Astrocyte Mediated Regulation of Activated Microglia: Implications for Exaggerated Microglial Activation in Aged Mice Following Peripheral Inflammatory Challenge

Dissertation

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Abstract

Older individuals are at greater risk for infection and concomitant with this they have a higher frequency for developing neurobehavioral complications that negatively affect health and lifespan. For instance, otherwise innocuous infections are triggers for cognitive decline and the onset of depressive disorders in the elderly. This is a major issue because of the expansion of the elderly population. Neuroinflammation may be the key mechanism underlying the development of these neuropsychiatric disorders. Crosstalk between glial cells of the brain, including microglia and astrocytes, is normally involved in propagating inflammatory signals that are initiated in the periphery with infection. For example, microglia and astrocytes increased expression of inflammatory mediators following peripheral immune challenge in mice (Chapter 2). These increases in cytokine expression correlated to active sickness behavior in mice injected with the bacterial component lipopolysaccharide (LPS). Our work in aged mice, however, shows that aged microglia are hyperactive during an immune challenge and this results in prolonged neuroinflammation and the development of depression and cognitive impairments in the older mice. To limit inflammation, glial cells produce anti-inflammatory cytokines including interleukin (IL)-10. While IL-10 is also elevated in the aged brain following immune challenge, microglial activation still goes unresolved. Our studies show that there are dynamic interactions between microglia and astrocytes mediated by IL-10. For example, in adult mice astrocytes decrease pro-inflammatory activation following IL-
10 signaling and also increase production of other regulatory molecules, including TGFβ, that feedback on microglia (Chapter 3). Therefore, IL-10 may act as a critical anti-inflammatory mediator that attenuates neuroinflammation and prevents the development of neurobehavioral complications. In aged mice, however, astrocytes are more inflammatory and less sensitive to IL-10 regulation. Aged astrocytes had increased GFAP and vimentin expression, and decreased IL-10R surface expression compared to adults. Following acute immune challenge in vivo, adult astrocytes up-regulated IL-10R and TGFβ mRNA. Aged astrocytes, however, failed to increase expression of these mediators. This lack of regulation by TGFβ was associated with exaggerated expression of pro-inflammatory mediators in aged microglia. Last, active microglia cultured ex vivo with adult astrocytes reduced inflammatory markers while those cultured with aged astrocytes did not (Chapter 4). In summary, these novel data indicate that astrocytes have an important role in regulating microglia via TGFβ signaling and that an impaired IL-10 response in aged astrocytes contributes to age-related deficits in the regulation of active microglia.
This thesis is dedicated to Jason, we started OSBP together and we will finish together.

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Chapter 1: *Introduction*

The immune system serves to protect the organism from disease and infection. Cells of the immune system recognize a variety of antigens, known as pathogen-associated molecular patterns (PAMPS), and upon recognition they mount an inflammatory response. During a peripheral infection, innate immune cells become activated and release inflammatory cytokines and mediators. This activation of the peripheral immune system elicits a coordinated response from the central nervous system (CNS). Key to this immune to brain communication is that glia, microglia and astrocytes, interpret and propagate inflammatory signals in the brain that influence physiological and behavioral responses. This low level of inflammation results in changes in neuronal activation leading to the physiological and behavioral components of the sickness response. Exaggerated or prolonged brain inflammation, however, can be detrimental. For example, circumstances that alter the immune response, such as aging, stress and disease, may cause a maladaptive sickness response that leads to behavioral and cognitive complications. In addition, inflammation contributes negatively to the progression of neurodegenerative diseases such as Alzheimer’s disease and prolongs recovery from CNS injuries. Therefore, research focusing on neuroinflammatory responses to systemic infection is of importance.
Introduction on Glial cells in the Brain

Microglia

Microglia are innate immune cells of the brain that mediate responses to pathogens and injury. In addition, microglia provide support, synaptic pruning and immunological activities within the central nervous system (CNS) (Schafer and Stevens, 2013). At one time, microglia were described as being in a ‘quiescent’ or ‘resting’ state. Recent evidence, however, indicates that microglia are constantly surveying their microenvironment (Davalos et al., 2005; Nimmerjahn et al., 2005). Moreover, it is clear that microglia are actively involved in maintaining homeostasis in the CNS. For instance, microglia have an important role in brain development and are critical in synaptic pruning and clearing debris (Schafer et al., 2012). Overall, emerging evidence indicates that microglia work to support normal CNS functions (Salter and Beggs, 2014).

Microglia are myeloid derived cells and have many immune functions related to innate immunity (Ransohoff and Perry, 2009). These immune related activities include key roles in immune surveillance and the interpretation and propagation of inflammatory signals that are initiated in the periphery (Dantzer et al., 2008). These responses are pivotal in the coordinated communication between the immune system and the brain. Microglia actively survey the brain microenvironment for disruptions in homeostasis. For example, in infection or disease, microglia become ‘activated’ and function as inflammatory cellular mediators. Activated microglia rapidly alter their transcriptional profile and produce inflammatory cytokines and chemokines. Active microglia also undergo cytoskeletal rearrangements that alter the pattern of receptor expression. This facilitates cytokine communication between cells. In addition, these alterations allow for microglia to migrate towards sites of injury or infection and to potentially increase their phagocytic efficiency (Davalos et al., 2005). In general, microglia activation and the increased expression of cytokines are aimed to be protective to the CNS and beneficial
to the host organism. This is represented in their role in mediating the behavioral symptoms of sickness following innate immune challenge (Corona et al., 2012). Nonetheless, when microglia profiles are altered or key regulatory systems are impaired, microglia activation may be maladaptive. Depending on the context, exaggerated microglial activation can lead to prolonged neuroinflammation and neurobehavioral complications. Here, we discuss changes that occur in microglia during aging and how these changes influence secondary inflammatory insult.

**Astrocytes**

Astrocytes are the most abundant cell in the brain and carry out many important functions within the central nervous system (CNS). Historically, astrocytes were viewed simply as supporting cells for neurons. Their functions included providing structural and functional support for the neuronal network and also maintaining metabolic and ion homeostasis of neurons. From more recent findings, however, it is now known that astrocytes carry out several important immunological functions (Kimelberg, 2010; Kimelberg and Nedergaard, 2010). For example, astrocytes express several immune receptors including scavenging receptors, toll-like receptors and complement receptors, and produce inflammatory cytokines upon activation. Under homeostatic conditions, astrocytes maintain an anti-inflammatory environment by producing mediators that regulate inflammation. Upon immune challenge, disease, or injury, astrocytes can be activated directly by cytokines produced by microglia or cytokines reaching the brain from the periphery. Astrocytes also act as “gate keepers” for leukocyte entry into the CNS (Abbott et al., 2006). These features of astrocytes have opened up a whole new spectrum of studies aimed at understanding the specific role of astrocytes during neuroimmune diseases and disorders. As our knowledge of astrocyte functions as
immune cells has expanded, exploration into their role during peripheral infection and in aging has followed.

**Introduction on the increased human aging population**

The elderly population in the US is increasing rapidly. It is estimated that by the year 2050, the population of seniors aged 65 and up will double (Ortman et al., 2014). The dramatic increase in the number of seniors living in the US shows that research into healthy aging has never been more important. Unfortunately, seniors continue to be afflicted with debilitating cognitive and neuropsychiatric disorders. For example, the number of seniors living with dementia is expected to triple in the next few decades (WHO, 2012). Clinical studies indicate that neuropsychiatric disorders in the aged, including depression and cognitive impairments, can be triggered by otherwise innocuous peripheral infections (Ahmed et al., 2014; Dunn et al., 2005; Penninx et al., 1999; Penninx et al., 2003). This may be because aging has a robust influence on both the immune system and the CNS. Because of this, the bi-directional communication between the immune system and the brain is altered with age (Corona et al., 2012; Jurgens and Johnson, 2010b).

The normal neuroimmune communication is essential for mounting the appropriate immunological, physiological, and behavioral responses to pathogens. The CNS interpretation of pathogen derived signals is essential for the induction of the behavioral symptoms of sickness (e.g., lethargy, anorexia, & social withdrawal) (Dantzer, 2001). This sickness response is mediated by cytokines, including interleukin (IL)-1β, and represents a beneficial reorganization of the host’s priorities to resolve infections (Kelley et al., 2003; Kelley et al., 1997). Altered neuroimmune communication in aging is clinically relevant because older individuals have an increased risk of infection with
concomitant neurobehavioral deficits (Jackson et al., 2004). For example, neuropsychiatric disorders are more frequent in older patients during an illness and positively correlate with increased morbidity and mortality (Godbout and Johnson, 2006; Koenig et al., 1988; Penninx et al., 1999). Importantly, transient immune challenges including urinary tract infections often present clinically in the elderly as acute cognitive impairment and altered mood (Ahmed et al., 2014; Dunn et al., 2005). Even after the infection has been resolved, these older individuals are at an increased risk of progressive dementia and functional disability (Iwashyna et al., 2010). These data indicate that acute immune challenges can be triggers for ongoing inflammatory processes that affect mood and cognition (Dantzer et al., 2008; Haroon et al., 2012). Therefore it is critical that we understand glial regulatory pathways in the brain and how normal aging influence glial responses and interactions.

Evidence of Increased Brain Inflammation with Age

There is significant clinical and experimental evidence that the inflammatory status of the brain increases as a function of normal aging. Hallmarks of brain aging include increased oxidative stress, lipid peroxidation and DNA damage (for a review see (Norden and Godbout, 2013)). Consistent with this premise, microarray studies indicate that there is an overall increase in inflammatory and pro-oxidant genes with a reduction in growth and anti-oxidant genes in the brain of older rodents compared to adults (Godbout et al., 2005b; Lee et al., 1999). In addition, two potent pro-inflammatory cytokines interleukin (IL)-1β and IL-6 are increased in the brain of aged rodents and humans (Fenn et al., 2013; Godbout et al., 2005b; Henry et al., 2009; Sheng et al., 1998). In addition, there are reductions in several regulatory molecules and anti-inflammatory cytokines including IL-10 and IL-4 (Maher et al., 2005; Ye and Johnson,
Because of these changes in the balance of pro- and anti-inflammatory profiles, microglia are implicated as the source of this inflammation.

Microglia are long-lived cells that have limited turnover from myeloid cells from the bone marrow during the course of a lifetime (Ajami et al., 2007; Ginhoux et al., 2010). Although microglia are not replaced from the bone marrow, a recent study shows that turnover may be from a progenitor source within the CNS (Elmore et al., 2014). In this study, intervention with a colony stimulating factor 1 receptor (CSF1R) antagonist effectively depleted the majority of microglia in the CNS. The removal of the CSF1R antagonist allowed for the robust repopulation of microglia from a previous unidentified progenitor source within the CNS (Elmore et al., 2014). It is still unclear however, the rate at which microglia would normally be turned over in the absence of CSF1R blockade. Overall, microglia appear to have a relatively low turnover rate. This stability and longevity of microglia makes them particularly sensitive to oxidative stress and inflammatory exposure over time.

**Evidence of Microglial Priming with Age**

As innate immune cells of the CNS, the increased pro-inflammatory phenotype of the brain that occurs with aging is linked to changes in microglia. With aging, microglia may develop an altered profile consistent with a more inflammatory state. This is also referred to as a primed profile. An inflammatory or primed microglia profile is defined by 1) higher baseline expression of markers of inflammation and inflammatory mediators, 2) a lower threshold to be activated and ‘switch’ to a pro-inflammatory state (Lull and Block, 2010), and 3) an exaggerated inflammatory response following immune activation. This primed phenotype of microglia is detected in models of aging and neurodegenerative disease. This phenomena of primed microglia was first described in a model of prion disease where microglia from prion infected mice produced exaggerated IL-1β following
both central and systemic immune challenges (Cunningham et al., 2005b). Overall, here we define microglial priming as microglia that have an increased inflammatory state at baseline and also produce an exaggerated response to immune challenge. Microglia priming has mainly been described in aging, but similar phenotypes of microglia are present during neurodegenerative disease and following traumatic brain injury. Although there are similarities between these disease models, no single source has been identified as the cause of priming. Therefore, microglial priming towards an inflammatory state is likely influenced by exposure to immune challenges, stressors and injury over time.

In the aged brain, microglial priming has been characterized by increased mRNA and protein expression of various inflammatory markers and alterations in morphology. For example, expression of the antigen presenting molecule major histocompatibility complex (MHC) II and complement receptor 3 (CD11b) were increased in the aged brain of rodents and humans (Frank et al., 2006; Streit and Sparks, 1997; VanGuilder et al., 2011). MHC II is increased specifically on microglia of the aged brain (Henry et al., 2009). MHC II is commonly used as a marker of microglial priming both in models of aging and injury because primed MHC II positive microglia produce exaggerated IL-1β following activation (Henry et al., 2009). Consistent with heightened MHC II expression, several other inflammatory markers are also increased in models of aging (for a review see (Norden and Godbout, 2013). In addition to immune markers, baseline expression of inflammatory cytokines tumor necrosis factor (TNF)-α, IL-1β and IL-6 are also increased in the brains of aged rodents and humans (Hickman et al., 2013; Sheng et al., 1998; Sierra et al., 2007; Youm et al., 2013).

These results are paralleled by morphological alterations detected using Iba-1 labeling. Microglia from non-diseased aged brains have shorter and less branched dendritic arbors than microglia of young adults (Streit et al., 2004). Microglia from the
brains of aged gerbils (Choi et al., 2007) and dogs (Hwang et al., 2008) also have a shift towards a more activated morphology (thickened and de-ramified processes) compared to young adults. This de-ramified morphology is comparable to the activated morphology of microglia. Moreover, the de-ramified morphology of microglia in aged rats corresponded with higher MHC II expression (VanGuilder et al., 2011). These data further support the hypothesis that MHC II is a marker of primed microglia. Recent studies have also used positron emission tomography (PET) to evaluate microglial activation in humans. The ligand PK [11C]PK11195 binds to translocator protein (TSPO) receptors that are expressed in mitochondria of activated microglia. PET imaging using this compound showed increased TSPO in older individuals, indicating that microglial activation was elevated with age (Schuitemaker et al., 2012). Taken together, the increase of inflammatory mediators and altered phenotype of microglia with age indicates that microglia develop a primed phenotype and this may contribute to the heightened inflammatory status of the aged brain.
In normal aging there is increased mRNA and protein expression of several inflammatory markers on microglia. In older rodents and non-human primates these include proteins associated with antigen presentation, (MHC II and CD86), scavenger receptors (CD68), pattern associated recognition receptors (Toll-like receptors), and integrins (CD11b and CD11c). There are also detectable increases in inflammatory cytokines and decreases in anti-inflammatory cytokines in the aged brain. Last, in several aging models the morphology of the microglia is more de-ramified. Collectively these findings are interpreted to indicate that microglia of the aged brain maintain a primed or activated immune profile.

As stated above, the source of the glial priming in the aged brain is unclear. A hallmark of brain aging is increased oxidative stress and free radical damage that may affect the profile of microglia. In addition, changes in microRNA regulation of multiple genes may play a role in brain aging (Fenn et al., 2013). Moreover, recent evidence indicates that immune sensors, such as inflammasomes, are involved in increased inflammatory status with age. The NLRP3 inflammasome is an immune sensor that is activated in response to a diverse array of signals. When the NLRP3 inflammasome was deleted, CNS inflammation was reduced in mice that were allowed to age (Youm et al., 2013). For example, nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), IL-1β, interferon, and complement pathways were all markedly increased in the
brains of aged wildtype mice compared to adult wildtype mice. In aged NLRP3 knockout mice, however, these pathways were significantly attenuated compared to aged wildtype mice (Youm et al., 2013). Thus, increases in oxidative stress, changes in microRNA regulation and increased inflammasome activity with age could drive mediators of inflammation.

**Similarities between microglial priming in aging and neurodegenerative disease**

In addition to aging, there is also increased priming of microglia in presymptomatic neurodegenerative disease. Several neurodegenerative diseases including Alzheimer’s disease (AD) increase in risk as a function of aging and may also be influenced by history of head injury (Nemetz et al., 1999). Therefore, it is not surprising that microglia in the aged brain and in neurodegeneration share similar phenotypes. For example, both prion disease (transmissible spongiform encephalopathies) and AD show marked microglial priming associated with an increased inflammatory status of the CNS. In a mouse model of prion disease, microglia have an activated morphology and increased expression of immune markers including MHC II (Betmouni et al., 1996; Cunningham et al., 2005b). In AD, microglia also acquire an activated morphological profile (McGeer et al., 1987). Furthermore, in aging combined with AD, microglia become even more activated. Microglia in aged AD mice were significantly more activated compared to wildtype controls, and these changes in microglia morphology were most evident in regions of high beta-amyloid plaque pathology (Koenigsknecht-Talbooo et al., 2008). In AD, microglia have similar or exacerbated increases in inflammatory surface markers (such as MHC II and CD68) as in aging (Cameron and Landreth, 2010). Importantly, these changes in microglial activation within the hippocampus of AD patients correlated to a precipitous loss in cognitive function and
memory (Cagnin et al., 2001). Immune changes that occur in the brain with AD have been linked to such functional impairments.

The most prominent pathology during AD is impaired clearance of neurotoxic Aβ plaques, which leads to impaired neuronal signaling and ultimately cognitive decline. The contribution of neuroinflammation to impaired clearance of Aβ has been extensively studied; unfortunately no conclusive results have been reported. It is well understood, however, that microglial clearance of Aβ plaques is impaired in AD (Floden and Combs, 2011). Recent genomic studies indicate that several AD risk genes such as TREM2 and CD33 are implicated in microglial phagocytosis (Griciuc et al., 2013; Jiang et al., 2013; Jonsson et al., 2013), supporting the idea that microglia in the aged or diseased brain have impaired phagocytosis functions. TREM2 was recently identified as a gene that is significantly down-regulated in microglia from aged mice (Hickman et al., 2013). This result indicates that loss of TREM2 expression is involved in both normal aging and neurodegenerative aging and may be a potential target of therapeutics for treatment of age-associated CNS complications. Another association between aging and AD is the immune modulator NLP3. As discussed above, recent evidence highlight the detrimental role of NLPR3 in normal, non-pathological aging (Youm et al., 2013). Similarly, in a mouse model of AD, the NLPR3 inflammasome also had detrimental effects. NLPR3 was activated in both human and mouse AD brains. Genetic deletion of NLPR3 reduced plaque burden and mice were protected from loss of spatial memory (Heneka et al., 2013). These recent studies indicate that NLPR3 contributes both to again and to pathology in AD. Overall, these studies suggest common pathways for the development of primed microglia in both aging and neurodegeneration.
Primed Microglia and Increased Reactivity to Secondary Challenge

A functional consequence of microglial priming in aging is an exaggerated neuroinflammatory response following a peripheral or central immune challenge (for a review see (Norden et al., 2014b)). This idea is illustrated in Fig.2.2. This hypothesis of microglia priming and immune reactivity is well supported in research using rodent models of aging. Lipopolysaccharide (LPS) is a bacterial endotoxin derived from the cell wall of *E. coli* commonly used to model systemic infection. Intraperitoneal (i.p.) injections of LPS have been shown to induce a heightened neuroinflammatory response and corresponding sickness-like behavior in BALB/c mice (Berg et al., 2004; Bluthe et al., 2000a). In addition, numerous studies have implemented intraperitoneal (i.p.) LPS injections to examine the deleterious effects of CNS inflammation following peripheral immune challenge in models of aging, depression, and neurodegenerative diseases, such as Parkinson’s and Alzheimer’s (Norden et al., 2014b).

Mixed glial cultures and coronal brain sections derived from the brains of aged mice had an exaggerated response to LPS stimulation and produced more IL-1β and IL-6 compared to cultures established from adults (Xie et al., 2003; Ye and Johnson, 2001). In addition, microglia cultured *ex vivo* from the brains of aged mice had increased IL-6 and TNF-α levels compared to microglia from adults (Njie et al., 2010). *In vivo* studies demonstrate that stimulation of the peripheral innate immune system by injection of LPS caused a prolonged and exaggerated neuroinflammatory cytokine response (IL-1β and IL-6) in aged BALB/c mice compared to young adults (Godbout et al., 2005b; Wynne et al., 2010). Similarly, peripheral injection of *Escherichia coli* (*E. coli*) promoted higher and prolonged levels of IL-1β in the hippocampus of aged rats compared to young adults (Barrientos et al., 2009a; Barrientos et al., 2006).

It is relevant to note that while a peripheral injection of LPS or *E. coli* induced higher levels of IL-1β in the aged brain compared to adult, plasma levels of IL-1β were
not amplified (2009a; Barrientos et al., 2006; Godbout et al., 2005b). The disconnection between peripheral IL-1β and brain IL-1β induction has been interpreted to suggest that an amplification of the immune response occurs within the brain. Indeed, central activation of the innate immune system by intracerebroventricular (i.c.v.) administration of LPS or GP120, a viral envelope glycoprotein, caused amplified mRNA expression of inflammatory cytokines, IL-1β, IL-6 and TNF-α in aged (22-24 mo) mice (Abraham et al., 2008; Huang et al., 2008). These studies provide evidence that increased pro-inflammatory cytokine production in the aged brain is related to an amplified response from the resident glia population.

This exaggerated cytokine production is attributed to the microglia. The priming effect in microglia of the CNS was first demonstrated in a mouse model of prion disease (Cunningham et al., 2005b). In this study, microglia from pre-symptomatic prion disease mice had exaggerated IL-1β production compared to non-prion mice following both central and systemic LPS challenges. This study provided direct evidence of heightened inflammatory response to secondary inflammatory challenge. There are several studies showing exaggerated activation of primed microglia in models of aging. For instance, ex vivo stimulation of microglia from aged mice with LPS (Frank et al., 2010a) or Pam3CSK4, a toll-like receptor-2 (TLR2) agonist (Njie et al., 2010), resulted in exaggerated production of IL-1β, IL-6 and TNF-α compared to adults. When microglia were isolated from whole brain homogenates after a peripheral LPS injection, microglia-specific mRNA levels of TLR2 and IL-1β were increased in aged compared to adult mice (Henry et al., 2009). It is important to note that aged microglial reactivity increased production of both pro-inflammatory cytokines and anti-inflammatory cytokines. For example, aged mice had amplified microglial specific mRNA induction and intracellular protein expression of both IL-1β and IL-10 compared with adult mice following LPS injection (Henry et al., 2009; Sierra et al., 2007). Furthermore, a key point was that the
reactive (MHC II⁺) microglia of aged mice had the most prominent IL-1β induction following immune challenge compared to non-reactive (MHC II⁻) microglia (Henry et al., 2009). These studies indicate that after a peripheral immune stimulus primed, MHC II⁺ microglia had an exaggerated and prolonged production of inflammatory cytokines.

Figure 1.2. Neurobehavioral complications associated with microglial reactivity in the brain of aged. Under normal conditions, microglia interpret and propagate inflammatory signals that are initiated either peripherally or centrally. Microglial activation increase cytokine and secondary messenger release that lead to transient physiological and behavioral responses that are beneficial to the host organism (top panel). A consequence of microglial priming with age, however, is a hyperactive response to an immune challenge with amplified and prolonged production of cytokines. In several models of aging, an exaggerated cytokine response is associated with the development of cognitive, behavioral, and physiological complications that are interpreted to be maladaptive to the host organism (bottom panel).
Behavioral Consequences of Microglia Priming and Immune Reactivity

*Sickness and depressive-complications following innate immune challenges*

The primed profile of microglia leads to an exaggerated response to a secondary challenge or insult. Importantly this exaggerated neuroinflammation negatively affects behavior and cognition and increases CNS pathology (Fig. 1.2). One potential consequence of this is the induction of prolonged sickness and depressive-like behaviors. While the induction of cytokine-mediated sickness behavior is a necessary and beneficial response to systemic infection, an amplified or prolonged response affects behavioral and cognitive processes (Jurgens and Johnson, 2010a). In the studies discussed above, peripheral or central immune stimulation with LPS caused protracted neuroinflammation in the brain of aged rodents. This was paralleled by a prolonged sickness response with protracted anorexia, lethargy and social withdrawal (Abraham et al., 2008; Godbout et al., 2005b; Huang et al., 2008). An amplified sickness response was also detected in older rats that were infected subcutaneously with *E. coli*. The aged rats displayed an altered febrile response including a blunted and delayed increase of core body temperature followed by a significant and prolonged increase of inflammatory cytokines (Barrientos et al., 2009b). Similar to the extended sickness behaviors in aged BALB/c mice, the increase of inflammatory cytokines were likely driven by exaggerated microglial IL-1β (Henry et al., 2009). In support of this notion, i.c.v. infusion of IL-1 receptor antagonist (IL-1RA) reversed the prolonged LPS-induced sickness behavior in aged mice (Abraham and Johnson, 2008). These findings indicate that the exaggerated sickness response in aged rodents was likely caused by the exaggerated and prolonged production of IL-1β by primed microglia.

Related to prolonged sickness behavior, there is also evidence of development of depressive-like complications in aged rodents following an immune challenge. Depressive-like complications in rodent models are reflected by increased resignation...
behavior (i.e. immobility) in the forced swim test, or tail suspension test. These behavioral assays are intended to model the aspect of despair displayed by depressed human patients (Cryan et al., 2005). In aged BALB/c mice, peripheral stimulation of the innate immune system with LPS caused prolonged depressive-like behavior 72 h after injection in aged mice after acute (Godbout et al., 2008) and chronic (Kelley et al., 2013) immune challenge. In one study aged mice showed increased resignation behavior in the forced-swim and tail-suspension tests even after the acute effects of LPS on lethargy and food intake were resolved. These protracted depressive-like behaviors are likely a direct consequence of the impaired regulatory mechanisms of microglia. As mentioned previously, microglia from aged BALB/c mice show extended down regulation of fractalkine receptor (CX$_3$CR1) on microglia that may be an underlying cause of prolonged microglial activation (Corona et al., 2010; Wynne et al., 2010). Consistent with this finding, adult CX$_3$CR1-deficient mice displayed prolonged social withdrawal and depressive-like behaviors 48 and 72 h after an LPS injection. The depressive-like behavior in CX$_3$CR1-deficient mice was paralleled by a prolonged activated morphology of microglia in the absence of apparent neuronal death (Corona et al., 2010).

**Cognitive complications following innate immune challenges**

As mentioned, increased cytokine expression is important in the behavioral response to inflammation in order to mount an adaptive sickness response. These transient increases in neuroinflammation also effect cognitive performance even in healthy rodents. For example, IL-1β administered into the hippocampus caused hippocampal-dependent memory deficits (contextual fear conditioning) but not deficits to hippocampal-independent memory tasks (auditory fear conditioning) (Rachal Pugh et al., 2001). Furthermore, a recent study in rats assessed neural circuit activity in the hippocampus in relation to context discrimination memory retrieval after systemic
administration of LPS (Czerniawski and Guzowski, 2014). They showed that along with eliciting an acute neuroinflammatory response in the hippocampus, LPS abolished retrieval of discrimination memory by disrupting cellular pattern separation processes within the hippocampus. Together these studies show that acute and transient increases in neuroinflammation can lead to cognitive impairments in healthy mice. Similar immune challenges, however, lead to amplified and exaggerated cognitive impairments in conditions where microglia are primed.

There are multiple studies indicating that increased cytokine production in the aged brain after peripheral innate immune challenge is associated with impaired cognitive function. For example, injection of LPS caused an amplified cytokine response in the hippocampus of older mice that was paralleled by impaired hippocampal-dependent spatial memory (Chen et al., 2008). Moreover, infection by *E. coli* led to prolonged production of IL-1β in the hippocampus of aged rats (Barrientos et al., 2009a) and reduced long-term contextual memory examined by context-dependent fear conditioning and Morris water maze (Barrientos et al., 2009a; Barrientos et al., 2006). When aged mice were fed a diet supplemented with resveratrol, a potent anti-oxidant, LPS-induced neuroinflammation and working memory deficits were attenuated (Abraham and Johnson, 2009b). In the absence of an immune stimulus there is not a significant effect of age on the acquisition of memory tasks. There is, however, evidence of age-associated memory problems in the reversal task of the Morris water maze (Jang et al., 2010). Nonetheless, age-related cognitive impairment is exaggerated when a secondary immune challenge is provided.

**Impaired anti-inflammatory regulation of activated and primed microglia**

The cause of this amplified microglial activation with age may be related to impairments in several key regulatory systems that make it more difficult to resolve
microglial activation. For example, recent evidence shows that microglia hyper-activity with age is associated with reduced activity of regulatory pathways. There are several mechanisms within the CNS to maintain a surveying and anti-inflammatory microglia phenotype including neuronal regulation, astrocytic regulation, and immune regulation. Within the aged brain, however, these regulatory networks begin to break down leaving microglia primed and more responsive to activating stimuli. Detailed discussion on these regulatory pathways are discussed in (Norden and Godbout, 2013) and are highlighted in Figure 1.3. Here we will discuss microglial regulation by the anti-inflammatory cytokines IL-10 and TGFβ.
Figure 3. Activated microglia from the aged brain are refractory to anti-inflammatory stimulus. The activation of microglia is tightlyregulated and there are several anti-inflammatory mediators thatmodulate microglial activation. For example, anti-inflammatory cytokinesincluding IL-10, TGF-β, and IL-4 modulate the activation of microglia andare decreased in the brain with age. In addition neuronally derived ligandsincluding CD200 and fractalkine (CX3CL1) are also decreased with age. Theroad panels depict the differences in expression of several regulatoryproteins and receptors with age. There are also several regulatory systemsin that the ligand and the receptor interaction that change whenmicroglia become activated. For example, compared to adult microglia,aged microglia have prolonged reduction of CX3CR1 and fail to increasesurface expression of the IL-4 receptor-a (bottom left panel). Taken altogether, the prolonged activation of microglia of the aged brain may bebecause they are less sensitive to the anti-inflammatory regulation thatnormally helps to resolve activation.

**IL-10**

IL-10 is a proposed regulator of microglial activation (Fiorentino et al., 1991;Kremlev and Palmer, 2005). IL-10 signals through the IL-10 receptor, which has two
major components: 1) the ligand binding domain (IL-10R1) and 2) the signaling domain (IL-10R2) (Moore et al., 2001). IL-10R2 is expressed constitutively by most cells. Expression of IL-10R1, however, varies by cell type and is expressed at low levels under homeostatic conditions (Moore et al., 2001). Other studies have established an anti-inflammatory and potentially neuroprotective role of IL-10 within the CNS in models of EAE (Cua et al., 2001), spinal cord injury (Bethea et al., 1999; Ishii et al., 2013), stroke (Frenkel et al., 2005) and Parkinson’s disease (Qian et al., 2006). In addition, i.c.v. infusion of IL-10 suppressed cytokine-mediated sickness behavior (Bluthe et al., 1999) and blocked IL-1β expression in the hippocampus after LPS challenge (Lynch et al., 2004). In IL-10KO mice, LPS challenge caused prolonged neuroinflammation and sickness behavior compared to wild-type mice (Richwine et al., 2009). In the absence of an immune challenge, the aged brain has decreased anti-inflammatory IL-10 expression (Ye and Johnson, 2001). Although there is pervasive support for a skew towards a pro-inflammatory phenotype in microglia with age, it is also apparent that this may not always be the case. After an immune challenge, for example, microglia from aged mice release exaggerated levels of both IL-1β and IL-10 (Henry et al., 2009; Sierra et al., 2007). This increased IL-10 response, however, was not sufficient to restore homeostasis and inflammation persisted (Fenn et al., 2012b; Henry et al., 2009). Recent evidence indicates that astrocytes are highly responsive to the anti-inflammatory effects of IL-10 (Norden et al., 2014a). Therefore, current research is investigating astrocyte responsiveness to these inflammatory and anti-inflammatory signals and how this may be altered with age.

**TGFβ**

Transforming growth factor beta (TGFβ) is an anti-inflammatory cytokine expressed at low levels by both neurons and glial cells (Flanders et al., 1991; Hamby et
al., 2010) and its expression is upregulated in response to a wide range of CNS insults and inflammatory challenges (Henrich-Noack et al., 1996; Wynne et al., 2010). TGFβ is an important regulator of microglia both during and after development. In recent studies, TGFβ was critical for microglia to populate the brain (Butovsky et al., 2014) and identified as a strong regulator of microglial quiescence (Abutbul et al., 2012; Butovsky et al., 2014; Norden et al., 2014a). Therefore, it is plausible that there are impairments in TGFβ regulation of primed microglia in the aged brain. In support of this notion, comparison of the cytokine expression profile of brain tissue from immune challenged aged and adult mice showed that TGFβ expression was increased after immune challenge only in adults (Tichauer et al., 2013; Wynne et al., 2010). This reduction in TGFβ could explain some of the hyper-responsiveness of aged microglia. For example, treatment with TGFβ in culture decreased IL-1β and increased CX3CR1 expression in microglia (Wynne et al., 2010). This finding provides a possible link between impaired TGFβ upregulation and prolonged downregulation of CX3CR1.

In addition to impaired upregulation of TGFβ following immune challenge, microglia from aged mice may also be less sensitive to TGFβ. A recent study found that microglia isolated from aged mice had deceased expression of TGFβ receptor compared to adults (Hickman et al., 2013). Furthermore, microglial cultures established from aged mice were less sensitive to the anti-inflammatory effects of TGFβ compared to microglia from adult mice (Rozovsky et al., 1998). Finally, when microglia were isolated from LPS-injected adult and aged mice, only microglia from adult mice were responsive to TGFβ treatment ex vivo (Tichauer et al., 2013). These studies provide strong support that TGFβ-mediated regulation of microglia becomes impaired with age.

Impaired regulation of microglia by TGFβ is significant in both normal aging and age associated neurodegenerative diseases such as Alzheimer’s disease. During Alzheimer’s disease, there is impaired clearance of neurotoxic amyloid-β plaques.
Specifically, microglial clearance of amyloid-β plaques is impaired in aging and Alzheimer's disease (Floden and Combs, 2011). Under normal conditions, TGFβ signaling in microglia promotes an anti-inflammatory microglial phenotype that is highly phagocytic (Tichauer et al., 2013; Wyss-Coray et al., 2001). In aged mice, however, microglia may be sensitive to TGFβ and did not increase phagocytosis following TGFβ treatment (Tichauer et al., 2013). Overall, impaired regulation of microglia by TGFβ may promote microglial priming and decrease the phagocytic efficiency of aged microglia.

Evidence of Astrocyte Changes with Age

As described above, the majority of aging studies examine the effect of age on microglia. It is important, however, to discuss that astrocytes become more inflammatory with age. For example, aging is associated with increased expression of glial fibrillary acidic protein (GFAP) and vimentin. Up-regulation of GFAP and vimentin filaments is associated with numerous functions, including increased astrocyte motility and vesicle trafficking (Lepekhin et al., 2001). Activated astrocytes following CNS injury rapidly upregulate both GFAP and vimentin. Therefore, these markers have been used to identify activated astrocytes. In aging, there are several reports showing that astrocyte inflammatory markers GFAP and vimentin are increased in the brains of aged rodents and humans (Cotrina and Nedergaard, 2002; Finch, 2003; Godbout et al., 2005b; Nichols et al., 1993; Porchet et al., 2003; Unger, 1998; VanGuilder et al., 2011). Similar to microglia, astrocytes have an increased hypertrophic morphology with a shift from resting/stellate to active in the brains of aged rats (VanGuilder et al., 2011).

Although not typically thought about as an immune cell, recent findings reveal that astrocytes provide several important immunological functions (Kimelberg, 2010; Kimelberg and Nedergaard, 2010). For example, astrocytes express several immune receptors including scavenging receptors, toll-like receptors and complement receptors
and produce inflammatory cytokines when activated. Under homeostatic conditions, astrocytes maintain an anti-inflammatory environment by producing mediators that regulate inflammation. Upon immune challenge, disease, or injury, astrocytes can be activated directly by cytokines produced by microglia or cytokines reaching the brain from the periphery. Activated astrocytes are also involved in propagating cytokine production and producing factors that resolve microglial activation (Tichauer et al., 2007). For example, astrocytes produce TGFβ which down-regulates inflammatory cytokine expression in microglia (Norden et al., 2014a; Ramirez et al., 2005) (Fig.1.4). While it is known that astrocytes have a more ‘reactive’ profile with higher GFAP expression in aging, the consequence of this reactive profile is less clear. We propose that these altered profiles of astrocytes effect the dynamic interactions between microglia and influences the ability to regulate microglia activation (Fig.1.4). These features of astrocytes have opened up new areas of research aimed at understanding the specific role of astrocytes and their dynamic interactions with microglia.
Microglia and Astrocyte Interactions: It Takes Two to Make a Thing go Right

Resolution of Microglial Activation

- IL-1β, TNF, IL-6
- CX3CR1, IL-4R
- IL-10
- TGFβ
- IL-4

Unresolved Microglial Activation

- IL-1β, TNF, IL-6
- CX3CR1 ↔ IL-4Rα
- IL-10

Pro-inflammatory Mediators

Normal Astrocyte

Reactive Astrocyte (GFAP++)

Figure 1.4. Primed Glia and Impaired Regulation of Active Microglia by Reactive Astrocytes. Astrocytes also have a more ‘reactive’ profile with higher GFAP expression after TBI or in neurodegenerative disease. The long-term consequence of this reactive astrocyte profile in the brain is not well understood. One idea that this altered profiles of astrocytes affects the dynamic interaction with active microglia. In this scenario, astrocytes help to regulate microglia activation. Thus it takes the appropriate interactions between these two glia cell types to make things go right.

Summary

Aging results in the loss of integrated regulatory systems designed to maintain microglia in a surveying and anti-inflammatory state. Loss of these regulatory pathways does not come from a single source, but instead represents a global loss in regulation from neurons, astrocytes, micro-RNAs, and autocrine immune mediators. As a result, microglia in the aged brain develop a primed phenotype and become hyper-inflammatory following challenge to the immune system. An exaggerated and prolonged microglial
response promotes the development of neuropsychiatric complications including depression and cognitive decline. Moreover, this hyper-inflammatory response can precipitate or contribute to the development of neurodegenerative diseases. Thus, efforts to restore proper microglia regulation and function may be the key to successful CNS aging.
Chapter 2: Induction of Glial Cytokine Expression and Sickness Behavior Precede Increased Iba-1 or GFAP Immunoreactivity Following Acute Immune Challenge

Abstract

Activation of the peripheral immune system elicits a coordinated response from the central nervous system. Key to this immune to brain communication is that glia, microglia and astrocytes, interpret and propagate inflammatory signals in the brain that influence physiological and behavioral responses. One issue in glial biology is that morphological analysis alone is used to report on the glial activation state. Therefore, our objective was to compare behavioral responses after in vivo immune (lipopolysaccharide, LPS) challenge to glial specific mRNA and morphological profiles. Here, LPS challenge induced an immediate (2h) but transient sickness response (24h) with decreased locomotion and social interaction. Corresponding with active sickness behavior (2-12h), inflammatory cytokine mRNA expression was elevated in both enriched microglia and astrocytes. Microglial pro-inflammatory cytokine expression peaked 2-4 h after LPS while cytokine and chemokine induction in astrocyte peaked at 12 h. Morphological alterations in microglia (Iba-1+) and astrocytes (GFAP+), however, were not detected during this 2-12 h timeframe. Increased Iba-1 immunoreactivity and de-ramified microglia were evident 24 and 48 h after LPS but corresponded with the resolution phase of activation. Morphological alterations in astrocytes were undetected after LPS. Additionally, glial cytokine expression did not correlate with morphology after repeated LPS challenge. In fact, repeated LPS challenge was associated with immune and behavioral tolerance and a less inflammatory microglial profile compared to acute LPS
challenge. Overall, induction of glial cytokine expression and sickness behavior preceded morphological alterations in microglia after LPS challenge. Thus Iba-1 and GFAP labeling are unreliable readouts of “glial activation”.

Introduction

Microglia are innate immune cells and are involved in immune surveillance within the central nervous system (CNS) (Davalos et al., 2005). For example, microglia play an important role in receiving and propagating inflammatory signals in response to activation of the peripheral immune system activation (Nguyen et al., 2002; Skelly et al., 2013). This response allows for functional and coordinated communication between the immune system and the brain. For instance, a peripheral immune challenge with lipopolysaccharide (LPS) activates the innate immune system which promotes microglial activation and the production of cytokines and chemokines within the brain (Henry et al., 2009). This activation profile is consistent with a M1 profile of microglia and macrophages (Mosser and Edwards, 2008). Cytokine induction by microglia, including IL-1β, TNFα and IL-6, helps to propagate this immune derived signal within the brain and mediate physiological and behavioral responses (Dantzer, 2001; Dantzer et al., 2008). Chemokine induction by active microglia also propagates neuroinflammatory signals and may represent a mechanism by which resident microglia signal to peripheral immune cells (Carson et al., 2006; Cazareth et al., 2014; Fenn et al., 2014b; Puntambekar et al., 2011; Wohleb et al., 2013). In addition, microglial activation following peripheral immune challenge is also associated with an acute phase response, which involves the sequestration of iron that reduces the bioavailability of these nutrients to limit the growth of pathogens (Feelders et al., 1998; Parrow et al., 2013). Collectively, peripheral immune challenge elicits transient neuroinflammation, which is mediated by microglia, and represents a coordinated response between the immune system and brain.
Because of the inflammatory capacity of microglia, these cells are under tight regulation provided by anti-inflammatory cytokines, neuropeptides, and hormones (Biber et al., 2007; Rivest, 2009). This regulation helps ensure that activated microglia return to a surveying state after the resolution of immune challenge (Norden and Godbout, 2013). While there is considerable interest in M1 and M2 (regulation/repair) phases of activation (Mosser and Edwards, 2008), it is unclear if a shift from this M1 to M2 profile is required as inflammation resolves after immune challenge (Fenn et al., 2012a). In addition, acute phase response by microglia with iron sequestration may serve as a mechanism of neuroprotection because excess iron exacerbates tissue damage and worsens outcome after traumatic CNS injury (Sauerbeck et al., 2013). Another related issue in the exploration of microglial activation phases is that morphological analysis with Iba-1 immunoreactivity is used to report on their activation state. While microglia undergo cytoskeletal rearrangements that alter their morphology (Davalos et al., 2005), these morphological changes may not accurately represent an “active” inflammatory profile. For example, Cunningham et al. 2005 showed, in a model of prion disease, that LPS altered microglia morphology without significant changes in cytokine profile (Cunningham et al., 2005b).

Astrocytes are also active participants in both propagating and regulating neuroinflammation (Bian et al., 2013; Farina et al., 2007; Liu et al., 2011; Norden et al., 2014a). Astrocytes become activated by inflammatory mediators, engagement of toll-like receptors TLRs (Bsibsi et al., 2006; Carpentier et al., 2005; Gurley et al., 2008) and cytokines, including IL-1β (John et al., 2004). Once activated, astrocytes produce many regulatory factors that may influence CNS immunity and provide negative feedback to activated microglia (Min et al., 2006). For example, astrocytes are responsive to IL-10 and feed back on microglia using TGFβ (Norden et al., 2014a). Thus, a secondary phase
of activation mediated by astrocytes may help to regulate microglial responses after immune challenge. In this regard, microglia and astrocytes both contribute to acute phase, inflammatory, and regulatory responses after peripheral immune challenge. Similar to microglia assessment of activation by morphology, activation states of astrocytes are determined based on increased GFAP labeling. Although the inflammatory potential of astrocytes has been assessed after CNS trauma (Myer et al., 2006; Sofroniew, 2005; Voskuhl et al., 2009), the activation profile of astrocytes during peripheral immune challenge is less understood.

One consequence of microglia and astrocyte activation after peripheral immune challenge with LPS is the induction of sickness behavior. This cytokine mediated response of glia leads to the physiological and behavioral components of the sickness response including fever, hypophagia, lethargy, listlessness, decreased activity, and reduced social interaction (Dantzer et al., 2008). These behavioral changes are evolutionarily adaptive and necessary to reallocate the host’s resources and to fight infection (Berg et al., 2004; Bluthe et al., 2000a). Recent studies also indicate that repeated LPS injections enhance neuroprotective properties of microglia during traumatic CNS injury, further extending the interest in understanding glial activation profiles (Chen et al., 2014; Chen et al., 2012). Overall, a single or repeated LPS i.p. injection can be used to examine the biochemical and morphological profiles of astrocytes and microglia in the context of functional behavioral responses.

The objective of this study was to compare behavioral responses after LPS challenge to glia specific levels of cytokine expression and morphology. We show that increased pro-inflammatory cytokine expression by microglia correlated with active sickness behavior. De-ramification of microglia and increased Iba-1 immunoreactivity, however, was delayed following the LPS injection and corresponded with the resolution phase of microglial activation. While mRNA profiling also detected astrocyte activation
after LPS challenge, morphological alterations in astrocytes (GFAP immunoreactivity) were not detected at any time. Repeated LPS injection also increased Iba-1 immunoreactivity that corresponded with the resolution of microglial activation. In summary, Iba-1 and GFAP immunoreactivity are unreliable readouts of “glial activation”. Therefore, a multipronged approach is necessary to report on the activation state of microglia and astrocytes.

**Materials and Methods**

*Mice*

Adult (3-4 months-old) BALB/c mice were obtained from our breeding colony kept and in barrier-reared conditions in a specific-pathogen-free facility at The Ohio State University. Mice were individually housed in polypropylene cages and maintained at 25°C under a 12 h light/12 h dark cycle with *ad libitum* access to water and rodent chow. All procedures were in accordance with the National Institute of Health Guidelines for the Care and Use of Laboratory Animals and were approved by The Ohio State University Institutional Laboratory Animal Care and Use Committee.

*Peripheral injection of LPS*

In the first set of studies, adult mice received a single intraperitoneal (i.p.) injection of either saline or 10 µg LPS (approximately 0.33 mg/kg). The LPS dosage was selected because it elicits a pro-inflammatory cytokine response in the brain resulting in a transient sickness response in adult mice (Berg et al., 2004; Godbout et al., 2005b). Body weight, locomotor activity, social exploratory behavior, and cytokine mRNA expression were determined at various time points after injection.

In the second set of studies, adult mice received either a single or four repeated intraperitoneal (i.p.) injections of saline or 20 µg LPS (approximately 0.66 mg/kg). For
repeated LPS challenge, mice received one injection of LPS every 24 h for four consecutive days. This LPS dosage and time course were selected because they are similar to previous studies that have administered multiple repeated injections to investigate microglial activation profiles (Bodea et al., 2014; Cardona et al., 2006; Chen et al., 2012; Puntener et al., 2012).

**Locomotor activity**

Mice were maintained in their home cage with a floor area of 26 x 20 cm, and activity was video recorded for 5 minutes. On the video records, cages were divided into 6 identical virtual rectangles, and the number of line crossings was determined during the last 3 minutes. Baseline locomotor activity was measured prior to experimental treatment (time 0).

**Social exploratory behavior**

Social exploratory behavior was determined as a measure of sickness behavior as previously described (Godbout et al., 2005b). A novel juvenile was introduced into the test subject’s home cage for a 10-min period. Behavior was videotaped, and the cumulative amount of time the experimental subject engaged in social investigation of the juvenile (e.g., anogenital sniffing, trailing) was determined. Baseline social behavior was measured prior to experimental treatment (time 0). Results are expressed as the percent of time engaged in social behavior compared to baseline.

**Microglia and astrocyte isolation from brain**

Microglia and astrocytes were isolated from brain homogenates using a Percoll density gradient as previously described (Norden et al., 2014a). In brief, tissues were homogenized and cell pellets were re-suspended in 70% isotonic Percoll. A
discontinuous Percoll density gradient (70%, 50%, 35%, 0%) was layered and centrifuged for 20 min at 2000xg. Enriched microglia were collected from the interphase between 70% and 50% Percoll. Of the cells recovered from this Percoll interphase, approximately 90% of the cells were CD11b⁺/CD45low microglia. Less than 1.5% of the enriched cells were CD11b⁺/CD45high macrophages. Enriched astrocytes were collected from the interphase between 50% and 35% Percoll. Of the cells recovered from this Percoll interphase, approximately 70% of the cells were CD11bneg/GLAST-1⁺ astrocytes. The purity of the glia after Percoll experiments was consistent with our flow cytometric analysis of Percoll enriched glia (Henry et al., 2009; Norden et al., 2014a; Sawicki, 2014).

**Immunohistochemistry and digital image analysis**

Mice were deeply anesthetized and transcardially perfused with PBS followed by 4% formaldehyde. Brains were post-fixed in 4% formaldehyde for 24 h and cryoprotected in 20% sucrose in PBS for 48 h. Preserved brains were frozen using dry-ice cooled isopentane (-165°C) and sectioned (30 µm) using a Microm HM550 cryostat. Brain sections were identified by reference markers in accordance with the stereotaxic mouse brain atlas (Paxinos and Franklin, 2004). Iba-1 or GFAP staining was performed as previously described (Fenn et al., 2014a). In brief, free-floating sections were blocked and then incubated with rabbit anti-mouse Iba-1 antibody (Wako Chemicals) or rabbit anti-mouse GFAP antibody (Dako) overnight at 4°C. Sections were washed with PBS and incubated with Alexa Flour 488/594 secondary antibodies. Sections were mounted, coverslipped using Flourmount G, and left to dry at room temperature.

Fluorescent images were visualized using an epifluorescent Leica DM5000B microscope and captured using a Leica DFC300 FX camera and imaging software. To quantify the phenotypic changes of microglia, digital image analysis (DIA) of Iba-1 staining was performed (Donnelly et al., 2009) in the frontal cortex and hippocampus.
Phenotypic changes in astrocytes were quantified through DIA of GFAP staining in the hippocampus. For each mouse, 4-6 representative images were taken at 20x magnification of the PFC, and 10-12 representative images were taken at 20x magnification in the dentate gyrus, CA1, and CA3 regions of the hippocampus. A threshold for positive staining was determined for each image that included all cell bodies and processes, but excluded background staining (ImageJ). Results were reported as the average percent area in the positive threshold for all representative pictures.

Determination of IL-6 protein levels in plasma

IL-6 was determined from plasma using the BD OptEIA Mouse IL-6 ELISA according to the manufacturer’s instructions (BD Biosciences). In brief, 96-well enzyme immunoassay plates were coated with anti-mouse IL-6 capture antibody and incubated overnight at 4°C. Samples and IL-6 standards (0–1000 pg/ml) were added and incubated for 2 h at room temperature (RT). Plates were washed and incubated with biotinylated anti-mouse IL-6 antibody. Plates were washed and incubated with streptavidin-horseradish peroxidase conjugate. After 1 h incubation at RT, plates were washed and incubated with tetramethylbenzidine liquid substrate for 15 min. Reactions were terminated and absorbance was read at 450 nm using a Synergy HT Plate Reader (Bio-tek instruments). The assay was sensitive to 10 ng/ml IL-6 and the interassay and intra-assay coefficients of variation were less than 10%.

RNA isolation and RT-PCR

RNA was isolated from a 1 mm coronal brain section using the Tri-Reagent protocol (Sigma-Aldrich). For Percoll enriched microglia and astrocytes, RNA was isolated using the PrepEase kit (USB, CA). RNA was reverse transcribed to cDNA and
real-time (RT)-PCR was performed using the Applied Biosystems Taqman® Gene Expression Assay-on-Demand Gene Expression protocol. In brief, experimental cDNA was amplified by real-time PCR. Target cDNA (e.g., IL-1β, IL-6, CCL2) and reference cDNA (glyceraldehyde-3-phosphate dehydrogenase; GAPDH) were amplified simultaneously using an oligonucleotide probe with a 5’ fluorescent reporter dye (6-FAM). Fluorescence was determined on an ABI PRISM 7300-sequence detection system (Applied Biosystems). Data were analyzed using the comparative threshold cycle (Ct) method and results are expressed as fold difference.

Statistical Analysis

To ensure a normal distribution, data were subjected to the Shapiro-Wilk test using Statistical Analysis Systems (SAS) statistical software (Cary, NC). To determine significant main effects and interactions between main factors, single LPS injection data were analyzed using one-way (i.e., Saline and LPS) or two-way (i.e., LPS x Time) ANOVA using the General Linear Model procedures of SAS. Repeated LPS injection data were analyzed using one-way (i.e., Saline and LPS 1x and LPS 4x) or two-way (Group x Time) ANOVA using the General Linear Model procedures of SAS. When appropriate, differences between treatment means were evaluated by an F-protected t-test using the Least-Significant Difference procedure of SAS. All data are expressed as treatment means ± standard error of the mean (SEM). Values were considered to be significantly different at p-values < 0.05 and a tendency at p-values = 0.06 to 0.1.
Results

*Acute LPS challenge promoted a transient sickness response that was resolved within 48 h*

In glial biology, analysis of morphology is commonly used as a single measure to report on the activation state of microglia (de-ramification, Iba-1 labeling) and astrocytes (astrogliosis, GFAP labeling). Morphological analysis alone may not be a reliable index of inflammatory profile, especially in the context of activation profiles and their influence on behavioral or physiological processes. Therefore, the objective of this study was to compare behavioral responses after LPS challenge to glial specific levels of cytokine expression and to glial morphological profiles.

In the first study, adult male BALB/c mice were injected i.p. with LPS (10 µg) and plasma IL-6 levels and several indices of sickness behavior (e.g., weight loss, lethargy, social interaction) were determined over a 72 h time course. Fig. 2.1A shows that LPS injection reduced body weight in a time-dependent manner ($F_{(5,71)}=7.27$, $p<0.001$). For example, body weight was reduced by 4 h after LPS ($p<0.01$) and mice continued to lose body weight through 48 h. LPS challenge also decreased locomotor activity in a time-dependent manner ($F_{(6,87)}=7.33$, $p<0.001$, Fig. 2.1B). For example, LPS injection initially reduced locomotor activity (2-12 h post injection, $p<0.001$), but locomotion steadily increased after 12 h and reached baseline activity by 72 h. LPS injection had a similar time-dependent effect on social exploratory behavior ($F_{(6,74)}=5.71$, $p<0.001$, Fig. 2.1C). The largest reductions in social exploratory behavior were evident between 2 and 4 h following LPS ($p<0.001$, for each). Social exploratory behavior in LPS injected mice steadily increased after 4 h and was at baseline levels by 24 h. Overall, acute injection of LPS induced an active sickness response in which mice recovered to baseline social exploration by 24 h and baseline locomotor activity by 48 h.
LPS injection causes the acute phase response with the release of inflammatory cytokines into circulation, including IL-6 (Skelly et al., 2013). Therefore, IL-6 was determined in the plasma of mice used in the behavioral studies above. Fig. 1D shows that IL-6 was increased in the plasma 2-12 h after LPS challenge \((p<0.02)\). These elevated IL-6 levels returned to baseline by 24 h after LPS and were undetectable 48 and 72 h after LPS (data not shown). Thus, there is a transient increase in plasma IL-6 after peripheral LPS injection.

**Figure 2.1.** Acute LPS challenge promoted a transient sickness response that was resolved within 48 h. Adult BALB/c mice were injected (i.p.) with saline or LPS \((10 \mu g)\). Over a 72 h time course after injection of saline or LPS, A) body weight, B) locomotor activity, and C) social exploratory behavior were determined. Data expressed as percent change from baseline values. D) IL-6 protein levels were determined in plasma collected 2, 4, 12, 24, and 48 h after LPS. Means with (*) are different from saline controls \((p<0.05)\) and means with (+) tend to be different from saline controls \((p=0.06-0.10)\).
Acute LPS challenge induced concomitant induction of inflammatory, regulatory and acute phase mediators in the brain

Using a similar time course as the sickness behavior, the mRNA expression of several inflammatory, regulatory and acute phase genes were determined in the brain 2, 4, 12, 24, and 48 h after LPS injection. To assess the overall inflammatory status of the brain, a coronal brain section was collected. In these samples, mRNA expression of several pro-inflammatory (IL-1β, IL-6, and TNFα), regulatory (IL-4Ra, IL-10, YM-1, TGFβ), and acute phase mediators (Serum amyloid 3, Ceruloplasmin, Haptoglobin) was determined (Table 2.1).

The mRNA levels of two markers associated with the identification of microglia (Iba-1) or astrocytes (GFAP) were determined. Iba-1 expression was increased in the brain 24 h after LPS (p<0.01) and GFAP expression was increased 12-24 h after LPS (p<0.01). Overall, the induction of these markers were modest and were not detectable until 24 h after LPS.

Similar to the enriched glia, brain expression of IL-1β, IL-6, TNFα showed a significant increase beginning at 2 h and continued through 4 h for IL-6 and 12 h for both IL-1β and TNFα (p<0.05). These pro-inflammatory mRNA profiles in the brain are similar to the profiles detected in microglia specifically. Two of the regulatory mediators had a rapid induction and two had a delayed induction after LPS. Consistent with the glia specific data, there was an early induction of IL-10 at 2 h (p<0.01) and IL-10 mRNA levels returned to baseline by 12 h. IL-4Ra was also increased by 2 h (p<0.03) and was maintained for 12 h after LPS (p<0.01). The upregulation of the other two regulatory mediators, TGFβ and YM-1, however, was delayed until 12-24 h after LPS (p=0.1). These data are consistent with previous findings that astrocytes increase TGFβ mRNA expression during resolution of inflammation following LPS (Norden et al., 2014a).
The acute phase proteins Saa3, CeruP, and HaptoG followed a similar time course. Saa3 was induced at 4 h \( (p=0.1) \), peaked at 12 h \( (p<0.01) \) and remained elevated 24 h after LPS \( (p<0.05) \). CeruP and HaptoG were increased by 4 h after LPS \( (p<0.01) \) and were maintained 24 h later \( (p=0.08) \). Overall, inflammatory, regulatory, and acute phase mediators were all enhanced in the brain by 4 h after LPS. Three mediators \( (\text{TGF} \beta, \text{YM}-1, \text{and Saa3}) \) all showed a delayed induction after LPS and were still elevated 24 h later.

<table>
<thead>
<tr>
<th>Brain mRNA levels (Fold Δ)</th>
<th>Gene</th>
<th>Saline</th>
<th>LPS (2 h)</th>
<th>LPS (4 h)</th>
<th>LPS (12 h)</th>
<th>LPS (24 h)</th>
<th>LPS (48 h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Label</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Iba-1</td>
<td>1.00 ± 0.06</td>
<td>0.92 ± 0.09</td>
<td>0.94 ± 0.18</td>
<td>0.88 ± 0.04</td>
<td>2.00 ± 0.22</td>
<td>1.36 ± 0.08*</td>
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</tr>
<tr>
<td>GFAP</td>
<td>1.03 ± 0.12</td>
<td>1.22 ± 0.12</td>
<td>1.24 ± 0.08</td>
<td>1.78 ± 0.16</td>
<td>1.92 ± 0.10</td>
<td>1.32 ± 0.22</td>
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</tr>
<tr>
<td>IL-1β</td>
<td>1.02 ± 0.09</td>
<td>13.8 ± 1.44*</td>
<td>24.9 ± 3.64*</td>
<td>9.58 ± 2.07</td>
<td>4.16 ± 0.45</td>
<td>3.78 ± 0.39</td>
<td></td>
</tr>
<tr>
<td>IL-6</td>
<td>1.00 ± 0.05</td>
<td>51.6 ± 12.5*</td>
<td>18.2 ± 1.27*</td>
<td>1.27 ± 0.14</td>
<td>1.10 ± 0.20</td>
<td>0.93 ± 0.05</td>
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</tr>
<tr>
<td>TNFα</td>
<td>1.03 ± 0.10</td>
<td>9.08 ± 1.48*</td>
<td>13.6 ± 3.74*</td>
<td>9.43 ± 2.20</td>
<td>3.54 ± 0.89</td>
<td>2.74 ± 0.57</td>
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<tr>
<td>IL-4Ra</td>
<td>1.04 ± 0.14</td>
<td>1.91 ± 0.14*</td>
<td>4.89 ± 0.55*</td>
<td>2.77 ± 0.46</td>
<td>1.66 ± 0.13</td>
<td>1.50 ± 0.12</td>
<td></td>
</tr>
<tr>
<td>IL-10</td>
<td>1.24 ± 0.35</td>
<td>15.9 ± 1.50*</td>
<td>10.5 ± 2.59*</td>
<td>3.29 ± 0.85</td>
<td>2.09 ± 0.48</td>
<td>1.23 ± 0.39</td>
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</tr>
<tr>
<td>TGFβ</td>
<td>1.00 ± 0.04</td>
<td>0.89 ± 0.05</td>
<td>0.90 ± 0.07</td>
<td>0.97 ± 0.05</td>
<td>1.36 ± 0.07</td>
<td>1.41 ± 0.12*</td>
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<tr>
<td>YM-1</td>
<td>1.37 ± 0.47</td>
<td>1.38 ± 0.26</td>
<td>3.17 ± 0.91</td>
<td>12.3 ± 4.21</td>
<td>5.54 ± 2.23*</td>
<td>1.06 ± 0.46</td>
<td></td>
</tr>
<tr>
<td>Saa3</td>
<td>1.37 ± 0.47</td>
<td>15.3 ± 3.78</td>
<td>66.3 ± 9.58*</td>
<td>231 ± 97.8</td>
<td>91.5 ± 16.2</td>
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<tr>
<td>CeruP</td>
<td>1.02 ± 0.07</td>
<td>2.43 ± 0.21*</td>
<td>3.45 ± 0.47*</td>
<td>2.61 ± 0.41</td>
<td>1.63 ± 0.19</td>
<td>1.11 ± 0.13</td>
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<tr>
<td>HaptoG</td>
<td>1.12 ± 0.21</td>
<td>3.30 ± 0.51</td>
<td>4.99 ± 0.31*</td>
<td>14.38 ± 3.30</td>
<td>5.60 ± 0.78*</td>
<td>2.23 ± 0.37</td>
<td></td>
</tr>
</tbody>
</table>

Table 2.1. Acute LPS challenge induced concomitant induction of inflammatory, regulatory and acute phase mediators in the brain. Adult mice were injected (i.p.) with saline or LPS \( (10 \mu g) \) and a 1 mm coronal brain section was collected 2, 4, 12, 24, and 48 h later. The mRNA expression of several markers associated with “Label” (Iba-1, GFAP) “Inflamm,” (IL-1b, TNFa, IL-6) “Regulatory,” (IL-4Ra, IL-10, TGFβ, YM-1) and “Acute Phase” (Saa3, CeruP, HaptoG) were determined. Means with (*) are different from saline controls \( (p<0.05) \) and means with (+) tend to be different from saline controls \( (p=0.06-0.10) \).

Rapid microglial cytokine induction after acute LPS challenge preceded astrocyte cytokine expression

We next sought to determine the mRNA profile of astrocytes and microglia over a 48 h time course after a single LPS challenge. Here mice were injected with 10 µg LPS and glial mRNA profiles were determined 2, 4, 12, 24, or 48 h later. Microglia and
astrocytes were isolated and enriched using a Percoll density gradient (Fig.2.2A), and mRNA levels of IL-6, IL-1β, TNFα, CCL2, IL-10 and TGFβ were determined.

Fig.2.2B shows representative bivariate dot plots of CD11b and CD45 labeling of Percoll enriched microglia and CD11b and Glast-1 labeling of Percoll-enriched astrocytes. As we have previously reported (Henry et al., 2009; Norden et al., 2014a), isolation from the 70-50 phase of Percoll preferentially enriched (over 85%) for microglia (CD11b+/CD45low). CNS macrophages (CD4 

high) were also present in the 70-50 interphase of Percoll but were less than 2% and this percentage of CD45 

high cells was not influenced by LPS injection. Furthermore, isolation from the 50-35 interphase phase of Percoll preferentially enriched (over 70%) for astrocytes (CD11b-/Glast-1).

In enriched microglia there was a rapid response to LPS with induction of pro-inflammatory cytokines IL-6, IL-1β and TNFα (main effect LPS F(5,40)=6.78, p<0.001, for each). Fig.2.2C shows an initial and peak 10-fold induction of IL-6 at 2 h (p<0.01). This higher IL-6 mRNA induction in microglia was maintained at 4 h (p<0.01) but was back to baseline expression by 12 h. Similarly, IL-1β mRNA induction in microglia was robustly increased by 40 fold at 2 h after LPS injection (p<0.01) and was highest with an 80 fold induction at 4 h (p<0.01, Fig.2.2D). In addition, microglial induction of IL-1β remained elevated 12 h after LPS (p<0.05) but was back to baseline expression by 24 h. Levels of TNFα and chemokine CCL2 mRNA were also elevated by 2 h in enriched microglia (p<0.01, for each) and were maintained up to 12 and 24 h after LPS, respectively (p<0.05, Figs.2.2E&F). In microglia, the peak of pro-inflammatory cytokine expression occurred 2 to 4 h after LPS injection and was back to similar levels as saline injected controls by 24 h.

There was also a rapid response to LPS with induction of the anti-inflammatory cytokine IL-10 in enriched microglia. Fig.2.2G shows an initial microglial induction of IL-
10 by 2 h \((p<0.05)\) after LPS. A 20 fold mRNA induction of IL-10 in microglia was maintained 4 h after LPS \((p<0.01)\) and tended to be higher up to 24 h later compared to saline injected controls \((p=0.10)\). TGF\(\beta\), another important anti-inflammatory cytokine, was not induced in enriched microglia by LPS injection (Fig.2.2H). These data show concomitant induction of IL-10 in microglia with other pro-inflammatory mediators including IL-1\(\beta\), IL-6, and TNF\(\alpha\).

In enriched astrocytes, LPS challenge also increased the mRNA expression of several inflammatory mediators in a time-dependent manner (Figs.2.2I-K). Fig.2.2I shows a modest induction of IL-6 mRNA in enriched astrocytes that was only elevated 2 h after LPS \((p<0.01)\). In addition, IL-1\(\beta\) mRNA in astrocytes was detected by 2 h \((p<0.03)\) after LPS and was highest at the 12 h time point \((p<0.01, \text{Fig.2.2J})\). IL-1\(\beta\) mRNA in astrocytes also tended to be higher 48 h later \((p=0.10)\). Fig.2.2K shows a robust 15-fold induction of TNF\(\alpha\) in enriched astrocytes at 12 h after LPS \((p<0.01)\). This up-regulation was maintained 24 h after LPS \((p<0.01)\), but returned to baseline expression by 48 h after LPS. Similar to the TNF\(\alpha\) induction, CCL2 induction was also robust (20-fold) in astrocytes at 12 h after LPS \((p<0.01)\) and was maintained 24 h later \((p<0.01, \text{Fig.2.2L})\). In astrocytes, the peak of pro-inflammatory cytokine expression occurred 12 h after LPS injection and was back to similar levels as saline injected controls by 48 h.

Contrary to the data from enriched microglia, there was not a significant induction of IL-10 in astrocytes after LPS i.p. challenge (Fig.2.2M). The mRNA expression of TGF\(\beta\) tended to be reduced in enriched astrocytes 2 and 4 h after LPS injection \((p=0.1, \text{Fig.2.2N})\). At 24 h following LPS, however, the expression of TGF\(\beta\) was significantly increased compared to saline injected controls \((p<0.05, \text{Fig.2.2N})\). Taken together, an acute LPS (i.p.) challenge was sufficient to induce cytokine expression in microglia and
astrocytes. In addition, the peak of pro-inflammatory cytokine and chemokine expression in astrocytes occurred between 12 and 24 h after LPS injection, which was delayed compared to the robust induction of cytokines in microglia at 2-4 h.
Figure 2.2. Rapid microglial cytokine induction after acute LPS challenge preceded astrocyte cytokine expression. Adult mice were injected (i.p.) with saline or LPS (10 μg) and A) astrocytes and microglia were isolated and Percoll-enriched 2, 4, 12, 24, and 48 h later. B) Representative bivariate dot plots are shown for CD11b and CD45 labeling of microglia and CD11b and Glast-1 labeling of astrocytes collected after Percoll enrichment. Levels of C) IL-6, D) IL-1β, E) TNFα, F) CCL2, G) IL-10, and H) TGFβ mRNA were determined in enriched microglia. The expression levels for the same markers I) IL-6, J) IL-1β, K) TNFα, L) CCL2, M) IL-10, and N) TGFβ were also determined in Percoll-enriched astrocytes. Means with (*) are different from saline controls (p<0.05) and means with (+) tend to be different from saline controls (p=0.06-0.10).
Fig. 2. Percoll Gradient Separation:

A. Percoll Gradient Separation:
   - 0%
   - 35%
   - 50%
   - 70%
   - Astrocyte Analysis
   - Microglia Analysis

B. Microglia (Saline)
   - CD45
   - CD11b

C. Microglia (LPS-24 h)
   - IL-6 mRNA (Fold Δ)
   - IL-10 mRNA (Fold Δ)
   - CCL2 mRNA (Fold Δ)

D. Astrocytes (Saline)
   - GLAST-1

E. Astrocytes (LPS-24 h)
   - CD11b
   - CD45

F. Microglia (LPS-24 h)
   - IL-6 mRNA (Fold Δ)

G. Microglia (LPS-24 h)
   - IL-10 mRNA (Fold Δ)

H. Microglia (LPS-24 h)
   - TNFα mRNA (Fold Δ)

I. Astrocytes (Saline)
   - IL-6 mRNA (Fold Δ)

J. Astrocytes (LPS-24 h)
   - IL-10 mRNA (Fold Δ)

K. Astrocytes (LPS-24 h)
   - TNFα mRNA (Fold Δ)

L. Astrocytes (LPS-24 h)
   - CCL2 mRNA (Fold Δ)
Iba-1 immunoreactivity of microglia was increased in the cortex and hippocampus 24 and 48 h after LPS challenge while GFAP immunoreactivity of astrocytes in the hippocampus was unaffected.

Here we show evidence of glial induction of cytokines within 2 h of a peripheral LPS injection that corresponded with an active sickness behavioral response. Therefore, we next sought to determine morphological alterations in microglia and astrocytes over the same time course. Representative labeling for either Iba-1 (cortex) or GFAP (hippocampus) is shown 0, 4, 12, 24, 48 and 72 h after LPS injection (Fig. 2.3A&B). It is important to note that GFAP labels astrocytes in the white matter tracts and the hippocampus of mice but does not label astrocytes in the cortex (Cahoy et al., 2008; Zhang and Barres, 2010). The hippocampus and cortex were selected for microglial morphological analysis because we have previously shown that microglia have increased Iba-1 immunoreactivity in these regions after peripheral LPS challenge (Wohleb et al., 2012). Fig. 2.3A shows that LPS injection was not associated with increased Iba-1 immunoreactivity at either 4 or 12 h. In fact, these microglia had a similar Iba-1 proportional area as the saline controls (Fig. 2.3C). At 24 and 48 h following LPS, however, there was a significant increase in Iba-1 immunoreactivity of microglia (p<0.05, Fig. 2.3A&C). Microglia at 24 and 48 h after LPS had larger cell bodies and thicker processes, consistent with a de-ramified morphological profile. Similar to the frontal cortex, increased Iba-1 proportional area was evident in microglia of the hippocampus 24-72 h after LPS (p<0.05, Fig. 2.3D). For astrocytes, there were neither differences in morphology differences (Fig. 2.3B) nor GFAP immunoreactivity at any time after LPS injection (Fig. 2.3E). Thus, immunoreactivity of Iba-1 was evident 24-48 h after LPS but delayed compared to the rapid cytokine mRNA induction. In addition, although there was a detectable increase in cytokine expression in enriched astrocytes after LPS
but there were no differences in GFAP labeling detected in astrocytes over the same time course.

Figure 2.3. Iba-1 immunoreactivity of microglia increased 24 and 48 h after acute LPS challenge while GFAP immunoreactivity of astrocytes was unaffected. Adult BALB/c mice were injected i.p. with saline or LPS (10 μg) and mice were perfused and PFA fixed 4, 12, 24, 48, and 72 h later. Brains were post-fixed, frozen, and sectioned, and Iba-1 expression was determined in the A) frontal cortex (shown above) and hippocampus (not shown). B) GFAP expression was determined in the hippocampus. White arrows indicate the enlarged insert of a representative cell. Proportional area of Iba-1 labeling in the C) frontal cortex and D) hippocampus. E) Proportional area of GFAP in the hippocampus. Means with (*) are different from saline controls (p<0.05).
Acute LPS challenge corresponded to a more pronounced active sickness response over 24 h compared to repeated LPS challenges

Our data indicate that an acute LPS i.p. challenge was sufficient to rapidly activate both microglia and astrocyte to induce cytokine expression. Nonetheless, there was no correlation between cytokine expression and morphological changes in glia after acute LPS challenge. Other studies have used a repeated LPS injection paradigm (i.e., LPS pre-conditioning) to investigate microglial activation and neuroprotective properties (Cardona et al., 2006; Chen et al., 2014; Chen et al., 2012). Therefore, we next sought to determine the degree to which an acute LPS injection differed from the repeated LPS injection in the context of sickness behavior, glial cytokine induction, and morphological changes. To be consistent with other repeated LPS injection models we used a higher dosage of LPS at 20ug per mouse, which was twice as high as the LPS dosage used in Figs.2.1-3. In these studies (Fig.2.4A), mice received either one (LPS 1x) or four daily injections (LPS 4x) of LPS (20 ug).

The percentage of body weight loss was determined over the 4 days of repeated LPS injections. Fig.2.4B shows mice injected repeatedly with LPS lost body weight ($p<0.01$). During the course of four daily injections, however, loss of body weight occurred only during the first two days. After two days of LPS injections, the body weight loss was maintained and not further reduced. Next, social exploratory behavior was determined at baseline and again 4, 12 and 24 h after the last LPS injection. In the social exploratory test, LPS 1x mice showed a time dependent reduction in social behavior after LPS injection ($F_{(2,66)}=23.36$, $p<0.01$, Fig.2.4C). In these mice, social exploratory behavior was reduced 4 and 12 h after LPS ($p<0.01$, for each) but was resolved by 24 h. This finding is similar to the transient behavioral response induced by 10 ug of LPS (Fig.2.1D). In the LPS 4x mice, however, there was no significant reduction in social
exploratory behavior at 4, 12, or 24 h. Thus, the LPS 4x mice no longer had any “active sickness response” by 4 h after the 4th injection of LPS.

To access the peripheral cytokine response, IL-6 levels were determined in the plasma 24 h after the last injection of LPS. Plasma levels IL-6 were elevated 24 h after LPS 1x (approximately 100 pg/ml) compared to saline controls (p<0.01, Fig.4.4D). There was significantly less IL-6 present in the plasma by 24 h after the last injection in the LPS 4x mice and these levels were not different from saline controls. Overall, LPS 1x mice had evidence “active sickness response” 4-12 h later but these differences in behavioral and were no longer evident after the 4th repeated injection of LPS.
A more pronounced active inflammatory response was evident in the brain 24 h after acute LPS challenge compared to repeated LPS challenges.

The behavioral data in Fig. 2.5 shows that while LPS 1x mice had a sickness response 4-12 h after LPS, LPS 4x mice no longer had any “active sickness response” by 4 h after the 4th injection of LPS. After completion of the behavioral testing (24 h), a coronal brain section was collected to assess the overall inflammatory status of the brain (Fig. 2.5A). In these samples, mRNA expression of several pro-inflammatory (IL-1β and TNFα), regulatory (IL-4Rα, YM-1, TGFβ), and acute phase genes (Saa3, CerulP,
HaptoG) were determined. Fig.2.5A shows that mRNA levels of IL-1β were elevated in both LPS 1x and LPS 4x mice ($p<0.01$) but were highest in LPS 1x mice ($p<0.02$). TNFα was elevated (21 fold) in the brain of LPS 1x mice ($p<0.01$) but was reduced (3 fold) in LPS 4x mice towards saline levels. The regulatory gene, IL-4Rα was also highest in the LPS 1x mice compared to all other groups ($p<0.01$). YM-1 and TGFβ were increased following both one and four injections of LPS ($p<0.01$). Similarly, the genes associated with acute phase response, Saa3, CerulP, and HaptoG, were increased in the brain 24 h after the last injection of LPS 1x and LPS 4x mice ($p<0.05$, for all).

Next, mRNA expression of two inflammatory mediators (IL-1β and TNFα), two regulatory genes (IL-4Rα and YM-1), and one acute phase gene (HaptoG) were determined in Percoll-enriched microglia. In enriched microglia, IL-1β mRNA was still elevated at 24 h in LPS 1x mice ($p<0.01$) but these IL-1β levels were attenuated in LPS 4x mice compared to saline controls ($p=0.06$, Fig.2.5B). In addition, TNFα was still increased 24 h after LPS in the LPS 1x mice ($p<0.03$) but these levels were no longer induced in the LPS 4x mice (Fig.2.5C). Fig.2.5D shows that IL-4Rα was still elevated 24 h after LPS compared to saline controls in LPS 1x mice ($p<0.04$) but these levels were attenuated in LPS 4x mice. Also, YM-1 mRNA levels tended to be increased 24 h after LPS in 1x mice ($p=0.1$) and these levels were augmented in LPS 4x mice compared to all other treatment groups ($p<0.02$, Fig.2.5E). The acute phase protein HaptoG was also elevated in both LPS 1x ($p<0.02$) and LPS 4x mice and these levels were augmented in LPS 4x mice compared to all other treatment groups ($p<0.01$, Fig.2.5F). Taken together, the expression of pro-inflammatory genes was decreased while the mRNA expression of YM-1 and HaptoG were enhanced in microglia from mice with repeated LPS injections.
A. Brain mRNA levels (Fold Δ)

<table>
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<tr>
<th></th>
<th>24 h</th>
<th>Saline</th>
<th>LPS 1x</th>
<th>LPS 4x</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inflam IL-1β</td>
<td>1.00</td>
<td>± 0.05</td>
<td>16.1 ± 1.90*</td>
<td>11.1 ± 1.01‡</td>
</tr>
<tr>
<td>Inflam TNFα</td>
<td>1.05</td>
<td>± 0.14</td>
<td>21.6 ± 2.64*</td>
<td>3.62 ± 0.42‡</td>
</tr>
<tr>
<td>Regulatory IL-4Rα</td>
<td>1.01</td>
<td>± 0.07</td>
<td>2.87 ± 0.10*</td>
<td>1.51 ± 0.12‡</td>
</tr>
<tr>
<td>Regulatory YM-1</td>
<td>1.19</td>
<td>± 0.33</td>
<td>44.1 ± 8.22*</td>
<td>36.5 ± 8.02‡</td>
</tr>
<tr>
<td>Regulatory TGFβ</td>
<td>1.00</td>
<td>± 0.02</td>
<td>1.37 ± 0.08*</td>
<td>1.23 ± 0.03‡</td>
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<tr>
<td>Acute Phase Saa3</td>
<td>1.12</td>
<td>± 0.21</td>
<td>2318 ± 323*</td>
<td>1585 ± 486‡</td>
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<tr>
<td>Acute Phase CerulP</td>
<td>1.00</td>
<td>± 0.04</td>
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<td>Acute Phase HaptoG</td>
<td>1.20</td>
<td>± 0.27</td>
<td>13.7 ± 0.83*</td>
<td>16.1 ± 3.32‡</td>
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Figure 2.5. A more pronounced active inflammatory response was evident in the brain 24 h after acute LPS challenge compared to repeated LPS challenge. Adult BALB/c mice were injected (i.p.) with saline, LPS 1x (20 μg), or LPS 4x (20 μg) and a 1 mm coronal brain section was collected 24 h after the last injection. A) The mRNA expression of several markers associated with "Inflam," (IL-1β, TNFα) “Regulatory,” (IL-4Rα, TGFβ, YM-1) and “Acute Phase” (Saa3, CerulP, HaptoG) were determined. In the same mice, mRNA levels of B) IL-1β, C) TNFα, D) IL-4Rα, E) YM-1, and F) HaptoG were determined in enriched microglia. Means with (*) are different from saline controls (p<0.05) and means with (‡) are different from LPS 1x mice (p<0.05).
Increased Iba-1 immunoreactivity of microglia in the cortex and hippocampus 24 h after acute and repeated LPS challenge with no change of GFAP immunoreactivity of astrocytes

The mRNA and behavioral data indicate that the LPS 4x mice do not have an active sickness response and the microglia have more of a resolution phase mRNA profile compared to the LPS 1x mice. The next question was to determine the degree to which morphology difference correlated with the resolution phenotype. Here we determined the morphological inflammatory profile of astrocytes and microglia 24 h after the last LPS injection (Fig.2.6A). Fig.2.6A shows representative Iba-1 labeling in the cortex of saline (0 h) and LPS (1x, 4x) injected mice 24 h after the final LPS injection. Microglia of both LPS 1x and LPS 4x mice had larger cell bodies and thicker processes. There was a significant increase in Iba-1 immunoreactivity of microglia following either LPS 1x or LPS 4x injection in both the frontal cortex ($p<0.05$, Fig. 2.6B) and hippocampus ($p<0.05$, Fig.2.6C) at 24 h following the last LPS injection. There was no difference in Iba-1 immunoreactivity of microglia between mice injected one or four days with LPS. Therefore, independent of repeated injection, microglia 24-48h after LPS had increased Iba-1 immunoreactivity.

Fig.2.6B shows representative GFAP labeling in the hippocampus of saline (0 h) and LPS (1x, 4x) injected mice 24 h after the last LPS injection. Again, there was no difference in GFAP morphology or immunoreactivity of astrocytes following one or four repeated LPS injections (Fig.2.6B&D). This is consistent with the data in Fig.2.3 with the LPS 10 ug injection. Overall, these data show that while there were behavioral differences and mRNA profile differences between one and four repeated LPS injected mice, there were no differences in glial morphology.
Figure 2.6. Similar increased Iba-1 immunoreactivity of microglia was detected at 24 h in the cortex and hippocampus after acute and repeated LPS challenge. Adult BALB/c mice were injected i.p. with saline, LPS 1x (20 µg), or LPS 4x (20 µg) and mice were perfused and PFA fixed 24 h later. Brains were post-fixed, frozen, and sectioned and Iba-1 and GFAP protein expression was determined. Representative images of labeling for A) Iba-1 and B) GFAP in the hippocampus are shown. White arrows indicate the cell represented in the inset. Proportional area of Iba-1 labeling in the C) hippocampus and D) cortex. E) Proportional area of GFAP labeling in the hippocampus. Means with (*) are different from saline controls (p<0.05).

Discussion

There is significant interest in understanding the role of glial cells under homeostatic and inflammatory conditions. Emerging evidence indicates that microglia and astrocytes may have specific and dynamic roles during immune activation. One issue in glia biology, however, is that morphological analysis alone is used to report on...
the activation state of microglia and astrocytes. Here we show that elevated cytokine expression by enriched microglia correlates to active sickness behavior and precedes alterations in glial morphology. For example, microglia had increased inflammatory cytokine mRNA expression during the active sickness behavior phase (Fig. 2.7). Increased Iba-1 immunoreactivity and morphological alterations in microglia, however, were delayed and were only evident during the resolution/recovery phase. While astrocytes also had increased inflammatory mRNA expression, these were delayed compared to microglia and there was no increase in GFAP immunoreactivity at any timepoint after LPS. Moreover, cytokine induction in glia also did not correlate with morphological changes after repeated LPS challenge. Thus, this study highlights the importance/necessity of using a multi-pronged approach to analyze glial activation.
Glial Activation after LPS i.p. challenge

<table>
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<tr>
<th>Timing</th>
<th>Behavior</th>
<th>Microglia Profile</th>
<th>Microglia Morphology</th>
<th>Microglia Biochem</th>
<th>Astrocyte Morphology</th>
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<td>2-4 h post LPS</td>
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<td>Active Sickness Behavior</td>
<td>Resolving Sickness Behavior</td>
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<td>“Active Phase” Microglia</td>
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<td>++IL-1β, TNFa, IL-6, CCL2 +IL-4Rα, IL-10 ++Acute phase</td>
<td>+IL-1β, TNFa, IL-6, CCL2 +IL-4Rα, IL-10, YM-1 ++Acute phase</td>
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<td>-TGFβ</td>
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Figure 2.7. Time course of biochemical and morphological alterations of microglial and astrocyte activation in the context of LPS-induced sickness behavior. This illustration summarizes the biochemical and morphological alterations of microglia and astrocytes after peripheral LPS challenge. Activation: Microglia were rapidly and robustly activated at 2-4 h after LPS to express cytokines, chemokines and acute phase mediators. This microglia activation was associated with a ramified Iba-1 morphology and an active sickness behavioral response. Astrocytes had a down regulation of TGFβ, limited cytokine induction and no alteration in GFAP morphology during this same 2-4 h time frame. Transition: By 12 h after LPS the profile of microglia was attenuated and this was associated with a ramified Iba-1 morphology and a resolving sickness behavioral response. Astrocytes had a peak activation of cytokine and chemokine expression 12 h after LPS but no alteration in GFAP morphology. Resolution: By 24-48 h after LPS microglia had baseline expression of cytokines and chemokines, but also had some level of maintenance of IL-10, YM1 and acute phase genes in acute and repeated LPS injection. This regulatory profile, however, was augmented by repeated LPS injection. In both cases, this resolution phase of microglia was associated with a de-ramified Iba-1 morphology and the resolution of sickness behavior. Again, astrocytes had an altered gene expression profile after LPS, but no changes were detected in their GFAP morphology.

One important component of this study is that LPS-induced sickness behavior is cytokine (IL-1, IL-6, TNF) mediated, (Bluthe et al., 2000a; Bluthe et al., 2000b; Kelley et al., 2003; Laye et al., 2000; Skelly et al., 2013) so it provides a functional basis to compare glial mRNA, morphological, and behavioral profiles to each other. We first show that genes associated with M1 (IL-1β, TNFα, IL-6), M2 (IL-10, IL-4Rα, YM-1) and acute phase (Saa3, CeruLP, HaptoG) responses were increased following LPS injection in
coronal brain sections. Consistent with previous studies, M2 markers IL-4Rα and IL-10 were significantly up-regulated alongside pro-inflammatory markers during active sickness behavior 2-4 h (Fenn et al., 2012a; Henry et al., 2009; Sierra et al., 2007). Overall there was not a clear shift from M1 to M2 markers with the resolution of LPS-induced sickness behavior. Nonetheless, there was a time dependent transition from an active to a resolution phase of glial activation, which is outlined in the diagram in Fig.7. For example, there was a persistence of regulatory factors including IL-10, YM-1 and HaptoG in microglia and TGFβ in astrocytes during the resolution phase. Despite these data, understanding differences in M1 and M2 activation profiles of microglia is relevant in neurotrauma, aging, and disease (Cunningham, 2013; Kumar et al., 2013; Norden et al., 2014b). For instance, a shift towards an M1 profile of microglia with age or after TBI is detrimental to recovery from LPS challenge and is associated with amplified microglia activation and prolonged sickness and depressive-like behaviors (Fenn et al., 2014a; Godbout et al., 2005b; Godbout et al., 2008; Wynne et al., 2010). In addition, reduced M2 microglia and macrophage responses after TBI or SCI was associated with more tissue damage and reduced functional recovery in aged rodents (Fenn et al., 2014b; Kumar et al., 2013). Overall, active sickness behavior after LPS was associated with parallel induction of M1 and M2 related genes.

Our data also highlight a direct association between the timing of microglial specific induction pro-inflammatory cytokines and the sickness behavior. As expected, LPS i.p. injection (10 or 20 μg) elicited a transient sickness behavioral response that was evident within 2 h and was resolved within 24 h (Berg et al., 2004). Sickness behavior at the acute time points (2-4 h) corresponded with heightened pro-inflammatory cytokine expression specifically by microglia. It is important to highlight that these cytokine mRNA data from enriched microglia are consistent with the data obtained from the coronal brain section. Because the glia contained in this section were not subjected to Percoll, this
limits the concern that Percoll isolation alone activates glia. Although mRNA expression was determined in this study, we have previously detected correspondingly higher protein levels of IL-1β in microglia 4 h after LPS (Henry et al., 2009). Moreover, we have previously reported rapid changes in surface expression of chemokine and cytokine receptors of active microglia. Consistent with the mRNA data presented here, LPS injection caused a substantial increase in surface expression of IL-4Rα protein at 4 h that was maintained 24 h after LPS (Fenn et al., 2012). In addition, we have reported a robust reduction of CX3CR1 (fractalkine receptor) on the surface of microglia at 4 h after LPS that was associated with higher intracellular expression of IL-1β protein (Wynne et al., 2010). In addition, CX3CR1 surface expression on microglia of adult mice returned to baseline at 24 h, parallel with resolution of pro-inflammatory cytokine expression and the recovery from sickness behavior (Wynne et al., 2010). Overall, the timing of microglial cytokine induction reflects active sickness behavior.

Novel data also shows that the peak of inflammatory cytokine expression by astrocytes occurred at 12 h after LPS and was delayed compared to microglia. This second phase mediated by astrocytes was associated with increased CCL2, TNFα, and TGFα. These data are interpreted to indicate that microglia become activated first, and that this is followed by astrocyte activation. Notably, astrocyte induction of CCL2 and TNFα was delayed but the relative fold induction was high and similar to what was detected in microglia. Part of this secondary activation of astrocytes with higher TGFβ expression may be to feedback on active microglia. For example, our previous work indicates that IL-10 produced by microglia stimulates astrocytic TGFβ, which in turn provides negative feedback on microglial activation (Norden et al., 2014a). Inhibition of TGFβ signaling after LPS injection was associated with prolonged microglial activation and protracted sickness behavior (Norden et al., 2014a). Here again we detected
delayed induction of TGFβ mRNA in astrocytes, but no relative induction in microglia. Also consistent with our previous study, microglia had strong induction of IL-10, but astrocytes did not upregulate IL-10 at any timepoint after LPS. Overall, we show key differential expression and timing of cytokine expression between microglia and astrocytes that are interpreted to indicate dynamic communication between two cell types.

Another relevant aspect of this study is the lack of connection between the morphological profile of glia and the corresponding increased inflammatory mRNA profile after LPS challenge. Importantly, neither behavior nor mRNA profiles of microglia corresponded with the increased de-ramified Iba-1 morphology of microglia. During the active cytokine expression phase 2-12 h after LPS, microglia from saline and LPS injected mice displayed similar Iba-1 immunoreactivity. Increased Iba-1 immunoreactivity and de-ramification was not detected in the hippocampus and cortex until 24-48 h after LPS. On an mRNA level, Iba-1 expression also had a delayed induction following LPS (24-48 h). These data are consistent with a previous study of cytokine induction in the absence of de-ramification after LPS injection in a model of preclinical prion disease (Cunningham et al., 2005b). Notably there are dynamic alterations in the surface expression of proteins in microglia (increased IL-4Rα and decreased CX3CR1) within 2-4 h after LPS (Fenn et al., 2012a; Wynne et al., 2010), but again these are not reflected by significant increase in Iba-1 immunoreactivity and morphological de-ramification. Similar to the microglial assessments, astrocytes had an increased mRNA expression profile associated with activation but did not have a corresponding increase in GFAP immunoreactivity. Furthermore, the disconnect between the activation mRNA profile of glia and morphological changes was also evident in the repeated LPS challenge. LPS (10 µg), LPS (20 µg), and LPS 4x (20 µg) all had different mRNA profiles but the same level of Iba-1 and GFAP immunoreactivity 24 h after the last LPS injection. Thus,
morphological analysis alone would not provide a representative assessment of activation following acute or repeated LPS challenge. Our collective data indicate that the de-ramified morphology after a peripheral challenge with LPS represents a resolution phase of microglia cytokine induction and corresponding sickness behavior.

Our study highlights the limitations of myriad studies that only use Iba-1 and GFAP labeling and report on the “inflammatory” states of glia. Indeed, the data from our time course provided in this study show the importance of using multiple approaches (behavior, glial specific mRNA, morphology) when reporting on the level of microglial activation. We acknowledge that when microglia are chronically activated in neurodegenerative disease (Bhaskar et al., 2010; Cameron et al., 2012; Cunningham et al., 2005a), CNS infection (Nayak et al., 2013), traumatic CNS injury (Cao et al., 2012; Detloff et al., 2008; Kumar et al., 2013), or stress (Kreisel et al., 2014; Wohleb et al., 2011) a de-ramified Iba-1 profile of microglia is likely a good representation of a pro-inflammatory profile. In addition, other more sensitive morphological approaches that include analysis of glial soma area, cell length, cell perimeter, process length and size may be more revealing about activation state (Kongsui et al., 2014b). Although, based on how rapidly active microglia express cytokines, there will be a significant time lag between activation and the detection of any of these morphological changes.

Another important finding is that repeated LPS injections did not amplify the response to LPS in the context of sickness behavior and biochemical or morphological analysis of glia. Our data indicate that four repeated injections of LPS (provided every 24 h) leads to immune tolerance and an overall less inflammatory profile in the brain compared to 24 h after an acute injection of the same LPS dose. In support of this idea, LPS 4x mice did not continue to lose weight and showed no sickness behavior in the social exploratory test 4 h after the last injection. In addition, plasma IL-6 levels were no longer elevated 24 h after the fourth injection of LPS. These data are consistent with
studies showing that multiple exposures to the same stimuli lead to immune tolerance of peritoneal macrophages (Biswas and Lopez-Collazo, 2009). Notably, LPS 1x and LPS 4x mice had a similar de-ramified morphology of microglia. Enriched microglia from LPS 4x mice, however, had lower inflammatory cytokine mRNA expression and maintained higher levels of YM-1 (M2) and HaptoG (acute phase) expression compared to microglia from LPS 1x mice. This anti-inflammatory profile of microglia associated with repeated LPS challenges is proposed to be neuroprotective (Chen et al., 2014). For example, repeated LPS injection prior to brain injury was associated with reduced cell death and lesion volume induced by cryogenic brain injury (Chen et al., 2012). Similar to our data, at 24 h after the last injection of LPS, microglia had pronounced de-ramified morphology, higher YM-1 and IL-4Rα, and a lower M1 profile (Chen et al., 2012). This increased YM-1 expression was important as it represents an enhanced M2a and repair supportive profile (Colton, 2009). Similar to repeated LPS injections, YM-1 expression is also enhanced in microglia by the anti-inflammatory agent minocycline (Fenn et al., 2012a) and by IL-4 administration (Pepe et al., 2014). The maintenance of elevated HaptoG expression in microglia may represent a profile consistent with neuroprotection and iron sequestration following CNS injury (Zhao et al., 2009). Overall, repeated injections of LPS does not amplify the inflammatory response, however, it may shift the microglia profile to be more neuroprotective.

It is important to highlight that the data collected in this repeated LPS paradigm was during the resolution phase of microglial activation (24 h). Thus, the potential neuroprotective properties of microglia after inflammatory resolution are likely time dependent and not solely an effect of repeated LPS injections. In fact, studies have also reported less extensive injury when pre-treated with acute LPS 24-72 h prior to CNS injury (Hayakawa et al., 2014; Stevens et al., 2011). At the height of inflammation, however, the potential of microglia to be neuroprotective may be altered. For instance,
cytokine levels in the brain were elevated after 2 injections of LPS compared to 1 injection at an acute time point after LPS (3 h) (Puntener et al., 2012). Notably, another paper indicated that microglia were more activated following four repeated injections of LPS compared to one injection (Bodea et al., 2014). In this study, however, microglia were analyzed by Iba-1 staining 5 days after the initial LPS challenge which corresponded to 4 days after the single injection and only 24 h after the last in the series of 4 injections. Overall, our study is consistent with the notion that the resolution phase of microglial activation, independent of acute or repeated LPS challenge, is anti-inflammatory and potentially neuroprotective.

In summary, neither Iba-1 nor GFAP immunoreactivity alone adequately inform on the pro-inflammatory state of microglia or astrocytes. In the context of a peripheral LPS challenge, Iba-1 immunoreactivity 24-48 h after LPS corresponded with the resolution phase of the cytokine and behavioral response. We also provide evidence of a delayed response of astrocytes that may correspond with anti-inflammatory feedback on microglia. In addition, mice with repeated LPS injections had a similar morphological profile of microglia as acute LPS injected mice but had reduced inflammatory mRNA profile and attenuated sickness behavior. Collectively, these data indicate that multiple approaches (behavior, glial specific mRNA, and morphology) are required to provide a reliable interpretation of the inflammatory profile and the activation state of glia.
Chapter 3: TGFβ produced by IL-10 re-directed Astrocytes Attenuates Microglial Activation

Abstract

While there clearly is an intimate relationship between astrocytes and microglia, few studies have examined these potentially dynamic interactions. In this study, cytokine-mediated communication between microglia and astrocytes under inflammatory conditions was investigated. We have previously shown that activated microglia produce Interleukin (IL)-10, a regulatory cytokine that plays an important role in resolving neuroinflammation. Nonetheless, the mechanism by which IL-10 attenuates pro-inflammatory cytokine expression in the brain is unclear. Here we show that IL-10 re-directed astrocytes regulate the activation of microglia in a Transforming growth factor (TGF)-β dependent manner. In support of this concept, astrocytes in the brain maintained higher IL-10 receptor (IL-10R1) expression and primary astrocytes in culture were markedly more sensitive to the anti-inflammatory effects of IL-10 compared to microglia. Moreover, studies using primary cultures and an astrocyte-microglia co-culture system revealed that astrocytes mediated the anti-inflammatory effects of IL-10 on microglia through the production of TGFβ. For instance, only when astrocytes were present did IL-10 stimulation reduce the expression of IL-1β and increase expression of anti-inflammatory mediators fractalkine receptor (CX₃CR1) and interleukin 4 receptor-α (IL-4Rα) in microglia. Importantly, these IL-10-astrocyte dependent effects on microglia were blocked by a TGFβ inhibitor. Furthermore, inhibition of TGFβ signaling in the brain resulted in prolonged sickness behavior and amplified pro-inflammatory cytokine expression in mice challenged with lipopolysaccharide (LPS). Taken together, IL-10...
stimulated the production of TGFβ by astrocytes, which in turn, attenuated microglial activation. Overall, these findings provide novel insight into the mechanisms by which astrocytes modulate microglia under inflammatory conditions.

**Introduction**

Innate immunity within the central nervous system (CNS) is primarily provided by resident microglia. Microglia are pivotal in immune surveillance and also facilitate the coordinated responses between the immune system and the brain (Davalos et al., 2005; Nimmerjahn et al., 2005). For example, microglia interpret and propagate inflammatory signals that are initiated in the periphery. This transient microglial activation is essential for the induction and maintenance of the behavioral symptoms of sickness. This term describes neurobehavioral symptoms associated with infection, including lethargy, listlessness, decreased activity and reduced social interaction (Dantzer et al., 2008). This sickness response is mediated by pro-inflammatory cytokines, including IL-1β and represents a reorganization of the host priorities to resolve infections (Kelley et al., 1997). Nonetheless, prolonged exposure to inflammatory cytokines in the brain has deleterious effects on neuronal plasticity, behavior, and cognition (Dantzer et al., 2008). For example, microglia in the aged brain develop a more “primed” or reactive profile (Cunningham, 2013; Sierra et al., 2007) and as a consequence of this there is amplified production of IL-1β following an innate immune challenge (Frank et al., 2010b; Henry et al., 2009). Amplified neuroinflammation induced by primed microglia is associated with cognitive impairment, prolonged sickness behavior, and depressive-like behavior (Barrientos et al., 2006; Godbout et al., 2008). Exaggerated microglial responses may be related to impairments in key regulatory systems that make it more difficult to resolve
microglial activation (Norden and Godbout, 2013). Thus, understanding how microglial activation is regulated in the brain is critical.

Recent findings indicate that astrocytes in the brain are active participants in both propagating and regulating neuroinflammation (Farina et al., 2007; Liu et al., 2011; Pekny and Nilsson, 2005). Astrocytes become activated by inflammatory mediators, engagement of TLRs (Bsibsi et al., 2006; Carpentier et al., 2005; Gurley et al., 2008) and cytokines including IL-1β (John et al., 2004). Once activated, astrocytes produce many regulatory factors that may influence CNS immunity and provide negative feedback to activated microglia. For instance, addition of conditioned media from astrocytes to microglia cultures increased antioxidant and anti-inflammatory gene expression (Min et al., 2006). In addition, several studies indicate that activated astrocytes release factors that are neuroprotective (Farina et al., 2007). Although these results were not directly linked to regulating microglial activation, it is plausible that this is a critical component to astrocyte-mediated neuroprotection.

Two key anti-inflammatory cytokines that modulate astrocyte and microglial activation are IL-10 and TGFβ. IL-10 is an anti-inflammatory cytokine that has immunoregulatory effects in the brain (Kremlev and Palmer, 2005). Direct administration of IL-10 into the brain suppressed LPS-induced IL-1β expression in the hippocampus and ameliorated sickness behavior (Bluthe et al., 1999; Lynch et al., 2004). In IL-10 deficient mice, peripheral LPS challenge caused prolonged neuroinflammation and sickness behavior (Richwine et al., 2009). IL-10 also has beneficial effects in neuroinflammatory disease models including experimental autoimmune encephalomyelitis (Cua et al., 2001), spinal cord injury (Ishii et al., 2013), and stroke (Frenkel et al., 2005). These findings support an anti-inflammatory and potential neuroprotective role of IL-10 within the brain. While active microglia and macrophages

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secrete IL-10 (Henry et al., 2009), the cellular target of IL-10 regulation in the brain is unclear.

TGFβ also has neuroprotective and anti-inflammatory effects in the CNS. These anti-inflammatory responses to TGFβ may be mediated, in part, by reducing microglial activation (Butovsky et al., 2014). For example, TGFβ inhibited pro-inflammatory cytokine and nitric oxide production in activated microglia (Orellana et al., 2013; Suzumura et al., 1993). In addition, TGFβ protected against amyloid-beta induced neurotoxicity (Caraci et al., 2008; Ramirez et al., 2005) and neuronal death in a model of Parkinson disease by inhibiting reactive oxygen species (ROS) production in microglia (Qian et al., 2008). Nonetheless, the mechanisms and cell-specific targets of IL-10 and TGFβ mediated suppression of neuroinflammation remain unknown.

Therefore, the objective of this study was to investigate microglia-astrocyte interactions under inflammatory conditions and focus on key anti-inflammatory cytokine signals between these two cell types. In this study, we provide several lines of evidence that IL-10 re-directs active astrocytes to produce TGFβ, which in turn, attenuates the activation of microglia. Inhibition of TGFβ signaling blocked these IL-10-astrocyte mediated effects on microglia. After peripheral immune challenge with LPS, we confirmed that increased IL-10 expression was specific to enriched microglia in the brain and the increased IL-10R1 and TGFβ expression was specific to enriched astrocytes. Moreover, inhibition of TGFβ signaling in the brain exaggerated microglial activation, neuroinflammation, and sickness behavior following LPS challenge. Collectively these data provide new insight into cytokine-mediated interactions between astrocytes and microglia.
**Materials and Methods**

**Animals**

Neonatal pups (postnatal day 1-3) and adult (3-4 months-old) BALB/c mice were obtained from our breeding colony kept in barrier-reared conditions in a specific-pathogen-free facility at the Ohio State University. Mice were individually housed in polypropylene cages and maintained at 25°C under a 12 h light/12 h dark cycle with *ad libitum* access to water and rodent chow. All procedures were in accordance with the National Institute of Health Guidelines for the Care and Use of Laboratory Animals and were approved by The Ohio State University Institutional Laboratory Animal Care and Use Committee.

**Microglia and astrocyte isolation from brain**

Microglia and astrocytes were isolated from brain homogenates using a Percoll density gradient as previously described (Fenn et al., 2012a). In brief, tissues were homogenized and cell pellets were re-suspended in 70% isotonic Percoll. A discontinuous Percoll density gradient (70%, 50%, 35%, 0%) was layered and centrifuged for 20 min at 2000xg. Enriched microglia were collected from the interphase between 70% and 50% Percoll. Of the cells recovered from this interphase, 90% of the cells were CD11b+ microglia (Henry et al., 2009). Enriched astrocytes were collected from the interphase between 50% and 35% Percoll. Of the cells recovered from this interphase, 60% of the cells were GLAST-1+ astrocytes.

**Flow cytometry**

Cells were assayed for surface antigens by flow cytometry as previously described (Fenn et al., 2012a; Henry et al., 2009). Percoll enriched microglia were incubated with rat anti-mouse CD11b-FITC, IL-10R1-PE, and CD45-PerCP-Cy5.5.
antibodies (eBioscience). Percoll enriched astrocytes were incubated with rat anti-mouse GLAST-1-APC (Miltenyi Biotec), CD-11b-FITC, and IL-10R1-PE antibodies (eBioscience). Expression was determined using a Becton-Dickinson FACSCaliber four color Cytometer. Ten thousand events characterized as microglia or astrocytes were recorded. Microglia were identified by CD11b⁺/CD45low expression and astrocytes were identified by GLAST-1⁺/CD11b⁻ expression. For each antibody, gating was determined based on appropriate negative isotype stained controls. Flow data were analyzed using FlowJo software (Tree Star, CA).

**BV2 and Primary Cell culture**

BV2 microglia, neonatal primary microglia and primary astrocyte cultures were established and maintained as previously described (Fenn et al., 2012a). Mixed primary glia cultures were shaken at 160rev/min and 37°C for 3.5h to harvest microglia. Remaining cells were treated with 50 mM l-leucine methyl ester (Sigma-Aldrich) for 45 min to deplete remaining microglia. After l-leucine incubation, astrocytes recovered in growth medium supplemented with 0.1 mM l-leucine for 1-3 days (Phulwani et al., 2008). BV2 and primary microglia were plated at a density of 75,000-100,000 cells per well on poly-L-lysine coated 24-well plates and left overnight to adhere. Primary astrocytes were plated at a density of 50,000 cells/well. Before treatment, cells were washed with serum-free medium. Primary microglia were activated with 10 ng/mL LPS for 1 h (stereotype 0127:B8, Sigma-Aldrich) and treated with recombinant mouse IL-10 (10 ng/mL) or human TGFβ (1 ng/mL) (R&D Systems) for an additional 3 h. BV2 microglia and primary astrocytes were activated with 100 ng/mL LPS for 1 h and treated with TGFβ (1 ng/mL) or IL-10 (10 ng/mL), respectively, for an additional 3 h. Conditioned media was collected and stored at -80°C and RNA was isolated using Tri-Reagent.
**Microglia-Astrocyte transwell co-cultures**

Primary microglia were plated at 50,000-75,000 cells/well in a 24-well transwell plate (Corning Life Sciences). After incubation for 3 hours, astrocytes were added to a removable 0.4 um polycarbonate membrane at an equal number as microglia. Following incubation overnight, cells were washed with serum-free media and activated with LPS (10 ngl/mL). After 1 hour, IL-10 (10ng/mL) and SB431542 (10 uM) (Sigma-Aldrich) was added for an additional 3 h. Conditioned media was collected and RNA was isolated from the microglia using Tri-Reagent.

**Immunohistochemistry and digital image analysis**

Primary astrocytes or microglia were grown on glass coverslips, washed with PBS and incubated with 4% formaldehyde for 10 minutes. Cells were blocked with 5% normal goat serum and 1% bovine serum albumin (BSA) then labeled using rabbit anti-mouse GFAP (Dako), Iba-1 (Wako Chemicals) or IL-10R1 (Millipore) antibodies overnight at 4° C. Next, cells were washed and incubated with a flurochrome-conjugated secondary antibody (Alexa Fluor, anti-rabbit 488 or 594) and counterstained with a DNA stain, 4′,6-diamidino-2-phenylindole (DAPI).

Mice were deeply anesthetized and transcardially perfused with PBS followed by 4% formaldehyde. Brains were post-fixed in 4% formaldehyde for 24 h and cryoprotected in 20% sucrose for 48 h. Preserved brains were frozen using dry-ice cooled isopentane (-165ºC) and sectioned (25 µm) using a Microm HM550 cryostat. Brain sections were identified by reference markers in accordance with the stereotaxic mouse brain atlas (Paxinos and Franklin, 2004). Iba-1 staining was performed as previously described (Wohleb et al., 2012). In brief, free-floating sections were blocked and then incubated with rabbit anti-mouse Iba-1 antibody (Wako Chemicals) overnight at
4°C. Sections were washed with PBS and incubated with antibody Alexa Flour 594 secondary.

Fluorescent images were visualized using an epifluorescent Leica DM5000B microscope and captured using a Leica DFC300 FX camera and imaging software. To quantify the phenotypic changes of microglia, digital image analysis (DIA) of Iba-1 staining was performed (Donnelly et al., 2009) in the hippocampus. 6-10 representative images were taken at 20x magnification in the dentate gyrus, CA1, and CA3 regions. A threshold for positive staining was determined for each image that included all cell bodies and processes, but excluded background staining (ImageJ). Results were reported as the average percent area in the positive threshold for all representative pictures.

**Determination of TGFβ protein**

TGFβ1 protein concentration was determined using the DuoSet ELISA Development System according to the manufacturer’s instructions (R&D Systems). In brief, 96-well enzyme immunoassay plates were incubated overnight with anti-mouse TGFβ capture antibody. Samples were activated with 1N HCl for 10 minutes and neutralized with 1.2N NaOH. Plates were incubated with samples and standards (0-320 pg/ml) for 2 h. Next, biotinylated detection antibody was added for 2 h. Bound TGFβ was detected using Streptavidin-HRP development and absorbance at 450 nm was read using a Snergy HT Plate Reader (Bio-tek instruments). Wavelength correction was subtracted at 540 nm. The assay was sensitive to 5 pg/mL TGFβ and the interassay coefficient of variation was <10%.
RNA isolation and RT-PCR

RNA was isolated from cell cultures and brain tissue using the Tri-Reagent protocol (Sigma-Aldrich). For Percoll enriched microglia and astrocytes, RNA was isolated using the PrepEase kit (USB, CA). RNA was reverse transcribed to cDNA and real-time (RT)-PCR was performed using the Applied Biosystems Taqman® Gene Expression assayAssay-on-Demand Gene Expression protocol as previously described (Wohleb et al., 2012). In brief, experimental cDNA was amplified by real-time PCR where a target cDNA (e.g., IL-1, IL-6, CCL2) and a reference cDNA (glyceraldehyde-3-phosphate dehydrogenase; GAPDH) were amplified simultaneously using an oligonucleotide probe with a 5' fluorescent reporter dye (6-FAM). Fluorescence was determined on an ABI PRISM 7300-sequence detection system (Applied Biosystems). Data were analyzed using the comparative threshold cycle (Ct) method and results are expressed as fold difference.

Intracerebroventricular cannulation

The i.c.v. cannulation was performed as previously described (Huang et al., 2008). In brief, mice were deeply anesthetized and positioned in a stereotaxic instrument so that the plane formed by the frontal and parietal bones was parallel to the table top. An incision was made on the cranium to reveal the bregma and a 26-guage stainless-steel guide cannula was placed in the lateral cerebral ventricle using the following stereotaxic coordinates: Lat 0.5 mm; and A-P 1.2 mm to the bregma; and Hor -2 mm from the dura mater. A dummy cannula was inserted in the guide cannula to prevent occlusion and infection. Mice were provided a minimum of 7 d to recover before any treatments were administered.
Peripheral and Central Injections

Adult mice were injected intraperitoneally (i.p.) with saline or 0.33mg/kg LPS. Mice with the indwelling cannula were injected i.c.v. with 2.5 nmoles SB431542 (Sigma-Aldrich) or vehicle (50% DMSO in PBS) one hour after i.p. injection of saline or LPS. The LPS dosage was selected because it elicits a pro-inflammatory cytokine response in the brain resulting in a transient sickness response in adult mice (Berg et al., 2004; Godbout et al., 2005b). Body weight, food intake, and social exploratory behavior were determined 0, 4, 8 and 24 h after injections.

Social Exploratory Behavior

Social exploratory behavior was determined as a measure of sickness behavior as previously described (Godbout et al., 2005b). A novel juvenile was introduced into the test subject’s home cage for a 10-min period. Behavior was videotaped and the cumulative amount of time the experimental subject engaged in social investigation of the juvenile (e.g., anogenital sniffing, trailing) was determined. Baseline social behavior was measured immediately before experimental treatment (time 0). Results are expressed as percent of time engaged in social behavior compared to baseline.

Statistical Analysis

To ensure a normal distribution, data were subjected to the Shapiro-Wilk test using Statistical Analysis Systems (SAS) statistical software (Cary, NC). To determine significant main effects and interactions between main factors, data were analyzed using one-way (i.e., Pretreatment and Treatment), two-way (i.e., Pretreatment x Treatment), and three-way (i.e., Pretreatment x Treatment x Time) ANOVA using the General Linear Model procedures of SAS. When appropriate, differences between treatment means were evaluated by an F-protected t-test using the Least-Significant Difference procedure.
of SAS. All data are expressed as treatment means ± standard error of the mean (SEM). Values were considered significant at p-values < 0.05 and a tendency at p-values ≤ 0.1.

Results

IL-10 receptor was expressed on astrocytes and microglia isolated from the brain.

Based on previous work, we are interested in how IL-10 mediates anti-inflammatory responses in the brain (Henry et al., 2009; Lynch et al., 2004; Richwine et al., 2009). Therefore, the ability of IL-10 to regulate microglia and astrocytes was investigated using adult mice and a series of primary cell culture experiments. IL-10 signals through the IL-10 receptor, which has two major components 1) the ligand binding domain (IL-10R1) and 2) the signaling domain (IL-10R2). IL-10R2 is expressed constitutively by most cells. Expression of IL-10R1, however, varies by cell type and is expressed at low levels under homeostatic conditions (Moore et al., 2001). Therefore, we focused our attention on the ligand binding domain of the IL-10 receptor (IL-10R1).

In initial studies, expression of IL-10R1 was determined on astrocytes and microglia collected from brain homogenates using a modified Percoll density gradient. The diagram in Fig.3.1A shows that enriched astrocytes were collected from the interphase between 35-50% Percoll and that enriched microglia were collected from the 50-70% interphase. Fig.3.1B shows representative dot plots of CD11b and GLAST-1 staining of astrocytes (top panel) and CD11b and CD45 staining of microglia (bottom panel). Consistent with our previous work (Henry et al., 2009), cells isolated from the 50%-70% Percoll interphase were enriched microglia (CD11b+/CD45low). In addition, the majority of cells collected from the 35%-50% Percoll interphase (over 60%) were astrocytes (CD11b-/GLAST+). Therefore, this modified Percoll gradient protocol yielded both enriched microglia and astrocytes.
Next, IL-10R1 expression was determined on astrocytes and microglia. Fig.3.1C shows representative dot plots of IL-10R1 staining on astrocytes and microglia. The black vertical bars (either CD11b or GLAST-1) and horizontal bars (IL-10R1) represent isotype staining. Representative histograms of mean fluorescence intensity (MFI) for IL-10R1 on astrocytes and microglia are shown in Fig.3.1D. Fig.3.1E shows the percentage of IL-10R1+ cells based on isotype labeling. These data indicate that astrocytes had increased expression of IL-10R1 compared to microglia ($p<0.01$). For example, 24 ± 1.0% of astrocytes were positive for IL-10R1 while only 10.8 ± 0.3% of microglia were positive for IL-10R1. Taken together, astrocytes maintained a relatively higher basal expression of IL-10R1 than microglia.
IL-10 receptor was expressed on the surface of primary astrocytes

We next sought to determine IL-10R1 expression in primary cultures of microglia and astrocytes. First, the purity of the primary cultures was determined. Fig.3.2A shows a representative image of primary astrocytes labeled with GFAP and DAPI. Consistent with previous reports, primary astrocytes were large and formed a sheet across the
bottom of the plate (Souza et al., 2013). This was a highly purified culture and every DAPI cell also co-labeled with GFAP. A similar high purity of primary microglia was detected. For example, every DAPI cell in the microglia culture also co-labeled with ionized calcium binding adapter molecule 1 (Iba-1) (Fig.3.2B).

Next, the expression of IL-10R1 was determined on primary cultures of astrocytes and microglia. Fig.3.2C shows that IL-10R1+ labeling was prevalent on the surface of GFAP+ astrocytes. Although IL-10R1 labeling was also observed on microglia, this labeling was inconsistent with cell surface labeling (Fig.3.2D). Overall, both primary cell types expressed IL-10R1 when cultured, but there was an enhanced prevalence of IL-10R1 on the surface of astrocytes.
Figure 3.2. IL-10 receptor was expressed on the surface of primary astrocytes. Primary astrocytes and microglia were plated on cover slips. A) Representative images of DAPI, GFAP and merged DAPI/GFAP labeling of primary astrocytes. B) Representative images of DAPI, Iba-1 and merged DAPI/Iba-1 labeling of primary microglia. Representative images of IL-10R1+ staining in C) primary astrocytes or D) primary microglia. Arrows depict surface IL-10R1 labeling.

**IL-10 suppressed LPS-induced IL-1b in mixed microglia-astrocyte cultures**

IL-10 has anti-inflammatory actions within the CNS (Lynch et al., 2004), but the cell-specific target of IL-10 regulation is unclear. Our data indicate that IL-10R1 was prevalent on the surface of astrocytes whereas surface expression of IL-10R1 on microglia was limited (Fig. 3.1&2). Thus, astrocytes may have a higher capacity to respond to IL-10. To test this hypothesis, the ability of IL-10 to reduce IL-1β mRNA
expression in primary astrocytes, microglia, or mixed microglia-astrocyte cultures was determined. In this experiment, primary cultures were activated with LPS, treated with IL-10, and IL-1β mRNA expression was determined 4 h later. IL-10 reduced the LPS-induced increase in IL-1β mRNA expression in primary astrocytes (Fig.3.3A, \( p<0.01 \)) and in mixed microglia/astrocyte cultures (Fig.3.3C, \( p<0.01 \)) but had no effect on the LPS-induced expression of IL-1β mRNA in primary microglia (Fig.3.3B). Fig.3.3D highlights the percent reduction of IL-1β mRNA by IL-10 treatment. For example, IL-10 reduced IL-1β mRNA expression in primary astrocytes by 26 ± 5% and mixed microglia/astrocytes by 55 ± 5%. Thus, IL-10 was most effective in reducing LPS-induced IL-1β mRNA when both microglia and astrocytes were present in the culture. Taken together, there is important interplay between microglia and astrocytes in responding to IL-10.

![Figure 3.3](image-url)

**Figure 3.3.** IL-10 suppressed LPS-induced IL-1β in mixed microglia-astrocyte cultures. LPS (10ng/mL) was provided to primary cultures for 1 h followed by vehicle or IL-10 (10ng/mL) for an additional 3 h. IL-1β mRNA was determined in A) primary astrocytes, B) primary microglia or C) mixed astrocytes/microglia cultures (n=6-9). Data are expressed as fold change from saline. Bars represent the mean ± SEM. Means with * are significantly different (\( p<0.05 \)) from the LPS-vehicle group. D) Percent reduction of LPS-induced IL-1β expression by IL-10.
**IL-10 did not re-direct activated microglia towards a less inflammatory profile**

To further understand the mechanism by which IL-10 reduces the inflammatory profile of glia, separate cultures of primary microglia and astrocytes were examined. We have previously shown that IL-10 was unable to re-direct activated BV2 microglia towards a less inflammatory profile (Fenn et al., 2012a). Therefore, we anticipated that IL-10 would have a limited effect on the inflammatory status of LPS activated primary microglia. In this experiment, primary microglia were activated with LPS, treated with IL-10, and expression of several inflammatory (IL-1β, IL-6), anti-inflammatory (IL-10, TGFβ) and immuno-regulatory markers (fractalkine receptor (CX₃CR1) and interleukin 4 receptor-α (IL-4Rα)) were determined. As expected, LPS increased IL-1β mRNA in primary microglia, but this induction of IL-1β was unaffected by IL-10 (Fig.3.4A). LPS also increased mRNA expression of IL-6 mRNA (p<0.0001). In this case, the LPS-induced expression of IL-6 was reduced by IL-10 (Fig.3.4B, F(1,18)=23.87, p<0.002). LPS also increased expression of the anti-inflammatory markers IL-10 (Fig.3.4C, p<0.02) and TGFβ (Fig.3.4D, p<0.02), but these increases were independent of IL-10. Moreover, Fig.3.4E shows IL-10 reduced mRNA expression of CX₃CR1 (p<0.05) and microglia treated with both LPS and IL-10 tended to have the lowest expression of CX₃CR1 compared to all other groups (p=0.1). IL-10 increased IL-4Rα expression in primary microglia (p<0.002), but this increase was independent of LPS (Fig.3.4F). Taken together, primary microglia had some responsiveness to IL-10 with reduction of LPS-induced IL-6 and increase in IL-4Rα, but overall IL-10 was limited in suppressing LPS activated primary microglia.
Figure 3.4. IL-10 did not re-direct activated microglia towards a less inflammatory profile. Primary microglia were pre-treated with saline or LPS (10ng/mL) for 1 h and vehicle or IL-10 (10ng/mL) was added for an additional 3 h. mRNA expression of A) IL-1β, B) IL-6, C) IL-10, D) TGFβ, E) CX3CR and F) IL-4Rα was determined (n=8, 2 independent experiments). Means with * are significantly different (p<0.05) from saline-vehicle, means with # are significantly different (p<0.05) from LPS-vehicle, means with + tend to be different (p=0.1) from saline.
**IL-10 lowered the pro-inflammatory profile of LPS-activated astrocytes**

In LPS-activated microglia/astrocyte co-cultures, IL-10 significantly decreased IL-1β expression (Fig. 3.3C&D). In microglia cultures, however, IL-10 had no effect on LPS-induced IL-1β mRNA expression (Fig. 3.4A). Therefore, we sought to determine the extent to which IL-10 re-directed activated primary astrocytes. In this experiment, primary astrocytes were activated with LPS, treated with IL-10, and mRNA expression of several inflammatory (IL-1β, IL-6, CCL2, TNFα) and anti-inflammatory (SOCS3, IL-10, TGFβ) markers was determined. Fig. 3.5A-D shows that LPS increased mRNA expression of IL-1β, CCL2, TNFα, and IL-6 (p<0.001, for all). Moreover, IL-10 attenuated the LPS-induced mRNA expression of IL-1β, CCL2, TNFα, and IL-6 (F(1,31)=6.62, p<0.02, for each). LPS also increased expression of the anti-inflammatory genes SOCS3 and IL-10 (Fig. 3.5E-F, p<0.0001, for each), but this was independent of IL-10 treatment. Fig. 3.5G shows that TGFβ mRNA was increased by LPS and was significantly augmented by IL-10 (F(1,40)=5.34, p<0.03). In addition, secretion of TGFβ protein by astrocytes was increased by LPS (p<0.002) and by IL-10 (p<0.003) (Fig. 3.5H). Furthermore, the highest level of TGFβ protein secretion was by astrocytes activated with LPS and stimulated with IL-10 (p<0.005). Overall, these data indicate that IL-10 decreased pro-inflammatory cytokine expression and increased TGFβ production in immune activated astrocytes.
Figure 3.5. IL-10 lowered the pro-inflammatory profile of LPS-activated astrocytes. Primary astrocytes were pre-treated with saline or LPS (100ng/mL) for 1 h and vehicle or IL-10 (10ng/mL) was added for an additional 3 h. mRNA expression of A) IL-1β, B) CCL2, C) TNFα, D) IL-6, E) SOCS3, F) IL-10, and G) TGFβ was determined. H) TGFβ protein levels in the conditioned media were determined (n=8-10, 2 independent experiments). Means with * are significantly different (p<0.05) from saline-vehicle, means with # are significantly different (p<0.05) from LPS-vehicle.
TGFβ promoted an anti-inflammatory phenotype in resting and activated microglia.

TGFβ mRNA and protein was augmented by IL-10 treatment of LPS-activated astrocytes (Fig.3.5). To determine the degree to which TGFβ has anti-inflammatory effects on microglia, BV2 and primary microglia were activated with LPS and treated with TGFβ. A summary of the gene expression changes in BV2 microglia is shown in Fig.3.6A. As expected, LPS increased IL-1β, IL-6, and TNFα mRNA expression (p<0.001, for each). Moreover TGFβ attenuated IL-1β, IL-6, and TNFα mRNA expression in LPS-activated BV2 microglia (F(1,22)=12.27, p<0.003, for each). TGFβ alone increased CX3CR1 (p<0.0001) and IL-4Rα (p<0.01) expression.

A similar experiment was completed in primary microglia. Fig.3.6B&C show that TGFβ attenuated the LPS-induced increase in IL-1β and IL-6 mRNA expression (F(1,32)=94.96, p<0.0001). Moreover, TGFβ alone increased CX3CR1 and IL-4Rα mRNA expression (F(1,31)=42.11, p<0.0001, for each). Taken together, TGFβ reduced pro-inflammatory cytokine expression in activated microglia and also enhanced the expression of receptors associated with microglial regulation.
IL-10 re-directed astrocytes regulated the activation of microglia in a TGFβ dependent manner

Based on these data, we hypothesized that astrocytes are directly responsive to the anti-inflammatory effects of IL-10 and, in turn, produce TGFβ to suppress microglial activation. To address this premise, a transwell co-culture system of microglia and astrocytes was developed where microglia were plated on the bottom of the well and
astrocytes were placed on a removable transwell insert. In this manner the cells shared media, but did not physically interact and the astrocytes could be readily removed. In this experiment, all wells were activated with LPS and after 1 hour, IL-10, an inhibitor of TFGβ (SB431542) (Inman et al., 2002), or respective vehicle controls were added for an additional 3 h. First, TFGβ protein levels were determined in the supernatants. Fig.3.7A shows that LPS-activated microglia cultured without astrocytes secreted low levels of TFGβ. When astrocytes were present on the transwell insert, TFGβ production was significantly increased (F(1,42)=33.04, p<0.0001). In addition, IL-10 tended to augment TFGβ production in LPS-activated astrocyte-microglia co-cultures (p = 0.09). This IL-10 mediated augmentation of TFGβ was absent in microglia cultures without astrocytes. The TFGβ signaling inhibitor SB431542 had no effect on the induction of TFGβ expression in the co-cultures treated with IL-10.

Next, microglial activation was examined by determining mRNA expression of IL-1β, CX3CR1 and IL-4Rα specifically in the cultured microglia. Because all groups were treated with LPS, the data is normalized to the levels of mRNA with LPS treatment alone. Fig.3.7B shows that IL-10 had no effect on LPS-induced expression of IL-1β in microglia. The addition of astrocytes in the transwells increased IL-1β expression in microglia 3-fold over LPS alone (p<0.0001). When IL-10 was added to microglia-astrocyte co-cultures, IL-1β expression was decreased (p<0.01). Importantly, the TFGβ inhibitor SB431542 restored the 3-fold induction of IL-1β expression in microglia to the same level of astrocyte-vehicle treated microglia. Thus, we interpret these data to indicate that IL-10 stimulates the release of TFGβ by astrocytes, which provides negative feedback on microglia to reduce IL-1β expression.

TFGβ secreted by IL-10 re-directed astrocytes also increased the mRNA expression of CX3CR1 and IL-4Rα in microglia. For example, CX3CR1 mRNA was
decreased in microglia following stimulation with IL-10 (Fig. 3.7C, F(1,39)=48.67, p<0.0001). Moreover, addition of the TFGβ inhibitor further reduced CX3CR1 mRNA expression (p<0.002). In a similar manner, microglia treated with IL-10 increased IL-4Rα mRNA expression (Fig. 3.7D, F(1,40)=19.04, p<0.0001) and when co-cultured with astrocytes, further increased IL-4Rα expression (p=0.08). Addition of the TFGβ inhibitor, however, decreased IL-4Rα mRNA expression to the same level as microglia cultured without astrocytes (F(1,42)=4.71, p<0.04). Taken together, IL-10 regulates microglial activation indirectly by increasing TFGβ production by astrocytes.
LPS injection increased IL-10R1 and TGFβ mRNA expression in enriched astrocytes

Our data indicate that TGFβ production by astrocytes following IL-10 stimulation is important for attenuating microglial activation in culture (Fig. 3.7). Therefore, we next...
sought to determine if similar interactions between astrocyte and microglia existed in the brain. In these studies, the innate immune system was activated by an i.p. LPS injection and IL-10R1 surface expression was determined on astrocytes and microglia 24 h later. Fig.3.8A-B shows representative dot plots of IL-10R1 on astrocytes and microglia. Consistent with Fig.3.1, these data indicate that astrocytes had higher IL-10R1 expression compared to microglia and show that IL-10R1 expression on either cell type was not significantly altered 24 h after LPS (Fig.3.8C).

Using the same LPS injection protocol, mRNA levels of IL-10, IL-10R1 and TGFβ were determined in enriched astrocytes and microglia. While IL-10 mRNA was increased only in microglia (Fig.3.8D, p<0.01), IL-10R1 and TGFβ mRNA levels were increased specifically in astrocytes 24 h after LPS (Fig.3.8D, p<0.05, for each). mRNA for both IL-10R1 and TGFβ were detectable in microglia, but they were unaltered by LPS. These data indicate that astrocytes, but not microglia, increase mRNA expression of TGFβ following peripheral immune challenge and suggest that IL-10 signaling is important for increasing TGFβ production by astrocytes.
Mice were injected i.p. with saline or LPS (0.33 mg/kg) and astrocytes and microglia were isolated 24 h later. IL-10R1 expression was determined on A) astrocytes and B) microglia. C) Quantification of IL-10R1 positive (IL-10R1+) astrocytes and microglia (n=10). D) mRNA expression of IL-10, IL-10R1, and TGFβ was determined in enriched astrocytes and microglia (n=5). Means with * are significantly different (p<0.05) from saline controls.

Figure 3.8. LPS injection increased IL-10R1 and TGFβ mRNA expression in enriched astrocytes. Mice were injected i.p. with saline or LPS (0.33 mg/kg) and astrocytes and microglia were isolated 24 h later. IL-10R1 expression was determined on A) astrocytes and B) microglia. C) Quantification of IL-10R1 positive (IL-10R1+) astrocytes and microglia (n=10). D) mRNA expression of IL-10, IL-10R1, and TGFβ was determined in enriched astrocytes and microglia (n=5). Means with * are significantly different (p<0.05) from saline controls.

Inhibition of TGFβ signaling in the brain prolonged sickness behavior and exaggerated neuroinflammation after peripheral LPS challenge

TGFβ decreased the activation profile of LPS activated primary microglia (Fig.3.6). In the brain, TGFβ mRNA was enhanced specifically in enriched astrocytes 24 h after LPS (Fig.3.8). Therefore, we next sought to determine if TGFβ regulation of microglia had a role for resolving microglial activation and reducing neuroinflammation after LPS challenge. In these studies, the innate immune system was activated by an i.p LPS injection and TGFβ signaling was blocked in the brain by a central injection (i.c.v) of...
the TGFβ inhibitor SB431542 (SB). Fig. 3.9A shows that mice injected i.p. with LPS lost weight ($F(1,70)=125.71, p<0.0001$) over 24 h and the LPS-SB group tended to have the most weight loss compared to all other groups ($p=0.10$).

To evaluate sickness behavior, social exploratory behavior was determined at baseline and again at 4, 8, and 24 hours after LPS. As expected, LPS injection reduced social exploratory behavior (Fig. 3.9B, $F(1,130)=128, p<0.0001$). Moreover, this was dependent on time ($F(1,130)=28.51, p<0.0001$) and TGFβ inhibition ($F(1,130)=3.18, p<0.03$). For example, both groups of LPS injected mice had decreased social exploratory behavior compared to controls 4 and 8 h after injection. The LPS-vehicle mice returned to baseline social exploratory behavior by 24 h. The LPS-SB mice, however, did not return to baseline behavior by 24 h and still maintained a 50% reduction in social exploratory behavior (Fig. 3.9B, $p<0.01$).

After the completion of behavioral testing 24 h after LPS, microglia and a coronal brain section (through the i.c.v. injection site) were collected for RNA analysis. Fig. 3.9C shows that LPS injection increased IL-1β mRNA expression in enriched microglia ($F(1,24)=57.3, p<0.001$). Moreover, TGFβ inhibition tended to increase microglial IL-1β expression ($F(1,24)=3.27, p=0.08$). Post hoc analysis revealed that microglia from LPS-SB mice had the highest expression of IL-1β 24 h after LPS ($p<0.04$). In addition, LPS injection increased TNFα expression ($F(1,21)=35.22, p<0.0001$) and inhibition of TGFβ tended to enhance TNFα mRNA expression ($F(1,21)=2.11, p=0.10$) in microglia. Post hoc analysis revealed that microglia from LPS-SB mice tended to have the highest expression of TNFα 24 h after LPS ($p=0.10$).

To supplement the data from enriched microglia, mRNA levels of several inflammatory markers associated with microglial activation were determined from the coronal brain section. Similar to the results from enriched microglia, LPS increased IL-1β
mRNA expression in the brain \((F(1,41)=15.53, p<0.0003)\) and TGF\(\beta\) inhibition tended to further increase IL-1\(\beta\) expression \((F(1,41)=2.73, p=0.09)\). For example, IL-1\(\beta\) mRNA expression was highest in the LPS-SB group compared to all other groups \((p<0.02, \text{Fig.}3.9D)\). TGF\(\beta\) inhibition also amplified IL-6 mRNA expression in the brain of LPS injected mice \((F(1,41)=2.79, p=0.1)\) and the LPS-SB mice had the highest expression of IL-6 compared to all other groups \((p<0.01)\). A similar pattern of expression was detected in TNF\(\alpha\) mRNA \((\text{tendency}, p=0.1)\) and CD14 mRNA \((p<0.02)\). CX\(_3\)CR1 mRNA was lower in the LPS-SB431542 group compared to the LPS-vehicle group \((p<0.04)\). IL-4R\(\alpha\), IL-10, and TGF\(\beta\) mRNA were all increased 24 h after LPS \((p<0.04 \text{ for all})\), but these increases were independent of TGF\(\beta\) inhibition.

In other models of amplified microglial responses to peripheral LPS challenge \((\text{e.g., } \text{CX}\(_3\)CR1\(^{\text{KO}}\) and stress) microglial Iba-1 immunoreactivity persisted 72 h after LPS injection \((\text{Corona et al., 2010; Wohleb et al., 2012})\). Therefore, to confirm that LPS-induced microglial activation was prolonged after TGF\(\beta\) inhibition, Iba-1 immunoreactivity was determined in the hippocampus (HPC) 72 h after LPS. Microglia in the HPC of LPS-SB mice had larger cell bodies and dense processes indicating a more activated phenotype \((\text{Fig.}3.10A)\). Iba-1 proportional area analysis confirmed increased Iba-1 immunoreactivity in the hippocampus of SB treated mice \((F(1,17)=6.56, p<0.02)\). The highest Iba-1 proportional area was in the mice treated with both LPS and SB compared to all other groups \((\text{Fig.}3.10B, p<0.04)\). Collectively these results indicate that interfering with TGF\(\beta\) signaling in the brain caused exaggerated LPS-induced neuroinflammation and sickness behavior.
Figure 3.9. Inhibition of TGFβ signaling in the brain prolonged sickness behavior and exaggerated neuroinflammation after peripheral LPS challenge. Mice were injected i.p. with saline or LPS (0.33mg/kg) and i.c.v. with vehicle (Veh) or 2.5 nmoles SB431542 (SB). A) Body weight was determined 24 h after LPS. B) Social exploratory behavior was determined at baseline and again at 4, 8, and 24 h after LPS. At 24 h, mice were sacrificed enriched microglia and a coronal brain section were collected. C) IL-1β mRNA and D) TNFα mRNA expression was determined in microglia. E) Expression of several inflammatory markers were determined in the coronal brain section (n=8-12). * p<0.05 from saline-vehicle, # p<0.05 from LPS-vehicle, +p=0.1 from LPS-vehicle.
Figure 3.10. Inhibition of TGFβ signaling in the brain prolonged microglial activation in the hippocampus of LPS injected mice. Mice were injected i.p. with saline or LPS (0.33mg/kg) and i.c.v. with vehicle (Veh) or 2.5 nmoles SB431542 (SB). After 72 h, brains were collected and Iba-1 immunoreactivity was determined. A) Representative Iba-1 labeling in the hippocampus. Inset includes enlarged image of Iba-1+ cell indicated by white arrow. B) Proportional area analysis of Iba-1 positive labeling (n=4). Means with * are significantly different (p<0.05) from saline.

Discussion

Bi-directional communication between microglia and astrocytes under inflammatory conditions is dynamic and complex. The primary objective of this study was to understand how IL-10 mediates its anti-inflammatory effects in the brain to attenuate pro-inflammatory cytokine expression. Here we report that LPS-activated astrocytes were attenuated by IL-10 to a greater extent than activated microglia. Moreover, activated astrocytes re-directed by IL-10 in culture responded with increased mRNA expression and protein production of TGFβ. Novel data are provided showing that this TGFβ production by astrocytes, in turn, modulated the activation state of microglia. For instance, only when astrocytes were present in co-cultures did IL-10 stimulation reduce
expression of IL-1β and increase expression of anti-inflammatory mediators CX3CR1 and IL-4Rα in primary microglia. Moreover, these IL-10-astrocyte mediated effects on primary microglia were blocked by inhibition of TGFβ signaling. Furthermore, blockade of TGFβ signaling in the brain (i.c.v.) resulted in exaggerated LPS-induced neuroinflammation and sickness behavior. Taken together, these findings outline a mechanism by which IL-10 suppresses astrocyte activation and stimulates the production of TGFβ, which attenuates microglial activation. These new insights into cytokine mediated interactions between astrocytes and microglia are summarized in Fig.3.11.

Proposed Mechanism:
Regulation of astrocytes and microglia following immune activation:

Activated Microglia
IL-1β, IL-6, IL-10

TGFβ-Resolved Microglia
IL-1β, IL-6, IL-10

CX3CR1, IL-4Rα

IL-10

Activated Astrocyte
IL-10

IL-10 Re-directed Astrocyte
IL-1β, IL-6

TGFβ

Figure 3.11. Proposed mechanism for IL-10 mediated regulation of astrocytes and microglia after immune activation. Immune activated microglia and astrocytes produce both pro- and anti-inflammatory cytokines. In this model of systemic LPS activation, microglia become activated and increase IL-10 expression. IL-10 has limited anti-inflammatory potential on microglia. In astrocytes, however, IL-10 decreases production of pro-inflammatory cytokines and increases TGFβ expression. TGFβ released by astrocytes signal to microglia to decrease production of IL-1β and IL-6 and increased expression of CX3CR1 and IL-4Rα.
One important aspect of this study was that IL-10R1 was more abundant on astrocytes than microglia. For example, by using a novel Percoll density gradient to separate both enriched microglia and astrocytes from the same mouse, we were able to show that astrocytes collected from the brain had higher relative surface expression of IL-10R1 compared to microglia (Fig.3.1). Primary astrocytes in culture also had higher levels of surface IL-10R1 expression compared to microglia (Fig.3.2). Examining these specific populations of microglia and astrocytes confirmed previous cell culture work showing that primary astrocytes isolated from rats had higher levels of IL-10R1 mRNA than primary microglia (Ledeboer et al., 2002). In addition, facial nerve axotomy and ischemia markedly increased IL-10R1 on astrocytes, but not microglia (Perez-de Puig et al., 2013; Xin et al., 2011). Here, LPS injection increased neuroinflammation and microglial expression of IL-10, but did not increase surface expression of IL-10R1 on either microglia or astrocytes. Nonetheless IL-10R1 mRNA was increased specifically in enriched astrocytes (Fig.3.8) and we surmise that IL-10R1 protein will be increased on the surface of astrocytes in a time dependent manner. It is also plausible that IL-10R1 protein was increased on astrocytes after LPS, but was internalized and degraded in the presence of IL-10 (Wei et al., 2006). Collectively these data support the premise that active microglia produce IL-10 and that astrocytes maintain a high level of IL-10R1 protein expression.

A key finding in the study was that astrocytes were identified as the cellular target of IL-10 regulation in the brain. Several reports indicate that IL-10 is an anti-inflammatory cytokine that reduces neuroinflammation (Bluthe et al., 1999; Lynch et al., 2004). The cellular target of IL-10 suppression, however, was unclear. Here, we show novel data that astrocytes respond to IL-10. For example, IL-10 attenuated LPS-induced IL-1β in mixed glia cultures and primary astrocyte cultures, but only had a limited effect on primary microglia (Figs.3.3&4). IL-10 reduced microglial mRNA expression of IL-6 after
LPS, but had no effect on the LPS-induced increase in IL-1β or the LPS-induced reduction of CX3CR1 mRNA expression (Fig. 3.4). These data are consistent with a previous study showing that IL-10 does not re-direct activated BV2 microglia (Fenn et al., 2012a). In primary astrocyte cultures activated by LPS, however, IL-10 reduced all the pro-inflammatory cytokines determined (IL-1β, IL-6, CCL2, TNFα) and further increased expression of the regulatory cytokine TGFβ (Fig. 3.5). In addition, analysis of enriched astrocytes/microglia after LPS challenge in vivo confirmed that TGFβ mRNA was increased specifically in enriched astrocytes and not enriched microglia (Fig. 3.8). Thus, IL-10 robustly re-directed activated astrocytes towards a less inflammatory profile and promoted expression of TGFβ.

Another relevant aspect of this study is that TGFβ produced by IL-10 re-directed astrocytes modulated microglial activation. For instance, IL-10 treatment in astrocyte-microglia co-cultures lowered IL-1β expression in microglia and this was blocked using SB4315342, a TGFβ signaling inhibitor (Inman et al., 2002). In addition, TGFβ mRNA was increased specifically in astrocytes isolated from the brain 24 h after LPS. When TGFβ was provided to LPS activated BV2 and primary microglia in culture, the LPS-induced inflammation was markedly attenuated (Fig. 3.6). These data are consistent with a recent study showing that astrocyte conditioned media containing TGFβ inhibited nitric oxide (NO) production in microglia. When TGFβ was neutralized, however, the conditioned media from astrocytes failed to inhibit microglial NO production (Orellana et al., 2013). It is important to mention that our results were obtained from primary cultures established from neonates, which may not fully represent the functions of mature microglia and astrocytes in vivo (Butovsky et al., 2014). Therefore, these data were supplemented by examining astrocytes in vivo in the context of a peripheral LPS challenge and also the effects of TGFβ inhibition on microglial activation. Consistent with
the *in vitro* results, inhibition of TGFβ signaling in the brain exaggerated microglial activation (IL-1β and TNFα) and corresponding neuroinflammation (i.e. higher IL-1β, IL-6, TNFα, CD14 mRNA expression) following LPS (Fig.3.9). Moreover, central TGFβ inhibition was associated with increased Iba-1 immunoreactivity in the hippocampus 72 h after LPS (Fig.3.10). These results parallel the microglia/astrocyte transwell culture data (Fig.3.7) and indicate that interfering with TGFβ signaling in the brain ablates the anti-inflammatory effects mediated by IL-10. Indeed, elevated IL-10 mRNA expression was maintained in the brain of mice injected with LPS independent of TGFβ inhibition. Thus, without TGFβ signaling, high IL-10 expression in the LPS-SB group was insufficient to resolve pro-inflammatory gene expression. Taken together, these data indicate that IL-10 dependent anti-inflammatory responses in the brain are mediated by astrocytes and their corresponding production of TGFβ.

Our findings indicate that an important consequence of impaired regulation of microglia by astrocytes is prolonged symptoms of sickness. For example, inhibition of TGFβ signaling in the brain contributed to more extensive weight loss, prolonged sickness behavior, and exaggerated neuroinflammation after a peripheral LPS challenge (Fig.3.9&3.10). These data are consistent with models of aging and impaired microglial regulation (i.e., CX3CR1KO) where exaggerated IL-1β expression in the brain was associated with prolonged sickness/depressive-like behaviors (Abraham and Johnson, 2009a; Corona et al., 2010; Godbout et al., 2005b; Wynne et al., 2010). Specifically, TGFβ increased CX3CR1 expression and reduced IL-1β expression in activated microglia. Both of these events are associated with the resolution of microglial activation and return to baseline behavior after LPS challenge (Henry et al., 2009; Wynne et al., 2010). Similar to our previous reports in aged mice, TGFβ inhibition in adult mice resulted in a more inflammatory phenotype of microglia with amplified expression of IL-
1β and down-regulated expression of CX₃CR1. These current data indicate that astrocytes contribute to the regulation of microglia and help restore the brain to a state of homeostasis after a transient inflammatory insult.

These new insights into the dynamic relationship between microglia and astrocytes are critically relevant to the study of neuroimmune regulation in injury, aging, and disease. For example, IL-10 signaling in astrocytes was important for neuroprotection following facial nerve axotomy and IL-10 deficient mice had decreased motor neuron survival following injury (Xin et al., 2011). Moreover, following peripheral LPS immune challenge, primed microglia from aged mice produce elevated levels of both IL-1β and IL-10 compared to adult microglia. Although IL-10 protein expression was high in the aged brain after LPS, it was insufficient to resolve microglial activation (Fenn et al., 2012a; Henry et al., 2009). These results suggest that responsiveness to IL-10 may be impaired in the aged brain. A connection with these previous findings to the current study is that astrocytes in the aged brain may not be responding appropriately to IL-10 produced by microglia. For instance, mRNA expression of astrocyte inflammatory markers GFAP and vimentin (Pekny and Nilsson, 2005; Zamanian et al., 2012) were increased in the brain of aged mice compared to adults (Cotrina and Nedergaard, 2002; Godbout et al., 2005b). In addition, TGFβ mRNA expression was increased in the brain of adult, but not aged mice 24 h after LPS challenge. This low TGFβ expression in the aged brain corresponded with high IL-1β expression and protracted CX₃CR1 downregulation (Wynne et al., 2010). The data provided in this study support the premise that aged astrocytes are less responsive to IL-10 and fail to produce sufficient TGFβ to resolve microglia activation after a peripheral immune challenge. It is important to note that in other disease models, TGFβ may promote inflammation. In experimental autoimmune encephalomyelitis, central TGFβ signaling initiated the onset of disease
(Luo et al., 2007) and aided in sustaining high levels of inflammation in the brain and spinal cord (Lanz et al., 2010). In this model however, mice are immunized against CNS which elicits an auto-immune response. Nonetheless, in the context of resolving the activation of microglia after peripheral innate immune challenge, our results indicate that TGFβ lowered microglial activation and that inhibition of TGFβ signaling in the brain amplified neuroinflammation.

In conclusion, we provide novel evidence that astrocytes interpret the anti-inflammatory signals provided by IL-10 and respond by producing TGFβ. This dynamic interaction of astrocytes provides feedback to attenuate microglial activation. These results provide a novel mechanism by which astrocytes and microglia use anti-inflammatory mediators to modulate cytokine production, and may provide new avenues to understand impaired regulation of inflammation in aging, neurological disease, and traumatic CNS injury.
Chapter 4: Age-Related Impairments in the Dynamic Regulation of Active Microglia by Astrocytes

Abstract

Older individuals are at greater risk for infection and concomitant with this they have a higher frequency for developing neurobehavioral complications that negatively affect health and lifespan. Amplified and unregulated neuroinflammation may be the key mechanism underlying the development of these neuropsychiatric disorders. In aged rodents, the anti-inflammatory cytokine IL-10 is highly expressed in the brain following immune challenge, yet microglial activation is prolonged and behavioral impairments develop. Recently we showed that astrocytes of adult mice express the IL-10 receptor (IL-10R) and that IL-10 re-directs active astrocytes to produce TGFβ, which in turn, attenuates the activation of microglia. Therefore, the purpose of this study was to investigate the degree to which these key cytokine interactions between glia are impaired in the aged brain. Here we report that along with microglia, astrocytes also had a reactive morphological profile in aged mice. In addition, aged astrocytes had increased GFAP and vimentin expression, and decreased IL-10R surface expression compared to adults. Following acute immune challenge in vivo, adult astrocytes up-regulated IL-10R and TGFβ mRNA. Aged astrocytes, however, failed to increase expression of these mediators. This lack of regulation by TGFβ was associated with decreased TGFβ signaling and exaggerated expression of pro-inflammatory mediators in aged microglia. Additionally, active microglia cultured ex vivo with adult astrocytes reduced inflammatory
markers while those cultured with aged astrocytes did not. In summary, these novel data indicate that astrocytes have an important role in regulating microglia via TGFβ signaling and that an impaired IL-10 response in aged astrocytes contributes to age-related deficits in the regulation of active microglia.

Introduction

As the population grows and the percentage of elderly people increases, with number of seniors aged 65 and up predicted to double by 2050 (Ortman et al., 2014), research into healthy aging has never been more important. Unfortunately, seniors continue to be afflicted with debilitating cognitive and neuropsychiatric disorders. For example, the number of seniors living with dementia is expected to triple in the next few decades (WHO, 2012). Recent studies indicate that neuropsychiatric disorders in the aged, including depression and cognitive impairments, can be triggered by otherwise innocuous peripheral infections (Ahmed et al., 2014; Dunn et al., 2005; Penninx et al., 1999; Penninx et al., 2003). During systemic inflammation, infection, or illness, peripheral inflammation is interpreted and propagated within the brain. Although normally adaptive to help fight infections, increased and prolonged brain inflammation in the elderly may lead to behavioral and cognitive complications, including long-lasting sickness behavior, depression, and cognitive decline (for a review see (Norden and Godbout, 2013)). The mechanisms underlying these complications are unknown. Therefore, a better understanding of how the immune system within the brain is dysregulated with age and how this leads to behavioral and cognitive complications is needed to improve the likelihood of successful aging.

Several studies show that transient activation of the immune system with E.coli or lipopolysaccharide (LPS), leads to exaggerated neuroinflammation and significant cognitive and behavioral complications in aged rodents (Barrientos et al., 2006; Godbout
et al., 2005b; Godbout et al., 2008). The same immune insult, however, induces no complications in adult mice. The reason neuroinflammation is prolonged in the aged after immune challenge may be related to changes in brain glia. Microglia, the principal immune cell of the central nervous system (CNS), interpret and propagate inflammatory signals that are initiated in the periphery (Nguyen et al., 2002). With age, however, microglia become “primed” and following immune activation they become hyperactive. Hyperactive microglia produce exaggerated levels of both pro- and anti-inflammatory cytokines, including the anti-inflammatory cytokine Interleukin (IL)-10 (Henry et al., 2009; Sierra et al., 2007). Exaggerated microglial responses may be related to impairments in key regulatory systems that make it more difficult to resolve microglial activation (Jurgens and Johnson, 2010b; Norden and Godbout, 2013). For example, the exaggerated IL-10 expression by active microglia was not accompanied by the expected anti-inflammatory action. We interpret these results to indicate that IL-10 signaling is impaired in the aged brain.

To understand the mechanism of this age-related impairment in IL-10 regulation, we have turned our attention to another glial cell type, astrocytes. Astrocytes produce several anti-inflammatory mediators that promote microglial quiescence and resolve microglial activation after immune challenge. We have previously shown that immune activated astrocytes preferentially respond to IL-10 and decrease microglial activation in a Transforming Growth Factor (TGF) β-dependent manner (Norden et al., 2014a). For example, astrocyte cultures activated with LPS and treated with IL-10 have reduced pro-inflammatory IL-1β expression and higher TGFβ protein secretion than those without IL-10 treatment. This is important because TGFβ is a regulatory cytokine with neuroprotective and anti-inflammatory effects in the CNS. In microglia, increased pro-inflammatory mediators and cytokine expression initiated by LPS challenge was attenuated by TGFβ (Butovsky et al., 2014; Herrera-Molina and von Bernhardi, 2005;
Norden et al., 2014a; Tichauer et al., 2014). These results indicate that TGFβ produced by activated astrocytes is important for regulating active microglia.

In this study, we compared IL-10 regulation and TGFβ expression in adult and aged mice. At baseline, astrocytes from aged mice had an activated morphological profile and decreased IL-10 receptor (IL-10R) surface expression compared to astrocytes from adult mice, indicating that aged astrocytes may be more inflammatory and less sensitive to IL-10. During systemic immune challenge, astrocytes from adult mice increased IL-10R and TGFβ mRNA expression. Astrocytes from aged mice, however, failed to upregulate both IL-10R and TGFβ. This lack of regulation by TGFβ was associated with exaggerated microglial activation. Collectively, this study indicates that prolonged microglial activation in the aged brain may be caused by impaired anti-inflammatory regulation by astrocytes.

**Materials and Methods**

**Mice**

Neonatal pups (postnatal day 1-3) and adult (3-4 months old) C57BL/6 and C57BL/6 IL-10R2−/− mice were obtained from our breeding colony kept in barrier-reared conditions in a specific-pathogen-free facility at the Ohio State University. Adult (3-4 months old) and aged (18-20 months old) Balb/c mice were purchased from the National Institute of Aging. Mice were individually housed in polypropylene cages and maintained at 25° C under a 12 h light/12 h dark cycle with *ad libitum* access to water and rodent chow. All procedures were in accordance with the National Institute of Health Guidelines for the Care and Use of Laboratory Animals and were approved by The Ohio State University Institutional Laboratory Animal Care and Use Committee.
Primary microglia and astrocyte cultures

Primary microglia and astrocyte cultures were established from WT and IL-10R2−/− neonatal mice and maintained as previously described (Godbout et al., 2004; Norden et al., 2014a). In brief, mixed glia cultures were shaken at 160rev/min and 37°C for 3.5h to harvest microglia from the confluent cell layer. Remaining cells were treated with 50 mM l-leucine methyl ester (Sigma-Aldrich) for 45 minutes to deplete remaining microglia from the astrocyte layer. After l-leucine incubation, astrocytes recovered in growth medium supplemented with 0.1 mM l-leucine for 1-3 days (Phulwani et al., 2008). Astrocytes were collected by incubating with 0.25% trypsin for 15 minutes, and then plated at a density of 50,000 cells/well in 24-well plates. Immediately before treatment, cells were washed with serum-free DMEM medium. Primary astrocytes were activated with 100 ng/mL LPS (stereotype 0127:B8, Sigma-Aldrich) for 1 h followed by IL-10 (10 ng/mL) (R&D Systems) addition for an additional 23 h. Following experimental treatments RNA was isolated using Tri-Reagent.

Microglia-Astrocyte transwell co-cultures

Microglia-Astrocyte transwell co-cultures were established from IL-10KO mice using a protocol similar to one previously described (Norden et al., 2014a). In brief, primary microglia were plated at 50,000-75,000 cells/well in a 24-well transwell plate (Corning Life Sciences). After incubation for 3 hours, astrocytes were added to a removable 0.4 um polycarbonate membrane at an equal number as microglia. Following incubation overnight, cells were washed with serum-free DMEM media prior to treatment. LPS was added to the shared medium at 10 ng/mL. After 1 hour, recombinant mouse IL-10 was suspended in PBS with 0.1%BSA and added at 10 ng/mL. Following experimental treatments RNA was isolated from the microglia using Tri-Reagent.
Peripheral Injections

Adult and aged Balb/c mice were injected intraperitoneally (i.p) with saline or *Escherichia coli* lipopolysaccharide (LPS) (0.33 mg/kg; serotype 0127:B8, Sigma, St. Louis, MO). Adult C57BL/c wildtype and IL-10R2<sup>−/−</sup> mice were injected i.p with 0.5mg/kg LPS. The LPS dosage was selected because it elicits a pro-inflammatory cytokine response in the brain resulting in a transient sickness response in adult mice (Berg et al., 2004; Godbout et al., 2005b; Henry et al., 2008; Wynne et al., 2010).

Microglia and astrocyte isolation from brain

Microglia and astrocytes were isolated from brain homogenates using a Percoll density gradient as previously described (Norden et al, 2014). In brief, tissues were homogenized and cell pellets were re-suspended in 70% isotonic Percoll. A discontinuous Percoll density gradient was layered and centrifuged for 20 min at 2000xg. Microglia were collected from the interphase between the 70% and 45% Percoll. Enriched microglia were washed and re-suspended in PBS for qPCR analysis or FACS buffer for flow cytometric analysis. These cells were referred to as enriched brain CD11b<sup>+</sup> cells based on previous studies demonstrating that viable cells isolated by Percoll density gradient yields greater than 90% CD11b<sup>+</sup> cells (Henry et al., 2009). Enriched astrocytes were collected from the interphase between 45% and 35% Percoll layers. Of the cells recovered from this Percoll gradient layer, 75-80% of the cells were GLAST-<sup>−1</sup> astrocytes. These enriched astrocytes were washed and re-suspended in PBS for qPCR analysis or FACS buffer for flow cytometric analysis.

IL-10 Receptor Surface Expression

Astrocytes were assayed for IL-10R1 surface expression by flow cytometry. Percoll enriched astrocytes were incubated with rat anti-mouse CD11b-FITC, IL-10R1-
PE (eBioscience), and GLAST-1-APC (Miltenyi Biotec) antibodies. Expression was
determined using a Becton-Dickinson FACSCaliber four color Cytometer. Astrocytes
were identified by GLAST-1⁺/CD11b⁻ expression. For each antibody, gating was
determined based on appropriate negative isotype stained controls. Flow data were
analyzed using FlowJo software (Tree Star, CA).

**FAC-Sorting**

Microglia and astrocytes were percoll enriched as described above. Microglia
and astrocyte fractions were pooled together and incubated with rat anti-mouse CD11b-
FITC, CD45-PerCP-Cy5.5 (eBioscience) and GLAST-1-APC (Miltenyi Biotec) antibodies.
Cells were sorted using a Becton-Dickinson FACSCaliber/Aria four color Cytometer at
the OSU Comprehensive core facility. Microglia were identified by CD11b⁺/CD45low
expression and astrocytes were identified by GLAST-1⁺/CD11b⁻ expression. Each
separate population was sorted into separate collection tubes. Samples were pelleted
and lysed immediately in RNA lysis buffer (PrepEase kit (USB, CA)).

**RNA isolation, RT-PCR**

RNA was isolated from a 1 mm coronal brain section, cell cultures, Percoll
enriched microglia and astrocytes, and sorted microglia and astrocytes. For cell cultures
and brain tissue, total RNA was isolated using the Tri-Reagent protocol (Sigma-Aldrich).
For percoll isolated and sorted microglia and astrocytes, RNA was isolated using the
PrepEase kit (USB, CA). RNA was reverse transcribed to cDNA and real-time (RT)-PCR
was performed using the Applied Biosystems Taqman® Gene Expression assayAssay-
on-Demand Gene Expression protocol. In brief, experimental cDNA was amplified by
real-time PCR where a target cDNA (e.g., IL-1β, IL-6, IL-10) and a reference cDNA
(glyceraldehyde-3-phosphate dehydrogenase; GAPDH) were amplified simultaneously using an oligonucleotide probe with a 5’ fluorescent reporter dye (6-FAM). Fluorescence was determined on an ABI PRISM 7300-sequence detection system (Applied Biosystems). Data were analyzed using the comparative threshold cycle (Ct) method and results are expressed as fold difference.

**NanoString analysis**

For sorted microglia and astrocytes, mRNA was isolated using PrepEase kit (USB, CA) and analyzed by NanoString nCounter technology (www.nanostring.com) which allows expression analysis of multiple genes from a single sample. NanoString analysis was performed by the Nucleic acid core facility at OSU. Briefly, RNA quality and integrity was assessed using Bioanalyzer 2100 (Agilent Technologies). We performed nCounter multiplexed target profiling of 100 inflammation genes. Customized plates were designed with selected astrocytic and microglia related gene. Overall the selected genes were relevant to glia identification (e.g., CD11b, Iba-1, GFAP, GLT-1), activation (e.g., IL-1β, TNFα, CCL2), regulation (e.g., TGFβ, IL-10R1/R2) and reactivity (e.g., CD68, vimentin).

**Ex vivo microglia-astrocyte transwell co-cultures**

Enriched microglia were isolated from saline (adult, aged mice) and LPS (adult) injected mice (4h post injection) using Percoll density gradient separation. Microglia were plated at the bottom of poly-L-lysine coated 24-well plates (Corning Life Sciences). After 30 minutes, enriched astrocytes were added to a removable 0.4 um polycarbonate membrane. After an additional 4 h, conditioned media was collected and stored at -80°C and RNA was isolated from microglia using PrepEase kit (USB, CA).
**Determination of IL-6 protein levels in conditioned media**

IL-6 was determined from conditioned media using the BD OptEIA Mouse IL-6 ELISA according to the manufacturer's instructions (BD Biosciences). In brief, 96-well enzyme immunoassay plates were coated with anti-mouse IL-6 capture antibody and incubated overnight at 4°C. Samples and IL-6 standards (0–1000 pg/ml) were added and incubated for 2 h at room temperature (RT). Plates were washed and incubated with biotinylated anti-mouse IL-6 antibody. Plates were washed and incubated with streptavidin-horseradish peroxidase conjugate. After 1 h incubation at RT, plates were washed and incubated with tetramethylbenzidine liquid substrate for 15 min. Reactions were terminated and absorbance was read at 450 nm using a Synergy HT Plate Reader (Bio-tek instruments). The assay was sensitive to 10 ng/ml IL-6 and the interassay and intra-assay coefficients of variation were less than 10%.

**Immunohistochemistry and digital image analysis**

Adult and aged mice were deeply anesthetized and transcardially perfused with sterile PBS followed by 4% formaldehyde. Brains were post-fixed in 4% formaldehyde for 24 h and then cryoprotected in 20% sucrose for an additional 48 h. Preserved brains were frozen using dry-ice cooled isopentane (-165°C) and then sectioned (20 µm) using a Microm HM550 cryostat. Brain sections were identified by reference markers in accordance with the stereotaxic mouse brain atlas (Paxinos and Franklin, 2004). Free-floating sections were washed in PBS and blocked with 5% normal goat serum (NGS) and 1% bovine serum albumin (BSA) for 1 h at room temperature. Sections were then stained using rabbit anti-mouse GFAP (Dako) or Iba-1 (Wako Chemicals) antibodies and incubated overnight at 4°C. Sections were washed and incubated with a fluorochrome-conjugated secondary antibody (Alexa Fluor, anti-rabbit 488 or 594) overnight at 4°C. Fluorescent images were visualized using an epifluorescent Leica DM5000B microscope.
and were captured using a Leica DFC300 FX camera and imaging software. To determine Iba-1 and GFAP phenotypes in the hippocampus, 6-10 representative images were taken at 20x magnification in the CA1, and CA3 regions.

**Microglia and Astrocyte Reconstruction**

Glia Reconstruct was used to analyze GFAP and Iba-1 labeled images as previously described (Kongsui et al., 2014a; Kongsui et al., 2014b; Walker et al., 2014) but with modifications. In brief, images were captured using a Leica DFC300 FX (20X) as described above. 3-5 representative sections per experimental mouse were used. Cumulative spectra analysis (Kongsui et al., 2014b) was used to find the appropriate pixel intensity for glial cell analysis. The data returned from this analysis were exported to Excel and a cumulative threshold spectrum was generated. This spectrum reflects the cumulative number of pixels that occurred at or below each of the 256 pixel intensities (0 = black and 256 = white) analyzed. A threshold for pixel intensity was determined for each image that included all cell bodies and processes, but excluded background staining. Glia were traced throughout the section and the trace information was used for analysis. Only microglia or astrocytes that displayed intact processes unobscured by background labeling or other cells were used. Morphological software was used to generate the metric analyses of the reconstructed glia. This analysis is based on a comprehensive set of algorithms for the morphological analysis of glia. These algorithms combine a variety of image analysis approaches including feature extraction using multipoint variance clustering (Radler et al., 2015). Using this approach the key morphological features of glia can be examined. For example, total cell perimeter, total cell length, cell body perimeter, number of primary processes, number of nodes (branch points), total length of all processes, and total volume of all processes were measured. In addition, the area encompassed by the entire cell was measured as the convex hull.
area, determined from the polygon created from straight lines connecting the most distal points of the microglial processes. Pixel information was converted into μm values to provide information on relative size, area or volume of microglia and astrocytes.

Statistical Analysis

To ensure a normal distribution, data were subjected to the Shapiro-Wilk test using Statistical Analysis Systems (SAS) statistical software (Cary, NC). To determine significant main effects and interactions between main factors, data were analyzed using one-way (i.e. Pretreatment and Treatment) or two-way (i.e. Pretreatment x Treatment) ANOVA using the General Linear Model procedures of SAS. When appropriate, differences between treatment means were evaluated by an F-protected t-test using the Least-Significant Difference procedure of SAS. All data are expressed as treatment means ± standard error of the mean (SEM). Values were considered significant at p-values < 0.05 and a tendency at p-values ≤ 0.1.

Results

Altered glia morphology in the aged brain

We recently reported that there is dynamic communication between astrocytes and microglia during immune challenge. For instance, immune challenge activated microglia to produce IL-10, which astrocytes responded to and increased TGFβ. In turn, TGFβ attenuated the activation of microglia. Thus astrocytes may provide essential negative feedback on active microglia using IL-10 and TGFβ (Norden et al., 2014a). Therefore, the purpose of this study was to investigate the degree to which these key cytokine interactions between glia are impaired in the aged brain.
First, we aimed to determine aged related changes in astrocytes and microglia that may explain dysfunction in anti-inflammatory regulation. Therefore, mRNA levels of Iba1, GFAP, vimentin mRNA were determined in the cortex, cerebellum and hippocampus of adult and aged mice. Iba1 mRNA expression was not altered with age in any of the brain regions (Fig.4.1A). GFAP mRNA expression was increased in the hippocampus ($p<0.02$) and cerebellum ($p<0.01$) of aged mice compared to adults (Fig.4.1B). GFAP mRNA expression, however was not increased in the cortex. Increased vimentin mRNA expression was only increased in the hippocampus of aged mice compared to adult controls ($p<0.002$, Fig.4.1C). Based on these data, we next examined if there were differences in cell number and structure that may account for age-associated changes in glia and the increased GFAP expression in the hippocampus.

Thus, Glia Reconstruct was used to determine cell number and several key morphological parameters of microglia and astrocyte including the perimeter of the cell, length and area. First, cumulative spectra analysis was used to find the appropriate pixel intensity for analysis. Images were then thresholded and analyzed. Fig.4.1D shows representative labeling (left) and the corresponding thresholded images (Right) for Iba1 and GFAP from the CA1 region for adult and aged mice. Fig.4.1E shows the results of Glia reconstruct analysis on microglial cell number, cell perimeter, cell length and convex cell area. There was no difference in cell number with age, but there were significant differences in microglial morphology. For example, microglia of aged mice had an increased perimeter ($p<0.001$), max length ($p<0.001$), and convex hull area ($p<0.002$). Similar data were obtained with astrocytes. Overall, astrocytes were larger with altered morphology, but there was no increase in the number of astrocytes in the hippocampus of aged mice compared to adult mice (Fig.4.1F). There was evidence of significant remodeling and cytoskeletal re-organization of aged astrocytes in the hippocampus. For
instance, aged astrocytes had larger perimeter ($p<0.02$), length ($p<0.03$), and a global enlargement of the GFAP cytoskeleton (convex hull) ($p<0.001$).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Adult</th>
<th>Aged</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number (per section)</td>
<td>11.0 ± 1.5</td>
<td>12.3 ± 2.0</td>
</tr>
<tr>
<td>Perimeter (um)</td>
<td>48.0 ± 4.7</td>
<td>58.5 ± 3.7*</td>
</tr>
<tr>
<td>Max Length (um)</td>
<td>13.2 ± 0.8</td>
<td>15.3 ± 0.7*</td>
</tr>
<tr>
<td>Hull Area (um²)</td>
<td>52.7 ± 5.5</td>
<td>63.1 ± 4.6*</td>
</tr>
</tbody>
</table>

**Figure 4.1. Altered glia morphology in the aged brain.** The cortex (CX), hippocampus (HPC), and cerebellum (CB) was dissected from adult (3mo) and aged (18mo) mice and mRNA expression of A) Iba1, B) GFAP, and C) vimentin was determined (n=3). D) Iba1 and GFAP expression was determined in the hippocampus of adult and aged mice. Glia Reconstruct analysis was used on thresholded images for E) microglia and F) astrocytes (n=5). * $p<0.05$ from Adult.

**Microglial and astrocyte gene expression profiles**

Next, we sought to determine gene expression profiles of aged microglia and astrocytes. In these studies, microglia and astrocytes were collected from brain
homogenates using a modified Percoll density gradient. Cells were pooled and then FAC sorted. The diagram in Fig. 4.2A shows the gating strategy for microglia (MGL, CD11b+ ) and astrocytes (AST, GLAST-1+). The microglia collected had minimal GFAP expression while the astrocytes had minimal CD11b mRNA expression (Fig. 4.2B).

**Figure 4.2. Percoll isolation and FAC sorting of microglia and astrocytes.** A) Percoll gradient separation was used to isolate microglia and astrocytes. Cells were pooled and stained with CD11b and GLAST-1 for FAC sorting. B) Relative expression of CD11b and GFAP in sorted populations. n=3

Using this gating strategy, microglia and astrocytes were collected and FAC-sorted from adult (3mo) and aged (18mo) mice. The mRNA was isolated and analyzed by NanoString gene array that determined mRNA copy numbers of selected genes.
Fig.4.3 shows that several genes associated with microglial identification (CX3CR1, CSF1R, TREM2, TGFβR1, CD11b, Iba1) were not altered by age. The mRNA copy numbers of CD86 and CD45 were increased in microglia from aged mice compared to microglia from adult mice (p<0.05 for each), indicating a primed profile of aged microglia.

Genes associated with common inflammatory signaling pathways were also determined. Most of the genes related to intracellular signaling were not altered with age (JUN, JAK, STAT, SMAD). Of these genes, only SMAD4 (common Smad) and SMAD7 (inhibitory Smad) were increased with age (p<0.05).

The mRNA copy numbers of several pro-inflammatory genes, however, were elevated in microglia from aged mice compared to microglia from adult mice. These included pro-inflammatory cytokines (IL-1β, IL-6, TNFα, Caspase1), chemokines (CCL3, CCL5, CXCL10) and Toll-like receptors (TLR1, TLR2). Microglia from aged mice also had elevated expression of several anti-inflammatory genes (IL-4Rα, Mrc1, IL-1RN). Overall these data indicate that microglia have a primed and pro-inflammatory gene expression profile in aged mice.
### Microglia Gene Expression (mRNA counts)

<table>
<thead>
<tr>
<th>Identification</th>
<th>Adult</th>
<th>Aged</th>
</tr>
</thead>
<tbody>
<tr>
<td>CX3CR1</td>
<td>2273 ± 1422</td>
<td>2258 ± 1445</td>
</tr>
<tr>
<td>CSF1R</td>
<td>15638 ± 187</td>
<td>14285 ± 3511</td>
</tr>
<tr>
<td>TREM2</td>
<td>3474 ± 93</td>
<td>3838 ± 308</td>
</tr>
<tr>
<td>CD11b</td>
<td>1814 ± 10</td>
<td>1819 ± 100</td>
</tr>
<tr>
<td>IBA1</td>
<td>14.4 ± 0.5</td>
<td>16.3 ± 2.5</td>
</tr>
<tr>
<td>P2RX7</td>
<td>436 ± 4.2</td>
<td>492 ± 21</td>
</tr>
<tr>
<td>CD200R1</td>
<td>147 ± 29</td>
<td>179 ± 20</td>
</tr>
<tr>
<td>Fcgr1</td>
<td>2091 ± 7.0</td>
<td>2383 ± 84</td>
</tr>
<tr>
<td>Fcgr3</td>
<td>2264 ± 231</td>
<td>2808 ± 163</td>
</tr>
<tr>
<td>CD45</td>
<td>356 ± 23</td>
<td>510 ± 14</td>
</tr>
<tr>
<td>CD48</td>
<td>794 ± 21</td>
<td>851 ± 22</td>
</tr>
<tr>
<td>CD68</td>
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**Figure 4.3. Microglia gene expression profile.** Microglia were isolated and FACsorted from adult and aged mice. mRNA copy numbers of genes associated with microglial identification, intracellular signaling, and pro- and anti-inflammatory processes were determined by NanoString analysis. (n=4) * p=0.1 from Adult
Astrocytes from adult and aged mice were also evaluated using this NanoString gene array. There were several differences detected in genes associated with astrocyte identification. For example, expression of two glutamate transport proteins, EEA2 and EEA1 were decreased in aged astrocytes. The gene array also confirmed increased GFAP, but not vimentin, expression in astrocytes from aged mice (Fig.4.4). There was decreased expression of S100A1 but increased expression of S100B. There was no difference in Aquaporin 4 expression. Similar to microglia, expression of genes involved in common inflammatory signaling pathways were not altered with age. The only intracellular signaling components that were different with age were STAT1, Myc, Jun, CCND1 and CDKN2B. Of these genes, Myc, CCND1 and CDKN2B are associated with IL-10 signaling. In addition, there was differential expression of the IL-10 receptor subunits. Although there was no difference in IL-10R2 expression, there was a selective decrease in IL-10R1 mRNA in astrocytes from aged mice. These data suggest that aged astrocytes may have impaired IL-10 responsiveness. Aged astrocytes also had decreased expression of several growth factors. BDNF, IGF1, VEGFA and the anti-inflammatory marker Fizz1 were all deceased in aged astrocytes (Fig.4.4). Most of the pro-inflammatory genes were not changed with age in astrocytes. IL-6 was decreased in aged astrocytes while TNF was increased. Overall, these data indicate that age had a differential effect on microglia compared to astrocytes. Aged microglia had a primed profile with higher expression of several pro-inflammatory cytokines. Aged astrocytes had a reactive profile with increased GFAP expression, but also a profile associated with impaired growth support.
### Astrocyte Gene Expression (mRNA counts)

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#### Intracellular Signaling

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<td>Jun</td>
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*Figure 4.4. Astrocyte gene expression profile.*

Astrocytes were isolated and FACsorted from adult and aged mice. mRNA copy numbers of genes associated with astrocyte identification, intracellular signaling, and pro- and anti-inflammatory processes were determined by NanoString analysis. (n=4) * p=0.1 from Adult
Decreased surface expression of IL-10R1 on aged astrocytes

The gene array revealed that astrocytes from aged mice had decreased mRNA expression of IL-10R1 compared to adults. Thus we sought to confirm that there was a corresponding reduction in IL-10R1 surface expression in aged astrocytes. Astrocytes were enriched from adult and aged mice and IL-10R1 protein expression was determined. Fig.4.5A shows representative dot plots of Percoll enriched astrocytes (Glast/CD11b) from adult and aged mice. There was no difference in the number of GLAST-1+ astrocytes with age (Fig.4.5B). There was, however, reduced number of astrocytes that were IL-10R1+. Fig.4.5C&D show a significant reduction in IL-10R1 surface expression in aged astrocytes (16%) compared to adult astrocytes (23%) (Fig.4.5D, p<0.03). Overall, these data are consistent with the mRNA data that IL-10R1 is decreased in astrocytes of aged mice.
Exaggerated and prolonged neuroinflammation following systemic immune challenge in the aged

We have previously reported that microglia of the aged brain are hyperactive following immune challenge and produce high levels of both IL-1β and IL-10 (Henry et al., 2009). In addition, the cytokine response in aged microglia is prolonged compared to adults (Wynne et al., 2010). To determine the degree to which anti-inflammatory
regulation is altered in the aged brain, adult and aged mice were injected i.p with LPS and the brain was collected 24 h later. This timepoint represents the transition from sickness to resolution in adult mice, but prolonged sickness in aged mice (Wynne et al., 2010). Several pro-inflammatory mediators (IL-1β and IL-6) and key anti-inflammatory regulatory cytokines and their corresponding receptors (IL-10/IL-10R1, TGFβ/TGFβR1) were determined. Similar to previous reports (Godbout et al., 2005b; Richwine et al., 2008b), IL-1β, IL-6, and IL-10 mRNA expression was higher in the aged brain 24 h after LPS than in the adult brain (F\(_{1,24}\)=6.56, \(p<0.02\) for each) (Fig. 4.6A-C). Expression of the IL-10 receptor IL-10R1, however, was only upregulated in the brain of adult mice (F\(_{1,24}\)=13.55, \(p<0.002\)) and there was no induction above baseline in the brain of aged mice (Fig. 4.6D). Similar to IL-10R1, TGFβ mRNA expression was increased following LPS injection in the brain of adult mice but not in the brain of aged mice (F\(_{1,24}\)=5.50, \(p<0.03\), Fig. 4.6E). Moreover, aged mice had prolonged downregulation of TGFβR1 (F\(_{1,24}\)=6.51, \(p<0.02\), Fig. 4.6F). These data indicate that prolonged cytokine expression in the aged brain following LPS is associated with reductions in IL-10R1, TGFβ, and TGFβR1.
Figure 4.6. Exaggerated and prolonged neuroinflammation following systemic immune challenge in the aged. Adult and aged mice were injected i.p with LPS. After 24 h, the brain was collected and mRNA expression of A) IL-6, B) IL-1β, C) IL-10, D) IL-10R1, E) TGFβ and F) TGFβR1 was determined (n=6). * p<0.05 from Adult-Saline, # p<0.05 from Adult-Saline, + p=0.10 from Adult-Saline
Prolonged microglial activation following LPS injection in aged mice

To determine specific gene expression profiles of astrocytes and microglia after LPS injection, we used novel enrichment, cell sorting, and gene expression techniques. Adult and aged mice were injected peripherally with saline or LPS. After 24 h, astrocytes and microglia were isolated by Percoll gradient separation and FAC sorted to yield pure populations of respective cell type. The mRNA was isolated and analyzed by NanoString array.

mRNA levels were determined for selected genes associated with microglia identification, intracellular signaling, inflammation, and regulation. To focus on the LPS induced changes, the fold change of adult LPS and aged LPS groups relative to microglia from saline injected adult mice are represented in the graphs (Fig.4.7). First, none of the genes associated with microglia identification were different with age 24 h after LPS. There was also no large induction of identification genes after the LPS injection. Several genes associated with TGFβ signaling were decreased following LPS injection (SMAD2, SMAD3, SMAD4, and the TGFβ receptor subunits) but there was no difference with age. Other intracellular signaling pathway proteins, such as STATs, Jak/Tyk, Mapk14, were also not different with age. There was an exaggerated induction of pro-inflammatory cytokines in microglia of aged mice (IL-1α, IL-1β, TNF, HMOX1) and also of several chemokines (CCL4, CCL5). There was also exaggerated induction of several inflammatory receptors (IL-1R1, TNFRSF1a, TLR1, TLR2). Consistent to what we have shown before, the exaggerated expression of pro-inflammatory genes was also associated with elevated anti-inflammatory gene expression. There was exaggerated expression of IL-4Rα, SOCS3, Arg1, CHI3L, and CD163 in aged microglia. Importantly, TGFβR1 was downregulated after LPS and aged microglia had significantly decreased TGFβR1 compared to adult microglia. These data indicate that microglia from aged mice...
have a prolonged activation state, and that following activation, there is decreased TGFβ signaling in microglia.
Figure 4.7. Exaggerated and prolonged microglial activation after immune challenge in the aged. Adult and aged mice were injected i.p with LPS. After 24 h, microglia were isolated by Percoll Density gradient and FACsorted by CD45 labeling. NanoString gene array was used to determine mRNA copy numbers of genes associated with microglial identification (purple), intracellular signaling (green), and pro- (red) and anti- (blue) inflammatory processes. Data expressed as fold change from adult-saline. (n=4). * p=0.1 from Adult-Saline
LPS injection increased IL-10R1 and TGFβ mRNA expression in enriched astrocytes of adult, but not aged mice

Using the same LPS injection protocol, mRNA levels of IL-1β, IL-6, IL-10, IL-10R1 and TGFβ were determined in enriched astrocytes at 24 h (Fig.4.8A). There was no increase in IL-6 mRNA expression in either astrocytes of adult or aged mice at the 24 h timepoint (Fig.4.8B). IL-1β expression was increased after LPS injection (F\(_{1,24}=19.35, p<0.001\), Fig.4.8C), however there was no exaggerated expression in astrocytes of LPS injected aged mice. IL-10 mRNA expression was also increased after LPS injection (F\(_{1,24}=7.49, p<0.02\)), but there was no effect of age (Fig.4.8D). Astrocytes of adult mice increased IL-10R1 and TGFβ expression following LPS (F\(_{1,24}=6.58, p<0.02\) for each). In aged astrocytes, however, there was no induction of either of these mediators 24 h after LPS (Fig.4.8E&F). The failure to induce IL-10R1 and TGFβ following induction of an immune response indicate that sensitivity of aged astrocytes to IL-10 may be impaired.
**Figure 4.8.** LPS injection increased IL-10R1 and TGFβ mRNA expression in enriched astrocytes of adult, but not aged mice. Adult and aged mice were injected i.p with LPS. After 24 h, astrocytes were isolated by Percoll Density gradient (A). mRNA expression of B) IL-6, C) IL-1β, D) IL-10, E) IL-10R1, and F) TGFβ was determined (n=6). * p<0.05 from Adult-Saline.

To confirm these results, we also FAC sorted GLAST-1+ astrocytes and performed NanoString on the isolated mRNA. To focus on the LPS induced changes, the fold change of adult LPS and aged LPS groups relative to astrocytes from saline injected adult mice are represented in the graphs (Fig.4.9). Several genes associated with astrocyte identification were not changed after LPS challenge and were not changed with age (AQP4, EAA1, EAA2, S100a1). S100b and GFAP were increased after LPS challenge and astrocytes from aged mice had the highest expression. Vimentin expression was increased after LPS, but astrocytes from aged mice had attenuated vimentin upregulation. Several genes associated with inflammatory signaling pathways were upregulated after LPS. Of these, several genes associated with IL-10 signaling...
(Myc, Jak1, Tyk2, PIK3R1 and CDKN2B) were attenuated in aged astrocytes. Furthermore, IL-10R1 and IL-10R2 were both upregulated in astrocytes from adult mice. Aged astrocytes, however, had decreased IL-10R1 upregulation compared to adult. There was a small induction of pro-inflammatory genes after LPS in astrocytes, and most of them were not changed with age. The only pro-inflammatory marker that had exaggerated expression in aged astrocytes was Casp1. There was also upregulation of several chemokines after LPS in astrocytes, but none of these had exaggerated expression with age. CCL1 and CXCL10 were expressed at decreased levels in aged astrocytes compared to adult. Expression of most anti-inflammatory genes was also not changed with age, except for CHI3L1 which was highest in aged astrocytes. Last, TGFβ1 mRNA expression was increased after LPS in adult astrocytes, but there was decreased upregulation in aged astrocytes. These data indicate that there is decreased IL-10 signaling after LPS challenge in aged astrocytes.
Figure 4.9. LPS injection increased IL-10R1 and TGFβ mRNA expression in sorted astrocytes from adult, but not aged mice. Adult and aged mice were injected i.p with LPS. After 24 h, astrocytes were isolated by Percoll Density gradient and FACsorted by GLAST-1 labeling. NanoString gene array was used to determine mRNA copy numbers of genes associated with astrocyte identification (purple), intracellular signaling (green), and pro- (red) and anti- (blue) inflammatory processes. Data expressed as fold change from adult-saline. (n=4). * p=0.1 from Adult-Saline
Astrocytes from IL-10R⁻/⁻ mice do not upregulate TGFβ and fail to reduce microglial activation

To determine a possible link between impaired IL-10 signaling and impaired upregulation of TGFβ following activation, we analyzed this relationship in IL-10R knockout mice. Similar to what we have previously shown (Norden et al., 2014a), LPS and IL-10 treatment increased TGFβ mRNA expression in primary astrocytes (p<0.02 from all groups, Fig.4.10A). In astrocytes from IL-10R⁻/⁻ mice, LPS increased TGFβ mRNA expression (F₁,₄₈=25.99, p<0.001) but there was no further augmentation by IL-10 treatment. Next, we used a transwell co-culture system to evaluate astrocyte regulation of microglial activation. In this setup, microglia were plated at the bottom of a well and astrocytes from either WT or IL-10R⁻/⁻ mice were added to a removable insert. In this manner, we were able to evaluate microglia specific mRNA expression. All wells were activated by LPS and after 1 h, vehicle or IL-10 was added. After an additional 4 h, mRNA expression of IL-1β was determined in the microglia. Fig.4.10B shows that there was no effect of IL-10 on IL-1β expression in microglia that were cultured without astrocytes. The addition of astrocytes increased IL-1β expression in microglia, however, IL-10 treatment attenuated IL-1β expression in microglia when WT astrocytes were present in the co-culutes (F₁,₂₈=10.05, p<0.004) (Fig.4.10B). When astrocytes from IL-10R⁻/⁻ mice were in the transwell, there was no effect of IL-10 treatment on microglial IL-1β mRNA expression (Fig.4.10B).

Last, we injected WT and IL-10R⁻/⁻ mice i.p with LPS and collected enriched microglia and astrocytes at 4 h after injection. Similar to a previous study (Richwine et al., 2009), there was exaggerated inflammation and microglial expression of IL-6 in IL-10R⁻/⁻ mice (F₁,₂₄=7.10, p<0.01) (Fig.4.10C). Here we show novel data that astrocytes from IL-10R⁻/⁻ mice fail to upregulate TGFβ mRNA expression following LPS injection.
TGFβ mRNA was increased almost 2-fold in astrocytes from WT mice \((p<0.02\) from all groups), but there was no significant induction in IL-10R\(^{-/-}\) mice (Fig.4.10D). Overall, astrocytes from IL-10R\(^{-/-}\) mice fail to increase TGFβ expression following LPS and IL-10 stimulation both in vitro and in vivo. Furthermore, this lack of TGFβ upregulation was associated with exaggerated microglial activation. These data provide a connection between lack of IL-10 signaling in astrocytes of IL-10R\(^{-/-}\) mice and aged mice.

![Bar graph showing TGFβ mRNA expression](image)

**Figure 4.10. Astrocytes from IL-10R\(^{-/-}\) mice do not upregulate TGFβ and fail to reduce microglial activation.** A) Primary astrocytes from WT and IL-10R\(^{-/-}\) mice were activated with LPS and treated with IL-10 for 24 h. mRNA expression of TGFβ was determined \((n=12)\). B) Microglia were co-cultured in a transwell with WT or IL-10R\(^{-/-}\) astrocytes. LPS and IL-10 was added for 5 h and microglial IL-1β mRNA expression was determined \((n=10)\) * \(p<0.05\). WT and IL-10R\(^{-/-}\) mice were injected i.p with LPS. C) After 4 h, microglial IL-6 mRNA expression and D) astrocyte TGFβ mRNA expression was determined \((n=8)\). * \(p<0.05\) from WT-Saline, # \(p<0.05\) from WT-LPS.
Astrocytes from aged mice fail to regulate activated microglia ex vivo

To further confirm that astrocytes from aged mice fail to regulate microglial activation, we next used the transwell co-culture system to determine the direct effect of aged astrocytes on microglial cytokine expression. Adult mice were injected i.p with LPS. At the height of inflammation (4h), microglia were isolated and plated. Astrocytes from adult and aged mice were added to a removable insert (Fig.4.11A). After 4 h, mRNA from microglia and the conditioned media was collected. Fig.4.11B shows that microglial expression of IL-1β was increased after i.p LPS challenge (p=0.06). The addition of adult astrocytes lowered IL-1β expression back to the same level as saline injected mice (p>0.4). The addition of aged astrocytes, however, did not reduce IL-1β expression in microglia (p<0.01 from saline). In a similar manner, IL-6 expression in microglia was increased after LPS activation (p<0.05) (Fig.4.11C). The addition of adult (p>0.4 from saline), but not aged (p<0.05 from saline), astrocytes lowered IL-6 expression in microglia (Fig.4.11C). IL-6 protein levels were determined in the shared conditioned media. Fig.4.11D confirms that the highest amounts of IL-6 protein were present in the wells where LPS activated microglia were cultured with aged astrocytes (p<0.001 from all). IL-6 levels were not significantly elevated in any other condition. These data show that astrocytes from adult mice regulate microglial activation ex vivo. Astrocytes from aged mice, however, amplify the inflammatory state.
Figure 4.11. Astrocytes from aged mice fail to regulate activated microglia ex vivo. A) Microglia were isolated from saline (CON) and LPS injected adult mice. Astrocytes were injected from saline injected adult and aged mice. Microglia were plated in a transwell and astrocytes were added after 30 min. After an additional 4 h, microglial mRNA and the conditioned media was collected. B) Microglial IL-1β mRNA expression. C) Microglial IL-6 mRNA expression. D) IL-6 levels in the conditioned media (n=8). * p<0.05 from control

Discussion

Older individuals are at greater risk for infection and concomitant with this they have a higher frequency for developing neurobehavioral complications that negatively affect health and lifespan. For instance, otherwise innocuous infections are triggers for cognitive decline and the onset of depressive disorders in the elderly (Godbout and Johnson, 2006). Exaggerated microglial activation may be the key mechanism underlying the development of these neuropsychiatric disorders. For example, our work in mice shows that aged microglia are hyperactive during an immune challenge and this results in prolonged neuroinflammation and the development of depression and cognitive impairments in the older mice (for a review see (Norden et al., 2014b). To limit inflammation, glial cells produce anti-inflammatory cytokines including interleukin (IL)-10.
While IL-10 is also elevated in the aged brain following immune challenge, microglial activation still goes unresolved (Henry et al., 2009). Our recent work shows that there are dynamic interactions between microglia and astrocytes mediated by IL-10 (Norden et al., 2014a). In adult mice, astrocytes decrease pro-inflammatory activation following IL-10 signaling and also increase production of TGFβ which provides negative feedback on microglia. Therefore, IL-10 may act as a critical anti-inflammatory mediator that attenuates neuroinflammation and prevents the development of neurobehavioral complications. We hypothesized that prolonged microglial activation and neurobehavioral complications in the aged are caused by impaired IL-10 signaling in astrocytes and subsequent TGFβ upregulation. Here we show data indicating that microglia and astrocytes have a more reactive morphology and gene expression profile in aged mice. Moreover, our findings suggest that astrocytes from aged mice have decreased IL-10 sensitivity and fail to increase TGFβ expression following activation. The decreased response to IL-10 and impaired upregulation of TGFβ was associated with heightened microglial activation in both aged and IL-10R<sup>−/−</sup> mice.

Here we provide data indicating that the reason neuroinflammation is prolonged in the aged after immune challenge may be related to changes in brain glia. Previous studies have shown morphological differences and enhanced Iba1 labeling in microglia of aged humans (Streit et al., 2004) and rodents (VanGuilder et al., 2011). In addition, previous studies have shown increased GFAP mRNA expression in the aged brain (Godbout et al., 2005b). Therefore, aged astrocytes may also develop morphological alterations, however, these have been less described. Here, we morphologically characterized microglia and astrocytes using Iba1 and GFAP labeling, respectively. Gene expression of GFAP and vimentin, two markers of astrocyte reactivity, were increased specifically in the hippocampus of aged mice. In addition, the majority of aging
research has focused on the hippocampus as an anatomical region for age-related changes (Lister and Barnes, 2009; Miller and O'Callaghan, 2005). The hippocampus is an area involved in both cognition and mood, therefore, age-related cognitive decline and mood disorders may be linked to changed that occur specifically in the hippocampus. For these reasons, we investigated glial changes in this region. Although there was no difference in overall cell number of microglia or astrocytes, there were significant differences in morphology. There were similar changes in both astrocytes and microglia where there was an overall size increase. Most importantly, there was an increase in cell body size, which would indicate “swelling” of the cells. Our detailed analysis confirms cellular changes in microglia morphology of aged mice. For example, microglia of aged mice had an increased perimeter, indicating that aged microglia had a larger overall cell body. Aged microglia also had an increased max length. This suggests that microglia from aged mice have longer processes. However, this result may also be due to thicker branches that give a positive threshold for a longer distance compared to thinner branches that do not give a positive threshold reading. Similar to microglia, our morphological analysis shows that astrocytes also become hypertrophic in the hippocampus of aged mice. A previous study has shown that astrocytes in aged rats have an increased hypertrophic morphology with a shift from resting/stellate to active (VanGuider et al., 2011). Moreover, the morphological alterations were pronounced in the hippocampus. The astrocytes appeared to have an early stage pathology profile with significant enlargement of the GFAP+ cytoskeleton. It is unknown if these changes in morphology lead to dystrophy and senescence or if they underlie the exaggerated inflammatory response to secondary insult. Overall, these results show that microglia and astrocytes undergo significant changes with age and we interpret these data to indicate that astrocytes and microglia of aged mice develop a “primed” or reactive morphological profile.
Gene expression profiling confirmed that microglia of aged mice have a more inflammatory or primed gene profile. There was upregulation of inflammatory associated genes in microglia of aged mice, while there was no difference in microglia signature genes. These data show a specific and inflammatory related effect of age and priming. Here we provide novel data on the gene expression profile of astrocytes from aged mice. Aged astrocytes had decreased expression of several genes associated with growth support. These data are consistent with a previous study that aged astrocytes were less supportive of neurogenesis compared to astrocytes from adult mice (Miranda et al., 2012). The decreased expression of growth factors was also associated with an increased expression of several inflammatory related genes including GFAP and S100b. Expression of glutamate transporters EAA2 and EAA1 were also decreased in aged astrocytes. These findings are consistent with other studies showing decreased EAA2 in inflammatory conditions such as TBI or viral infection (van Landeghem et al., 2006; Wang et al., 2003). Important for our study was that genes associated with IL-10 signaling were reduced in aged astrocytes. There was decreased expression of both IL-10R1 mRNA and cell surface protein. Aged astrocytes also had decreased expression of genes associated with IL-10 signaling such as Myc, CCND1 and CDKN2B (Donnelly et al., 1999; O'Farrell et al., 2000).

Using an LPS injection paradigm, we show that neuroinflammation is exaggerated and prolonged in aged mice during systemic immune challenge. Consistent with previous studies, we show that IL-1β and IL-6 mRNA expression is both exaggerated and prolonged in the brain of aged mice following peripheral immune challenge (Henry et al., 2009; Wynne et al., 2010). However, we also provide novel data that the problem of exaggerated neuroinflammation is two-fold. Not only was pro-inflammatory mediators highly expressed, we also show impaired anti-inflammatory induction in the aged brain. Although IL-10 ligand mRNA was highly expressed in the
aged brain following LPS, induction of IL-10R1, TGFβ and TGFβR1 was impaired. IL-10 receptor (IL-10R1) and TGFβ were increased during the resolution phase in adult mice. At this same timepoint, however, there was no induction of either of these mediators. A previous study has shown that microglia decrease TGFβR1 expression upon immune activation and that TGFβ1 ligand can induce the expression of its receptor (Mitchell et al., 2014). Here we show prolonged downregulation of TGFβR1 in the brains of adult mice, indicating a lack of TGFβ signaling.

These new insights into microglia and astrocyte age related reactivity are critically relevant to the study of neuroimmune regulation. We have previously reported that IL-10 protein expression was high in the aged brain after LPS, however, it was insufficient to resolve the LPS-induced microglial activation (Fenn et al., 2012a; Henry et al., 2009). These results were interpreted to indicate that responsiveness to IL-10 was impaired in the aged brain. A connection with these previous findings to the current study is that astrocytes in the aged brain may not be responding appropriately to IL-10 produced by microglia. The data provided in this study support the premise that aged astrocytes are less responsive to IL-10 and fail to produce sufficient TGFβ to resolve microglia activation after a peripheral immune challenge. Astrocytes in aged mice had decreased IL-10R1 mRNA and protein expression. After LPS challenge, adult astrocytes upregulated IL-10R1 but there was no induction in aged astrocytes. Furthermore, aged astrocytes had attenuated Myc, Jak1, Tyk2, CDKN2B, and TGFβ mRNA expression. Importantly, all of these genes have been associated with IL-10 signaling (Donnelly et al., 1999; Norden et al., 2014a).

The association between impaired IL-10 signaling and decreased TGFβ upregulation is further confirmed by our finding that after LPS challenge, enriched astrocytes from both aged and IL-10R−/− mice failed to upregulate TGFβ. Other studies
have shown decreased TGFβ expression and signaling in the aged brain following LPS injection or TBI (Kumar et al., 2013; Tichauer et al., 2014; Wynne et al., 2010). Here, we provide novel evidence that astrocytes, specifically, may be the cell responsible for this lack of TGFβ upregulation. This lack of regulation was associated with high microglial pro-inflammatory cytokines. In addition, IL-10 lowered microglial activation when astrocytes from wild-type but not IL-10R-/- astrocytes were present in a co-culture system. Furthermore, when activated microglia were cultured with astrocytes isolated from adult mice, microglial activation was decreased. Addition of astrocytes isolated from aged mice, however, further augmented microglial activation. These current data indicate that astrocytes provide important regulatory feedback to activated microglia. Astrocytes from aged or IL-10R-/- mice, however, do not regulate microglial activation. Therefore, this study reveals a novel intervention to enhance astrocyte sensitivity to IL-10 to restore proper regulation of microglia and attenuate cognitive and behavioral complications in the aged.

Exaggerated microglial activation was associated with prolonged sickness/depressive-like behaviors (Corona et al., 2010; Godbout et al., 2005b; Huang et al., 2008; Wynne et al., 2010). Our data indicate that TGFβ reduced IL-1β expression in activated microglia (Norden et al., 2014a; Wynne et al., 2010). In aged mice following LPS injection, microglia had exaggerated levels of IL-1β and other pro-inflammatory mediators. In addition, microglia decreased expression of TGFβR1 following LPS injection and this downregulation was prolonged in aged microglia. Other studies have shown that microglia in aged mice have a decreased TGFβ profile (Hickman et al., 2013). Together these studies show that there is decreased TGFβ signaling in activated aged microglia, and that this lack of regulation contributes to heightened and prolonged pro-inflammatory expression.
It is important to note that we did not select for GFAP$^+$ astrocytes during the sorting and that we also did not collect astrocytes only from the hippocampus. Therefore, the region of the astrocytes used for the gene array is not controlled for. This is important as astrocytes display significant heterogeneity throughout the brain (Cahoy et al., 2008; Zhang and Barres, 2010). It is possible that different results would have been obtained if select brain regions or GFAP phenotypes were used. More detailed anatomical analysis of these changes would be beneficial for future studies. Similarly, we have previously shown that primed MHCII positive microglia are the cells responsible for exaggerated IL-1$\beta$ protein production after LPS challenge in aged mice (Henry et al., 2009). In this study, however, MHCII positive microglia were not selected for. In addition, microglia were isolated from the whole brain and not in a brain region dependent manner. Nonetheless, this study provides an overview of astrocyte and microglia inflammatory-related changes that occur with age that may influence astrocyte-dependent regulation of activated microglia.
Astrocytes regulate microglial activation by increasing TGFβ production

This thesis provides novel evidence of astrocyte mediated regulation of microglia during systemic inflammatory challenge. Prior to this thesis, there was little data showing astrocyte responses in the absence of neurodegeneration or CNS injury. Here we show that peripheral immune challenge with LPS is sufficient to activate astrocytes. Following LPS injection, enriched astrocytes increase expression of several inflammatory mediators including IL-1β and CCL2. These are important novel findings indicating responsiveness of astrocytes to systemic inflammation (Chapter 2). Perhaps more important is the data presented that astrocytes provide anti-inflammatory feedback to activated microglia. As previously shown, activated microglia produce high levels of pro-inflammatory cytokines but also the anti-inflammatory cytokine IL-10. It was believed that IL-10 acted as a negative regulator of microglia in an autocrine manner. Although this is still a possibility, data presented here shows that astrocytes are more reactive to IL-10 compared to microglia. IL-10 lowered pro-inflammatory cytokine expression in astrocytes and also increased expression of the anti-inflammatory mediator TGFβ. This is an important mediator in the process of microglia returning to a homeostatic state following activation. When TGFβ signaling was blocked during LPS challenge, microglial activation was prolonged with heightened expression of IL-1β and TNFα. Associated with prolonged microglia activation was also prolonged sickness behavior in mice injected with a TGFβ inhibitor (Chapter 3). Similar results were obtained in IL-10R
knockout mice. Following LPS challenge in vivo, astrocytes from IL-10R KO mice failed to upregulate TGFβ mRNA. This lack of TGFβ upregulation was associated with heightened microglial activation and prolonged sickness behavior (Chapter 4). These data are further supplemented with in vitro studies where microglial activation is attenuated with TGFβ treatment. Last, in a co-culture experiment, microglial activation was exaggerated when TGFβ signaling was blocked even though IL-10 was present in the system (Chapter 3). These data show that IL-10 alone is insufficient to reduce microglial activation during LPS challenge in vitro and in vivo. Furthermore, the data presented show that IL-10 receptor must be present on astrocytes. In a co-culture system, IL-10 only attenuated microglial activation when IL-10 receptor was present on astrocytes. When microglia with IL-10R were cultured with astrocytes that lacked IL-10R, there was no effect of IL-10 treatment (Chapter 4). Overall, microglia are highly responsive to LPS challenge both in vivo and in vitro and produce both pro- and anti-inflammatory cytokines during activation. Although microglia produce IL-10, it is not sufficient to attenuate microglial activation. Astrocytes must have functional IL-10 receptor and increase TGFβ expression for microglia to decrease their activation state. When either astrocyte IL-10 receptor expression or TGFβ signaling was blocked, microglial activation was exaggerated and prolonged. These novel findings highlighted here are important for understanding immune regulation in the brain. Furthermore, prior to these studies, astrocytes were not considered highly active during systemic inflammatory challenge. With this data, astrocytes can now be used as a target to control of inflammation. These data can be applied not only to circumstances of systemic inflammation, but during all circumstances of high CNS inflammation.
Impaired astrocyte mediated regulation of microglia in the aged brain

This thesis also provides novel data that astrocytes in the aged brain have impaired regulatory capacity of microglia. As discussed, astrocytes provide negative feedback to microglia during immune challenge. In aged mice, however, astrocytes have a more reactive morphology and gene expression profile (Chapter 4). For example, GFAP+ astrocytes in the hippocampus had a larger cell area and length. In addition, aged astrocytes had decreased expression of several genes associated with growth support. These data are consistent with a previous study that aged astrocytes were less supportive of neurogenesis compared to astrocytes from adult mice (Miranda et al., 2012). The decreased expression of growth factors was also associated with an increased expression of several inflammatory related genes including GFAP and S100b. Important for our study was that genes associated with IL-10 signaling were reduced in aged astrocytes. There was decreased expression of both IL-10R1 mRNA and cell surface protein and decreased expression of several genes associated with IL-10 signaling (Myc, Jak1, Tyk2) (Donnelly et al., 1999). These age-related changes in astrocytes set the stage for impaired anti-inflammatory regulation after immune challenge.

Using our LPS injection paradigm (Chapter 2), we show that neuroinflammation is exaggerated and prolonged in aged mice during systemic immune challenge. Consistent with previous studies, IL-1β and IL-6 mRNA expression was both exaggerated and prolonged in the brain of aged mice following peripheral immune challenge (Henry et al., 2009; Wynne et al., 2010) (Chapter 4). However, we also provide novel data that the problem of exaggerated neuroinflammation is two-fold. Not only was pro-inflammatory mediators highly expressed, we also show impaired anti-inflammatory induction in the aged brain. Although IL-10 ligand mRNA was highly expressed in the aged brain following LPS, induction of IL-10R1, TGFβ and TGFβR1
was impaired. IL-10 receptor (IL-10R1) and TGFβ were increased during the resolution phase in adult mice. At this same timepoint, however, there was no induction of either of these mediators. We interpreted the lack of IL-10R1 and TGFβ upregulation in the aged brain to indicate that responsiveness to IL-10 was impaired in the aged brain. The data provided in this thesis support the premise that aged astrocytes are less responsive to IL-10 and fail to produce sufficient TGFβ to resolve microglia activation after a peripheral immune challenge. Enriched and sorted astrocytes in aged mice had decreased IL-10R1 mRNA and protein expression at baseline. At 24 h after LPS challenge, astrocytes from aged mice failed to upregulate both IL-10R1 and TGFβ (Fig.5.1). The correlations between IL-10R1KO and aged astrocytes support our hypothesis that impaired IL-10 signaling in aged astrocytes leads to impaired TGFβ upregulation. Other studies have shown heightened TGFβ signaling (phosphorylated SMAD2) during resolution after LPS challenge in adult mice, and a lack of TGFβ signaling after LPS in aged mice. In addition, there was decreased TGFβ upregulation in the aged brain after TBI compared to adult mice. All of these studies reported heightened pro-inflammatory profiles in the aged brain associated with the lack of TGFβ regulation. These studies, however, did not address which cell type was responsible for the impaired TGFβ upregulation (Kumar et al., 2013; Tichauer et al., 2014; Wynne et al., 2010). Here, we provide novel evidence that astrocytes, specifically, fail to upregulate TGFβ expression after immune challenge. This is a very important finding; it shows a specific lack of anti-inflammatory regulation in aged astrocytes. This lack of regulation was associated with high microglial pro-inflammatory cytokine expression in vivo, including IL-1β and IL-6. Astrocytes from aged mice also failed to regulate microglial activation in a direct ex vivo setting. When activated microglia were cultured with astrocytes isolated from adult mice, microglial activation was decreased. Addition of astrocytes isolated from aged mice, however,
further augmented microglial activation. These current data indicate that astrocytes provide important regulatory feedback to activated microglia. Astrocytes from aged mice, however, do not regulate microglial activation. Therefore, this study reveals a novel intervention to enhance astrocyte sensitivity to IL-10 to restore proper regulation of microglia and attenuate cognitive and behavioral complications in the aged.

Exaggerated microglial activation can be detrimental and lead to behavioral complications. For example, high microglial activation was associated with prolonged sickness/depressive-like behaviors (Corona et al., 2010; Godbout et al., 2005b; Huang et al., 2008; Wynne et al., 2010). Our data indicate that TGFβ reduced IL-1β expression in activated microglia (Norden et al., 2014a; Wynne et al., 2010) (Chapter 3). In aged mice following LPS injection, microglia had exaggerated levels of IL-1β and other pro-inflammatory mediators. In addition, microglia decreased expression of TGFβR1 following LPS injection and this downregulation was prolonged in aged microglia. A previous study has shown that microglia decrease TGFβR1 expression upon immune activation and that TGFβ1 ligand can induce the expression of its receptor (Mitchell et al., 2014). Therefore, the prolonged downregulation of TGFβR1 of microglia from aged mice indicate that there is a lack of TGFβ signaling. Other studies have shown that microglia in aged mice have a decreased TGFβ profile (Hickman et al., 2013). Together these studies show that there is decreased TGFβ signaling in activated aged microglia, and that this lack of regulation contributes to heightened and prolonged pro-inflammatory expression. Therefore, enhancing TGFβ signaling in aged microglia may be of therapeutic value (Fig.5.1).
Fig. 5.1. *Impaired Astrocyte-Dependent Regulation of Microglia in the Aged*? 1) Age-related problems with regulation of microglia leads to prolonged neuroinflammation and corresponding neurobehavioral deficits after immune challenge. 2) Following immune challenge, active microglia secrete IL-10 independent of age, but neuroinflammation persists in the aged. 3) There is a failure to increase IL-10R1 on aged astrocytes after immune challenge. 4) IL-10 re-directed astrocytes produce TGFβ which modulates microglia activation, but aged astrocytes do not have increased induction of TGFβ.

**Astrocytes as anti-inflammatory regulators in aging and Alzheimer’s disease**

During normal, non-pathological conditions the brain maintains an anti-inflammatory environment. There is high expression of anti-inflammatory regulatory molecules that restrict microglial, macrophage, astrocyte and neuronal activation. This is significant because an increase in inflammation within the brain can have detrimental
effects on neurons that are for the most part non-regenerative. As the innate immune cell of the brain, microglia have the highest capacity of eliciting an immune response in within the CNS. Therefore, microglial regulation is of high interest. Microglia are regulated by both neuronal factors such as CX3CL1 and CD200 and also by anti-inflammatory cytokines such as TGFβ and IL-10. These anti-inflammatory cytokines, especially TGFβ are mainly produced by astrocytes (Ramirez et al., 2005). A lack of anti-inflammatory communication by astrocytes to microglia can lead to microglial dysregulation. As discussed, this phenomenon of microglial dysregulation is observed in models of aging. In addition, there is evidence of dysregulated microglia in Alzheimer’s disease. In both AD mouse models and humans there is a marked increase in neuroinflammation. There is much debate whether this increase in inflammatory status is a cause or effect and also whether it is beneficial. During AD, impaired clearance of neurotoxic Aβ plaques leads to impaired neuronal signaling. The contribution of neuroinflammation to impaired clearance of Aβ has been extensively studies, but unfortunately no conclusive results have been reported. It is well understood, however, that microglial clearance of Aβ plaques is impaired in AD. Post-mortem histological staining showed that activated microglia cluster around Aβ plaques, but do not seem to be able to internalize and degrade the plaques. Activated microglia can have several phenotypes depending on the microenvironment and the stimulus provided. Stimulating microglia with TGFβ activates them towards an anti-inflammatory phenotype that has high phagocytosis potential. This was shown by Wyss-Coray et al. using an animal model in which astrocytes produce TGFβ under the GFAP promoter. They found that TGFβ promoted Aβ clearance by microglia which reduced total Aβ plaque burden in transgenic AD mice (Wyss-Coray et al., 2001). In addition, it was later reported that
TGFβ signaling in neurons protects against neurodegeneration (Brionne et al., 2003; Tesseur et al., 2006).

The findings suggest a beneficial and protective role of CNS TGFβ in AD models. Therefore, several studies used astrocyte TGFβ overexpression mice to further understand the protective role of TGFβ. The results of these findings, however, were not those expected as TGFβ overexpression also had detrimental effects on AD. For example, genetically engineered mice in which astrocytes overexpress TGFβ showed increased T-cell recruitment and worsened disease pathology (Buckwalter et al., 2006). Similarly, another study showed that inhibiting TGFβ signaling in CD11c-positive innate immune cells reduced cognitive deficits associated with AD pathology (Town et al., 2008). Blocking TGFβ signaling in immune cells also decreased Aβ deposition. The increased clearance of Aβ plaques was explained by an increased infiltration of peripheral macrophages. It is important to note that in this study, TGFβ signaling was blocked in peripheral macrophages but not microglia. These data suggest that blockade of TGFβ signaling in peripheral macrophages would be beneficial for Aβ clearance in AD (Town et al., 2008). Therefore, targeting astrocytes to produce TGFβ centrally would still be beneficial for enhancing microglial clearance of Aβ, but this has to be done with caution to prevent T-cell recruitment to the brain.

**Therapeutic value of AAV astrocyte targeting**

Novel methods using gene-therapy have the potential to restore function in aged astrocytes. Adeno-associated viruses (AAV) are small viruses which infects humans that only elicit a mild immune response and do not cause disease. These features of AAV have made it an attractive candidate for creating viral vectors for gene delivery. There
are several types of viral vectors that transduce various cell types. Recently, an AAV vector type-9 (AAV9) was reported to cross the blood-brain barrier and infect astrocytes (Foust et al., 2009). Moreover, AAV9 has now been used in both mouse models and non-human primates without pathological consequences and more importantly, have shown astrocyte transduction in both species (Samaranch et al., 2012). Therefore, the use of AAV9 for delivery of genes into the CNS is of much current interest. This type of research and intervention could have great potential for treating aging disorders. As shown in this thesis, TGFβ production by reactive astrocytes is impaired in aged mice. AAV gene delivery of IL-10R1 could restore IL-10 sensitivity and TGFβ upregulation. Restored TGFβ production by astrocytes could inhibit microglial hyperactivity and resolve neuroinflammation before damage to the CNS occurs. In addition, astrocyte production of TGFβ could increase microglial clearance of Aβ peptides and inhibit neurodegeneration in Alzheimer's Disease (as discussed above). As our understanding of astrocytes is increasing, it is important to consider this cell type as active immune regulators with a broad spectrum of functions. Overall, many possibilities lie in targeted gene-expression in astrocytes.

**Senescent versus Primed Microglia: Two Sides of the Same Coin?**

In the glial biology field and in this thesis, we describe long-term changes in microglia profiles as primed or pro-inflammatory. This is because there are increased markers of inflammation (IL-1β, MHC II, CD68) on microglia with age and neurodegenerative disease and when these cells are activated by an innate immune challenge (i.e., LPS) they have an amplified activation profile (Cunningham et al., 2009; Fenn, 2013; Henry et al., 2008). Nonetheless, several other studies have described microglia of the aged brain to be anti-inflammatory or neuroprotective, and also as
senescent (Streit and Xue, 2009). For example, a recent study using RNA sequencing shows a microglial profile interpreted as an anti-inflammatory or neuroprotective profile (Hickman et al., 2013). The transcriptional profile of microglia from aged mice had decreased expression of proteins involved in sensing endogenous ligands whereas transcripts for sensing immune pathogens were elevated. There was also a significant increase of alternative or neuroprotective pathways including IL-4. In the same mice, however, elevated expression of pro-inflammatory mediators such as IL-1β and TNF-α remained. As discussed previously, increased activation of NF-κB, IL-1β, and NLP3 inflammasome pathways is consistent with other studies (Godbout et al., 2005a; Youm et al., 2013) and these data are interpreted to show that aging is associated with a more inflammatory profile. Therefore, it is difficult to categorize the activation of glia based on RNA analysis alone.

Consistent with a senescence profile of microglia, microglia from older rodents have functional impairments in proliferation, motility and phagocytosis (Hefendehl et al., 2014). For example, there was reduced phagocytosis of beta-amyloid by microglia from older AD transgenic mice (Hickman et al., 2008; Lee et al., 2010). Recent genomic studies indicate that several AD risk genes such as TREM2 and CD33 are implicated in microglial phagocytosis (Griciuc et al., 2013; Jiang et al., 2013; Jonsson et al., 2013), supporting the idea that microglia in the aged brain have impaired phagocytosis functions. In addition, microglia from aged rats showed delayed recruitment of phagocytic cells and less clearance of myelin after a toxin-induced demyelination lesion (Zhao et al., 2006). In a focal laser injury model, microglia from aged mice migrated at a slower velocity towards the site of injury and also aggregated at the injury site for a longer duration than that of adult mice (Damani et al., 2011). In this thesis, we characterized the morphology of microglia from adult and aged mice and found that aged microglia had a "reactive" morphological profile (Chapter 4). It is unknown,
however, if these changes in morphology lead to dystrophy and senescence or if they underlie the exaggerated inflammatory response to secondary insult. Overall, these results show that microglia undergo significant changes with age and we interpret these data to indicate that microglia of aged mice develop a “primed” or reactive morphological profile. Further research is needed to fully understand why microglia undergo these changes with age, and what the consequences are.

Microglia may also appear ‘senescent’ in the context of anti-inflammatory feedback. For instance, microglia from aged mice are less sensitive to specific regulatory feedback from anti-inflammatory mediators including IL-4 and transforming growth factor beta (TGFβ). Active microglia from aged mice failed to up-regulate the expression IL-4Rα after LPS injection or spinal cord injury (SCI). In both cases this was associated with reduced sensitivity to IL-4 dependent programming towards an M2a or repair profile of microglia in vivo (Fenn et al., 2014b) and ex vivo (Fenn et al., 2012a). In addition, microglia isolated from aged mice had reduced expression of TGFβ receptor compared to adults (Hickman et al., 2013) and aged microglial cultures were less sensitive to the anti-inflammatory effects of TGFβ (Rozovsky et al., 1998). Finally, when microglia were isolated from LPS-injected adult and aged mice, only microglia from adult mice had increased phagocytic activity in response to TGFβ treatment ex vivo (Tichauer et al., 2013). Although functional impairments may be considered indicators of microglial senescence, these same cells may be highly inflammatory when activated by immune challenge or injury. Therefore, the terminology of microglial priming or microglial senescence both reflect age-related differences in microglia function, but are related to the context in which they are examined (Fig.5.2).
Figure 5.2. Microglial Priming and Senescence in the Aged Brain: Two Different Sides of the Same Coin? This diagram illustrates that microglia in the aged brain have been described as either primed (left side) or senescent (right side). While the two are often considered to be contrasting issues, it is more likely that they are related issues and that it depends on the context in which the function of aged microglia is being evaluated. On one hand, aged microglia have a more inflammatory profile and this is exaggerated after a transient immune challenge (peripheral or central). On the other hand, aged microglia have a reduction in proliferation, motility and ability to clear debris. In both sides of the coin, regulation of these microglia is impaired.

From these studies discussed above, it may seem that microglia in the aged brain are primed to become activated and resistant to regulation. The regulatory capacity of TGFβ is of interest to this thesis and needs to be further discussed. In the Tichauer et
al., 2013 study, TGFβ was provided to adult and aged microglia plated *ex vivo* and the phagocytic efficiency was determined after a few hours. This represents an important function in microglia, but it does not fully represent all the aspects of TGFβ signaling. In a similar experiment, microglia were isolated from adult and aged mice and treated with TGFβ *ex vivo*. After 4 hours, mRNA expression of CX3CR1 and IL-1β was determined. Fig.5.3A shows that TGFβ increased CX3CR1 expression in adult microglia, but not in aged microglia. These data suggest that aged microglia are not responsive to TGFβ.

Fig.5.3B however, shows that IL-1β expression was decreased in both adult and aged microglia after TGFβ treatment. These data suggest that aged microgli are responsive to TGFβ. These two gene expression results were obtained from the same samples, yet they yielded contradictory results. Therefore, further downstream pathways need to be evaluated to fully understand how aged microglia have an impaired response to TGFβ.

![Figure 5.3.](image)

**Figure 5.3.** *Ex vivo* TGFβ treatment of adult and aged microglia give differential responses. Microglia were isolated by percoll density gradient from adult and aged mice and plated *ex vivo*. Microglia were treated with vehicle or TGFβ for 4 hours and A)CX3CR1 and B)IL-1β mRNA expression was determined. *p<0.05
Canonical TGFβ signaling involves the SMAD proteins. Upon TGFβ binding to its receptor, SMAD2 or SMAD3 is phosphorylated. SMAD2/3 binds the common SMAD4 and is translocated to the nucleus to act as a transcription factor. Blocking SMAD2 or 3 has shown to block TGFβ activity in multiple studies and is the most common pathway for TGFβ signaling (Shi and Massague, 2003). More recent studies, however, have discovered several other pathways activated as a result of TGFβ binding to its receptor. TGFβ signaling can activate the Pi3k pathway, MAPK pathway, JNK pathway and the ERK1/2 pathway (Massague, 2012). These are classified as non-canonical TGFβ signaling pathways. Given all these possibilities, it is likely that multiple pathways are activated by direct TGFβ treatment. It is also possible that activation of specific pathways are impaired in aged microglia. For example, the downregulation of IL-1β mRNA following TGFβ treatment of both adult and aged microglia may be mediated by Pi3K activation. However, the upregulation of CX3CR1 and increased phagocytic activity associated with TGFβ treatment may be mediated by SMAD2 activation. This is likely as a previous study has shown decreased SMAD2 phosphorylation in the aged brain after immune challenge (Tichauer et al., 2014). Future studies are necessary to determine specific deficits in TGFβ signaling. Activating the deficit could provide therapeutic treatment; however, activating all possible pathways may not be beneficial.

Primed, reactive and senescent astrocytes

The same discussion must be had about terminology used to describe aged astrocytes. The age-related changes reported here can be used to classify astrocytes as either primed, reactive, or senescent, or all three. Therefore, it is critical that researchers use the correct terminology when describing phenotypes of astrocytes. These phenotypes will depend upon the context in which astrocytes are analyzed. Here, we
show that aged astrocytes are reactive by having increased GFAP, vimentin and S100b mRNA expression. This generalization is built upon CNS injury studies where GFAP expression is dramatically upregulated (Faulkner et al., 2004). Aged astrocytes can also be characterized as primed because they had increased TNF and Casp1 expression at baseline. However, aged astrocytes did not have an amplified pro-inflammatory response after LPS challenge. Therefore, the terminology of “primed” may not be fully accurate for astrocytes compared to the way it accurately describes aged microglia. Perhaps most importantly, our data suggest that aged astrocytes have a senescent phenotype. Astrocytes express high levels of glutamate receptors, including EAA1 and EAA2. Aged astrocytes, however, had decreased expression of these genes which may indicate senescence towards maintaining glutamate homeostasis. Aged astrocytes also showed evidence of senescence when evaluating IL-10 responsiveness and TGFβ upregulation. After immune challenge, aged astrocytes had impaired IL-10R1, Myc, Jak1, Tyk2, CDKN2B, and TGFβ upregulation. Impaired upregulation of these genes all suggest impaired IL-10 signaling in aged astrocytes (Donnelly et al., 1999; Norden et al., 2014a). Other examples of senescence include decreased growth support by aged astrocytes. Here we report decreased BDNF, IGF1, VEGFA and Fizz1 expression in aged astrocytes. We and others have previously reported decreased expression on several of these mediators in the aged brain (Chapman et al., 2012; Fenn et al., 2013). When astrocytes were cultured with neuronal progenitor cells, aged astrocytes had decreased growth support towards the progenitors compared to adult astrocytes (Miranda et al., 2012). Overall, it appears as though aged astrocytes have a senescent profile. However, these examples described above were specific towards expression of growth factors and responsiveness to IL-10. It is important to not classify aged
Importance of glial heterogeneity

A limitation of these studies is that microglia and astrocytes were isolated by Percoll density gradient from whole brain homogenates. This raises questions of cell population purity and brain region location. The Percoll isolation yields a cell population with approximately 85% microglia. For sorting experiments, microglia were sorted based on CD11b and CD45 expression to exclude macrophages. However, many of the results presented here were generated from Percoll enriched microglia that were not sorted. Therefore, there may be contaminating cells that alter the mRNA profile of our “microglia”. These contaminations should be negligible, given the consistent results between enriched microglial gene expression and FACSsorted microglia gene expression at the 24 h timepoint after LPS (Chapter 2 and 4). However, these points still need to be considered and caution should be made not neglect the issue of cellular contamination.

The same issue is also relevant for our astrocyte analysis. Astrocytes were also collected from whole brain using Percoll separation. The astrocyte cell population was approximately 80% pure prior to sorting (Chapter 4) and this non-sorted population was used and referred to as enriched astrocytes in chapters 2 and 3. The other 20% of this population is still unknown. There was a small amount of microglia contaminating this population, but the majority of cells were both GLAST and CD11b negative. These may be another astrocyte population or an oligodendrocyte population. Given these unknowns, any major findings that appear to be attributed to astrocytes should be confirmed in a FACSsorted astrocyte population. Here, our finding that astrocytes fail to upregulate IL-10R1 and TGFβ after immune challenge was replicated in enriched and
sorted astrocytes. Therefore, we feel confident that the astrocytes impairments described in this thesis are attributed to astrocytes specifically.

The other important issue that needs to be addressed is the issue of glial location. The percoll density isolations were made from whole brain homogenates. However, the histological data was performed in a brain region dependent manner. In the hippocampus, astrocytes have strong GFAP labeling. We did not select for GFAP+ astrocytes during the sorting and we also did not collect astrocytes only from the hippocampus. Therefore, the region of the astrocytes used for the gene array is not controlled for. This is important as astrocytes display significant heterogeneity throughout the brain (Cahoy et al., 2008; Zhang and Barres, 2010). It is possible that different results would have been obtained if select brain regions or GFAP phenotypes were used. Here we show a decrease in the number of IL-10R1 positive astrocytes in the aged brain. It would be of interest to know if these astrocytes that lack IL-10R1 are found in a specific location or if they are the astrocytes that have higher GFAP or vimentin expression. Therefore, more detailed anatomical analysis of these age-related changes would be beneficial for future studies. Similarly, we have previously shown that primed MHCII positive microglia are the cells responsible for exaggerated IL-1β protein production after LPS challenge in aged mice (Henry et al., 2009). In this study, however, MHCII positive microglia were not selected for. In addition, microglia were isolated from the whole brain and not in a brain region dependent manner. The majority of aging research has focused on the hippocampus as an anatomical region for age-related changes (Lister and Barnes, 2009; Miller and O'Callaghan, 2005). The hippocampus is an area involved in both cognition and mood, therefore, age-related cognitive decline and mood disorders may be linked to changes that occur specifically in the hippocampus. Therefore, future studies should investigate microglia and astrocyte changes that occur within the aged hippocampus. Nonetheless, this study provides an overview of astrocyte
and microglia inflammatory-related changes that occur with age that may influence astrocyte-dependent regulation of activated microglia.

How increased neuroinflammation leads to behavioral complications

As described in this thesis, microglia from aged mice have an exaggerated and prolonged activation following immune challenge. As previously shown, this high level of inflammation in the aged brain is associated with prolonged sickness behaviors and the development of depressive-like behavior and cognitive impairments. Although microglia are the cells producing inflammatory cytokines and mediators, it is ultimately the neurons that are responsible to eliciting the behavioral response. So how does increased neuroinflammation lead to behavioral complications? There are several answers to this question.

Sickness behaviors

The time course for the development of sickness behavior correlated with the induction of cytokine expression within the brain (Chapter 2). In addition, neurons express several receptors for cytokines and secondary mediators, such as IL-1b and prostaglandins (Dantzer et al., 2000; Srinivasan et al., 2004). Therefore, the acute sickness response is likely mediated by direct effects of cytokines. To prevent pathogen invasion and excessive immunological signals, the brain is protected by the blood-brain barrier (BBB) (Bechmann et al., 2007). During a peripheral infection or illness, however, the brain must still receive the appropriate signals because the brain is responsible for critical components of the immune response. Therefore, the bi-directional communication between the immune system and the brain is essential for mounting the appropriate immunological, physiological, and behavioral response to an infectious pathogen or an injury. This communication is, in part, mediated through inflammatory
cytokines. Cytokines produced in the periphery can relay their message across the BBB through several distinct pathways. First, there are a few, relatively porous regions of the BBB adjacent to circumventricular organs where circulating cytokines can diffuse into the brain (Lacroix et al., 1998; Laflamme et al., 1999). Second, endothelial cells throughout the brain vasculature express IL-1 receptors (Ching et al., 2007). Through IL-1b binding to its receptor, the endothelial cells can relay the signal by producing IL-1b de novo and release it into the CNS. It is also known that the endothelial cells can actively transport IL-1B from circulation into the brain (Quan and Banks, 2007). Third, the cytokine signal can also be propagated to the brain by direct activation of the vagus nerve (Goehler et al., 1998; Konsman et al., 2000; Marvel et al., 2004) or catecholaminergic circuits of the sympathetic nervous system (Johnson et al., 2008). Once the peripheral signals reach the CNS, microglia become activated and propagate cytokine production (Chapter 2). These cytokine signals trigger the release of secondary inflammatory mediators including prostaglandins and nitric oxide (Ericsson et al., 1997; Konsman et al., 1999; Marty et al., 2008). Together, the cytokines and secondary mediators within the CNS target neuronal substrates and initiate the brain response to peripheral infection and to elicit the “sickness response.”

The sickness response describes the nonspecific symptoms of infection, including lethargy, listlessness, decreased activity and loss of interest in social interaction (Dantzer and Kelley, 2007; Maier and Watkins, 1998). Physically, the sickness response is characterized by fever, increased sleep, reduced food and water intake, and activation of the hypothalamic-pituitary-adrenal (HPA) axis (Maier and Watkins, 1998). The sickness response is evolutionarily adaptive to reset the priorities in order to fight and clear infection and also promote recovery. For example, increased fever and reduced plasma iron concentrations will attenuate pathogen replication. The metabolic changes associated with the sickness response are aimed at both clearing the
pathogen by increased metabolic demands and conserving energy by increasing the amount time spent sleeping, suppressing appetite and social interaction. While the induction of cytokine-mediated sickness behavior is a necessary and beneficial response to systemic infection, an amplified or prolonged response negatively affects behavioral processes (Jurgens and Johnson, 2010a). Peripheral or central immune stimulation with LPS caused protracted neuroinflammation in the brain of aged rodents. This was paralleled by a prolonged sickness response with protracted anorexia, lethargy and social withdrawal (Abraham et al., 2008; Godbout et al., 2005b; Huang et al., 2008). An amplified sickness response was also detected in older rats that were infected subcutaneously with *E. coli*. The aged rats displayed an altered febrile response including a blunted and delayed increase of core body temperature followed by a significant and prolonged increase of inflammatory cytokines (Barrientos et al., 2009b). Similar to the extended sickness behaviors in aged BALB/c mice, the increase of inflammatory cytokines were likely driven by exaggerated microglial IL-1β (Henry et al., 2009). In support of this notion, i.c.v. infusion of IL-1 receptor antagonist (IL-1RA) reversed the prolonged LPS-induced sickness behavior in aged mice (Abraham and Johnson, 2008). Furthermore, when microglial activation is inhibited by minocycline treatment, sickness behaviors are also improved (Henry et al., 2008). These findings indicate that the exaggerated sickness response in aged rodents was likely caused by the exaggerated and prolonged production of IL-1β by primed microglia.

*Depressive-like behaviors*

Depressive-like behaviors are delayed and separate from the sickness response. Depressive-like complications in rodent models are reflected by increased resignation behavior (i.e. immobility) in the forced swim test, or tail suspension test. These behavioral assays are intended to model the aspect of despair displayed by depressed human patients (Cryan et al., 2005). In aged BALB/c mice, peripheral stimulation of the
innate immune system with LPS caused prolonged depressive-like behavior 72 h after injection in aged mice after acute (Godbout et al., 2008) and chronic (Kelley et al., 2013) immune challenge. Depressive-like behaviors are independent of malaise and lethargy and therefore should only be determined once sickness behaviors have passed. Therefore, depressive-like behaviors are not mediated directly by cytokines since cytokine expression has also returned to normal. The high cytokine expression acutely after LPS challenge, however, activates other inflammatory mediated pathways. One of these mediators is IDO. Cytokines activate IDO expression and activity. As a result IDO converts TRP into KYN. This is important as KYN and other downstream KYN products can have neurotoxic and depressive effects. This increased activity of IDO also indirectly decreases serotonin synthesis (for reviews see (Dantzer et al., 2008; Haroon et al., 2012)). Decreased serotonin availability has been known for several decades now as an underlying cause for depression. Therefore, IDO is a possible therapeutic target for preventing the development of depressive-like behaviors during immune challenge. This has currently not been tested in aged mice. This approach, however, has been explored in other models of heightened microglial activation. In fractalkine deficient mice, microglial activation was prolonged after immune challenge and depressive-like behaviors developed (Corona et al., 2010; Corona et al., 2013). These mice also had unbalanced TRP levels with higher concentrations of KYN and KYN metabolites and decreased serotonin availability. Treatment of the fractalkine deficient mice with an IDO competitive inhibitor restored TRP turnover and decreased depressive-like behaviors (Corona et al., 2013). It is important to note that microglial cytokine expression was not decreased with treatment (data not shown), therefore the anti-depressant effects were due specifically to IDO inhibition and not anti-inflammatory effects. This study is important to current aging work because IDO expression and activity was increased in the brain of aged mice following immune challenge (Godbout et al., 2008; Henry et al.,
2009). Therefore, IDO activity provides a novel therapeutic avenue for preventing depression associated with inflammation in the aged. The benefits of blocking IDO activity should be validated in mouse models where it can be determined if it is sufficient of if other treatments, such as an anti-inflammatory agent, need to be given as an adjuvant.
Figure 5.4. Proposed Role of the IDO pathway in Depressive-like behavior following LPS challenge in aged mice. In aged mice, LPS causes amplified microglial activation with prolonged and exaggerated production of pro-inflammatory cytokines. These inflammatory cytokines activate IDO, which in turn, shifts tryptophan metabolism away from the production of serotonin (5-HT) and towards the production of kynurenine (KYN). 5-HT can be degraded to 5-HIAA by monoamine oxidase (MAO) or by IDO to 5-hydroxykynuramine. Active KMO in microglia converts kynurenine into 3-HK, which is a pro-oxidant. This also effectively shifts KYN away from the production of KYNA. 3-HK can then be degraded into quinolinic acid (QUIN) by kynureninase. QUIN is an NMDA receptor agonist and pro-oxidant. The influence of 1-MT on this pathway is also shown. The inflammatory associated changes in TRP metabolism are highlighted using black arrows and boxes. Overall, it is hypothesized that the IDO-initiated increases in tryptophan affect both serotonergic and glutamatergic neurotransmission and leads to the development of depressive-like behavior.

**Cognitive impairments**

Related to depressive-like behaviors, cognitive impairments can persist even after inflammatory levels in the brain have returned to homeostasis. Therefore,
cognitive impairments can be mediated directly by cytokines and by secondary mediators. There are multiple studies indicating that increased cytokine production in the aged brain after peripheral innate immune challenge is associated with impaired cognitive function. For example, injection of LPS caused an amplified cytokine response in the hippocampus of older mice that was paralleled by impaired hippocampal-dependent spatial memory (Chen et al., 2008). Moreover, infection by *E. coli* led to prolonged production of IL-1b in the hippocampus of aged rats (Barrientos et al., 2009a) and reduced long-term contextual memory examined by context-dependent fear conditioning and Morris water maze (Barrientos et al., 2009a; Barrientos et al., 2006). When aged mice were fed a diet supplemented with resveratrol, a potent anti-oxidant, LPS-induced neuroinflammation and working memory deficits were attenuated (Abraham and Johnson, 2009b). In the absence of an immune stimulus there is not a significant effect of age on the acquisition of memory tasks. There is, however, evidence of age-associated memory problems in the reversal task of the Morris water maze (Jang et al., 2010). Nonetheless, age-related cognitive impairment is exaggerated when a secondary immune challenge is provided.

The mechanisms by which cognitive complications develop and persist in these models is unclear. A potential explanation is that neuroinflammatory pathways can impact neuronal plasticity (e.g, neurogenesis, long-term potentiation and dendritic restructuring). For example, when neuroinflammation was prolonged in aged mice, increased dendritic atrophy was detected in the CA1 region of the hippocampus (Richwine et al., 2008a). In addition, neurogenesis steadily decreases throughout life in mouse models of aging (Ben Abdallah et al., 2010) and may be further disrupted by inflammation (Ekdahl et al., 2003; Monje et al., 2003). It is expected that age-related decreases in neurogenesis would be exaggerated during an inflammatory challenge, but to our knowledge, this has not been determined. Nevertheless, it is possible that
impaired microglial regulatory processes in the aged brain would negatively impact neurogenesis. For example, Bachstetter et al. (2011) have shown that CX3CR1-deficient mice show profound deficits in neurogenesis. Furthermore, infusion of recombinant CX3CL1 into the brain of the aged rats reversed this decrease (Bachstetter et al., 2011). Therefore, it is plausible that a prolonged impairment of CX3CR1 on the microglia of aged mice after an LPS injection (Corona et al., 2010) may cause impaired neuroplasticity, leading to cognitive complications.

Increased pro-inflammatory cytokines and other neuroinflammatory pathways also suppress long-term potentiation (LTP) (Griffin et al., 2006; Kelly et al., 2001; Murray and Lynch, 1998; Vereker et al., 2000). LTP is a key mechanism involved in memory formation and can have different manifestations, including early and late-phase LTP. A recent study examined different types of LTP in hippocampal slices prepared from young or aged rats after recovery from E. coli infection or no infection. Early-phase LTP was not different with age, but late-phase LTP was significantly suppressed in aged rats 4 days after E. coli infection in hippocampal area CA1 (Chapman et al., 2010). These electrophysiological data correspond with observed deficits in long-term memory with age. Suppression of LTP is likely caused by enhanced IL-1β expression as i.c.v. administration of IL-1RA reversed the E. coli induced suppression of late-phase LTP in aged rats (Chapman et al., 2010). Taken together, these data show that impaired regulation of the neuroimmune response results in inflammatory cytokine-mediated suppression of neuronal plasticity to cause cognitive deficits.

**Conclusions**

In conclusion, there are inflammatory alterations in microglia biology with aging. Microglia of the aged brain are termed primed with a higher expression of MHC II and pro-inflammatory cytokines including IL-1β. This shift towards priming is associated with
a prolonged and amplified response to an immune challenge. Aging is also associated with dysregulation of microglia; for example, deficits in astrocyte mediated regulation of microglia. Specifically, this thesis provides novel data that there is dynamic communication between microglia and astrocytes. Activated microglia produce the anti-inflammatory cytokine IL-10. In response to IL-10 signaling, astrocytes increase TGFβ production which attenuates microglia. In aged mice however, astrocytes are less sensitive to IL-10 and do not upregulate TGFβ after immune challenge. As a result, microglial activation is prolonged and this sets the stage for behavioral impairments to develop. What causes these impairments in astrocytes sensitivity and what gives rise to a primed microglial population, however, remains to be elucidated. Therefore, a better understanding of the pathways by which microglia become dysregulated with age is needed to improve our understanding of neuroinflammatory complications associated with age and lead to the development of therapeutic interventions. Here we provide some evidence of impaired astrocyte dependent regulation of microglia. Therefore, targeting astrocyte sensitivity to IL-10 may provide one possible therapeutic approach.
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