly increased in cultures comprised of approximately 1% contaminating microglia as compared to highly-enriched astroglial cultures ($P < 0.0001$; **Figure 2.4B**). TNF-α and NO_2^- levels continued to increase with respect to the presence of microglia in a cell number-dependent manner (TNF-α, $P < 0.0001$; NO_2^-, $P < 0.0001$; **Figure 2.4**). These results demonstrate that even a miniscule number of microglia in highly-enriched astroglial cultures can significantly alter the outcome of a study in according to the sensitivity of the assay.
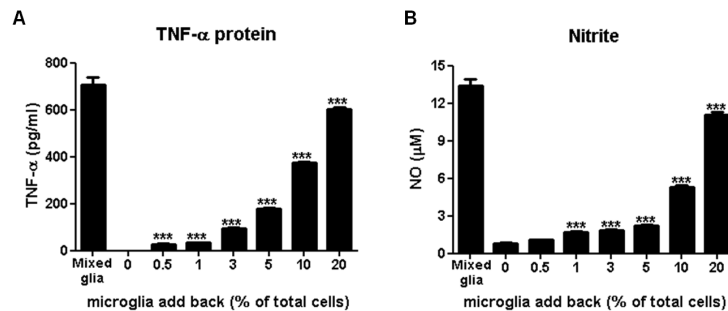
Figure 2.4 The major source of LPS-elicited TNF-α and nitrite in 'enriched astroglial' cultures is attributed to contaminating microglia. Different amounts of microglia ranging from 0.5 to 20% were seeded atop highly-enriched astroglial cultures and treated with 100 ng/ml of LPS 24 hours after being reconstituted with microglia. TNF-α (A) and NO_2^- (B) were determined from supernatant collected at 3 and 24 hours after LPS treatment, respectively. Data were represented as mean ± SEM from three independent experiments, each done in triplicate. *** $P < 0.0001$, one-way ANOVA followed by Bonferroni's *post hoc* multiple comparison test.

Microglia required for astroglial production of proinflammatory factors upon LPS stimulation

Although our data shown above demonstrated that LPS failed to induce production of proinflammatory factors in highly-enriched astroglia cultures, we still cannot exclude the possibility that cell-to-cell interactions between microglia and astroglia may trigger pro-inflammatory gene expressions in astroglia. We used immunofluorescence staining to examine the iNOS distribution in mixed glia and highly-

enriched astroglia cultures after LPS stimulation. The results showed that iNOS can be detected in mixed glia cultures as early as 24 hours after LPS stimulation, and all iNOS positive cells co-express Iba-1, a marker for microglia (data not shown). Figure 5 shows the iNOS distribution at 72 hours after LPS stimulation in mixed glia cultures; iNOS was mostly labeled in microglia and less than 1% of iNOS-positive cells were positive for GFAP; iNOS was not detected at any time point in highly-enriched astroglia cultures.

Microglia released numerous soluble factors, such as cytokines and chemokines, in response to LPS stimulation. To elucidate whether microglia induced proinflammatory gene expressions in astroglia by these soluble factors, we collected microglia conditioned medium [309] from LPS-treated microglia-enriched cultures and added it to highly-enriched astroglial cultures. The results showed that MCM could induce TNF-α and iNOS mRNA expression from highly-enriched astroglial cultures in a concentration dependent manner (TNF-α, $P < 0.0001$; iNOS, $P < 0.0001$; **Figures 2.5C and 2.5D**). These results further support that paracrine signals released by microglia were responsible for stimulating the release of pro-inflammatory factors by astroglia.
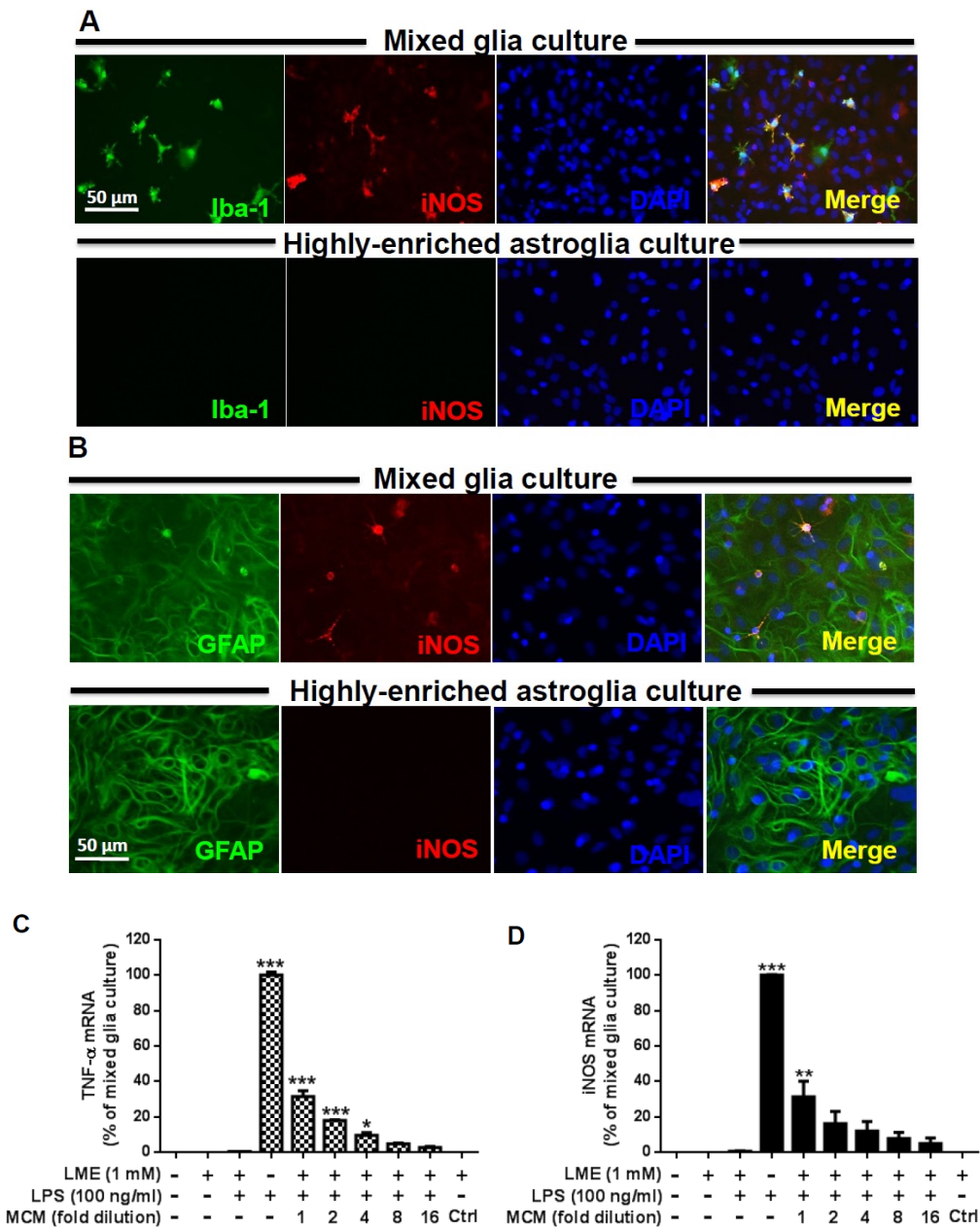
Figure 2.5 Microglia were required for the production of proinflammatory factors by astroglia after LPS stimulation. M/G or highly-enriched astroglial cultures were treated with 100 ng/ml of LPS for 72 hours. Cultures underwent immunofluorescence staining using antibodies against (A) Iba-1 (green, for microglia), iNOS (red) and DAPI (blue); or (B) GFAP (green, for astroglia), iNOS (red) and DAPI (blue). Scale bar: 50 μm. M/G and highly-enriched astroglial cultures were treated with vehicle, LPS, microglia conditioned medium [309] which was collected from enriched microglia cultures at 24 hours after LPS treatment, and control conditioned medium (ctrl). After 3 and 6 hours, cultures were subjected to real-time PCR assay for (C) TNF-α and (D) iNOS mRNA expressions. Value of M/G cultures-treated with LPS was set as 100%. Data shows mean ± SEM from three independent experiments, each done in duplicate. ***$P<0.001$, **$P<0.01$ Bonferroni's t-test compared to vehicle treated highly-enriched astroglial cultures.

Astroglia attenuated LPS-mediated dopaminergic neurodegeneration

Reactive astrogliosis is observed in neuroinflammation and serves as a marker for several neuropathologies [310, 311]. But the roles of reactive astrogliosis are not fully understood. To address this issue, we treated midbrain-derived neuron-glia (~50% astroglia, ~10% microglia, and ~40% neurons) and neuron-microglia cultures (~10% microglia and ~90% neuron) with LPS and assessed the function of DA neurons by measuring their [3H] DA uptake capacity. LPS-treated neuron-microglia cultures had significantly decreased [3H] DA uptake capacity compared to neuron-glia cultures (P = 0.012; **Figure 2.6A**).

Neurotrophic factors released predominantly from astroglia, such as glial-derived neurotrophic factor (GDNF), are known to promote dopaminergic neuronal survival [312]. To confirm whether GDNF participated in astroglia-mediated neuroprotection, GDNF neutralization antibody was added into cultures before LPS treatment. ANOVA analysis showed that there was a significant difference between groups (P< 0.0001), and *post hoc* test revealed that GDNF-neutralizing antibody significantly reduced DA uptake capacity (LPS vs. LPS + GDNF Ab, P<0.05; LPS + GDNF Ab vs. LPS + isotype control Ab, P<0.05), whereas the antibody isotype control had no effect (Figure 6B). Collectively, these results demonstrated that astroglia play a neuroprotective role via the release of neurotrophic factors under neuroinflammatory condition.
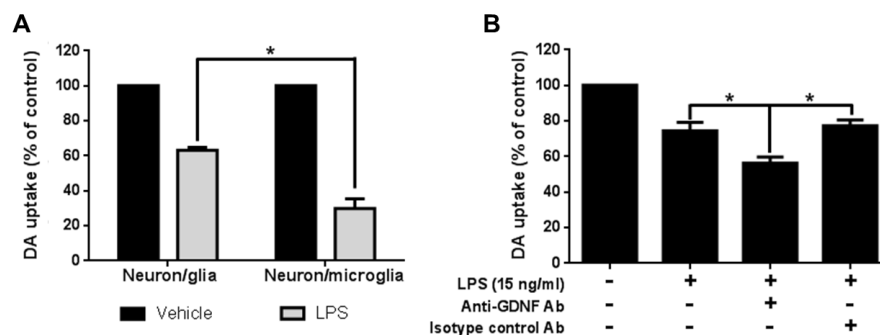
Figure 2.6 Astroglia are beneficial for the survival of DA neurons by releasing GDNF after LPS stimulation in neuron-glia cultures. (A) Neuron-glia and neuron-microglia cultures were treated with vehicle or 15 ng/ml of LPS. After 7 days, [3H] DA uptake assay was utilized to assess DA neuron function. Data show mean ± SEM from four experiments, each done in triplicate. *P < 0.05, Bonferroni's *t*-test. (B) Neuron-glia cultures were incubated with 20 μg/ml anti-GDNF or isotype control antibodies and then exposed with 15 ng/ml of LPS. After 7 days, [3H] DA uptake assay was used to determine DA neuron function. Data show mean ± SEM from three experiments, each done in duplicate. *P < 0.05, Bonferroni's *t*-test.

Microglia required for increased release of neurotrophins by astroglia after LPS stimulation

To further investigate how GDNF was up-regulated after LPS stimulation, microglia enriched, M/G and highly-enriched astroglial cultures were treated with LPS to determine the expression profiles of GDNF mRNA. The data show that LPS failed to induce GDNF mRNA expression in microglia enriched cultures. Interestingly, although astroglia constitutively express basal levels of GDNF mRNA, LPS failed to further increase this expression. In contrast, GDNF mRNA was significantly increased in M/G cultures after LPS treatment, suggesting that the interaction between microglia and astroglia is necessary for the induction of GDNF expression (**Figure 2.7A**).

Previous data demonstrated that soluble factors released from activated microglia in response to LPS stimulation could regulate proinflammatory gene expressions in astroglia. Thus, we tested the involvement of these soluble factors in the up-regulation of GDNF expression in astroglia. The data show that GDNF mRNA was significantly increased in highly-enriched astroglial cultures treated with microglia conditioned medium [309]($P < 0.0001$), and this phenomenon was abolished when the MCM was boiled. The control conditioned media [309] from microglia cultures without LPS stimulation or LPS was added into CM before incubating with highly-enriched astroglia cultures also failed to induce GDNF mRNA expression (**Figure 2.7B**).
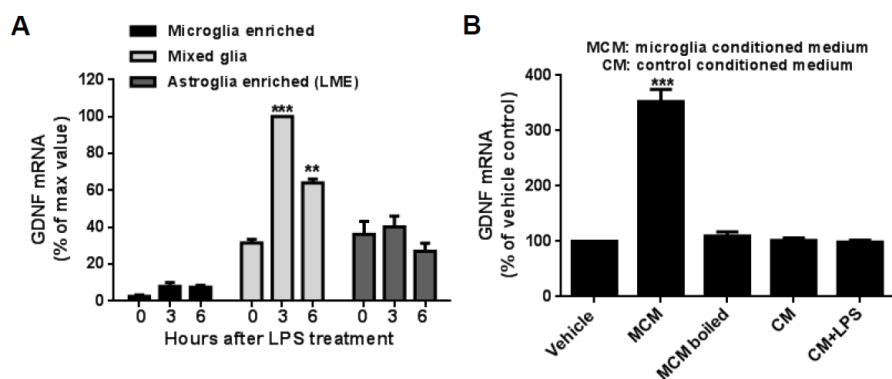
Figure 2.7 Soluble factors released from microglia are important in the induction of GDNF in astroglia. Real-time PCR of GDNF mRNA (A) in enriched microglia, M/G and highly-enriched astroglial cultures were treated with 15 ng/ml of LPS for 0, 3 and 6 hours. Data are presented as percent of maximum value, whereby GDNF mRNA in 3 hours LPS-treated M/G cultures was set as 100%. Data represent mean ± SEM from three independent experiments, each done in duplicate. **$P<0.01$, and ***$P<0.0001$, Bonferroni's t-test compared to 0 hour value. (B) Highly-enriched astroglial cultures were treated with vehicle, microglia conditioned medium [309] which was collected from enriched microglia cultures at 24 hours after LPS treatment, boiled MCM, control conditioned medium [309], and CM plus LPS (15 ng/ml). After 3 hours, cultures were subjected to real-time PCR assay for GDNF mRNA expression. Value of vehicle control cultures was set as 100%. Data shows mean ± SEM from three independent experiments, each done in duplicate. **$P<0.01$, Bonferroni's t-test compared to vehicle treated cultures.

Microglia stimulated the release of neurotrophic factors by astroglia through TNF-α

Among these soluble factors, TNF-α is one of the earliest and most abundant cytokines released by microglia after LPS stimulation. To assess the role of TNF-α in the induction of GDNF, we first compared GDNF mRNA expression between wild type and TNF-α knockout (KO) M/G cultures after LPS treatment (**Figure 2.8**). GDNF mRNA was significantly decreased in TNF-α KO M/G cultures ($P < 0.05$). TNF R1/R2 KO also revealed a similar phenomenon (**Figure 2.8B**), whereby GDNF mRNA expression was significantly attenuated after LPS treatment ($P < 0.05$). These results indicated that TNF-α plays a pivotal role in the regulation of GDNF expression by astroglia.
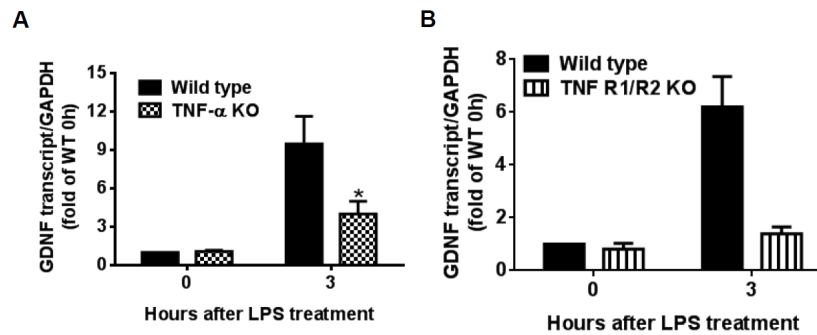
Figure 2.8 Microglia-released TNF-α is one of the key factors in the induction of GDNF in astroglia. M/G cultures were prepared from wild type and TNF-α KO pups, the cultures were treated with 15 ng/ml of LPS for 0 and 3 hours and then pelleted for mRNA analysis. Data were presented as fold increase of value compared to 0 hour wild-type cultures. Data show mean ± SEM from three independent experiments, each done in duplicate. **$P<0.01$, Bonferroni's t-test compared to 0 hour cultures.

Discussion

Many studies support astroglia as active players in the detection and generation of innate immune responses in the brain [159, 313, 314]. Yet with the exception of a few recent publications utilizing highly-enriched astroglial cultures, the majority of these studies could not properly differentiate the contribution of microglia in their enriched astroglial cultures and may have inadvertently attributed their findings to astroglia. Using a highly-enriched astroglial culture, we demonstrate here that astroglia are incapable of detecting and undergoing activation upon stimulation with LPS—thus, bringing to question their role as innate immune cells of the CNS.

Among the many methods developed to generate nearly purified astroglial cultures (>99%), enrichment methods have focused on either the application of chemical or mechanical systems to selectively deplete microglia or have generated cultures from transgenic mice with microglial deficiencies [220, 237, 250-255, 303]. However, many of these methods are costly, require specialized equipment or transgenic mice and may take as long as three to four weeks to enrich. In addition, some enrichment methods are applied after the astroglial monolayer has been formed and thus may physically shield microglia trapped beneath from chemical or mechanical removal [239], resulting in a rapid repopulation of surface microglia shortly after enrichment [254]. For this reason, we modified an established protocol [300] by treating M/G cultures with 1 mM of LME for 5-6 days prior to the monolayer formation—resulting in a quick, thrifty method to

produce highly-enriched astroglial cultures (>99.9%) with ordinary cell culture equipment. Furthermore, we confirmed that the prolonged exposure to LME had no effect on astroglial function and resulted in very little microglial repopulation beginning approximately 7 days after LME removal.

Several studies have concluded that astroglia can produce and secrete potentially cytotoxic pro-inflammatory factors such as TNF-α and •NO upon stimulation with LPS [217, 218, 270, 271, 278, 281, 298, 315]. Hence, similar to microglia [138], the release of cytotoxic factors by astroglia is thought to play a detrimental role during chronic neuroinflammation [293]. Although TNF-α and NO_2^- were detected in the supernatant of LPS-stimulated enriched astroglial cultures, only a few studies used double-immunofluorescent staining or *in situ* hybridization to validate that TNF-α and iNOS were produced by astroglia. Interestingly, the expression of TNF-α [266, 298] and iNOS [239, 279, 280, 282, 316] were almost exclusively co-localized to cells expressing microglial markers and only a few studies noted a delayed, faint and heterogenous expression of iNOS in astroglia [278, 281]. In consensus with the majority of these studies, we found that highly-enriched astroglial cultures were incapable of generating TNF-α or NO_2^- upon stimulation with LPS. Reconstituting microglia into these cultures clearly revealed that as few as 0.5% and 1% microglia were capable of confounding the results of TNF-α and iNOS mRNA expression, respectively. Furthermore, the presence of microglia during LPS treatment was sufficient to induce a weak expression of both iNOS and TNF-α mRNA in astroglia, which is most likely a result of secondary signaling mediated by microglial-derived paracrine factors or complex cell-to-cell contact interactions.

So why is LPS recognized, bound to, and/or sequestered by a combination of TLR-4/MD2, membrane bound CD14, the β2-integrin receptor CD11b/CD18 and class A scavenger receptors (SR-As) that are all localized almost exclusively on the surface of innate immune cells? Not surprisingly, microglia constitutively express high levels of these receptors [203, 205-207, 210-212, 317], whereas astroglia lack membrane bound CD14 [220], CD11b/CD18 [318] and SR-As receptors [317]. Yet, the expression of TLR-4 on astroglia remains controversial. Although several groups have published that TLR-4 is undetectable on astroglia [205, 206, 213, 215, 216], one study was able to detect low-levels of TLR-4 mRNA expression in astroglia using *in situ* hybridization [207]. Regardless of these mixed findings, the most likely explanation is that astroglia either

lack or insufficiently express membrane levels of TLR-4 to generate a signal transduction to LPS. The most compelling evidence in support of this theory is that biotinylated [220] and FITC-labeled LPS [205, 237] are neither bound nor internalized by astroglia.

In pathological conditions of the CNS, astroglia activation (reactive astrogliosis) is maintained in response to sustained endogenous innate immune stimuli [319]. However, the role of reactive astrogliosis as supportive or detrimental to neuronal survival remains to be defined [310, 311]. Here, we demonstrate that activated astroglia protected DA neurons against LPS-induced neuroinflammation, whereby more DA neuron loss was observed in LPS-treated neuron-microglia cultures compared to neuron-glial cultures. During inflammatory conditions, neurons downregulate their constitutive expression of neurotrophic factors such as GDNF, that are necessary to maintain neurite outgrowths and synapses [320]. Under these conditions, astroglia are known to secrete enhanced levels of GDNF [321]. To determine whether astroglia mediate their DA neuron protection by compensating for the reduction in neuronal GDNF by secreting GDNF, we show that LPS-treated neuron-glia cultures treated with GDNF neutralization antibody exacerbated the loss of DA neurons. Interestingly, exogenous GDNF can also serve a dual purpose by directly acting on microglia to suppress their activation and inhibit •NO generation in LPS-treated midbrain organotypic cultures, resulting in reduced inflammation-mediated DA neurodegeneration [322]. In addition, GDNF can reduce the production of extracellular ROS and microglial phagocytic activity in Zymosan A-stimulated cultures [323].

Although LPS is thought to up-regulate the expression of GDNF in primary astroglial cultures [244, 321, 324], we suspected this mechanism might be a misinterpretation due to the inability of LPS to activate highly-enriched astroglial cultures. We investigated three potential scenarios that could explain this up-regulation of GDNF. First, contaminating microglia generate the increased GDNF production detected in enriched astroglial cultures. Second, LPS can directly stimulate astroglia through a putative LPS receptor to produce GDNF. Third, activated microglia can stimulate astroglia through paracrine signaling to produce GDNF. We found that microglia, both non-stimulated and stimulated with LPS, did not express GDNF mRNA, whereas highly-enriched astroglial cultures constitutively express GDNF mRNA yet showed no difference in expression after LPS stimulation. To our surprise, our study showed that astroglia incubated with LPS-treated microglial conditioned medium significantly increased GDNF mRNA expression. This effect could be reversed

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by boiling the conditioned medium (i.e., denaturing the proteins), thus we suspect that cytokines released by LPS-activated microglia may trigger astroglial activation and induce the expression of GDNF. Among these factors, TNF-α has previously been shown to partake in the autocrine regulation of GDNF production in activated astroglia [244, 325]. By treating mixed glial cultures derived from transgenic mice deficient in either TNF-α or TNF-α receptors 1 and 2, we showed that microglial-derived TNF-α is a key modulator of GDNF regulation in astroglia.

In summary, this study demonstrates that highly-enriched astroglial cultures are incapable of detecting LPS, but instead become activated through their interactions with microglia to release neurotrophic factors to protect DA neurons and modulate neuroinflammation—supporting our glial crosstalk activation hypothesis [326]. Although LPS only rarely penetrates the blood-brain barrier [127], other endogenous innate immune stimuli released during stroke, traumatic brain injury, and exposures to xenobiotics could function in a very similar manner by activating microglia to release TNF-α, resulting in the subsequent activation of astroglia to release neurotrophic factors. The results derived from this study suggests that upon activation, astroglia most likely play a far more important role regulating inflammation and reducing neuronal injury than partaking in their collateral damage. Above all, we hope that this study may serve as a caution to those that investigate the function of glial cells that even a miniscule number of microglia may confound the results of a study.

CHAPTER 3: CONCLUSIONS

In the past decade our ability to diagnose PD and other parkinsonian syndromes has improved greatly with the use of brain imaging equipment, yet with this improvement variable pathologies and symptoms continue to be identified—further confounding our efforts to isolate causative environmental agents involved in the etiology of PD. Among the many *in vivo* and *in vitro* models that exist for PD, toxicant-induced models (e.g., Reserpine, 6-OHDA, Methamphetamine, MPTP/MPP+, Paraquat, Rotenone, Maneb, 3-Nitrotyrosine, Dieldrin, Endosulfan, Ziram) and transgenic mouse models (e.g., Park1, Park2, Pink1, Lrrk2, SCNA, VMAT2 and Uchl1) mimic acute atypical parkinsonianism and familial parkinsonianism, respectively. Together these forms of parkinsonianism are thought to represent ~15-20% of the total cases of PD [327]. In contrast to these models, the chronic neuroinflammatory model induced by a systemic injection of LPS is the only xenobiotic exposure-based model that induces progressive pathologies and motor-deficits similar to those of idiopathic PD.

Prior to the development of the chronic neuroinflammatory model induced by a systemic injection of LPS, inflammation-induced PD models were generated by directly injecting LPS into the brain resulting in acute parkinsonianism. The nearly immediate onset of PD-like motor deficits by this model are reminiscent to the case reports of the female laboratory technician exposed to LPS and elderly African man with septicemia that acquired similar deficits within three weeks of exposure—suggesting the rare possibility of a BBB disruption severe enough to allow for direct LPS exposure in the CNS. The work conducted in this thesis attempts to understand the CNS response to this ubiquitous toxin. By isolating astrocytes from microglia, we were able to confirm that astrocytes are incapable of detecting and subsequently generating an innate immune response to LPS. Interestingly, we found that the presence of microglia was required in order for astrocytes to generate any form of response—further suggesting a direct link between microgliosis and astrogliosis. Lastly, by determining that the bulk of the cytotoxic factors generated in the CNS are derived by

microglia, we confirmed that astrogliosis, as induced by the secondary messenger TNF-α from microglia, plays a neuroprotective role by releasing neurotrophins to protect the integrity of neural dendritic outgrowths and synapses and by modulating microglial activation. Together, the work in this thesis helps better define astrocytes as accessary cells that help mediate the inflammatory response by microglia to LPS. Furthermore, our findings suggest that the role of astrogliosis may be more beneficial than originally thought.

Beyond having clarified a potential misconception in the field of neuroimmunology, the results of this study highlight that targeting astrocytes to release neurotrophins should be exploited for therapeutic benefits in CNS conditions with persistent neuroinflammation. Although no therapy has currently been developed to directly target astrocytes to release neurotrophins, many groups are using neurotrophins and peptides/non-peptide neurotrophin analogues for AD and PD therapy with mixed, yet predominantly favorable, success. For this reasons, under the guidance of Dr. Jau-Shyong Hong, our research has been focusing on developing a new class of neuropharmaceuticals that can both inactivate reactive microglia and induce the release of neutrophrins to protect neurons. The hopes of this new therapeutic strategy, which seems to be rather promising in pilot studies of our animal models, is to slow any further neurodegeneration that becomes accelerated in chronic neuroinflammatory environments. Thus our goal, by better understanding the mechanisms that governs environmentally-driven chronic neuroinflammation, is to develop therapies that may improve the lives of PD patients.

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