Mechanisms and Consequences of Microglial Priming and Dysregulated M2a Responses with Age and Central Nervous System Injury

DISSERTATION

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Abstract

Aging is associated with the development of primed and hyper-reactive microglial population within the central nervous system (CNS). After an immune challenge these primed microglia release exaggerated levels of inflammatory cytokines into the CNS resulting in the development of neuropsychological complications including cognitive decline and depression. How and why these primed microglia develop in the aged brain is unclear, but recent studies indicate that microglia in the aged brain have reduced exposure and are more resistant to anti-inflammatory regulation. Rather than impairment in a single regulatory pathway, the aged brain demonstrates a loss in almost every regulatory system investigated including neuronal regulation by CD200 and fractalkine (CX3CL1), anti-inflammatory regulation by interleukin (IL)-10 and IL-4, and global regulation by micro-RNAs (miRs). Of these regulatory pathways, research into mechanisms for reduced CX3CL1 expression, reduced responsiveness to IL-4, and aberrant miR expression in the aged brain is lacking. Moreover, research into the degree to which the development of this primed microglia phenotype can be accelerated by CNS trauma or the degree to which primed microglia contribute to neuropsychiatric complications experienced by persons with traumatic brain injury (TBI) has not been performed. To address these holes in our understanding of microglia regulation and priming in the aged and injured CNS four primary studies were performed.

In the first study, miRs associated with the immune response were measured in adult and aged mouse and human brain, and mouse microglia. In both mouse and human samples miR-29a/b-1 was significantly increased with age associated with
reduced expression of CX3CL1 and insulin-like growth factor (IGF)-1, two confirmed
targets of miR-29a/b-1 that negatively regulate microglia activation. Thus, age-
associated increases in miR-29a/b-1 could contribute to reduced microglial regulation
and the development of a primed microglia phenotype (Chapter 2).

Following the development of a primed phenotype, these microglia may be more
resistant to regulation by anti-inflammatory factors including IL-4. In the second study,
upregulation of the receptor for IL-4, IL-4 receptor (IL-4R)α, was reduced in microglia of
aged mice corresponding to reduced responsiveness to IL-4 and a failure to induce an
arginase+/iNOS- anti-inflammatory phenotype (Chapter 3). Because an IL-4-induced
arginase phenotype is implicated in improved repair and recovery after a traumatic CNS
injury (e.g., spinal cord injury (SCI)), the ability of microglia from aged mice to develop
an IL-4-directed M2a phenotype after SCI was evaluated in the third study.
Corresponding to our immune challenge studies, microglia from aged mice had reduced
expression of IL-4Rα after SCI compared to microglia from adult mice and this was
associated with increased pathology and worsened functional recovery (Chapter 4).
Intriguingly, reduced IL-4-signaling in the aged was associated with an attenuated
inflammatory response and reduced recruitment of peripheral macrophages to the CNS
after SCI or peripheral LPS challenge (Chapter 4).

Finally, evidence of activation markers associated with microglia priming (e.g., major
histocompatibility complex (MHC) II) has been observed in animal modes of TBI and in
TBI patients suggesting that microglia priming can be accelerated by a traumatic CNS
injury. Thus, the degree to which TBI-primed microglia are hyper-reactive to an
inflammatory challenge promote neuropsychiatric complications after TBI was
investigated in the fourth study. One month after TBI microglial MHC II expression was
increased corroborating previous studies. Moreover, these primed microglia were hyper-
reactive to an inflammatory challenge inducing prolonged sickness behavior and the development of depressive-like behavior in TBI mice. These studies indicate that age-associated microglia priming can be accelerated by TBI and is likely responsible for increased TBI-associated cognitive impairment and depression (Chapter 5).

Taken together, these studies uncovered a mechanism for reduced CX3CL1 and IGF-1 in the aged brain corresponding with increased microglial priming, identified a deficit in IL-4Rα expression by aged microglia resulting in reduced responsiveness to IL-4 and increased morbidity after SCI, and demonstrated that microglial priming is accelerated by a TBI leading to increased sensitivity to secondary challenges (e.g., stress, infection, second injury) and precipitation of neuropsychiatric complications including cognitive decline and depression. Collectively these studies provide novel insight into the mechanisms of microglial priming and regulation with age and injury.
This thesis is dedicated to my husband, my parents, and my sister. Without you I could have never dreamed so high or accomplished so much. Thank you for your continued support, encouragement, friendship, and love.
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Publications


Fenn A.M., Hall J.C., Gensel J.C., Popovich P.G., and Godbout J.P. (In Review). IL-4 signaling drives a unique arginase+/IL-1β+ microglia phenotype and recruits macrophages to the inflammatory CNS: consequences of age-related deficits in IL-4Rα after traumatic spinal cord injury.


the number of inflammatory CNS macrophages, and prolonged social withdrawal in socially defeated mice. *Psychoneuroendocrinology*, 37: 1491-1505.


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**Field of Study**

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Table of Contents

Abstract .............................................................................................................................. ii
Dedication .......................................................................................................................... v
Acknowledgements ........................................................................................................... vi
Vita ..................................................................................................................................... x
List of Tables .................................................................................................................... xv
List of Figures .................................................................................................................. xvi

Chapter 1: Introduction

Psychoneuroimmunology – Immune-to-Brain communication ...................................... 1
Age-associated changes in Neuroinflammation ............................................................. 3
Microglia priming with age ........................................................................................... 5
Neuronal and micro-RNA-mediated regulation of microglia in the aged brain .......... 7
Anti-inflammatory-mediated regulation of microglia in the aged brain ....................... 11
IL-4 signaling in monocytes, in vitro and in vivo .......................................................... 14
IL-4 signaling – 4 pathways in brief .............................................................................. 16
IL-4 signaling in the brain ............................................................................................... 20
M1 vs M2 phenotypes – role of M2a after CNS injury ............................................... 21
Shift away from an M2 toward an M1 phenotype with age ........................................ 24
Is microglia priming specific to aging? ........................................................................ 26
Connection between traumatic brain injury and age-associated neurodegenerative diseases .......................................................... 28
Exaggerated brain inflammation after an immune challenge can lead to the development of neuropsychiatric complications – in brief ........................................ 29
Study Objectives ............................................................................................................. 31
Chapter 6: Discussion and Conclusions

Amalgamation of neuronal-, immune-, and miR-mediated regulation.................165
Site specific microglia priming..............................................................................168
What is the true M2a phenotype in vivo?.................................................................171
The role of IL-4 in the CNS....................................................................................175
IL-4 induced polyamines – potential for inflammatory regulation......................180
Differentiation between aging, TBI, and neurodegenerative disease...............184
Examples of successful aging..................................................................................187

References..............................................................................................................189
List of Tables

Table 2.1 Postmortem brain tissue samples .................................................................40
Table 2.2 Murine 3’ UTR sequences for luciferase assay ...........................................43
Table 3.1 IL-4 re-directed active BV-2 microglia towards an M2 profile ......................78
Table 3.2 IL-10 did not re-direct active BV-2 microglia towards an M2 profile ...........79
Table 3.3 IL-4 re-directed active primary microglia towards an M2 profile .................80
Table 5.1 TBI results in robust mRNA expression of several immunomodulatory genes .....................................................................................................................147
Table 5.2 GFAP and Iba-1 threshold area 1 m after TBI ..............................................156
Table 5.3 Gene expression in enriched CD11b+ cells 72 h after LPS .........................160
List of Figures

Figure 1.1  Factors involved in microglia priming with age...........................................11
Figure 1.2  The IL-4/IL-4Rα/γc signaling pathway........................................................18
Figure 1.3  M1 and M2 phenotypes and shift towards M1 with age.............................25
Figure 1.4  CNS disease/injury and aging synergize to induce depression after an immune challenge.........................................................................30
Figure 2.1  Increased expression of miR-146a, miR-155, miR-29a, and miR-29b in the brain of aged mice............................................................................45
Figure 2.2  Increased expression of miR-155, miR-29a, and miR-29b in the microglia of aged mice...............................................................................46
Figure 2.3  Peripheral injection with LPS increased miR-29b expression in the brain...........................................................................................................47
Figure 2.4  miR-29a and miR-29b suppressed the expression of IGF-1 and CX3CL1......................................................................................................49
Figure 2.5  IGF-1 was decreased in the brain after peripheral injection with LPS and suppressed by miR-29b in microglia...................................................51
Figure 2.6  miR-29a and miR-29b were increased in the brains of aged humans.................................................................53
Figure 2.7  The aged-associated increase in brain levels of miR-29a and miR-29b was negatively correlated with reduced expression of CX3CL1 and IGF-1...........................................................................................................55
Figure 3.1 Peripheral LPS injection promoted an M1 and M2c mRNA profile in microglia...........................................................................................................75
Figure 3.2 Peripheral LPS injection increased microglial surface expression of IL-4Rα, but not IL-10R1..............................................................................77
Figure 3.3 M1, M2a, and M2c profile in adult and aged microglia after peripheral LPS injection...............................................................................................82
Figure 3.4 Microglia from aged mice do not have increased surface expression of IL-4Rα following LPS..............................................................................85
Figure 3.5 IL-4 mRNA is decreased in the brain of adult and aged mice after LPS.........................................................................................................................86
Figure 3.6 Ex vivo treatment with IL-4 re-directed active microglia from adult, but not aged, mice towards an M2 profile.................................88
Figure 4.1 Age-related impairment in the induction of IL-4Rα surface expression on microglia after SCI

Figure 4.2 Recruitment of IL-4Rα+ cells to the injured spinal cord was impaired in aged mice

Figure 4.3 Enhanced IL-1β and CCL2 mRNA expression in adult WT mice after SCI

Figure 4.4 Arginase protein expression was reduced in the injured spinal cord of aged and IL-4RαKO mice

Figure 4.5 Aged mice had impaired functional recovery and extended lesion length after SCI

Figure 4.6 IL-4Rα induction on activated microglia was required for IL-4-dependent arginase expression

Figure 4.7 IL-4 mediated re-direction of activated microglia supported neurite growth ex vivo

Figure 4.8 IL-4 mediated re-direction of activated microglia augmented mRNA expression of IL-1β and CCL2

Figure 4.9 IL-4Rα was upregulated 24 h after LPS on microglia of C57BL/6 mice

Figure 4.10 IL-4 mediated re-direction of activated microglia increased the recruitment of CCR2+ macrophages to the brain

Figure 4.11 CD11b+/CD45high cells were peripherally derived

Figure 5.1 Diffuse TBI promoted neuroinflammation in mice in a time-dependent manner

Figure 5.2 Diffuse TBI promoted transient deficits in body mass, motor coordination and depressive-like behavior

Figure 5.3 TBI-associated astrogliosis 30 dpi

Figure 5.4 TBI-associated microglia priming 30 dpi

Figure 5.5 Peripheral LPS injection caused exaggerated microglia cytokine expression associated with protracted social withdrawal and depressive-like behavior

Figure 6.1 Proposed mechanism for IL-4 signaling after SCI
Chapter 1: Introduction

The immune system and central nervous system (CNS) engage in constitutive bidirectional communication to alter physiological and behavioral responses to our environment. Within the CNS a specialized immune cell, microglia, has developed to coordinate the responses between the peripheral immune and central nervous systems by initiating and propagating inflammatory signals. The role of microglia expands far beyond immunological regulation, however, as microglia are critical for proper neuronal targeting and synaptic pruning during development, maintaining a functional neurovascular unit, phagocytosis and removal of debris and amyloid peptides, and initiating reparative responses in the CNS during trauma or disease. To accomplish these discrete functions microglia, like their peripheral mononuclear counterparts, take on distinct phenotypes with separate cytokine, chemokine, and enzymatic profiles. These profiles may be sensitive to changes in microenvironment and could be differentially regulated in the hyper-inflammatory milieu of the aged CNS. Thus, the ability to promote a beneficial microglial response to infection or injury may be impaired in the aged, or in individuals that maintain a chronically inflammaed CNS.

Psychoneuroimmunology – immune-to-brain communication

Early descriptions of the immune system and CNS characterized these organs as segregated and distinct. The immune system existed to fight infection whereas the CNS existed to dictate behavior - two seemingly unrelated functions.
In 1964, however, Drs. George Solomon and Rudolf Moos published a hallmark paper proposing that the immune system and the CNS are integrated (Solomon and Moos, 1964). This hypothesis was based off of work done in their labs showing immunological perturbations in mental health patients, and personality shifts in patients with autoimmune disease. The term “Psychoneuroimmunology” and creation of a field dedicated to interactions between the immune, nervous, and endocrine systems was developed in 1975 by Drs. Robert Ader and Nicholas Cohen. Their seminal work demonstrated classical conditioning of the immune system through CNS taste receptor signaling (Ader and Cohen, 1975). During the interim 40 years, the Psychoneuroimmunology field has rapidly expanded and demonstrated interactions between the immune system and CNS for almost every condition and disease.

Fundamentally, Psychoneuroimmunology explains a prevalent and reoccurring condition for all organisms: sickness behavior. During the course of a bacterial or viral infection the peripheral immune system is activated. Innate immune cells respond first, trafficking to the affected tissue and releasing inflammatory mediators (e.g., cytokines, chemokines, reactive oxygen species) to further enhance cellular recruitment, clear the pathogen, and promote antigen presentation to adaptive immune cells. These inflammatory mediators, however, also travel via humoral and neuronal pathways to activate brain resident microglia (Corona et al., 2012; Dantzer et al., 2008; Godbout and Johnson, 2009). Microglia then propagate these inflammatory signals throughout the CNS to alter neuronal physiology and affect behavior. For example, inflammatory cytokines including interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF)-α bind to cytokine receptors present on temperature-sensitive neurons within the anterior hypothalamus increasing their temperature-set point and inducing a fever response (Shibata, 1990; Stefferl et al., 1996). Other components of sickness behavior include
reduced appetite and sexual drive, increased slow wave sleep, and social withdrawal. Importantly, these physiological and behavioral changes are evolutionarily adaptive and necessary to reallocate the host’s resources to fight infection (Corona et al., 2012; Dantzer et al., 2008; Godbout and Johnson, 2009; Norden and Godbout, 2013). Indeed, when lizards (*Dipsosaurus dorsalis*) were challenged with an active bacterial infection and given low doses of the antipyresis sodium salicylate, mortality rate increased from 0% to 100% (Bernheim and Kluger, 1976). Thus, elevations in brain inflammation are beneficial during an active immune challenge. Prolonged or repeated increases in brain inflammation, however, can lead to detrimental behavioral outcomes. Exaggerated brain inflammation and the resulting behavioral complications is now the focus of Psychoneuroimmunology research.

*Age-associated changes in neuroinflammation*

Aging is associated with a gradual decrease in immune function coinciding with a steady increase in global inflammation. For example, although neutrophils are more responsive to bacterial infection in the aged and release higher levels of reactive oxygen species, bacterial clearance is reduced (Nomellini et al., 2008; Wenisch et al., 2000). Aging is also associated with a shift in the bone marrow away from lymphopoesis in favor of myeloipoiesis (Beerman et al., 2010; Sansoni et al., 1993). This shift results in fewer numbers of B- and T-cells and increased numbers of monocytes and granulocytes. Critically, a reduction in B- and T-cell number results in reduced B/T-cell receptor diversity and reduced recognition and response to infiltrating pathogens (Yager et al., 2008). In addition, T-cell phenotype is also affected by age with T-cells gradually developing a more inflammatory, Th1 profile at the expense of the less inflammatory, Th2 profile (Sakata-Kaneko et al., 2000).
Increased inflammatory potential with concurrent immune senescence are well characterized in the periphery, but we now know that these age-associated complications extend to the brain. Indeed, several studies demonstrate increased expression of inflammatory-associated and pro-oxidant genes within the aged CNS and reduced expression of anti-inflammatory, regulatory, and anti-oxidant genes (Godbout and Johnson, 2004; Godbout and Johnson, 2009; Hickman et al., 2013; Jurgens and Johnson, 2012; Lyons et al., 2009a; Maher et al., 2005; Norden and Godbout, 2013). Moreover, inflammation is further exaggerated in the brain of aged animals after an immune challenge. For example, following a peripheral infection with the gram negative bacteria *Escherichia coli*, aged rats have exaggerated expression of IL-1β in the CNS that persists for a longer period of time compared to adult rats (Barrientos et al., 2006; Barrientos et al., 2009b). This has been replicated using injections of lipopolysaccharide (LPS), a cell wall component of gram negative bacteria (Godbout et al., 2005b). Although these data are convincing, the immune challenges were initiated in the periphery and thus, an exaggerated CNS response could merely be a reflection of an exaggerated peripheral response. Therefore, studies using injections of IL-1β or LPS directly into the brain have also been performed. Similar to the studies described earlier, central injection of IL-1β or LPS induces a heightened inflammatory reaction in the brain of aged rodents (Abraham and Johnson, 2009a; Cunningham et al., 2005; Fu et al., 2010; Huang et al., 2008). Collectively, these studies indicate that the CNS becomes hyper-inflammatory as a function of age leading to increased inflammatory cytokine production after a peripheral or central immune challenge.
Microglia priming with age

Microglia, the resident innate immune cell of the CNS, are thought to underlie heightened CNS inflammation with age. Microglia derive from the embryonic yolk sac and seed the brain early in embryogenesis (ED 9.25-9.5) (Ginhoux et al., 2010) similar to other tissue resident macrophages (Yona et al., 2013). Critically, microglia are considered long-lived cells with little to no turnover in the absence of gross CNS disease or injury (Ajami et al., 2007; Ginhoux et al., 2010). Because of their long-lived nature, these cells may become particularly sensitive to the increased oxidative environment of the aged brain, or become sensitized as a result of a life-time of immune activation (Streit et al., 2004). Alternatively, microglia may proliferate in response to successive immune challenges resulting in telomere shortening over time (Flanary and Streit, 2004; Rozovsky et al., 1998). Whatever the cause, during the course of normal, non-pathologic aging microglia develop an altered phenotype similar to that observed in peripheral mononuclear cells. Indeed, microglia from an aged brain become hyper-inflammatory, but are less efficient at performing normal homeostatic functions. For example, microglia from aged mice in the PS1-APP transgenic mouse model of Alzheimer’s disease exhibit reduced phagocytosis of fibrillar amyloid-beta peptides and increased inflammatory cytokine production (Hickman et al., 2008). This creates a caustic cycle because increased inflammation further enhances fibrillar amyloid aggregation, which further enhances microglial expression of inflammatory cytokines (Hickman et al., 2008; Rogers et al., 2002). Over time, microglia from PS1-APP mice become more major histocompatibility complex (MHC) II positive and inflammatory with a reduced phagocytic capacity resulting in the accumulation of amyloid plaques (Rogers et al., 2002).

Increased MHC II expression on microglia, however, is not restricted to transgenic PS1-APP mice. In fact, increased MHC II expression on microglia is noted on
almost every aged vertebrate examined including mice, rats, dogs, non-human primates, and humans (Frank et al., 2006; Henry et al., 2009; Perry et al., 1993; Sheffield, 1998; Streit et al., 2004). While increased MHC II expression alone does not indicate a change in activation, several studies indicate that heightened MHC II expression corresponds with a shifted microglial phenotype from anti-inflammatory and homeostatic, to more inflammatory. For example, 24 h after a peripheral (intraperitoneal) injection of LPS, microglia from adult mice express low levels of IL-1β and do not express MHC II. Microglia from aged mice, however, are 25% MHC II⁺ and IL-1β protein expression is significantly increased compared to adults. Moreover, the MHC II⁺ microglia, specifically, produce the exaggerated levels of IL-1β (Henry et al., 2009). Because multiple studies have repeatedly demonstrated that MHC II⁺ microglia have a heightened inflammatory response to immune challenge, they are now considered “primed” (Dilger and Johnson, 2008; Godbout et al., 2005b; Norden and Godbout, 2013). Importantly, these primed microglia are also characterized as senescent due to their dystrophic appearance (Streit et al., 2004), reduced responsiveness to growth and proliferating factors (e.g., granulocyte colony stimulating factor) (Rozovsky et al., 1998), slower process motility during steady state (Damani et al., 2011), and reduced mobilization toward a focal CNS injury (Damani et al., 2011). Both terms, however, are thought to describe the same cells within the aged brain (Fenn et al., 2014a).

Functional consequences of microglial priming in the aged are apparent in that aging is associated with increased prevalence of neurodegenerative diseases including Alzheimer’s disease, increased morbidity and mortality after CNS injury (Injury-Statistical-Center, 2013; Kumar et al., 2012; Onyszchuk et al., 2008), and increased mental health diseases including depression (Corona et al., 2012; Godbout and Johnson, 2009; Vescovini et al., 2010). Critically, each of these age-associated complications
coincides with elevations in CNS inflammation and microglial activation. Although the exact cause of microglia priming is unclear, there are numerous regulatory mechanisms that become impaired in the aged brain coinciding with a dysregulated microglial phenotype (Fig.1.1).

Neuronal and micro-RNA-mediated regulation of microglia in the aged brain

Because microglia activation can have robust effects on neuronal physiology and resulting behavior (Tremblay et al., 2011; Wynne et al., 2010), the brain maintains a dense network of regulatory mechanisms to precisely control microglia activity. These include neuronal and immune mediators, and micro-RNAs. Most researchers consider these regulatory systems as repetitive and overlapping thus maintaining microglia in a surveying state, often called M0. In this state microglia produce very few cytokines and low levels of neurotrophic factors (Choi et al., 2008a; Henry et al., 2009). Brain-associated regulatory pathways are also responsible for returning microglia to an M0 state after inflammatory-induced activation. The heightened inflammatory status of microglia in the aged brain suggests a deficit in these critical regulatory systems.

The proteins involved in neuronal regulation of microglia are constitutively expressed at high levels and rarely change in concentration. These include CD200 and fractalkine ligand (CX3CL1) (Jurgens and Johnson, 2012). Within the CNS, neurons are the exclusive producers of CD200 and CX3CL1 protein, whereas microglia are the sole expressers of CD200 receptor (CD200R) and fractalkine receptor (CX3CR1) (Cardona et al., 2006; Jurgens and Johnson, 2012). Thus, CD200-CD200R and CX3CL1-CX3CR1 interactions represent a unique relationship between neurons and microglia to maintain microglia in an M0, or surveying, state. Critically, several studies report reduced levels of CD200 and CX3CL1 mRNA and protein in the aged brain, particularly in the
hippocampus (Bachstetter et al., 2011; Cox et al., 2012; Frank et al., 2006; Wang et al., 2011; Wynne et al., 2010). This is critical because the hippocampus is particularly sensitive to inflammatory activation and excitotoxic damage due to the high number of inflammatory receptors (Ban et al., 1991). Moreover, the hippocampus is integrated into the limbic system and involved in the development of depressive-like behavior, and is also the primary source for memory and learning. Therefore, damage to this area of the brain is particularly debilitating. Loss of CD200 and CX3CL1 signaling in the aged hippocampus corresponds to increased microglial priming in this brain region and increased expression of several inflammatory mediators including IL-1β (Barrientos et al., 2009a; Cox et al., 2012; Murray and Lynch, 1998; Nolan et al., 2005; Richwine et al., 2008; Rogers et al., 2011). Unfortunately, our understanding of how CD200 and CX3CL1 signaling are reduced with age is incomplete. Several studies suggest that a reduction in CD200 is connected with a reduction in IL-4 protein in the brain (Lyons et al., 2007a; Lyons et al., 2009b). Indeed, in IL-4 deficient mice, CD200 expression is reduced similar to expression levels in aged mice and corresponds with exaggerated microglial activation after a peripheral LPS challenge (Lyons et al., 2009b). In addition, intracerebroventricular (i.c.v.) injection of IL-4 in rats results in increased CD200 protein expression in neurons (Lyons et al., 2007a). No studies, however, have determined why CX3CL1 is reduced with age.

Along with CD200 and CX3CL1, other regulatory proteins including IL-10 and growth factors (BDNF, IGF-1, TGFβ) are significantly reduced in the aged brain corresponding to increased microglia priming (Cortese et al., 2011; Deak and Sonntag, 2012; Llorens-Martin et al., 2009; Wynne et al., 2010). The loss of so many regulatory systems with age suggests an overarching or global regulatory modulator that is reduced with age (Chen et al., 2010). One such modulator is micro-RNA (miR). miRs are short
(19-22 nt) non-coding strands of RNA that bind to and inhibit the translation of mRNA (Ambros, 2004; O'Connell et al., 2010). miRs are powerful regulators of gene expression because a single miR can target 100s of genes (Lim et al., 2005). Indeed, it is estimated that over 30% of our genome is regulated by miRs. Although the majority of studies investigating immune-associated miRs are in the context of peripheral inflammation, new studies have elucidated the important role of miRs in CNS inflammation. A miR that has become a central focus of microglia regulation is miR-124. miR-124 is more highly expressed in the brain than in the periphery and can be expressed by microglia (Cheng et al., 2009; Ponomarev et al., 2011). A recent study showed that transfection of peripheral macrophages with exogenous miR-124 induces a “microglia-like” phenotype with reduced MHC II and CD45 expression (Ponomarev et al., 2011). Moreover, peripheral induction of miR-124 arrests macrophage and microglial activation in a model of experimental autoimmune encephalomyelitis and reduces disease pathology (Ponomarev et al., 2011). This study was paramount in demonstrating that a CNS-specific cell phenotype can be induced by upregulating a single miR. Importantly, some studies suggest that miR-124 is reduced in the brain with age (Inukai et al., 2012). Intriguingly, IL-4/IL-13 pathways can induce miR-124 (Veremeyko et al., 2013) suggesting that immune and miR regulatory system deficits may be closely connected in the aged. Altered miR-124 expression in microglia of the aged brain may partially explain the observed increases in MHC II expression and development of phenotypic characteristics normally associated with peripheral macrophages. Indeed, miR-124 targets a transcription factor necessary for PU.1 expression (Ponomarev et al., 2011), a gene necessary for myeloid cell development and proliferation typically expressed in high levels within peripheral monocytes and macrophages (Celada et al., 1996; McKercher et al., 1996). Thus, loss of miR-124 in aged microglia may allow for
upregulation of PU.1 and the development of a more inflammatory, peripheral-associated phenotype.

Another miR that has received attention in the field of aging is miR-29. miR-29 is expressed in two genomic clusters, the miR-29a/b-1 cluster and the miR-29b-2/c cluster. Of these, previous studies demonstrate that the miR-29a/b-1 cluster is upregulated in immune cells after activation and acts as a negative regulator to suppress IFN-γ production by Th1 cells (Steiner et al., 2011a). Importantly, the actions of miR-29 may be dysregulated in chronic inflammatory diseases (Smith et al., 2012) and in the aged (Hebert et al., 2008; Shioya et al., 2010). miR-29 is normally expressed within the CNS (Hebert et al., 2008), and has been found in high levels within the glial population (i.e., astrocytes and oligodendrocytes) (Lau et al., 2008; Ouyang et al., 2013), but evidence of microglial expression is lacking. miR-29 became a focus of the aging community because of its ability to inhibit beta-secretase (BACE)1 expression in vitro. BACE1 cleaves amyloid precursor protein allowing for accumulation of amyloid-beta fibrils, a hallmark of Alzheimer’s disease. Indeed, one of the most noted changes within the brain of Alzheimer’s disease patients is BACE1 upregulation (Hebert et al., 2008). Critically, miR-29 is markedly down-regulated in patients with sporatic Alzheimer’s disease (Hebert et al., 2008; Shioya et al., 2010) corresponding to BACE1 upregulation. Surprisingly, the opposite was found for normal non-pathological aging. Indeed, miR-29 expression was increased in the liver and lung of mice in an accelerated model of aging (Ugalde et al., 2011) and in normal aged mice (Bates et al., 2010). Moreover, miR-29 expression was reduced in these same tissues in a model of delayed aging (Bates et al., 2010). The extent to which miR-29a/b-1 regulates brain or microglia inflammation in the normal aged brain is currently unknown.
Figure 1.1. Factors involved in microglia priming with age

Anti-inflammatory-mediated regulation of microglia in the aged brain

In addition to external regulation, the immune system contains an internal “checks and balances” scheme such that induction of inflammatory mediators promotes expression of anti-inflammatory mediators. For example, after a peripheral LPS injection inflammatory factors including IL-1β, TNF-α, and IL-6 will be upregulated, followed by the delayed upregulation of several anti-inflammatory mediators including IL-1 receptor antagonist (IL-1RA) and IL-10 (Arend, 2002; Lawrence et al., 2002). Although IL-10
levels are reduced in the brain of aged rodents at baseline suggesting a breakdown in the normal checks and balances system (Frank et al., 2006; Ye and Johnson, 2001a), after an immune challenge microglia from aged mice and rats produce exaggerated levels of IL-10 coinciding to exaggerated production of IL-1β (Henry et al., 2009; Sierra et al., 2007). This exaggerated IL-10 expression, however, is insufficient to regulate inflammation in the aged brain and inflammation persists. Historically IL-10 was thought to act as an autocrine signal, binding to IL-10R on microglia and restricting inflammatory-associated gene expression. Recent evidence, however, suggests that IL-10 acts more on astrocytes and regulates microglia through astrocyte-produced TGFβ (Norden et al., 2014) (Fig.1.1). Thus, exaggerated levels of IL-10 may not be able to influence microglia activation because of a reduced sensitivity to IL-10 by aged astrocytes. Support for this idea comes from studies showing that TGFβ production in the aged brain is attenuated after an inflammatory challenge (Wynne et al., 2010), suggesting that astrocytes are unable to respond appropriately to IL-10 and fail to produce TGFβ. Further studies will be needed to identify how astrocyte senescence can be reversed with age.

Because microglia are not as sensitive to IL-10 (Fenn et al., 2012; Norden et al., 2014), other anti-inflammatory cytokines are likely better able to regulate microglia. In addition to classical IL-10- and TGFβ-mediated regulation, there is increasing support for microglial regulation by the alternative-activation cytokine, IL-4. IL-4 is historically affiliated with the adaptive immune response and was initially called “B-cell growth factor” or “B-cell stimulatory factor-1” given its role in the activation and class switching of B-cells (Hudak et al., 1987). In the past decade, however, it has become apparent that IL-4 can alter the activation status of innate immune cells within the periphery resulting in an “alternative-activated” or M2a phenotype more conducive to fighting parasitic infections and promoting wound healing (Mantovani et al., 2004; Mosser and Edwards,
2008). Since the discovery of IL-4-responsive innate immune cells, scientists are now researching how the M2a phenotype contributes to normal homeostasis and disease in both peripheral monocytes/macrophages and brain resident microglia. In addition, the degree to which IL-4 influences normal brain function and cognition is also being investigated. As stated earlier, IL-4 protein may be reduced in the hippocampus of aged rats (Maher et al., 2005; Nolan et al., 2005) contributing to a dysregulated microglial phenotype in the aged brain.

Impaired IL-4 signaling in the aged brain may extend beyond reductions in protein levels, however, as a recent study showed that the aged brain is less responsive to IL-4. Responsiveness to inflammatory promoting (TNF-α, IL-1β, IL-12) and alternative activation promoting (IL-4, IL-13) factors was tested in the hippocampus of young (6 mo), middle aged (12 mo), and aged (24 mo) mice (Lee et al., 2013). Cocktails of these inflammatory or alternative-activation factors were injected into the hippocampus and gene transcript expression was measured. In response to the inflammatory cocktail (TNF-α, IL-1β, IL-12), gene expression of CXCL13, haptoglobin, macrophage receptor with collagenous structure (MARCO), and calgranulin B was induced in all age groups. In contrast, the alternative activation cocktail (IL-4, IL-13) promoted gene expression of FIZZ-1, IGF-1, and EAR-11 and these factors were significantly attenuated in the aged brain (Lee et al., 2013). This study indicated that inflammatory-associated signaling remains intact in the aged brain whereas there is reduced responsiveness to IL-4/IL-13. Why the aged brain has reduced responsiveness to these alternative activation signals is unknown. But to fully understand microglia regulation by IL-4 in the aged brain, we must first understand how IL-4 signals in normal innate immune cells.
IL-4 signaling in monocytes, in vitro and in vivo

The potential for monocytes to be influenced by the lymphokine IL-4 was first noted in 1987 when expression of “B-cell stimulating factor (BSF-1)” receptor, known today as IL-4Rα, was found in monocytes (Crawford et al., 1987). The ability of IL-4 to influence monocyte phenotype was demonstrated one year later when monocytes were cultured with IL-4. Monocytes treated with IL-4 increased in size and upregulated several markers associated with differentiation, including MHC II (te Velde et al., 1988). Moreover, monocytes treated with IL-4 were less effective at inhibiting melanoma cell growth in culture and promoting chemotaxis. These deficits were associated with reduced expression of IL-1 (te Velde et al., 1988). Finally, in 1989 monocytes co-treated with LPS and varying concentrations of IL-4 were found to reduce expression of several inflammatory markers including TNFα, IL-1β, and prostaglandin E (Hart et al., 1989).

As a result of these initial studies, IL-4 is considered an anti-inflammatory cytokine for innate immune cells. Indeed, treatment of monocytes with IL-4 in vitro limits LPS-induced mRNA transcription of IL-1β while promoting IL-1RA expression (Allen et al., 1993) and reducing IFN-γ-dependent production of TNF-α (Nguyen and Benveniste, 2000). Moreover, IL-4 treatment of primary microglia and a cell line of human monocytes attenuates Aβ-induced IL-1β and TNF-α expression (Szczepanik et al., 2001) and reduces nitric oxide production (Zhao et al., 2006). The anti-inflammatory properties of IL-4 have also been observed in vivo. Mice deficient in IL-4 develop a more severe form of experimental autoimmune encephalomyelitis associated with increased levels of the inflammatory cytokines IL-1β, IL-6, and to a lesser extent, TNF-α (Falcone et al., 1998). Moreover, mice deficient in IL-4 have increased levels of IL-1β in the cortex and hippocampus after a peripheral LPS challenge corresponding with intensified sickness behavior (i.e., significantly reduced social exploratory behavior and mobility) (Lyons et al., 2000).
Loss of IL-4 signaling in the brain is also associated with reduced levels of CD200 and increased microglial activation (Lyons et al., 2009b). In addition, viral-mediated induction of IL-4 in the hippocampus of APP-PS1 mice limits microglia activation and reduces Aβ oligomerization and deposition (Kiyota et al., 2010). Although IL-4 could affect numerous cell types in the in vivo studies, there are only a handful of published reports showing IL-4Rα on astrocytes (Brodie et al., 1998; Hulshof et al., 2002) or neurons (Maher et al., 2005), and the predominant thought is that within the CNS, IL-4Rα expression is highest on microglia (David and Kroner, 2011; Hulshof et al., 2002; Nguyen and Benveniste, 2000; Zhao et al., 2006). Thus, a strong body of evidence supports the idea that IL-4 is a potent anti-inflammatory mediator for monocytes and microglia.

Despite the overwhelming evidence for an anti-inflammatory role of IL-4, there are still several published studies that indicate IL-4 may have pro-inflammatory physiological properties. In 2002 a study investigated the role of IL-4 in regulating the sickness response by giving an i.c.v. injection of IL-4 before or concurrently with a peripheral LPS injection and measuring sickness behavior. Pre-treatment with IL-4 12 h before LPS blocked LPS-induced sickness behavior. Injection of IL-4 simultaneously with LPS, however, exaggerated LPS-induced sickness behavior (Bluthé et al., 2002). In a separate study, injection of IL-4 into the hypothalamus of rats fed a high fat diet exacerbated hypothalamic inflammatory cytokine production and increased weight gain (Oh et al., 2010). There are also noted cases of “IL-4-priming” in which IL-4 potentiates the inflammatory response in vitro and in vivo. For example, mouse peritoneal macrophages treated with IL-4 in vitro and then challenged with the Th1-type pathogen, Neisseria meningitidis, have exaggerated inflammatory cytokine production compared to treatment with N. meningitidis alone (Varin et al., 2010). In this study IL-4-induced
inflammation relied on functional MyD88 signaling (Varin et al., 2010). A similar study showed that if peripheral blood mononuclear cells are pre-treated with IL-4 for more than 20 h before a challenge with LPS or *Staphylococcus aureus*, production of IL-12 and TNF-α are enhanced (D'Andrea et al., 1995). Further characterization into the pro-inflammatory effects of IL-4 found that prolonged pre-treatment with IL-4 enhances LPS- and double-stranded RNA-induced TNF-α and IL-1α expression (Major et al., 2002; Varin et al., 2010). Thus, the ability to promote an inflammatory response by IL-4 seems to rely on TLR stimulation and the MyD88 pathway. Little work, however, has denoted how IL-4 post-treatment affects the inflammatory response. This is of particular interest because in several CNS diseases (e.g., multiple sclerosis, Alzheimer's disease), and following CNS trauma, an inflammatory response would arise first followed by increased IL-4 from a central or peripheral source. The one study examining post-inflammatory IL-4 signaling demonstrated that treatment of cultured microglia with IL-4 2 h after LPS was more effective in limiting LPS-induced neuronal death compared to pre-treatment with IL-4 (Zhao et al., 2006). The exaggerated effects of IL-4 post-treatment were attributed to increased IL-4Rα expression on microglia and reduced nitric oxide production (Zhao et al., 2006). Thus, it appears that monocytes may be more sensitive to the anti-inflammatory effects of IL-4 if it is delivered after an inflammatory stimulus. Nonetheless, *in vivo* studies using post-treatment with IL-4 and further characterization of the IL-4-induced immune profile are required.

*IL-4 signaling – 4 pathways in brief*

To explain how IL-4 may induce both anti-inflammatory and inflammatory mediators, an understanding of the IL-4 signaling pathway is required (Fig.1.2). The IL-4
signaling cascade has been characterized in an outstanding review (Nelms et al., 1999), and will only be briefly introduced here (Fig.1.2).

The best known signaling cascade for IL-4 is the JAK/STAT signaling pathway. Binding of IL-4 to the IL-4Rα in hematopoietic cells induces the association of IL-4Rα with the gamma-c (γc) receptor (Murata et al., 1999). Binding of these receptors promotes the association of two janus-associated kinase (JAK) proteins: JAK-1 which binds to IL-4Rα, and JAK-3 which binds to the γc receptor (Nelms et al., 1999; Witthuhn et al., 1994). These JAKs then phosphorylate several tyrosine residues on the cytosolic component of IL-4Rα allowing the association of several proteins. There are 4 primary tyrosine residues associated with IL-4 signaling. Of these, three are grouped together in the middle of the cytosolic tail of IL-4Rα. Here, the transcription factor STAT-6 binds to IL-4Rα and is phosphorylated by the kinase activity of JAK-1/JAK-3. Once phosphorylated, STAT-6 will homodimerize and translocate to the nucleus to induce transcription of IL-4-associated genes including, but not limited to, arginase-1, mannose receptor, FIZZ-1, suppressor of cytokine signaling (SOCS)-1, and its own receptor IL-4Rα (Abu-Amer, 2001; Nguyen and Benveniste, 2000).

The three remaining pathways of IL-4 signaling have received far less attention, likely due to their variability. Indeed, IL-4 signaling invariably results in the phosphorylation and activation of STAT-6 independent of cell type, microenvironment, or species (Takeda et al., 1996). Thus, IL-4 signaling can reliably be tested through STAT-6 phosphorylation and transcription of STAT-6 genes. The other three pathways of IL-4 signaling can vary depending on cell type and microenvironment (Nelms et al., 1999; Welham et al., 1994). Nonetheless, understanding when these signaling cascades are preferentially induced is critical in identifying the role for IL-4 signaling in disease and injury. In addition to the binding and activation of STAT-6, IL-4Rα tyrosine
Figure 1.2. The IL-4/IL-4Rαγc signaling pathway. There are 4 pathways associated with IL-4 signaling. (1) The most well known pathway is the JAK-1/JAK-3/STAT-6 pathway that induces transcription of Th2/M2-associated genes including arginase-1 and mannose receptor. (2) IRS-1/2 is also associated with the IL-4Rα cytoplasmic domain at the first phosphorylated tyrosine. IRS-1/2 will then associate with Shc to promote Grb2-Sos interactions and the phosphorylation and activation of Ras. Alternatively, IRS-1/2 can directly associate with Grb2-Sos to promote this interaction. Activation of Ras promotes the Ras/MAPK pathway and phosphorylation of the transcription factors ERK-1/2, c-Jun, and JNK to induce several genes associated with proliferation, differentiation, and inflammation. (3) IRS-1/2 will also activate PI3K to phosphorylate PIP2 into PIP3. PIP3 can then activate Akt which will phosphorylate Iκκ. Iκκ functions to phosphorylate and degrade IκB which releases and activates NF-κB allowing for translocation to the nucleus and transcription of several inflammatory mediators. (4) PIP3 can also phosphorylate and activate PDK-1 which will activate PKC. PKC can induce reactive oxygen species production and activate the transcription factor CREB, which will translocate into the nucleus to promote neurotrophic and growth factor transcription.
phosphorylation induces the binding of insulin receptor substrate (IRS)-1/2 (Barry et al., 2005; Hartman et al., 2004; Keegan et al., 1994; Kelly-Welch et al., 2003). In contrast to STAT-6, IRS-1/2 binds to the first tyrosine in the IL-4Rα cytoplasmic domain (Keegan et al., 1994). Once bound, IRS-1/2 is phosphorylated by JAK-1 or JAK-3. From here, IRS-1/2 can activate two separate pathways, the Ras/MAPK pathway or the PI3K pathway.

To induce the Ras/MAPK pathway IRS-1/2 associates with the signaling protein, Shc. Shc can then associate with growth factor receptor bound protein (Grb)2 to induce the binding of Grb2 to son of sevenless (Sos) resulting in phosphorylation and activation of Ras. Alternatively, IRS-1/2 can bind Grb2 directly to induce association with Sos. Once activated, Ras activates several transcription factors including the mitogen associated protein kinase (MAPK) ERK-1/2, c-Jun, and c-Jun N-terminal kinase (JNK) (Nelms et al., 1999). These transcription factors perform a host of functions within the cell, but in general, induce genes associated with proliferation, differentiation, and inflammation.

IRS-1/2 can also promote the phosphatidyl inositol 3 kinase (PI3K) pathway by binding to PI3K directly and inducing its phosphorylation. PI3K adds a phosphate group to phosphatidylinositol 4,5-bisphosphate (PIP$_2$) to make PIP$_3$. From here PIP$_3$ can signal the two remaining pathways of the IL-4 signaling cascade. The first of which is activation of Akt. Although Akt activation is normally associated with the inhibition of apoptosis and induction of cell survival (Downward, 2004), in immune cells it can also activate the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-$\kappa$B) by binding to and inducing the degredation of the NF-$\kappa$B inhibitory molecule IκB (Ozes et al., 1999). NF-$\kappa$B translocates to the nucleus and upregulates transcription of several inflammatory-associated factors including IL-6 and IL-1β. The second pathway PIP$_3$ activates is the protein kinase C (PKC) pathway through phosphoinositide-dependent
kinase (PDK)-1. On its own, PKC can promote the upregulation of reactive oxygen species typically affiliated with inflammation (Inoguchi et al., 2003). PKC can also activate cAMP-response element binding protein (CREB) which translocates to the nucleus to upregulate transcription factors associated with growth (neurotrophic factors, growth factors) (Finkbeiner et al., 1997; Wen et al., 2010). Thus, through these four signaling pathways, IL-4 has the potential to induce genes associated with growth and repair (arginase, growth factors, neurotrophins), survival and proliferation (Bcl, c-fos), and inflammation (IL-1β, IL-6, TNF-α). These signaling cascades are complex and regulated by a host of factors not mentioned here, and thus, IL-4 signaling may be dynamic depending on the microenvironment and cell type (Fig.1.2).

*IL-4 signaling in the brain*

From the previous sections it is clear that IL-4 has the potential to alter the phenotype of mononuclear cells through 4 distinct signaling pathways. From *in vitro* work we know that this signaling capacity extends to microglia. But in an *in vivo* system does IL-4 actually play a role in modifying microglia physiology? To what extent is IL-4 involved in any brain functions?

A key transcription factor that promotes IL-4 expression and the differentiation of Th2 T-cells is GATA-3. Early work into GATA-3 deficient mice revealed that, in addition to hematopoietic deficiencies, these mice had several neural abnormalities including neural tube deformities and collapsed ventricles (Pandolfi et al., 1995). Although GATA-3 can regulate multiple genes aside from Th2 cytokines, its involvement in neural disorganization at least suggests that IL-4 may be involved in normal brain homeostasis. In support of this notion, a paper by Lovett-Racke’s group demonstrated that IL-4 protein is markedly induced in the developing CNS of mice (Lovett-Racke et al., 2000).
postnatal day 14, however, IL-4 protein was almost undetected in the CNS and by 6 weeks of age was completely absent. These results coincided with the upregulation of GATA-3 in the CNS. In contrast to GATA-3 knockout mice, however, IL-4 knockout mice are viable and develop normally without gross mutations in CNS structure (Derecki et al., 2010; Kopf et al., 1993). Nonetheless, these studies suggest a role for IL-4 in the CNS during early development. Whether or not IL-4 also maintains a role in the adult CNS, however, was still unclear.

In 2010 a novel role for IL-4 in the adult CNS was proposed. Using IL-4 deficient mice, researchers demonstrated that mice lacking IL-4 have impaired learning and memory in the Morris Water Maze task. Thus, they postulated that the importance of IL-4 in the CNS extends beyond development and helps regulate normal cognitive processing in adults. They went on to show that mice injected with an antibody against VLA-4, a transmembrane protein required for T-cell localization to the CNS, also show similar cognitive impairments in the Morris Water Maze. Moreover, replacement of IL-4 knockout bone marrow with wild type bone marrow restores learning and memory to levels observed in wild type mice (Derecki et al., 2010). These results indicate that IL-4 production by meningeal T-cells is responsible for mitigating changes in cognition. Nonetheless, few T-cells normally associate with the CNS (Kivisäkk et al., 2009) and IL-4 levels are still low to undetectable in the normal adult brain (Lovett-Racke et al., 2000). Therefore, a clear connection between IL-4 and the homeostatic adult CNS is still lacking.

**M1 vs. M2 phenotypes – role of M2a after CNS injury**

The connection between IL-4 and the brain appears the most robust after severe trauma (e.g., spinal cord injury) or CNS disease (e.g., multiple sclerosis). Increased IL-4
protein in the CNS has been noted, for example, within 1 day post injury in a mouse model of spinal cord injury (Guerrero et al., 2012; Lee et al., 2010). Curiously, the source of IL-4 protein within the CNS is still debated. One study demonstrated that IL-4 was produced by granulocytic-type cells that referred to as neutrophils (Lee et al., 2010), although capacity for neutrophils to produce IL-4 in any other paradigm has not been demonstrated. Studies investigating IL-4 regulation in the normal brain suggest that the small population of meningeal-associated T-cells produce CNS-associated IL-4 (Derecki et al., 2010). Still others have demonstrated that microglia upregulate IL-4 protein after injury and are the endogenous source of IL-4 in the CNS (Guerrero et al., 2012). Regardless of the source, all available evidence demonstrates that IL-4 concentrations are increased in the CNS after a gross inflammatory event.

Increased IL-4 in the CNS after spinal cord injury (SCI) is associated with the development of a microglia/macrophage phenotype beneficial for repair. In vitro IL-4 is associated with the development of an M2a, or alternative activation, monocytic phenotype. This is in contrast to the M1, or classically activated, phenotype. After the discovery that lymphocyte-associated cytokines alter the phenotypic responses of monocytes in culture, monocytes were classified into two primary states, M1 and M2 (Goerdt and Orfanos, 1999; Mantovani et al., 2004; Mills et al., 2000). These were named because of their similarity to and interactions with the Th1 and Th2 T-cell phenotypes. The M2 phenotype has been further broken down into M2a, M2b, and M2c. These different monocyte phenotypes are discussed in detail in several outstanding reviews (David and Kroner, 2011; Mantovani et al., 2004; Mosser and Edwards, 2008), and will be briefly introduced here (Fig.1.3).

An M1, or classical activation state, is induced by the prototypic inflammatory stimuli and cytokines including LPS, IL-1β, IFN-γ, and TNF-α. M1 cells are characterized
by upregulation of toll-like receptors (TLRs) 2 and 4 and expression of inflammatory cytokines including IL-1β, IL-6, and TNF-α (Mantovani et al., 2004). An M2a, or alternative activation state, is induced by IL-4 or IL-13 and results in the upregulation of the arginase I enzyme, chitinase-like receptor (Ym-1), antigen presenting proteins (e.g., MHC II), and scavenger receptors including mannose receptor and scavenger receptor-A (Fairweather and Cihakova, 2009; Mantovani et al., 2004). M2a cells are associated with increased wound repair and the clearance of parasites. An M2b phenotype is less well defined and induced by immune complexes and TLR agonists. An M2b phenotype is characterized by high levels of both IL-1β and IL-10, and is purported to function in immunoregulation (Mantovani et al., 2004). An M2c, or classical deactivation state, is induced by IL-10 and TGFβ and characterized by the lack of IL-1β expression and high IL-10 expression. These cells are important in the resolution of inflammation (Mantovani et al., 2004). After CNS injury, the M1 and M2a phenotypes are significantly elevated and thought to contribute differentially to damage and repair (Kigerl et al., 2009).

An M2a response after injury is primarily characterized by the induction of arginase and is thought to participate in endogenous repair processes. Arginase is important to the alternative activated phenotype because its enzymatic reaction can result in the creation of proline (Albina et al., 1993; Wu and Morris, 1998), an essential component of collagen (Barbul, 2008; Curran et al., 2006). Moreover, arginase is the rate-limiting enzyme for the production of polyamines (Cai et al., 2002; Curran et al., 2006). After injury, polyamines are thought to promote neuronal survival and increase neurite growth due to their inhibitory effects on myelin associated glycoprotein (Cai et al., 2002). Moreover, a recent study indicated that polyamines are required for CCL2 induction and recruitment of peripheral monocytes to the CNS (Puntambekar et al., 2011). This is important because peripheral monocytes are actively recruited to the CNS
after injury (Shechter et al., 2013) and can take on distinct phenotypes that may contribute to pathogenesis (CX₃CR₁⁺/Ly6C⁺/iNOS⁺) or repair (CCR2⁻/Ly6C⁺/iNOS⁻) (Donnelly et al., 2011). Thus, polyamines may recruit or maintain a particular population that would be CCR2⁺ and beneficial for repair after injury.

Importantly, the inflammatory M1 and alternative activation M2 phenotypes are both observed acutely after injury (i.e., first 14 days post injury) (David and Kroner, 2011; Kigerl et al., 2009). Thus, in the context of CNS trauma, increased IL-4 levels are present in the context of active and aggressive inflammation. It is unclear how the IL-4 signaling cascade is altered by this microenvironment and what effect it has on inflammation.

*Shift away from an M2 toward an M1 phenotype with age*

Because of the implication of an M2a response in the promotion of endogenous repair, an inability to induce or respond to IL-4 may increase damage associated with CNS injury or disease. In a study examining M1 vs. M2a responses following traumatic brain injury (TBI) in adult and aged mice, researchers found that aged mice had reduced expression IL-4Rα and the downstream signaling molecule STAT6 compared to adults. This reduction in IL-4 signaling corresponded with exaggerated expression of inflammatory mediators, increased lesion size, and more functional deficits (Kumar et al., 2012). Indeed, a clear concept in the field of aging is that as an individual ages, microglia/macrophage phenotypes are skewed away from the M2a and M2c phenotypes towards an M1 and inflammatory phenotype (Henry et al., 2009; Lee et al., 2013; Sierra et al., 2007; Ye and Johnson, 2001a) (Fig.1.3). Indeed, age-associated microglia priming (discussed earlier) is coupled with a reduced ability to promote a reparative or alternatively activated phenotype (Kumar et al., 2012; Lee et al., 2013). The reason for
this is still unknown. A shift away from the M2a phenotype may result from impairments in IL-4 protein or IL-4 responsiveness in the aged brain. As discussed earlier, IL-4 protein is reportedly reduced in the aged hippocampus (Maher et al., 2005), and IL-4/IL-13 signaling is attenuated in the aged brain (Lee et al., 2013). Further studies are required to assess why IL-4 responsiveness is reduced in the aged brain and the degree to which an impaired M2a microglial response affects recovery after SCI.

Figure 1.3. M1 and M2 phenotypes and shift towards M1 with age.
Is microglia priming specific to aging?

Increased MHC II expression and a hyper-active microglial response to an immune challenge are well noted in the aged, but can this process be accelerated or amplified and does MHC II priming exist outside of the aged? Studies using a mouse model of prion disease would suggest the answer to these questions is yes. In a mouse model of ME7 prion disease, microglia priming develops relatively quickly after inoculation with high expression of MHC II and increased Iba-1 immunoreactivity by 12 weeks after inoculation (5-5.5 months of age) (Murray et al., 2012). These primed microglia also become hyper-inflammatory following an immune challenge resulting in impaired working memory (Murray et al., 2012) and an exaggerated sickness response (Combrinck et al., 2002). Thus, microglia priming can be induced earlier in life as a result of neurodegenerative disease. Similar results are observed in several other models of neurodegeneration including multiple sclerosis (Benveniste, 1997; Ramaglia et al., 2012), amyotrophic lateral sclerosis (Liao et al., 2012; Weydt et al., 2004), and Parkinson’s disease (Kohutnicka et al., 1998; McGeer et al., 1988).

Age-associated microglial priming can also be amplified. For example, examination of post-mortem brain tissue from Alzheimer’s disease patients revealed MHC II⁺ (HLA-DR⁺) microglia concentrated around amyloid plaques (McGeer et al., 1987) producing elevated levels of IL-1β beyond that observed in non-neurodegenerative age-matched controls. Critically, an amplified inflammatory response over that of healthy aged-matched controls was associated with the onset of dementia (Engelhart et al., 2004) and increased prevalence of depression (~75%) (Craig et al., 2005). Thus, neurodegenerative disease can also amplify microglial priming to induce hyper-inflammatory responses that are exaggerated compared to those in healthy aged persons.
Microglia priming may also exist outside of neurodegenerative disease. Indeed, studies using a murine model of psychosocial stress found that repeated exposure to an aggressor resulted in a primed microglia state (Wohleb et al., 2012). Although the psychosocial stress model was not associated with increased MHC II expression on microglia, if stressed mice were given an immune challenge with LPS following the cessation of the stressor, they had an exaggerated inflammatory response associated and prolonged sickness behavior compared to non-stressed controls. Indeed, microglia from stressed mice showed over a 140-fold increase in IL-1β expression after LPS compared to only a 40-fold increase in non-stressed controls (Wohleb et al., 2012). Whether or not this primed phenotype is maintained for an extended period of time is unclear, but it demonstrates that priming can exist outside of aging or CNS degeneration.

Age-associated microglia priming may also be accelerated. A study in 1995 reported that a single traumatic brain injury (TBI) in young adult rats promoted the development of MHC II⁺ cells in the brain that persisted for at least 16 days post injury (Holmin et al., 1995). Moreover, in clinical cases of TBI, persons that suffered a TBI had increased microglia activation (i.e., increased CD68, CR3/43 staining) 16 years (Gentleman et al., 2004), and even up to 47 years after injury (Johnson et al., 2013). Importantly, persons with increased CD68 immunoreactivity had evidence of ongoing axonal and myelin degeneration (Johnson et al., 2013). Increased microglial activation is also noted in live TBI patients. Using the ligand [11C](R)PK11195, which binds to the peripheral benzodiazepine receptor upregulated by active microglia, PET imaging demonstrated that patients with a TBI had increased [11C](R)PK11195 expression up to 17 years after injury compared to non-injured controls (Ramlackhansingh et al., 2011). Whether these MHC II⁺ microglia observed after TBI are classically primed and demonstrate hyper-reactivity to immune challenge to precipitate behavioral disturbances
is unknown. Nonetheless, these studies suggest that trauma to the CNS could potentially accelerate microglia priming and exacerbate neurobehavioral complications normally associated with advanced age.

**Connection between traumatic brain injury and age-associated neurodegenerative diseases**

One of the most compelling lines of evidence linking CNS trauma (e.g., TBI) with accelerated microglia priming and age-associated brain dysfunction is that TBI is one of the primary risk factors for the development of Alzheimer’s disease (Fleminger, 2008; Lye and Shores, 2000). Indeed, history of head trauma increases an individual’s risk for developing Alzheimer’s disease by twofold (Mortimer et al., 1991). In further support of this connection, head trauma is the only known cause for the development of the chronic neurodegenerative disorder called chronic traumatic encephalopathy (CTE). CTE is characterized by accumulation of hyper-phosphorylated tau tangles in neurons, similar to those observed in Alzheimer's disease (Lucke-Wold et al., 2014).

The neuropsychological effects of TBI are also very similar to those observed with age or neurodegenerative disease. For example, it is documented that between 30 and 60% of TBI patients suffer from depression during their life-time (Hibbard et al., 1998; Holsinger et al., 2002; Jorge et al., 1993; Jorge et al., 2004; Kreutzer et al., 2001). This is significantly more than the 10% rate among the general population. Rates of TBI-associated depression are similar to those observed in aging patients with an active infection and in Alzheimer disease patients as discussed earlier.
Exaggerated brain inflammation after an immune challenge can lead to the development of neuropsychiatric complications – in brief

So how would microglia priming associated with aging and a TBI promote these neuropsychological complications? Several elegant studies and reviews have demonstrated a strong connection between elevated brain inflammation and the onset of psychological complications, including depression and cognitive decline (Arfanakis et al., 2013; Barrientos et al., 2009a; Chen et al., 2008; Corona et al., 2012; Cunningham et al., 2009; Dantzer et al., 2008; Ehlenbach et al., 2010; Godbout et al., 2008; Miller and Raison, 2008; Pollak and Yirmiya, 2002; Raison et al., 2006; Richwine et al., 2008; Steiner et al., 2011b; Villeda et al., 2011; Yirmiya et al., 2000). Thus, although aging and TBI may not promote depression or cognitive impairment alone, activation of resident primed microglia that exist in aged and injured brain may trigger the onset of these neurobehavioral impairments by promoting exaggerated expression of inflammatory mediators in the CNS (Fig.1.4). For example, studies using aged mice (22 mo – median lifespan 26 mo) found that aging alone was not associated with depressive-like behavior or cognitive impairment (Chen et al., 2008; Godbout et al., 2008). Following an immune challenge, however, aged mice had prolonged sickness behavior and, after the resolution of sickness, developed depressive-like behavior and cognitive impairments (Chen et al., 2008; Godbout et al., 2008). Similar results were demonstrated in a rat model of active immune challenge with *E. coli* (Barrientos et al., 2009a; Barrientos et al., 2009b). Evidence for this immune-associated hyper-CNS inflammation as the underlying mechanism for behavioral and cognitive impairments in the aged is also prevalent within the clinical literature. For example, a recent study found that compared to older individuals without hospitalization, those that had been hospitalized for a noncritical illness had a significantly greater risk for developing long-term cognitive decline.
Figure 1.4. CNS Disease/Injury and Aging synergize to induce depression after an immune challenge

Whether or not microglia priming is also the underlying mechanism for TBI-associated depression is unknown. Nonetheless, TBI-associated depression shares many of the characteristics of inflammatory-associated depression in the aged. For instance, the few studies investigating TBI-associated depression indicate that classical anti-depressant therapies are less efficacious in TBI patients (Ashman et al., 2009; Saran, 1988). A similar condition is seen in patients with chronically elevated levels of
circulating inflammatory cytokines (e.g., c-reactive protein, IL-6), in whom classical anti-depressants fail to reduce inflammation and do not induce behavioral improvements (Lanquillon et al., 2000; Miller and Raison, 2008). Moreover, TBI-associated depression often develops months to years after the initial injury suggesting a role of age-associated microglial priming (Hibbard et al., 1998; Holsinger et al., 2002), and often waxes and wanes suggesting an external cause, like spikes in inflammation from illness or extreme stress (Hibbard et al., 1998; Holsinger et al., 2002; Jorge et al., 1993). Understanding the mechanism of TBI-associated depression is critical so that we may begin to address how to treat it. With over 2 million people each year in the US suffering from a concussion and a rate of depression between 30 and 60%, this is a serious concern.

**Study Objectives**

In summary, aging is associated with microglial priming and, following an immune challenge, these primed microglia become hyper-inflammatory resulting in neuropsychological complications. Moreover, primed microglia may be more difficult to regulate and become resistant to anti-inflammatory or alternative activation signals, including IL-4. The role for IL-4 in the CNS remains unclear, but is implicated in reparative processes after CNS trauma. Thus, the aged may have reduced repair and exhibit increased morbidity associated with CNS injury as a result of their insensitivity to IL-4 regulation. Finally, this age-associated primed phenotype may be accelerated by factors including traumatic CNS injury, making patients that have suffered a head trauma more vulnerable to secondary challenges like stress or infection. Thus, the objectives of my dissertation were four-fold: (1) Identify a potential mechanism for reduced regulatory factors (e.g., CX₃CL1) in the brain with age that may lead to increased microglia priming (Chapter 2). (2) Identify the underlying mechanism for reduced sensitivity of aged
microglia to the alternative activating signal IL-4 (Chapter 3), and identify the degree to which reduced sensitivity to IL-4 impairs functional recovery after a CNS injury (Chapter 4). (3) Further characterize IL-4 signaling in the CNS in the context of active inflammation to better assess the role of IL-4 in CNS repair (Chapter 4). (4) Determine the extent to which microglia become primed and hyper-reactive after TBI and the degree to which this results in depressive-like behavior (Chapter 5).
Chapter 2: Increased miR-29b in the aged brain correlates with the reduction of IGF-1 and CX3CL1

Abstract

Microglia develop an inflammatory phenotype during normal aging. The mechanism by which this occurs is not well understood, but may be related to impairments in several key immunoregulatory systems. Here we show that miR-29a and miR-29b, two immunoregulatory micro-RNAs, were increased in the brain of aged BALB/c mice compared to adults. Insulin-like growth factor-1 (IGF-1) and fractalkine ligand (CX3CL1) are negative modulators of microglial activation and were identified as targets of miR-29a and miR-29b by luciferase assay and primary microglia transfection. Indeed, higher expression of miR-29b in the brain of aged mice was associated with reduced mRNA levels of IGF-1 and CX3CL1. Parallel to these results in mice, miR-29a and miR-29b were also markedly increased in cortical brain tissue of older individuals (mean 77 yrs) compared to middle-aged adults (mean 45 yrs). Moreover, increased expression of miR-29b in human cortical tissue was negatively correlated with IGF-1 and CX3CL1 expression. Collectively these data indicate that an age-associated increase in miR-29 corresponded with the reduction of two important regulators of microglia, IGF-1 and CX3CL1.

Introduction

An increase in the inflammatory potential of the brain is a normal consequence of aging. For example, inflammatory cytokines including interleukin (IL)-1β, tumor necrosis
factor (TNF-α, and IL-6 are increased in the brain of aged mice (Godbout et al., 2005b; Njie et al., 2012; Ye and Johnson, 1999). Microglia are resident innate immune cells of central nervous system (CNS) and contribute to the increased level of pro-inflammatory cytokine expression in the aged brain (Corona et al., 2012). In support of this notion, several studies indicate that microglia from aged rodents have a “primed” phenotype with increased expression of major histocompatibility complex (MHC)II (Frank et al., 2006; Henry et al., 2009). This is important because primed (MHCII⁺) microglia produce exaggerated levels of IL-1β in the brain of aged mice after a peripheral immune challenge (Henry et al., 2009). Increased IL-1β production is associated with dendritic atrophy, acute cognitive impairment, and prolonged sickness and depressive-like complications (Chen et al., 2008; Godbout et al., 2005b; Godbout et al., 2008; Richwine et al., 2008). While the cause of microglial priming with age is unclear, several studies indicate that a decrease in microglial regulatory systems may be involved. For instance, brain aging is associated with reduced expression of several mediators of microglial regulation including anti-inflammatory cytokines (e.g., IL-10, IL-4) (Maher et al., 2005; Ye and Johnson, 2001a), neuronal-derived proteins (CD200, CX3CL1) (Jurgens and Johnson, 2012; Lyons et al., 2007a; Wynne et al., 2010), and growth factors (IGF-1, NGF) (Deak and Sonntag, 2012; Larkfors et al., 1987; Sonntag et al., 2005b).

In rodent models of aging, fractalkine (CX3CL1) is an integral modulator of microglia activation that is decreased in the brain with age (Bachstetter et al., 2011; Deak and Sonntag, 2012; Lyons et al., 2009a; Wynne et al., 2010). Within the brain, CX3CL1 is a chemokine constitutively expressed by neurons that binds to the fractalkine receptor (CX3CR1) on microglia (Harrison et al., 1998). Thus, CX3CL1-CX3CR1 binding creates a unique regulatory relationship between neurons and microglia (Cardona et al., 2008). Disruption of this CX3CL1-CX3CR1 signaling pathway by loss of either CX3CL1
or CX$_3$CR1 allows for inflammatory-induced activation of microglia (Cardona et al., 2006; Lyons et al., 2009a; Wynne et al., 2010). For example, an age-associated reduction in CX$_3$CL1 corresponds with an increased number of primed/MHCII$^+$ microglia (Bachstetter et al., 2011), increased IL-1$\beta$ production in the hippocampus (Lyons et al., 2009a), and increased reactivity of microglia to a secondary immune challenge (Wynne et al., 2010). This increased microglial activity has functional consequences in reduced learning and memory (Rogers et al., 2011), reduced neurogenesis (Bachstetter et al., 2011), and increased depressive-like behavior (Godbout et al., 2008). Moreover, central infusion of CX$_3$CL1 reduced the primed MHCII$^+$ microglia profile in the brain of aged rats (Bachstetter et al., 2011; Lyons et al., 2009a). The idea that CX$_3$CL1 is important in modulating microglia responses within the brain is also supported by studies using mice deficient in the fractalkine receptor (CX$_3$CR1$^{KO}$). CX$_3$CR1$^{KO}$ mice have a hyper-reactive microglial response to an inflammatory challenge (Cardona et al., 2006) that results in amplified pro-inflammatory cytokine production, prolonged sickness behavior, and the development of depressive-like behavior (Corona et al., 2010). Therefore, CX$_3$CL1-CX$_3$CR1 interactions are critical in the modulation of microglial activation.

IGF-1 is another modulator of microglia activation that is reduced in aged brain. Classically, IGF-1 is a growth factor that increases neuroprotection (Sonntag et al., 2005a; Sonntag et al., 2005b), neurogenesis (Llorens-Martin et al., 2009), long-term potentiation (Maher et al., 2006; Sonntag et al., 2005b), and dendritic growth and complexity (Niblock et al., 2000). IGF-1, however, can also modulate immune function. For example, IGF-1 reduces inflammatory cytokine responses in the brain (O'Connor et al., 2008) and ameliorates LPS-induced sickness behaviors (Dantzer et al., 1999) that are primarily driven by microglia-dependent production of IL-1$\beta$ and TNF-α (Dantzer et al., 2008). Moreover, central injection of a viral vector that upregulated IGF-1 in a mouse
model of amyotrophic lateral sclerosis (ALS) reduced microglial secretion of TNF-α and nitric oxide (NO) (Dodge et al., 2008). Thus, age-related reduction in IGF-1 may also contribute to the enhanced inflammatory profile of microglia in the aged brain.

We hypothesize that the reduction of multiple microglial regulatory pathways with age, including CX3CL1 and IGF-1, indicate that there is loss of a global regulator of gene and protein expression. One possibility is that micro-RNA (miRNA) regulation is altered in the aged brain. miRNAs are small (19-24 nucleotides in length) non-coding RNAs that reduce post-transcriptional gene expression by binding to complementary target regions on mRNA to inhibit translation or promote mRNA degradation (Ambros, 2004; Bartel, 2004). miRNAs provide global regulation of gene expression and influence inflammatory processes (Baltimore et al., 2008; O'Connell et al., 2010). Target prediction algorithms of CX3CL1 and IGF-1 revealed that both of these genes are potentially regulated by the miR-29 cluster. We have previously shown that the miR-29a/b cluster is upregulated in immune cells during the course of chronic inflammation, including multiple sclerosis (MS) and the animal model of MS, experimental autoimmune encephalomyelitis (EAE) (Smith et al., 2012). Moreover, miRNAs in the miR-29 cluster were increased in the liver and muscle in a rodent model of accelerated aging (Zmpste24-null mice) (Ugalde et al., 2011). Thus, increased expression of miR-29 in the more inflammatory aged brain may contribute to the progression of microglial dysregulation and hyperactivity.

The purpose of this study was to determine the degree to which immunomodulatory miRNAs were altered in the aged brain and investigate their potential influence on microglial regulatory systems. Here, we show that miR-29a and miR-29b were increased in the brain of both aged mice and older humans. Moreover, this increase in miR-29a and miR-29b expression was associated with the down-regulation of specific CNS targets involved in the modulation of microglial activation, IGF-1 and
CX₃CL1. Indeed, increased expression of miR-29b in the brains of older humans significantly and negatively correlated with IGF-1 and CX₃CL1 expression. We interpret these results to suggest that age-associated increases in miR-29 in the brain suppress multiple factors, including IGF-1 and CX₃CL1, contributing to the development of an inflammatory profile of microglia in the aged brain.

Materials and Methods

Mice
Adult (2-3 mo) male BALB/c mice were obtained from a breeding colony kept in barrier-reared conditions in a specific pathogen-free facility at the Ohio State University. Aged (18-20 mo) male BALB/c mice were obtained from the National Institute on Aging specific pathogen-free colony (maintained at Charles River Laboratories, Inc., MA). Aged mice were allowed one week to acclimate to the facility prior to experimentation. All 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.

Postmortem human brain tissue
Postmortem human brain tissue was obtained from the Harvard Brain Tissue Resource Center (Belmont, MA, supported by the PHS grant R24 MH068855) (10 samples) and the Human Brain and Spinal Fluid Resource Center (VA West Los Angeles Healthcare Center, Los Angeles, CA which is sponsored by NINDS/NIMH, National Multiple Sclerosis Society and Department of Veterans Affairs) (17 samples).
The tissue consisted of eleven adult (14 – 55 years old with a mean age of 45 years old) and sixteen aged (58 – 91 years old with a mean age of 73 years old) brain samples (Table 2.1). The differentiation between “Adult” or “Aged” brain tissue was 57 yrs (<57 = Adult; >57 = Aged). Tissue sections were collected from the normal appearing white matter or postcentral parietal area (Brodmann 3, 1, 2, 5) and all samples were immediately flash frozen in liquid nitrogen (-196° C) after collection, shipped on dry ice, and stored at -80° C until use. All brains were designated healthy and non-CNS diseased controls with the exception of four samples obtained from epileptic patients (3 adult, 1 aged).

**Microglial isolation**

An enriched population of microglia was isolated from whole brain homogenates of mice as previously described (Fenn et al., 2012; Wynne et al., 2010). In brief, brains were homogenized in PBS through a 70 μm nylon cell strainer. Resulting homogenates were centrifuged and cell pellets were re-suspended in 70% isotonic Percoll. A discontinuous Percoll density gradient was layered, centrifuged, and enriched microglia were collected from the interphase between the 70% and 50% Percoll layers. Microglia were washed and re-suspended in PBS. Each brain extraction yielded approximately 3 x 10^5 viable cells. We have previously characterized these cells enriched (~85%) microglia (CD11b+/CD45low) (Henry et al., 2009).

**miRNA/mRNA isolation and real-time PCR**

miRNA and mRNA were isolated from enriched microglia using either a miRNA isolation kit (AM1561; Ambion, Austin, TX) or a PrepEase mRNA kit (USB, CA). From the coronal brain section, both miRNA and mRNA were isolated using the Tri Reagent
miRNA was reverse transcribed to cDNA using miRNA specific primers from Taqman® (Applied Biosystems; Foster, CA) for each miRNA of interest (e.g., miR-29a, miR-29b, etc). mRNA was reverse transcribed to cDNA using the high capacity protocol (Applied Biosystems; Foster, CA) according to manufacturer instructions.

Real-time (RT)-PCR was performed for both miRNA and mRNA using the Applied Biosystems (Foster, CA) Taqman® Gene Expression assay as previously described (Wohleb et al., 2011). Fluorescence was determined on an ABI PRISM 7300-sequence detection system (Applied Biosystems, CA). Data were analyzed using the comparative threshold cycle (Ct) method and results are expressed as the relative expression across both groups or fold difference from control. To reduce inter-test variability, relative expression and fold change for miRNA and mRNA expression was evaluated after each individual experiment and values were then combined.
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**Table 2.1.** The sample number, age, sex, description, and tissue section are listed for each sample used. The tissue consisted of eleven adult (14 – 55 years old with a mean age of 45 years old) and sixteen aged (58 – 91 years old with a mean age of 73 years old) brain samples. Abbreviations: BA = Brodmann’s Area; EP = Epileptic; HC = non-CNS disease control; NAWM = normal appearing white matter.
**Luciferase assay**

The 3’ untranslated region (3’UTR) segments containing the target sites for miR-29a and miR-29b from the murine IGF-1 and CX3CL1 gene were amplified from genomic DNA isolated from the brains of BALB/c mice (Table 2.2), and inserted into the PGL3 control vector (Promega) using the XBA1 site immediately downstream from the luciferase stop codon. HEK-293 cells were transfected with 800 ng firefly luciferase vector, 100 ng Renilla luciferase control vector, and 200 nM of precursor miR-29a, miR-29b, or scrambled oligonucleotides (negative control precursor; Ambion) using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol. Cells were lysed in Passive Lysis Buffer and assayed in duplicate using the Dual-Luciferase Reporter Assay System (Promega). Firefly luciferase activity was normalized to Renilla luciferase activity as measured by a Veritas microplate luminometer (Promega) and was then calculated relative to the scrambled control in each independent replicate.

**Primary cultures and miR Transfection**

Primary microglia cultures were established from neonatal mice (P0-P3) as previously described (Godbout et al., 2004). After 10-12 d in culture primary microglia were shaken at 180 rpm for 3.5 h and plated at 300,000 cells / well in a 12-well plate and used the following day for transfection.

Transfections were performed using the TransIT-TKO Transfection Reagent according to manufacturer’s protocol with minor modifications (Mirus Bio LLC, WI). In brief, primary microglia cells were maintained in 500 μL of 20% growth medium (DMEM supplemented with 20% FBS, sodium bicarbonate 3.7 g/L, 200 mM glutamine, 100 U/ml penicillin G, 100 Ag/mL streptomycin, 50 Ag/mL gentamicin) and medium was changed just prior to transfection. 100 μL of the transfection complexes (100 μL serum-free
medium + 2.5 μL TransIT-TKO Reagent + siRNA [50 nM final concentration]) was added to the appropriate wells. siRNAs included miR-29b and miR-542-5p (Thermo Fisher Scientific, MA). For controls, 100 μL of serum-free medium + 2.5 μL TransIT-TKO was added. Primary microglia were incubated with transfection complexes at 37° C / 5% CO2 for 24 h. After 24 h supernatant was aspirated and 500 μL of fresh growth medium was added.

**IGF-1 ELISA Assay**

Twenty-four h after transfection, primary microglia were treated with vehicle (0.1% BSA) or 5 ng/mL recombinant IL-4 (R&D Systems, MN). After 24 or 48 h supernatants were collected and stored at -80° C until use. IGF-1 ELISA was performed on undiluted supernatants according to the manufacture’s protocol (Abcam, MA). Detection limits were 50 pg/mL ± 5 pg/mL.

**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 with a one-way (i.e. Age) 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. Correlation analyses were performed by calculating the Pearson correlation coefficient (R) and determining p-value based on sample size \( t = R/(\sqrt{(1-R^2)/n-2}) \). 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. Statistics are represented as $F_{(\text{degrees of freedom, sample size}-1)} = f$-value, p-value.

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Table 2.2. cDNA from the brain sections of BALB/c mice was used to clone the UTR sequences for CX3CL1 and IGF-1. Sequences were placed into a vector downstream of the luciferase stop codon and transfected into HEK-293 cells for the luciferase assay. The forward and reverse sequences used for each UTR site investigated are shown.

**Results**

*Increased expression of miR-146a, miR-155, miR-29a, and miR-29b in the brain of aged mice*

miRNAs are important immunoregulatory factors (Baltimore et al., 2008; O'Connell et al., 2010) and aging is associated with an increase in brain inflammation (Fenn et al., 2012; Godbout et al., 2005b; Henry et al., 2009). Thus, we sought to determine the extent to which miRNA expression was altered in the brain with normal aging. Expression of miRNAs associated with modulating inflammation (i.e., miR-146a, miR-155, miR-124, miR-29a, miR-29b, and miR-29c) was determined in a 1 mm coronal brain section (~0.5 Bregma) from adult (3-4 mo) and aged (20 mo) BALB/c mice. This 1 mm section was selected because it contains cortex and striatum to provide a cortical
representation of alterations in miRNA or mRNA levels. Fig.2.1 shows that miR-146a (Fig.2.1A, $F_{(1,23)}=2.33$, $p=0.1$) and miR-155 (Fig.2.1B, $F_{(1,21)}=2.66$, $p=0.1$) tended to be increased in the brain with age, but miR-124 was unchanged (Fig.2.1C). Moreover, there were significant alterations in the miR-29 cluster in the brain with age. For instance, miR-29a was increased with age (Fig.2.1D, $F_{(1,31)}=22.97$, $p<0.0001$), miR-29b tended to be increased with age (Fig.2.1E, $F_{(1,34)}=2.71$, $p=0.1$) and miR-29c was reduced with age (Fig.2.1F, $F_{(1,21)}=7.78$, $p<0.02$). Overall, miRNAs known to modulate inflammatory responses (miR-146a, miR-155, miR-29a, and miR-29b) were increased in the brain of aged BALB/c mice.
Figure 2.1. Increased expression of miR-146a, miR-155, miR-29a, and miR-29b in the brain of aged mice. A 1 mm coronal brain section (-0.5 Bregma) was collected from adult (n=12-17) and aged (n=12-18) mice and levels of A) miR-146a, B) miR-155, C) miR-124 D) miR-29a, E) miR-29b, and F) miR-29c were determined. Samples represent three independent experiments and are presented as fold change compared to Adult. Horizontal bars represent the mean with each dot depicting a separate sample.

Increased expression of miR-155, miR-29a, and miR-29b in microglia of aged mice

Because microglia develop a more inflammatory and primed phenotype with age, these same miRNAs were determined in enriched CD11b cells (~85% microglia) that were collected by Percoll gradient separation from whole brain homogenates (Fenn et al., 2012). Fig.2.2A&B shows that both miR-29a (Fig.2.2A, F_{(1,20)}=8.51, p<0.009) and miR-
29b (Fig. 2.2B, $F_{(1,19)}=8.09$, $p<0.02$) were increased in the microglia of aged mice compared to adult controls. Fig. 2.2C shows the average relative expression of miR-146a, miR-155, miR-29c, and miR-124 in microglia of adult and aged mice. These data indicate that miR-155 tended to be increased in the microglia of aged mice ($F_{(1,6)}=3.06$, $p=0.1$), but that no other miRNA was altered in microglia of aged mice compared to adults. Taken together these data indicate that miR-29a, miR-29b, and miR-155 were increased in microglia of aged BALB/c mice.

![Figure 2.2. Increased expression of miR-155, miR-29a, and miR-29b in the microglia of aged mice.](image)

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<thead>
<tr>
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<th>Adult</th>
<th>Aged</th>
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<tr>
<td>miR-146a</td>
<td>0.90 ± 0.02</td>
<td>1.43 ± 0.67</td>
</tr>
<tr>
<td>miR-155</td>
<td>1.06 ± 0.26</td>
<td>4.78 ± 1.79*</td>
</tr>
<tr>
<td>miR-29c</td>
<td>1.15 ± 0.29</td>
<td>2.02 ± 0.68</td>
</tr>
<tr>
<td>miR-124</td>
<td>1.13 ± 0.11</td>
<td>1.27 ± 0.13</td>
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Peripheral injection with LPS increased miR-29b expression in the brain

Our previous studies show that a peripheral injection with lipopolysaccharide (LPS) resulted in microglial activation with increased IL-1β production in the brain after 4 h (Fenn et al., 2012; Henry et al., 2009). Our current results indicate that miR-29a and...
miR-29b were increased in the aged brain (Figs. 2.1&2.2), but the degree to which this related to increased inflammatory potential in the aged brain was unclear. Therefore, we next assessed the degree to which miR-29a and miR-29b were increased in the brain following a peripheral injection of LPS. Adult mice were injected i.p. with LPS (0.33 mg/kg), a 1 mm coronal section was collected after 4 h, and expression of IL-1β mRNA, miR-29a, and miR-29b were determined. As expected, LPS injection increased IL-1β mRNA expression in the brain (Fig. 2.3A, F(1,17)=61.49, p<0.0001). miR-29a was unaffected by LPS (Fig. 2.3B) and miR-29b was increased by LPS (Fig. 2.3C, F(1,13)=5.85, p<0.04). These results indicate that brain inflammation associated with a peripheral LPS injection corresponded with increased expression of miR-29b, but not miR-29a.

Figure 2.3. Peripheral injection with LPS increased miR-29b expression in the brain. BALB/c mice received an intraperitoneal (i.p.) injection of saline or LPS (0.33 mg/kg) (n=9). After 4 h a 1 mm coronal brain section (-0.5 Bregma) was collected and A) IL-1β mRNA expression, along with expression of B) miR-29a, and C) miR-29b was determined. Results represent two independent experiments and are presented as fold change from Saline. Means with * are significantly (p<0.0001) different from Saline controls.
IGF-1 and CX3CL1 were targets of miR29a/b mediated suppression

Because miR-29a and miR-29b were increased in the aged brain (Figs.2.1&2.2) we sought to identify targets that: 1) are regulated by miR-29a/b and 2) promote increased microglia reactivity when suppressed. Using the online TargetScan program (www.targetscan.org, release 6.2, MIT, Cambridge, MA) insulin-like growth factor (IGF)-1 and fractalkine ligand (CX3CL1) were identified as potential targets of miR-29a and miR-29b (Fig.2.4A). For example, TargetScan analysis of the possible targets for miR-29a and miR-29b showed that IGF-1 was a strongly projected target with a Total Context score below -0.3 and two conserved sites, while CX3CL1 was a projected target with a Total Context score of -0.09 and one conserved site. IGF-1 and CX3CL1 are relevant because both modulate the activation of microglia (Dodge et al., 2008; Lyons et al., 2009a) and are influenced by normal aging (Bachstetter et al., 2011; Deak and Sonntag, 2012; Wynne et al., 2010).

While TargetScan predicts the likelihood that a miRNA binds to the 3'UTR of a target gene, it is important to validate these predictions. To confirm that IGF-1 and CX3CL1 were targets of miR-29a and miR-29b, the 3'UTRs of murine IGF-1 or CX3CL1 were cloned downstream of the firefly luciferase gene. In these assays, if the transected miRNA binds the 3'UTR of the target gene then luciferase expression is suppressed. HEK-293 cells were co-transfected with miR-29a, miR-29b, or a negative control miRNA together with the firefly luciferase-IGF-1 or CX3CL1 3'UTR construct. Fig.2.4B shows that miR-29a and miR-29b repressed luciferase activity of the IGF-1 3'UTR more than 50% at two independent sites (p<0.005 for all). Luciferase activity of the constructs containing the CX3CL1 3'UTR was reduced by 30% by miR-29a (p=0.08), but was not reduced by miR-29b. These results indicate that miR-29a and miR-29b directly target the 3'UTR of IGF-1 and that miR-29a targets the 3'UTR of CX3CL1.
Next, mRNA expression of IGF-1 and CX3CL1 was determined in the brains of adult and aged mice. Corresponding with increased miR-29a and miR-29b (Figs.2.1 & 2.2), mRNA levels of IGF-1 (Fig.2.4C, F(1,34)=20.36, p<0.0001) and CX3CL1 (Fig.2.4D, F(1,35)=6.21, p<0.02) were significantly reduced with age. Taken together, these data indicate that the age-associated increase in brain levels of miR-29a and miR-29b coincided with the reduction of two confirmed targets, IGF-1 and CX3CL1.

<table>
<thead>
<tr>
<th>Projected targets of miR-29a/b</th>
<th>Target region</th>
<th>Total Context+ score</th>
<th>Conserved sites</th>
</tr>
</thead>
<tbody>
<tr>
<td>IGF-1</td>
<td>3' UTR</td>
<td>-0.37</td>
<td>2</td>
</tr>
<tr>
<td>CX3CL1</td>
<td>3' UTR</td>
<td>-0.09</td>
<td>1</td>
</tr>
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Figure 2.4. miR-29a and miR-29b suppressed the expression of IGF-1 and CX3CL1. A) TargetScan analysis of the possible targets for miR-29a and miR-29b showed that IGF-1 was a strongly projected target and CX3CL1 was a projected target. B) HEK-293 cells were transfected with a firefly luciferase vector and the 3'UTR segments containing the target sites for miR-29a and miR-29b for murine IGF-1 and CX3CL1 immediately downstream from the luciferase stop codon. Cells were co-transfected with miR-29a or miR-29b. The ratio of firefly to renilla luciferase activity was normalized to the control miRNA within each experimental replicate. Values represent the normalized luciferase activity of the constructs. C) A 1 mm coronal brain section was collected from adult (n=16-18) and aged (n=18) BALB/c mice and mRNA levels of IGF-1 and D) CX3CL1 were determined. Results represent two (B) or three (C,D) independent experiments and are presented as fold change compared to Control or Adult. Bars represent the mean ± SEM. Means with * are significantly (p<0.05) different and means with + tend (p=0.08) to be different from Control.
IGF-1 was decreased in the brain after peripheral injection with LPS and suppressed by miR29b in microglia

We show that LPS injection increased miR-29b expression in the brain, but had no effect on miR-29a (Fig. 2.3B&C). In addition, IGF-1 was a confirmed target of miR-29b as transfection with miR-29b repressed luciferase activity of the IGF-1 3'UTR (Fig. 2.4B). Therefore, to continue to investigate the relationship between increased miR-29a/b and reduced expression of its predicted targets, mRNA expression of IGF-1 and CX3CL1 in the brain of mice injected with saline or LPS was determined. Fig. 2.5A shows that IGF-1 mRNA was significantly reduced in the brain of mice injected with LPS compared to saline controls (p<0.02). CX3CL1 mRNA expression, however, remained unchanged after LPS injection (Fig. 2.5B). These data are consistent with previous data that LPS resulted in increased expression of miR-29b, but not miR-29a (Fig. 2.3C), and that IGF-1, but not CX3CL1, is a target of miR-29b (Fig. 2.4B). Overall, these data indicate that the LPS-induced miR-29b expression in the brain corresponded with reduced mRNA expression of IGF-1.

Next, the ability of miR-29b to suppress IGF-1 expression specifically in microglia was assessed. In these experiments, primary microglia were untransfected or transfected with either miR-29b or the non-specific miR-542-5p. Microglia were stimulated with interleukin (IL)-4, an inducer of IGF-1 (Zhao et al., 2006), and IGF-1 protein concentration was determined 24 and 48 h later. Fig. 2.5C&D show that IL-4 increased IGF-1 protein secretion by microglia 24 h and 48 h later (main effect of IL-4: F(1,15)=14.52, p<0.003). Transfection with miR-29b reduced IL-4 induced IGF-1 protein levels in primary microglia by 15% at 24 h and by 30% at 48 h (p=0.08). Thus, by 48 h levels of IGF-1 returned to baseline in the microglia transected with miR-29b. The non-specific miR control (542-5p) had no effect on IL-4-induced IGF-1 levels in microglia at
either time point. Taken together, these data indicate that miR-29b directly suppresses IGF-1 production by microglia, as predicted by target scan analysis and luciferase assay.

Figure 2.5. IGF-1 was decreased in the brain after peripheral injection with LPS and suppressed by miR-29b in microglia. BALB/c mice received an intraperitoneal (i.p.) injection of saline or LPS (n=9). After 4 h a 1 mm coronal brain section (-0.5 Bregma) was collected and A) IGF-1 and B) CX3CL1 mRNA expression was determined. In a separate set of studies, primary microglia cultures were established and then transfected with miR-29b or a control miR (n=2-3). Protein levels of IGF-1 were determined C) 24 or D) 48 h after IL-4 stimulation. Results represent two independent experiments and are presented as fold change compared to Control or Adult. Bars represent the mean ± SEM. Means with * are significantly (p<0.05) different and means with + tend (p=0.1) to be different from Control. Means with # tend (p=0.08) to be different from Control-IL-4.
miR-29a and miR-29b were increased in the brain of aged humans

To determine the degree to which increased miR-29a and miR-29b levels were relevant to human brain aging, cortical brain tissue was obtained from brain bank donor adults and older individuals (Harvard Brain Tissue Resource Center and the Human Brain and Spinal Fluid Resource Center). The tissue consisted of eleven adult (14 – 55 years old with a mean age of 45 ± 4.2 years old) and sixteen aged (58 – 91 years old with a mean age of 73 ± 2.2 years old) brain samples (Table 2.1).

Expression of miR-29a tended to be increased in the brain of older individuals compared to middle aged controls (Fig.2.6A, $F_{(1,24)}=2.87$, $p=0.1$), and miR-29a expression was positively correlated with advancing age (Fig.2.6B, $R=0.41$, $p=0.07$). Similarly, miR-29b expression was significantly increased in the brains of older individuals compared to the middle aged controls (Fig.2.6C, $F_{(1,24)}=5.28$, $p<0.04$), and miR29b expression was positively correlated with increasing age (Fig.2.6D, $R=0.45$, $p<0.04$). Taken together, these data indicate that miR-29a and miR-29b are increased in the brains of older individuals and increase as a function of advancing age.
Figure 2.6. miR-29a and miR-29b were increased in the brains of aged humans. Postmortem brain tissue was acquired for both adult (n=11) and aged (n=16) age groups and A) miR-29a and C) miR-29b expression was determined. In addition, the correlation between the individual's age and B) miR-29a and D) miR-29b expression are shown. Bars represent the mean ± SEM. Relative expression is compared to the average comparative Ct for all samples. Means with * are significantly (p<0.04) different and means with + tend (p<0.08) different from Adult controls.

The age-associated increase in brain levels of miR-29a and miR-29b was negatively correlated with reduced expression of CX3CL1 and IGF-1

Using the same post-mortem brain tissue as above, mRNA expression of IL-1β, IGF-1, and CX3CL1 was determined. Fig.2.7A shows that IL-1β mRNA levels appeared to increase in the brain with age, but this increase in older individuals was not statistically different from middle aged controls (Fig.2.7A). Consistent with the increase in miR-29a and miR-29b (Fig.2.7), there was a robust reduction in the mRNA levels of
IGF-1 (Fig. 2.7B, $F_{(1,18)}=14.17$, $p<0.002$) and CX3CL1 (Fig. 2.7C, $F_{(1,20)}=5.41$, $p<0.04$) in the brains of older individuals compared to controls. To begin to address the relationship between increased miR-29a and miR-29b and reduced IGF-1 and CX3CL1 expression, a correlation analysis was performed. Fig. 2.7D shows that increased miR-29a expression negatively correlated with reduced expression of IGF-1 ($R=-0.42$, $p<0.05$). Increased miR-29a, however, was not correlated with a reduction in CX3CL1 expression (Fig. 2.7E). Similar to miR-29a, Fig. 2.7F shows that increased miR-29b expression negatively correlated with reduced IGF-1 mRNA expression ($R=-0.74$, $p<0.0002$). In contrast to miR-29a, increased miR-29b expression also negatively correlated with reduced CX3CL1 mRNA expression (Fig. 2.7G, $R=-0.64$, $p<0.002$). Taken together, age-associated increases in miR-29b correlated with reduced expression of both IGF-1 and CX3CL1 in the brains of older individuals.
Figure 2.7. The aged-associated increase in brain levels of miR-29a and miR-29b was negatively correlated with reduced expression of CX3CL1 and IGF-1. Postmortem brain tissue was acquired for both adult (n=9) and aged (n=13) age groups. From the human brain tissue mRNA expression of A) IL-1β, B) IGF-1, and C) CX3CL1 was determined. In addition, a correlation plot was created for IGF-1 and CX3CL1 compared to miR-29a or miR-29b levels determined in Fig.5. Plots depict expression levels of miR-29a versus D) IGF-1 and E) CX3CL1 in the human brain tissue. In addition, levels of miR-29b versus F) IGF-1 and G) CX3CL1 are shown. Bars represent the mean ± SEM. Relative expression is compared to the average comparative Ct for all samples. Means with * are significantly (p<0.04) different from Adult controls.
Discussion

We have previously reported that microglia from aged mice have a primed (MHCII⁺) phenotype (Henry et al., 2009; Wynne et al., 2010). This is important because a primed microglial profile leads to an exaggerated inflammatory response following a central or peripheral immune challenge (Fenn et al., 2012; Henry et al., 2009; Huang et al., 2008; Wynne et al., 2010). The mechanisms underlying this age-associated increase in brain inflammation may be related to deficient regulation of microglia by anti-inflammatory mediators, including IGF-1 and CX₃CL1. Here we present novel evidence that the age-associated reduction in IGF-1 and CX₃CL1 expression in the brain corresponded with increased expression of the miR-29a/b cluster. Moreover, the miR-29a/b cluster was found to target IGF-1 and CX₃CL1 by luciferase assay and transfection studies confirmed that miR-29b attenuated IGF-1 protein expression in primary microglia. Parallel to the results in the brain of aged mice, miR-29a and miR-29b were increased in the brains of older humans and these increases were correlated with reductions in IGF-1 and CX₃CL1 mRNA expression. Collectively, the results of this study indicate that the miR-29a/b cluster is increased in the brain with age corresponding in the reduced expression of two key mediators of microglial regulation.

One important finding of this study was that the expression of several miRNAs was increased in the brain of aged mice compared to adults. While there are numerous identified miRNAs, we focused on immunomodulatory miRNAs including miR-146a, miR-155, and the miR-29 cluster (Steiner et al., 2011a; Taganov et al., 2006; Tili et al., 2007). For instance, our data indicate that miR-146a tended to be increased in the brain of aged BALB/c mice compared to adults. This is consistent with a previous report using aged C57BL/6J mice (Li et al., 2011). In addition, increased miR-146a levels support the notion of a more inflammatory CNS environment with age as miR-146a is increased by
inflammatory-induced NF-κB (Labbaye and Testa, 2012). Indeed, several studies in aged rodents indicate that NF-κB nuclear binding and subsequent gene expression are increased in the aged brain (Kim et al., 2000; Korhonen et al., 1997; Ye and Johnson, 2001b). Similar to the results with miR-146a, miR-155 was also increased in the brain of aged mice. Higher miR-155 expression was also detected specifically in microglia. Previous work denotes that the expression of miR-155 is increased by c-Jun N-terminal kinase (JNK) (O'Connell et al., 2007) and functions to suppress genes involved in toll like receptor signaling (e.g., fas associated protein with death domain and IKKε), but also to increase the production of TNF-α (Sonkoly et al., 2008). Thus, higher miR-155 in microglia is relevant as microglia from aged mice show higher production of TNF-α (Njie et al., 2012). Overall, an increase in both miR-146a and miR-155 is indicative of an increased inflammatory state within the aged brain.

Our results indicate that miR-29a and miR-29b are increased in the brain with age. A previous study showed that miR-29 expression gradually increased in the human brain from birth until puberty, but no further elevation was noted with age (Somel et al., 2010). Nonetheless, in animal models, miR-29a and miR-29b were increased in the liver and lung of aged mice and in the Zmpste24-null mouse model of accelerating aging (Ugalde et al., 2011). Moreover, miR-29b was decreased in these same tissues in a rodent model of delayed aging (Bates et al., 2010). Here, our data support that miR-29a and miR-29b are increased in the brain as a function of age. It is important to highlight that this age-associated increase in miR-29a and miR-29b expression was reflected in microglia. Microglia are the primary immune cell within the CNS and, thus, increases in immunoregulatory miRNAs likely contribute to microglial dysregulation and an enhanced inflammatory profile in the aged brain. It is also important to mention that miR-29c is part of the miR-29 cluster, but was reduced in the brain of aged mice. This is similar to our
previous report showing that the miR-29a/b cluster was elevated in memory T-cells of patients with MS, but expression of the miR-29b/c cluster was unchanged (Smith et al., 2012). Differential regulation of the miR-29 isoforms is related to the finding that miR-29a/b cluster is on chromosome 7 and the miR-29b/c cluster is on chromosome 1.

A key finding of this study was that increased miR-29a and miR-29b in the rodent brain was paralleled in the cortical brain tissue of human subjects. Therefore, these data support the relevance of using rodent models to study brain aging. In addition, brain tissue used was from adult (mean 45 yrs) and aged (mean 77 yrs) individuals who did not have a neurodegenerative disease. This is relevant to point out because a previous study indicates that miR-29a/b were decreased in the brain of aged patients with Alzheimer’s disease (Hebert et al., 2008). miR-29 was found to target BACE1 expression in vitro and indeed, patients with sporadic Alzheimer’s disease that exhibited reduced levels of miR-29a and miR-29b had abnormally high BACE1 expression (Hebert et al., 2008). In the current study, however, miR-29a and miR-29b were elevated in the brain of older individuals compared to adults, and increased expression of miR-29a and miR-29b was positively correlated with advancing age. Reductions in miR-29a/b may, therefore, indicate a dysfunctional response to increased neuroinflammatory load and a pre-disposition to Alzheimer’s disease (Blasko et al., 2004), whereas increased miR-29a/b would suggest a normal response to increased inflammation.

Multiple studies indicate brain aging is associated with increased DNA damage, oxidative stress, and inflammation (e.g., elevated NF-κB, IL-1β, TNF-α). Several of these factors have been reported to increase miR-29 expression in the periphery and in vitro (Smith et al., 2012; Ugalde et al., 2011). Although the function of the miR-29a/b cluster is to provide negative feedback and reduce inflammation (Ma et al., 2011; Smith et al., 2012; Steiner et al., 2011a), this miR-mediated response is less effective in the aged
brain. We have demonstrated a similar relationship with miR-29a/b expression and its target IFNγ in MS patients in which T-cells from MS patients had higher levels of IFNγ concordant with higher expression of miR-29b (Smith et al., 2012). A possible explanation for this relationship in the aged brain is that miR-29-dependent repression of key immuno-regulatory genes, including IGF-1 and CX3CL1, may potentiate microglial priming and contribute to increased inflammatory load. A similar relationship also exists between inflammatory induced IL-10 in the aged brain. Indeed, inflammatory activation of aged microglia promotes exaggerated expression of inflammatory (e.g., IL-1β) and anti-inflammatory (e.g., IL-10) cytokines (Henry et al., 2009). Despite this exaggerated increase in anti-inflammatory mediators, heightened inflammation persists. The cause for this is unknown, but contributes to the idea that negative regulatory systems within the aged brain are impaired on multiple levels.

Our results showing age-associated reductions in brain levels of IGF-1 and CX3CL1 mRNA are consistent with previous studies investigating IGF-1 (Deak and Sonntag, 2012; Llorens-Martin et al., 2009; O’Connor et al., 2008; Sonntag et al., 2005b) and CX3CL1 with age (Lyons et al., 2009a; Wynne et al., 2010). Here we extend these previous findings to show that the reductions of IGF-1 and CX3CL1 were significantly correlated with an increase in the miR-29a/b cluster in microglia. These correlations are consistent with the evidence that both of these genes are confirmed targets of the miR-29a/b cluster by luciferase assay. Moreover, transfection with miR-29b reduced IGF-1 protein secretion specifically in microglia. Of note, even if miR-29a/b was only increased within microglia, it could still influence CX3CL1 expression by neurons through the transfer of miRNAs in exosomes (Valadi et al., 2007). Nonetheless, one discrepancy between the luciferase studies and human brain correlations regarding CX3CL1 was that luciferase assay confirmed CX3CL1 as a target of miR-29a suppression, but CX3CL1
was not negatively correlated with miR-29a in the brain of aged humans. This could be explained by the actions of miR-29a on CX3CL1, which may be to inhibit translation rather than cause mRNA degradation (Valencia-Sanchez et al., 2006). Another unexpected finding was that although miR-29b did not repress CX3CL1 expression in the luciferase experiments, miR-29b negatively correlated with CX3CL1 expression in human cortical tissue. A potential explanation for these results is that murine sequences, rather than human sequences, were used for the target UTR sites and thus target sites may differ in humans. Alternatively, miR-29b-promoted reductions in IGF-1 could indirectly contribute to CX3CL1 reductions. Localized IGF-1 release by microglia prevents neuronal apoptosis (Galli et al., 1995) and sustains neurogenesis (Choi et al., 2008b; Lichtenwalner et al., 2001), primarily under conditions of inflammation (Ekdahl et al., 2009). Thus, reduced neuronal support by IGF-1 may contribute to reduced CX3CL1 levels as CX3CL1 is primarily released by healthy neurons in the brain (Cardona et al., 2006). Indeed, IGF-1 significantly and negatively correlated with CX3CL1 in the current study (data not shown).

Importantly, the data obtained using human cortical tissue indicates that the increase in miR-29b with age correlated with the reduction of both IGF-1 and CX3CL1. The relevance of miR-29b in the targeting of IGF-1 and CX3CL1 in the aged is consistent with its induction by inflammatory stimuli (Smith et al., 2012). For example, our data indicate that a peripheral challenge with LPS increased IL-1β and miR-29b expression in the brain, but did not affect the expression miR-29a. Moreover, there was a 2-fold increase in IL-1β expression in the brain of the older individuals compared to the middle aged controls. Therefore, we interpret these data to indicate that age-associated increases in pro-inflammatory cytokines results in increased expression of miR-29b in rodents and humans. Of note, miR-29a was not increased in the brain after a peripheral
injection of LPS, but was increased in the brain of aged mice and humans. One possibility for the age-related increase in miR-29a is increased DNA damage in the aged brain. Indeed, miR-29a is preferentially induced by DNA damage in a p53-dependent manner independent of an inflammatory response (Ugalde et al., 2011). Taken together, these findings support our hypothesis that the reduction of CX3CL1 and IGF-1 result from an increase in miR-29 dependent regulation.

In conclusion, miRNA-mediated immune regulation in the brain is altered as a function of normal aging. While increased miR-29a/b expression with age may represent a homeostatic negative feedback response to increased inflammatory potential within the aged brain, a consequence is the suppression of IGF-1 and CX3CL1. Down-regulation of IGF-1 and CX3CL1 contribute to an increased inflammatory profile of microglia in the aged brain. These findings are important because a heightened inflammatory profile in aged microglia may also lead to other impairments including reduced neuronal plasticity, cognitive impairment, and hyperactivity of microglia following central or peripheral immune challenges. Therefore, understanding how miRNAs regulate microglial activation is critical in identifying novel therapies to reduce microglial priming with age.
Chapter 3: Lipopolysaccharide-induced interleukin (IL)-4 receptor-α expression and corresponding sensitivity to the M2 promoting effects of IL-4 are impaired in microglia of aged mice

Abstract

In several models of aging, microglia become more inflammatory and reactive to immune challenges. For example, peripheral LPS injection causes exaggerated microglial activation associated with prolonged sickness and depressive-like behavior in aged BALB/c mice. Therefore, the purpose of this study was to determine the extent to which age-related amplified microglial activation was associated with reduced sensitivity to the anti-inflammatory and M2 promoting cytokines interleukin (IL)-10 and IL-4. In initial studies with adult mice, LPS induced a time-dependent increase in M1 and M2 mRNA profiles in microglia. Furthermore, peripheral LPS injection markedly increased surface expression of IL-4 receptor-alpha (IL-4Rα), but not IL-10 receptor-1 (IL-10R1) on microglia. In BV-2 cells, IL-4, but not IL-10, re-directed LPS-activated microglia towards an M2 phenotype. Based on these findings, comparisons of M1 and M2 activation profiles, induction of IL-4Rα, and sensitivity to IL-4 were determined in microglia from adult (3-4 mo) and aged (18-22 mo) mice. In aged microglia, LPS promoted an exaggerated and prolonged M1 and M2 profile compared to adults. Moreover, IL-4Rα protein was not increased on aged microglia following LPS injection. To determine the consequence of impaired IL-4Rα upregulation, adult and aged mice were injected with LPS and activated microglia were then isolated and treated ex vivo.
with IL-4. While ex vivo IL-4 induced an M2 profile in activated microglia from adult mice, activated microglia from aged mice retained a prominent M1 profile. These data indicate that activated microglia from aged mice are less sensitive to the anti-inflammatory and M2-promoting effects of IL-4.

**Introduction**

Within the central nervous system (CNS) microglia are responsible for the induction of an innate immune response by receiving and propagating inflammatory signals (Nguyen et al., 2002). Even in the absence of inflammatory stimuli, microglia are actively surveying their local microenvironment (Nimmerjahn et al., 2005). Once activated by an immune stimulus, microglia perform several macrophage-like functions including phagocytosis, inflammatory and anti-inflammatory cytokine production, and antigen presentation (Garden and Moller, 2006). This classical activation (M1) profile is transient with microglia returning to a surveying state as the immune stimulus is resolved. Key to this transition is regulation by several anti-inflammatory mediators (Biber et al., 2007) including neuronal factors, hormones, and cytokines that attenuate microglial activation and promote anti-inflammatory or repair (M2) profiles in microglia (Mantovani et al., 2004; Mosser and Edwards, 2008).

In rodent models of normal and non-neurodegenerative aging, there is an increase in “primed or reactive” microglia that have increased expression of a number of M1 and inflammatory markers including CD86 (Downer et al., 2010), CD68 (Wong et al., 2005), MHC II (Frank et al., 2006; Godbout et al., 2005b; Henry et al., 2009), and toll-like receptors (Letiembre et al., 2007). A consequence of a more inflammatory microglial profile is an exaggerated inflammatory response following peripheral immune activation (Godbout et al., 2005b; Perry et al., 2003). In support of this notion, central (Abraham et
al., 2008; Huang et al., 2008) or peripheral innate immune challenges (Chen et al., 2008; Godbout et al., 2005a; Henry et al., 2009; Wynne et al., 2010) lead to amplified and prolonged neuroinflammation (oxidative stress and cytokines) mediated, in part, by a hyperactive MHC II⁺ microglial population (Henry et al., 2009). An exaggerated M1 microglial response in aged mice is relevant because it is coupled with a myriad of complications including cognitive impairment (Barrientos et al., 2009a; Barrientos et al., 2006; Chen et al., 2008), exaggerated and prolonged sickness behavior (Abraham et al., 2008; Barrientos et al., 2009a; Godbout et al., 2005a; Huang et al., 2008), and protracted depressive-like behavior (Godbout et al., 2008) following an innate immune challenge (for reviews see (Dantzer et al., 2008; Godbout and Johnson, 2009; Jurgens and Johnson, 2012)). This is paralleled in clinical studies in which elderly patients with peripheral infections or other illnesses have an increased frequency of concomitant neurobehavioral complications including delirium (Lipowski, 1983; Mulsant et al., 1999) and depression (Alexopoulos, 2005; Godbout and Johnson, 2009; Koenig et al., 1988; Yirmiya et al., 2000) compared to younger adults with the same peripheral insults.

The reason aged mice have a reduced capacity to resolve amplified microglial activation after an immune challenge is unknown, but may be related to a reduced sensitivity to anti-inflammatory feedback by neuronal regulators (CD200, CX₃CL1) (Lyons et al., 2007a; Wynne et al., 2010) and anti-inflammatory cytokines. Previous studies demonstrate that IL-10 and IL-4, two key anti-inflammatory and M2-promoting cytokines (Mantovani et al., 2004; Strle et al., 2001) are reduced within the brain of older rodents (Nolan et al., 2005; Szczepanik et al., 2001; Ye and Johnson, 2001a). In the context of M2 activation profiles, IL-10 promotes an M2c (classical deactivation) profile and IL-4 promotes an M2a (alternative activation) profile in macrophages. Both M2a and M2c phenotypes reduce M1 cytokines and other inflammatory mediators (Mantovani et
al., 2004; Mosser and Edwards, 2008). An M2b activation profile has also been shown with expression of both M1 and M2c markers. The degree to which these activation profiles are conserved in microglia is less understood. In our work, peripheral injection of LPS amplified mRNA and intracellular protein expression of both IL-1β and IL-10 in microglia from aged mice (Henry et al., 2009). Despite exaggerated IL-10 production by microglia from aged mice, neuroinflammation persisted and corresponded with prolonged sickness and depressive-like behaviors (Godbout et al., 2005b; Godbout et al., 2008). In addition, other reports indicate that decreased IL-4 in the brain of aged rats was associated with reduced long term potentiation (LTP) (Maher et al., 2005), impaired neurogenesis in the hippocampus (Ziv et al., 2006), and increased brain inflammation (Maher et al., 2005; Nolan et al., 2005).

The purpose of this study was to determine the degree to which activated microglia from adult and aged mice were sensitive to anti-inflammatory effects of IL-10 and IL-4. Initial studies were completed using adult mice, BV-2, and primary microglia to determine microglial M1 and M2 profiles, expression of the receptors for IL-4 (IL-4Rα) and IL-10 (IL-10R1), and sensitivity to IL-10 and IL-4 following LPS. Collectively, these initial experiments demonstrated that LPS-activated microglia shifted towards an M2b phenotype, markedly upregulated IL-4Rα protein expression, and were redirected towards a less inflammatory M2a profile following IL-4 post-treatment. These studies also indicated that IL-10R1 was not induced following LPS-associated activation and IL-10 had little effect in redirecting activated microglia towards a less inflammatory phenotype. In age comparisons, LPS injection induced an exaggerated M2b phenotype in microglia from aged mice compared to adults, but IL-4Rα surface expression was not increased. Moreover, when LPS-activated microglia were isolated and treated ex vivo with IL-4, only microglia from adult mice successfully transitioned from an M1 towards an
M2 profile. Thus, failure to increase IL-4Rα surface expression on aged microglia was associated with a reduced sensitivity to the M2 promoting effects of IL-4.

Materials and Methods

Animals

Adult (3-4 month-old) male BALB/c mice were obtained from a 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. For age comparisons, male BALB/c mice (18-22 mo) were purchased from the National Institute on Aging specific-pathogen-free colony (maintained at Charles River Laboratories, Inc., MA). The median lifespan for BALB/c mice is approximately 26 months (Morley and Trainor, 2001). Aged mice were acclimated to the facilities for one week prior to experimentation. To investigate changes that occur from adulthood to what is considered aged, 3-4 month-old (adult) and 18-22 month-old (aged) male BALB/c mice were used. Upon arrival, mice were individually housed as described above. 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.

Experimental Protocols

In the first experiment adult (3-4 mo) male BALB/c mice were injected intraperitoneally (ip) with saline or *Escherichia coli* lipopolysaccharide (LPS) (0.33 mg/kg; serotype 0127:B8, Sigma, St. Louis, MO) and euthanized 4 h or 24 h later by CO₂ asphyxiation (n=5). This 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., 2005a; Henry et al., 2008). In a related study, adult (3-4 mo) male BALB/c mice were injected ip with vehicle or minocycline (50 mg/kg, Sigma, St. Louis, MO) for 3 consecutive days (Henry et al., 2008). Twelve hours following the last injection mice were injected ip with saline or LPS and were euthanized 4 h later (n=9). In both sets of experiments, brains were homogenized and microglia were isolated using a discontinuous Percoll density gradient. RNA isolation from enriched microglia was performed and M1, M2a, and M2c gene expression was determined.

In the second experiment adult (3-4 mo) male BALB/c mice were injected ip with saline or LPS (0.33 mg/kg). Brains were homogenized and microglia were isolated using a discontinuous Percoll density gradient either 4 h or 24 h later. CD11b, CD45, IL-10R1 and IL-4Rα were determined on enriched microglia by flow cytometry (n=6).

In the third experiment the BV-2 and primary microglia were used. Microglia were treated with either saline or LPS (10 ng/mL) for 1 h. Next, cells were incubated for an additional 3 h with the appropriate vehicle or recombinant cytokine: 10 ng/ml of IL-10 (n=9), or 20 ng/ml of IL-4 (n=6) (R&D Systems, MN). Concentrations of these cytokines were selected based on previous reports using IL-10 (Frei et al., 1994; Sheng et al., 1995) and IL-4 (Chao et al., 1993; Haque et al., 1998) in macrophage and microglial cultures. After 3 h cell lysates were collected. RNA was isolated and M1, M2a, and M2c gene expression was determined by real-time PCR (RT-PCR). Results represent two independent experiments.

In the fourth experiment, adult (3-4 mo) or aged (18-22 mo) male BALB/c mice were injected ip with saline or LPS (0.33 mg/kg) and euthanized 4 h or 24 h later by CO2 asphyxiation. The brain was collected and a 1 mm coronal brain section (+0.38 mm from
Bregma) (Paxinos and Franklin, 2004) was taken using a rodent brain matrix (ASI instruments, Warren, MI). Brain sections were used for analysis of mRNA levels of IL-4 (n=5). The remainder of the brain was homogenized and microglia were isolated using a discontinuous Percoll density gradient. Half of the microglia from each sample were used for RNA isolation and the other half for flow cytometry analysis. RNA was isolated and M1, M2a, and M2c gene expression was determined by RT-PCR (n=6). CD11b, CD45, and IL-4Rα surface expression was determined by flow cytometry (n=4).

In the fifth experiment, adult (3-4 mo) and aged (18-22 mo) male BALB/c mice were injected ip with saline or LPS (0.33 mg/kg). After 4 h brains were homogenized and microglia were isolated using a discontinuous Percoll density gradient and plated on poly-L-lysine coated plates for 1 h. After 1 h, enriched microglia were treated with vehicle or IL-4 (20 ng/ml) for an additional 3 h. After 3 h, RNA was isolated and M1 (IL-1β, iNOS) and M2a (Arg) gene expression was determined by RT-PCR.

**BV-2 cell culture**

BV-2 cells were cultured in growth medium (DMEM (Bio-Whittaker, Walkersville, MD) supplemented with 10% FBS (Hyclone, Logan, UT), 3.7 g/L sodium bicarbonate, 200 mM glutamine, 100 U/ml penicillin G, 100 μg/ml streptomycin, 0.25 μg/ml fungizone) as previously described (Wynne et al., 2010). Cultures were maintained and incubated at 37°C with 95% humidity and 5% CO₂ and growth medium was replenished every third day until confluence. Prior to experimentation cells were plated at 1x10⁵ cells per well in 24-well plates and allowed to adhere for 20 h. Immediately before treatment, cells were washed twice with serum-free DMEM medium and supplemented with warm serum-free DMEM medium containing experimental treatments. Following experimental treatments cells were homogenized and RNA or protein was isolated.
**Primary microglia culture**

Microglia cultures were established from neonatal mice as previously described (Godbout, 2004). In brief, whole brains were aseptically removed and mechanically dissociated after a 15 min trypsinization (0.25% trypsin) and passed through a 100 mm nylon mesh, washed twice in D-HBSS, and plated on poly-L-lysine coated 162 cm² culture flasks in growth medium (DMEM supplemented with 20% FBS, 3.7 g/L sodium bicarbonate, 200 mM glutamine, 100 U/ml penicillin G, 100 μg/ml streptomycin, 0.25 μg/ml fungizone, 50 μg/ml gentamicin). Mixed glia cultures were maintained at 37°C with 95% humidity and 5% CO₂ and growth medium was replenished every third day until confluence. Mixed glia cultures were shaken at 120 rev/min and 37°C for 3.5 h to harvest microglia from the confluent cell layer. Cells were collected, counted by trypan blue staining, and plated at a density of 1 x 10⁵ cells per 500 μl on poly-L-lysine coated 24-well plates. After 48 h, microglia were washed twice with serum free DMEM medium (growth medium without FBS) and supplemented with warm serum free DMEM medium containing experimental treatments.

**Microglial isolation**

Microglia were isolated from whole brain homogenates as described previously (Henry et al., 2009; Wynne et al., 2010). In brief, brains were homogenized in 1x phosphate buffered saline (PBS, pH 7.4) by passing through a 70 μm nylon cell strainer. Resulting homogenates were centrifuged at 600 x g for 6 min. Supernatants were removed and cell pellets were re-suspended in 70% isotonic Percoll at room temperature. A discontinuous Percoll density gradient was layered as follows: 70%, 50%, 35%, and 0% isotonic Percoll. The gradient was centrifuged for 20 minutes at 2000 x g and microglia were collected from the interface between the 70% and 50% Percoll layers.
Microglia were washed and re-suspended in sterile filtered PBS or FACS buffer. Each brain extraction yielded approximately $3 \times 10^5$ viable cells. We have previously characterized these cells as approximately 85% CD11b⁺/CD45low microglia (Henry et al., 2009). Based on this previous characterization, cells isolated by Percoll density separation will be referred to as “enriched microglia”.

*Ex vivo microglia cultures*

Enriched microglia were washed with PBS and counted by hemocytometer. Cells from each animal were then plated at $1 \times 10^5$ cells/well into two wells of a poly-L-lysine coated 24-well plate in serum-free DMEM medium. Cells were incubated at 37°C with 95% humidity and 5% CO₂ and allowed to adhere for 1 h. After 1 h cells were treated with vehicle or IL-4 (20 ng/mL) and incubated at 37°C with 95% humidity and 5% CO₂ for 3 h. After 3 h cells were homogenized and RNA was isolated.

*RNA isolation and RT-PCR*

RNA was isolated from a coronal brain slice, BV-2 cells, primary microglia, or enriched microglia. For the coronal brain slice, BV-2 cells, and primary microglia total RNA was isolated using the Tri-Reagent protocol (Sigma, MO) and subjected to the DNA-free™ RNA clean up procedure (Ambion, TX). For enriched microglia, RNA was isolated using the RNeasy plus mini kit (Qiagen, CA) or the PrepEase kit (USB, OH). In all RNA isolation procedures, RNA concentration was determined by spectrophotometry (Eppendorf, NY) and RNA was reverse transcribed to cDNA.

Real time PCR (RT-PCR) was performed using the Applied Biosystems (Foster, CA) Taqman® Gene Expression assay as previously described (Godbout et al., 2005b). In brief, cDNA was amplified by RT-PCR where a target cDNA 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, CA). Data were analyzed using the comparative threshold cycle (Ct) method and results are expressed as fold difference from saline controls (Henry et al., 2009). In the microglia ex vivo cultures established from the brains of adult or aged mice, fold change results are expressed as fold change from the age-matched saline controls.

Flow cytometry

Enriched microglia were assayed for surface antigens by flow cytometry as previously described (Henry et al., 2009). In brief, Fc receptors were blocked with anti-CD16/CD32 antibody. Next, enriched microglia were split for staining using two separate panels of antibodies. Cells for Panel 1 were incubated with rat anti-mouse antibodies (eBioscience, CA): CD11b-APC, CD45-PerCP-Cy5.5, and IL-10R1-PE. Cells for Panel 2 were incubated with rat anti-mouse antibodies (eBioscience, CA): CD11b-APC, CD45-PerCP-Cy5.5, and IL-4Rα-PE. Expression of these surface receptors was determined using a Becton-Dickinson FACSCaliber four color Cytometer. Twenty thousand events were recorded and microglia were identified by CD11b+/CD45low expression (Wohleb et al., 2011). For each antibody, gating was determined based on appropriate negative isotype stained controls. In age comparisons of IL-4Rα, separate isotypes were used for adult and aged mice to control for the increased non-specific staining detected in microglia from aged mice. Flow data were analyzed using FlowJo software (Tree Star, San Carlos, CA).
**Statistical Analysis**

To ensure a normal distribution, data were subjected to the Shapiro-Wilk test using Statistical Analysis Systems (SAS) software (Cary, NC). To determine significant main effects and interactions between main factors, data were analyzed using one- (i.e., Age, Pretreatment, Treatment), or two-way (i.e., Age x Pretreatment, Age x Treatment, 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 or individual student’s $t$-tests. 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**

*Peripheral LPS injection promoted an M1 and M2c mRNA profile in enriched microglia*

We have previously reported that peripheral LPS injection causes amplified and prolonged microglial activation (Henry et al., 2009; Wynne et al., 2010) in aged BALB/c mice that corresponds with protracted sickness and depressive-like behaviors (Godbout et al., 2005b; Godbout et al., 2008). We hypothesize that exaggerated microglial activation in aged mice is related to a reduced sensitivity to anti-inflammatory cytokines that impair the transition from an M1 phenotype to an M2 phenotype. To begin to address this hypothesis, adult mice were used in an initial set of studies to determine the expression of microglial genes associated with classical activation (M1), alternative activation (M2a), and classic deactivation (M2c) (Mantovani et al., 2004) at two time-points following peripheral LPS injection. These time-points correspond to the peak of neuroinflammation (i.e., 4 h) (Wynne et al., 2010) and the resolution of the sickness
response (i.e., 24 h) (Godbout et al., 2005b). Fig. 3.1A shows the relative microglial mRNA expression of M1 genes: CD86, inducible nitric oxide synthase (iNOS), and IL-1β, M2a genes: mannose receptor (Mrc), arginase (Arg) and chitinase3-like 3 (Ym-1) and M2c genes IL-10, IL-4 receptor-alpha (IL-4Rα) and suppressor of cytokine signaling (SOCS3) in adult mice 4 h after ip injection of LPS. As expected, LPS increased the mRNA levels of all M1 genes (CD86, iNOS, IL-1β) in microglia from adult mice (main effect of LPS, p<0.05 for each). Moreover, M2c genes (IL-10, IL-4Rα, SOCS3) were also increased in enriched microglia 4 h after LPS injection (main effect of LPS, p<0.001 for each). LPS injection did not increase the expression of alternatively activated (M2a) genes Mrc-1 and Arg, but did increase Ym-1 mRNA expression (main effect of LPS, p<0.005). These results indicate that M1 and M2c related genes are both increased in microglia within 4 h after peripheral injection of LPS. Because markers of both classical activation and classic deactivation are simultaneously increased, this activation profile is consistent with the proposed M2b monocyte phenotype (Mantovani et al., 2004; Mosser and Edwards, 2008).

At the 24 h time-point, higher levels of IL-1β and iNOS were still evident in the microglia from LPS treated mice (Fig. 3.1B, main effect of LPS, p<0.01 for each). These levels, however, were reduced compared to levels detected 4 h after LPS injection (Fig. 3.1A). Similar to the results at 4 h, Ym-1 was the only M2a gene that was elevated 24 h following LPS treatment (main effect of LPS, p<0.001). All M2c-related genes including, IL-10, IL-4Rα, and SOCS3, remained elevated 24 h after LPS (Fig. 3.1B, main effect of LPS, p<0.05 for each). These data indicate that M1-related genes were decreased by 24 h after LPS and that M2c-related genes were maintained.

Because minocycline pretreatment attenuates microglial activation and sickness behavior associated with LPS injection (Henry et al., 2008), the degree to which
minocycline alters the M1 and M2 profile of microglia after LPS challenge was determined in adult mice (Fig. 3.1C). As anticipated, LPS injection markedly increased IL-1β mRNA in enriched microglia (main effect of LPS, \( p < 0.0001 \)) and this induction was attenuated by minocycline (minocycline x LPS interaction, \( F(1,33)=4.24, p<0.05 \)). In addition, there was a significant interaction of minocycline and LPS on mRNA expression of Ym-1 \( (F(1,30)=8.15, p<0.009) \). The LPS-induced increase of IL-10 in microglia was also enhanced by minocycline pretreatment \( (p<0.02) \). Other M2c genes including IL-4Rα and SOCS3 were induced by LPS (main effect of LPS, \( p<0.008 \) for each), but were not affected by minocycline. These data indicate that minocycline attenuates the induction of an M1 activation profile after LPS and enhances an M2 profile with increased expression of Ym-1 and IL-10.
Figure 3.1. Peripheral LPS injection promoted an M1 and M2c mRNA profile in microglia. Adult (3-4 mo) BALB/c mice were injected ip with saline or LPS and M1, M2a and M2c related genes were determined from enriched microglia isolated A) 4 h or B) 24 h later (n=6). Bars represent the mean ± SEM. Means with * are significantly different (p<0.05) from Saline controls. C) Adult (3-4 mo) BALB/c mice were injected with vehicle or minocycline for 3 consecutive days. On the third day mice were injected ip with saline or LPS and M1- , M2a-, and M2c-related genes were determined from enriched microglia isolated 4 h later (n=9). Bars represent the mean ± SEM. Means with * are significantly different (p<0.05) from Vehicle Saline and means with # are significantly different (p<0.05) from Vehicle LPS.
Peripheral LPS injection increased microglial surface expression of IL-4Rα, but not IL-10R1

Because LPS injection increased several M2 genes in enriched microglia, including IL-10 and IL-4Rα, surface expression of IL-4Rα and IL-10R1 were determined on microglia of adult mice 4 or 24 h after LPS injection. Fig.3.2A shows a representative bivariate dot plot of CD11b and CD45 staining of Percoll isolated cells. Microglia were gated based on CD11b+/CD45low staining and IL-4Rα and IL-10R1 protein expression was determined. Representative histograms of the mean fluorescence intensity (MFI) for IL-4Rα-PE or IL-10R1-PE are shown in Figs.2B&C. There was a robust increase in MFI for IL-4Rα on microglia after LPS injection, but the MFI for IL-10R1 was not increased after LPS. To further support these data, representative bivariate dot plots of IL-4Rα and IL-10R1 staining on microglia (CD11b+/CD45low), are shown in Fig.3.2D and Fig.3.2E, respectively. These dot plots confirm that IL-4Rα, but not IL-10R1, protein expression on the surface of microglia was markedly increased 4 h after LPS injection (main effect of LPS, p<0.0001). Moreover, the increase in IL-4Rα surface expression was maintained 24 h after LPS (main effect of LPS, p<0.003).
IL-4 re-directs active BV-2 and primary microglia towards an M2 profile

Next, a series of experiments were completed using the BV-2 microglial cell line to determine the ability of IL-4 or IL-10 to re-direct active, M1 microglia towards a repair
or anti-inflammatory M2 profile. In the first experiment, BV-2 cells were stimulated with LPS to induce a M1 phenotype and then treated with recombinant IL-4 (20 ng/mL). Table 3.1 shows that LPS stimulation (LPS Vehicle) increased mRNA levels of M1 genes iNOS and IL-1β (main effect of LPS, \( p<0.0001 \) for each) and increased M2 genes IL-10 and SOCS1 (main effect of LPS, \( p<0.0003 \) for each). Arg mRNA levels were reduced following LPS (main effect of LPS, \( p<0.009 \)). These data are consistent with microglia expression of M1 and M2 related genes after peripheral injection of LPS (Fig. 3.1A). Moreover, IL-4 treatment alone decreased iNOS mRNA and increased mRNA levels of Arg, IL-10, and SOCS1 (main effect of IL-4, \( p<0.0001 \) for each). In this experiment, SOCS1 was selected as a target gene over SOCS3 because it is an M2c gene that is strongly and more specifically induced by IL-4 (Losman et al., 1999). IL-4 reduced the LPS dependent increase in iNOS mRNA (IL-4 x LPS interaction, \( F(1,23)=338.91, p<0.0001 \)) and IL-1β mRNA (IL-4 x LPS interaction, \( F(1,23)=28.91, p<0.0001 \)). Furthermore, pretreatment with LPS enhanced the IL-4-induced increase in SOCS1 mRNA (IL-4 x LPS interaction, \( F(1,11)=10.06, p<0.02 \)). IL-4 induced Arg and IL-10 mRNA expression were unaffected by LPS (Table 3.1).

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<tr>
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<th>mRNA</th>
<th>Saline Vehicle</th>
<th>Saline IL-4</th>
<th>LPS Vehicle</th>
<th>LPS IL-4</th>
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<tbody>
<tr>
<td><strong>M1</strong></td>
<td>iNOS</td>
<td>1.00 ± 0.4 (a)</td>
<td>0.28 ± 0.02 (b)</td>
<td>9.37 ± 0.22 (c)</td>
<td>3.88 ± 0.14 (d)</td>
</tr>
<tr>
<td></td>
<td>IL-1β</td>
<td>1.00 ± 0.02 (a)</td>
<td>0.80 ± 0.04 (a)</td>
<td>21.76 ± 0.64 (b)</td>
<td>16.54 ± 0.68 (c)</td>
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<tr>
<td><strong>M2</strong></td>
<td>Arg</td>
<td>1.02 ± 0.09 (a)</td>
<td>29.38 ± 2.37 (b)</td>
<td>0.68 ± 0.05 (c)</td>
<td>28.80 ± 1.92 (b)</td>
</tr>
<tr>
<td></td>
<td>IL-10</td>
<td>1.01 ± 0.07 (a)</td>
<td>3.22 ± 0.22 (b)</td>
<td>1.84 ± 0.07 (c)</td>
<td>3.11 ± 0.22 (b)</td>
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<td></td>
<td>SOCS1</td>
<td>1.00 ± 0.03 (a)</td>
<td>58.56 ± 6.42 (b)</td>
<td>3.06 ± 0.17 (c)</td>
<td>85.63 ± 4.57 (d)</td>
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**Table 3.1.** IL-4 re-directed active BV-2 microglia towards an M2 profile. BV-2 cells were plated and pre-treated with saline or LPS (10 ng/mL) for 1 h. After 1 h cells were treated with vehicle or IL-4 (20 ng/mL) for an additional 3 h (n=6). RNA was isolated and M1, M2a, and M2c gene expression was determined. These results represent 2 independent experiments. Table represents the mean ± SEM. Means with different letters (a,b,c,d) are significantly different (\( p<0.05 \)) from each other.
In the second experiment, BV-2 cells were activated with LPS (as above) and then treated with recombinant IL-10 (10 ng/mL). Table 3.2 shows a similar pattern of LPS-induced gene expression (LPS Vehicle) consistent with the data provided in Table 3.1. IL-10 alone increased IL-1β, IL-10 and SOCS3 (main effect of IL-10, p<0.02). IL-10, however, was ineffective in re-directing active microglia towards an M2 profile. For instance, co-treatment of IL-10 and LPS induced the highest level of iNOS gene expression (p<0.04) and did not reduce LPS-associated IL-1β. Although, IL-10 did enhance LPS-induced SOCS3 expression (F(1,35)=7.21, p<0.02). This is relevant because SOCS3, much like SOCS1 for IL-4, is strongly induced by IL-10 (Ito et al., 1999). Thus, an increase in SOCS3 following treatment with IL-10 and further enhancement with LPS and IL-10 co-treatment provides evidence that BV-2 cells have the capacity to respond to IL-10 and promote certain aspects of an M2 profile, but not to the same extent as IL-4. Taken together, these data indicate that following classical activation (M1) with LPS, IL-4, but not IL-10, was effective in reducing M1 genes and enhancing M2 genes in BV-2 cells.

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<tr>
<th>mRNA</th>
<th>Saline Vehicle</th>
<th>Saline IL-10</th>
<th>LPS Vehicle</th>
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<tr>
<td><strong>M1</strong></td>
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<tr>
<td>iNOS</td>
<td>1.09 ± 0.16 (a)</td>
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<td>IL-1β</td>
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<td>12.95 ± 3.72 (c)</td>
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<td><strong>M2</strong></td>
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<td></td>
</tr>
<tr>
<td>Arg</td>
<td>1.06 ± 0.13 (a)</td>
<td>1.16 ± 0.04</td>
<td>0.97 ± 0.16</td>
<td>0.78 ± 0.09</td>
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<tr>
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<td>1.93 ± 0.36 (a,b)</td>
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<tr>
<td>SOCS3</td>
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<td>3.09 ± 0.55 (b)</td>
<td>2.62 ± 0.31 (b)</td>
<td>8.25 ± 1.18 (c)</td>
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Table 3.2. IL-10 did not redirect active BV-2 microglia towards an M2 profile. BV-2 cells were plated and pre-treated with saline or LPS (10 ng/mL) for 1 h. After 1 h cells were treated with vehicle or IL-10 (10 ng/mL) for an additional 3 h (n=9). RNA was isolated and M1, M2a, and M2c gene expression was determined. These results represent 3 independent experiments. Table represents the mean ± SEM. Means with different letters (a,b,c) are significantly different (p<0.05) from each other.
Next, primary microglia were established from the brains of neonatal mice to confirm that IL-4 promotes an M2 phenotype in LPS activated microglia. Similar to the above studies, microglia were activated by LPS for 1 h and then treated with recombinant IL-4 (20 ng/mL) for an additional 3 h. Table 3.3 shows that LPS increased M1 markers iNOS and IL-1β (main effect of LPS, p<0.0001 for each) and M2 markers IL-10 and SOCS1 (main effect of LPS, p<0.0001 for each). IL-4 alone increased M2 markers Arg, SOCS1 (main effect of IL-4, p<0.02), and IL-10 (trend for IL-4, p=0.08) and decreased basal expression of M1 marker iNOS (trend for IL-4, p=0.1). IL-4 reduced the LPS-dependent increase in iNOS mRNA (trend for IL-4 x LPS, F(1,20)=2.58, p=0.1), but had no effect on LPS-induced IL-1β. IL-4 promoted Arg in the presence of LPS and enhanced SOCS1 induction compared to IL-4 and LPS treatments alone (IL-4 x LPS interaction, F(1,21)=4.98, p<0.04). Overall, these results support that LPS-activated microglia are successfully re-directed towards an anti-inflammatory and M2 phenotype following treatment with IL-4.

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<th>mRNA</th>
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<th>Saline IL-4</th>
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</tr>
</thead>
<tbody>
<tr>
<td>M1</td>
<td>iNOS</td>
<td>1.00 ± 0.05 (a)</td>
<td>0.76 ± 0.08 (b)</td>
<td>270.85 ± 43.97 (c)</td>
<td>187.95 ± 31.65 (g)</td>
</tr>
<tr>
<td></td>
<td>IL-1β</td>
<td>1.03 ± 0.13 (a)</td>
<td>0.64 ± 0.07 (b)</td>
<td>114.57 ± 10.99 (c)</td>
<td>102.87 ± 10.7 (c)</td>
</tr>
<tr>
<td>M2</td>
<td>Arg</td>
<td>1.19 ± 0.38 (a)</td>
<td>123.89 ± 44.13 (b)</td>
<td>1.06 ± 0.43 (a)</td>
<td>14.35 ± 4.16 (c)</td>
</tr>
<tr>
<td></td>
<td>IL-10</td>
<td>1.03 ± 0.11 (a)</td>
<td>2.71 ± 0.39 (b)</td>
<td>5.65 ± 0.65 (c)</td>
<td>7.18 ± 1.41 (c)</td>
</tr>
<tr>
<td></td>
<td>SOCS1</td>
<td>1.00 ± 0.03 (a)</td>
<td>13.36 ± 0.73 (b)</td>
<td>23.15 ± 3.97 (c)</td>
<td>52.25 ± 4.99 (d)</td>
</tr>
</tbody>
</table>

Table 3.3. IL-4 re-directed active primary microglia towards an M2 profile. Primary microglia were collected from neonatal mice, plated, and pre-treated with saline or LPS (10 ng/mL) for 1 h. After 1 h cells were treated with vehicle or IL-4 (20 ng/mL) for an additional 3 h (n=5). RNA was isolated and M1, M2a, and M2c gene expression was determined. These results represent 2 independent experiments. Table represents the ± SEM. Means with different letters (a,b,c) are different (p<0.1) from each other. Underlined letters trend towards a difference (p<0.1) from the same letter (c, g).
**M1, M2a, and M2c profile in adult and aged microglia after peripheral LPS injection**

Based on M1 and M2 microglial profiling (Fig.3.1&3.2) and the effects of IL-4 on activated BV-2 and primary microglia (Table 3.1-3.3), we next sought to determine differences in M1 and M2 microglial profiles in adult (3-4 mo) mice compared to aged (18-22 mo) mice. In these experiments, mice were ip injected with saline or LPS and enriched microglia were collected 4 h and 24 h later. Fig.3.3A shows the relative expression of M1 (iNOS, IL-1β), M2a (Ym-1, Arg) and M2c (IL-4Rα, SOCS3) genes 4 h after LPS. Consistent with our previous work in aged mice, LPS caused an amplified increase in IL-1β (age x LPS interaction, F(1,26)=4.13, p<0.05) and iNOS (age x LPS interaction, F(1,26)=4.58, p<0.05) gene expression. There were no significant differences in LPS associated M2a induction between age groups 4 h after LPS. LPS injection did, however, increase M2c (IL-4Rα, SOCS3) gene expression (main effect of LPS, p<0.01). The highest levels of microglial mRNA expression for both IL-4Rα and SOCS3 were detected in microglia from LPS treated aged mice (age x LPS interaction, IL-4Rα (F(1,25)=4.43, p<0.05; SOCS3 (F(1,25)=2.46, P=0.10) (Fig.3.2A). These data indicate that LPS caused an exaggerated induction in both M1 and M2c genes in aged mice compared to adult controls (Fig.3.3A).

Twenty-four hours following LPS injections microglia from aged mice maintained elevated expression of iNOS compared to adults treated with LPS (age x LPS interaction (F(1,31)=8.37, p<0.008, Fig.3.3B). IL-1β was also increased 24 h following LPS compared to saline controls, but this difference was independent of age (main effect of LPS, p<0.0001). Levels of IL-1β gene expression in activated microglia from adult mice, however, were higher than we have reported previously. Our previous studies support prolonged IL-1β expression in microglia of aged mice compared to adults (Wynne et al., 2010). At the 24 h time-point, aged mice treated with LPS had a significant increase in
M2a gene expression of Arg (age x LPS interaction, \( F(1,31)=6.40, p<0.02 \)) and Ym-1 (age x LPS interaction, Ym-1, \( F(1,31)=2.32, P=0.10 \)) that was not detected in microglia from adult mice injected with LPS. Furthermore, 24 h after LPS, increased M2c gene expression was maintained, but this was independent of age (Fig.3.3B, main effect of LPS, \( p<0.01 \)). These results indicate that microglia from aged mice have more profound increases in M1 and M2c gene expression 4 h after LPS compared to adults. In addition, both adult and aged mice maintain elevated M2c gene expression 24 h following LPS injection, but only microglia from aged mice also have prolonged induction of the M1-related gene, iNOS, and M2a-related genes.

**Figure 3.3.** M1, M2a, and M2c profile in adult and aged microglia after peripheral LPS injection. Adult (3-4 mo) and aged (18-22 mo) BALB/c mice were injected ip with saline or LPS and M1-, M2a-, and M2c-related genes were determined from enriched microglia isolated A) 4 h or B) 24 h later (n=6). Bars represent the mean ± SEM. Means with * are significantly different (\( p<0.05 \)) from Adult Saline and means with # are significantly different (\( p<0.05 \)) from Adult LPS.
LPS-induced surface expression of IL-4Rα on microglia is impaired in aged mice

The mRNA data from the microglia profiling between adult and aged microglia indicates an increase in the M2 response in aged microglia compared to adults. We have previously reported a similar pattern for IL-10 mRNA and protein expression in active microglia from aged mice (Henry et al., 2009). Despite the enhanced M2 profile, however, inflammatory responses persist in the brain of aged mice. Therefore, exaggerated and prolonged activation in microglia from aged mice may involve insensitivity to anti-inflammatory feedback. In these age comparison studies we focused our attention on IL-4. This is because IL-4Rα was markedly upregulated on the surface of microglia from adult mice following activation with LPS and IL-4 had more profound M2 promoting capabilities in activated BV-2 cells compared to IL-10. Adult and aged mice were injected ip with saline or LPS and enriched microglia were collected 4 h and 24 h later for analysis of IL-4Rα protein expression. Fig.3.4A&B are representative bivariate dot plots for CD11b and CD45 staining in enriched microglia from adult and aged mice used in the analysis for IL-4Rα expression. Fig.3.4C&D shows representative histograms of mean fluorescent intensity (MFI) for isotype control, saline, and LPS in these same enriched microglia. Because there was higher nonspecific staining of the isotype for IL4Rα in aged mice, separate isotype controls were used for adult and aged mice. After normalizing to these separate isotypes, saline treated adult and aged mice had the same percentage of IL-4Rα+ microglia (10.0% ± 1.0% and 10.2% ± 0.6%, respectively). Representative dot plots for IL-4Rα protein expression on microglia (CD11b+/CD45low) of adult and aged mice are shown in Fig.3.4E and Fig.3.4G, respectively. Consistent with the data provided in Fig.3.2, LPS injection markedly increased IL-4Rα surface expression on CD11b+/CD45low microglia from adult mice (main effect of LPS, p<0.0001) (Fig.3.4E&F). In aged mice, however, LPS injection did
not increase the percentage of IL-4Rα⁺ microglia at either the 4 h or 24 h time-point (Fig.3.4G&H). MFI for IL-4Rα in adult and aged microglia confirms that only microglia from adult mice had increased expression of IL-4Rα after LPS injection (Fig.3.4C&D). Taken together, these results indicate that IL-4Rα protein expression is not induced on microglia from aged mice after peripheral LPS injection.
IL-4 mRNA levels decrease in the brain of adult and age mice after LPS

Next, we sought to determine the extent to which IL-4 mRNA levels change within the brain with age and LPS injection. In this experiment, adult and aged mice were injected ip with saline or LPS and IL-4 mRNA expression was determined from a 1 mm
coronal brain section through the prefrontal cortex (+0.38 mm from Bregma) collected either 4 or 24 h later. These brain slices were collected from the same mice that were used in the RT-PCR and flow cytometry experiments. Fig.3.5 shows that IL-4 mRNA levels were decreased in both adult and aged mice 4 h after LPS injection (main effect of LPS, \( p<0.001 \)). IL-4 mRNA expression returned to baseline levels within 24 h after the injection of LPS independent of age. Overall, these data indicate that LPS decreased IL-4 mRNA expression in a time-dependent but age-independent manner.

![Graph showing IL-4 mRNA expression](image)

**Figure 3.5.** IL-4 mRNA is decreased in the brain of adult and aged mice after LPS. Adult (3-4 mo) and aged (18-22 mo) BALB/c mice were injected ip with saline or LPS and IL-4 mRNA was determined from a coronal brain slice 4 h and 24 h later (n=5). Means with * are significantly different \((p<0.05)\) from Adult Saline.

*Ex vivo treatment with IL-4 re-directed active microglia from adult, but not aged, mice towards an M2 profile*

To determine the degree to which reduced IL-4R\(\alpha\) on the surface of microglia from aged mice corresponded with impaired sensitivity to IL-4, a series of *ex vivo* experiments were conducted. In these experiments, adult and aged mice were injected
ip with saline or LPS to activate microglia *in vivo*. After 4 h, enriched microglia were isolated and plated on poly-L-lysine coated 24-well plates. Cells were treated *ex vivo* with vehicle or IL-4 (20 ng/mL) and RNA was collected 3 h later. Based on the results of the BV-2 and primary microglia experiments with LPS and IL-4, the M1 markers IL-1β and iNOS and the M2 marker Arg were selected for analyses.

In microglia from adult mice, mRNA levels of IL-1β and iNOS (M1 genes) were increased in mice injected with LPS compared to saline (main effect of LPS, *p*<0.0001). Thus, the microglia cultured *ex vivo* retained a degree of activation associated with the ip injection of LPS. Following treatment with *ex vivo* IL-4, microglia from adult mice had a significant reduction in LPS-induced iNOS mRNA expression (LPS x *ex vivo* IL-4 interaction, *F*(1,30)=18.6, *p*<0.0002), but no change in IL-1β levels (Fig.3.6A). The effects of IL-4 on active microglia are similar to the results obtained in primary microglial cultures (Table 3.3). Moreover, *ex vivo* IL-4 alone strongly promoted Arg mRNA expression (main effect of IL-4, *p*<0.0001). This Arg induction was potentiated when microglia from LPS injected mice were stimulated with IL-4 (Fig.3.6A, LPS x IL-4 interaction, *F*(1,36)=6.74, *p*<0.01). Because adult and aged microglia are inherently different, the results for age x LPS x IL-4 interactions were not directly compared. Nonetheless, IL-4-mediated effects in the aged mice are significantly different than results obtained from the adult experiment.

*Ex vivo* cultures of microglia from LPS injected aged mice also had increased mRNA levels of IL-1β and iNOS (M1 genes) compared to saline-injected aged controls (trend for main effect of LPS, *p*<0.07). Furthermore, in contrast to adult microglial cultures, *ex vivo* IL-4 further enhanced LPS-associated IL-1β mRNA expression (LPS x IL-4 interaction, *F*(1,34)=4.21, *p*<0.05) and did not reduce LPS-associated iNOS mRNA induction in aged microglia (Fig.3.6B). Arg mRNA expression was increased by IL-4
alone in aged *ex vivo* microglia (main effect of IL-4, *p*<0.003), but this Arg mRNA induction was not amplified in microglia isolated from the LPS injected aged mice. Taken together, a lack of IL-4Rα upregulation of microglia from aged mice (Fig. 3.4) is associated with reduced sensitivity to *ex vivo* IL-4 stimulation.

**Figure 3.6.** *Ex vivo* treatment with IL-4 re-directed active microglia from adult, but not aged, mice towards an M2 profile. Adult (3-4 mo) and aged (18-22 mo) BALB/c mice were injected ip with saline or LPS. Four hours later microglia were isolated, plated for 1 h, and treated *ex vivo* with IL-4 (20 ng/mL). RNA was isolated 3 h and expression of IL-1β, iNOS and Arg were determined from A) adult and B) aged mice (n=9). Bars represent the mean ± SEM. Means with different letters (a,b,c) are different (*p*<0.07) from each other.
**Discussion**

Previous studies from our lab and others indicate that aged but otherwise healthy rodents have prolonged sickness behavior (Godbout et al., 2005b; Huang et al., 2008), cognitive impairment (Abraham and Johnson, 2009b; Barrientos et al., 2006; Chen et al., 2008), and depressive-like behavior (Godbout et al., 2008) following peripheral or central immune challenge. These behavioral deficits correspond to exaggerated and protracted microglial activation in aged mice (Henry et al., 2009; Wynne et al., 2010). Here we demonstrate that microglia from adult and aged mice express an M2b phenotype following an LPS injection, but only adult mice showed a clear shift towards an M2c phenotype within 24 h. Furthermore, IL-4Rα, but not IL-10R1, was increased on the surface of microglia from adult mice after activation with LPS. In accordance, IL-4, but not IL-10, successfully re-directed activated microglia in culture towards an M2a/M2c profile. In age comparisons, microglia from aged mice did not upregulate surface expression of IL-4Rα following peripheral LPS injection. Furthermore, when activated microglia from adult and aged mice were isolated and treated *ex vivo* with IL-4, only microglia from adult mice were successfully re-directed towards an anti-inflammatory and M2 profile with reduced iNOS and increased Arg mRNA expression.

One key component of this study was that following a peripheral injection of LPS, microglia simultaneously induced both M1 (IL-1β, iNOS) and M2c (IL-10, IL-4Rα) markers consistent with an M2b phenotype (Mantovani et al., 2004). In microglia from aged mice, there was an exaggerated increase in both the M1 and M2c markers. These data are in line with previous studies showing elevated IL-1β and IL-10 mRNA and intracellular protein expression in microglia 4 and 8 h after LPS injection, and further enhancement of both IL-1β and IL-10 in aged mice (Henry et al., 2009; Sierra et al., 2007). By 24 h after LPS, microglia from adult mice shifted to a predominately M2c phenotype whereas microglia from aged mice maintained an M2b profile. In our earlier
studies enhanced IL-1β expression was also retained in LPS-treated aged mice 24 h after LPS. In the current study, however, IL-1β mRNA levels were higher in LPS-treated adult mice than we have previously reported (Godbout et al., 2005b; Henry et al., 2009; Wynne et al., 2010). A prolonged M2b phenotype in aged microglia is important because the shift towards an M2c phenotype 24 h after LPS corresponded with the resolution of the sickness response in adult mice in previous studies (Godbout et al., 2005b; Huang et al., 2008). Consistent with this notion, the present study indicates that pre-treatment with minocycline reduced IL-1β, maintained IL-4Rα and SOCS3, and significantly enhanced IL-10 and Ym-1 mRNA 4 h after LPS. This accelerated shift towards an anti-inflammatory, M2a/M2c phenotype corresponded with previous reports of minocycline-mediated ameliorated sickness response in adult mice (Henry et al., 2008). Based on a previous study (Lyons et al., 2007b) it is plausible that minocycline directly increased IL-4 expression to help promote an anti-inflammatory and M2 phenotype. Taken together these data indicate the time dependent transition of an M2b towards an M2c profile in microglia coincides with the resolution of LPS-induced sickness response.

Another difference between the M1 and M2 profile of microglia was that 24 h after LPS M2a markers were enhanced only in LPS-treated aged mice. Therefore, an increase in the M2a phenotype in microglia from aged mice may represent a greater degree of tissue injury associated with an exaggerated inflammatory response to LPS. In support of this notion, Arg was the most upregulated gene in the spinal cord immediately following induction of experimental autoimmune encephalomyelitis (EAE) (Xu et al., 2003) and was also promoted in mononuclear cells following spinal cord injury (Kigerl et al., 2009; Ochoa et al., 2001). Consequently, a promotion of the M2a phenotype within the aged CNS may be in response to overt CNS damage rather than promotion of an anti-inflammatory state.
Higher induction of mRNA for M2a-related genes may also be misleading because the increase in mRNA for IL-4Rα in microglia from aged mice did not correspond with increased IL-4Rα protein expression. The discrepancy between LPS-induced increased IL-4Rα mRNA levels with a corresponding failure to increase IL-4Rα surface expression in aged microglia may be related to problem with translation, post-translational modifications, or shuttling the receptor subunit to the cell membrane. For example microRNAs block the translation of genes into proteins (Bell, 2007) and multiple sequences and signaling cascades are involved in shuttling a translated protein from the endoplasmic reticulum to the Golgi and then to the cell membrane (Cho and Stahelin, 2005). Thus, it is plausible that a failure to increase IL-4Rα protein expression can occur independent of gene transcription.

Another important finding of this study is that an ip injection of LPS markedly increased protein expression of IL-4Rα, but not IL-10R1, on the surface of microglia from adult mice. IL-4Rα may be upregulated on microglia in preparation for increased levels of IL-4 to promote a microglial phenotype permissive to tissue repair and the resolution of inflammation (Mantovani et al., 2004; Mosser and Edwards, 2008). This may be particularly important in brain injury, EAE, and neurodegenerative diseases where there are tissue damage and repair processes associated with invading immune cells (Popovich and Jones, 2003; Schwartz and Kipnis, 2005). In addition, IL-4Rα is also a co-receptor for IL-13 (Nelms et al., 1999) and therefore microglia may be preparing to encounter IL-13. In the context of the induction of sickness behavior, microglia may enhance IL-4Rα surface expression to respond to a transient reduction in IL-4 mRNA levels after an LPS injection. Alternatively, IL-4Rα may be upregulated to maintain neurotrophic factor expression and support hippocampal neurogenesis in an effort to prevent inflammatory-induced deficits in learning and memory. For example, IL-4
production by T-cells is purportedly involved in increasing brain derived neurotrophic factor (BDNF) and nerve growth factor (NGF) during memory tasks (Derecki et al., 2010). Furthermore, IL-4 deficient mice had reduced learning and memory that was reversed following reconstitution with wild type bone marrow (Derecki et al., 2010).

Consistent with a main effect of LPS on microglial induction of anti-inflammatory cytokine receptors, IL-4, but not IL-10, was sufficient to redirect LPS-activated BV-2 and primary microglia towards an M2 phenotype. It is important to note that IL-4 did not reduce LPS-induced IL-1β expression in primary or ex vivo cultures. Several studies have reported no effect of IL-4 on IL-1β mRNA (Allen et al., 1993; Ledeboer et al., 2000), but rather reduced IL-1β bioactivity by enhancing IL-1 receptor antagonist (IL-1RA) through a PI3-kinase dependent mechanism (O’Connor et al., 2007). Nonetheless, these data are consistent with a previous study showing that LPS increased IL-4Rα mRNA on microglia and that both pre- and post-treatment of IL-4 reduced LPS-associated nitric oxide production in microglial and motoneuron co-cultures (Zhao et al., 2006). In contrast to IL-4, IL-10 had little effect in reducing LPS-associated M1 activation in BV-2 and ex vivo microglial cultures (data not shown). The current study was focused on how to re-direct the inflammatory profile of microglia following activation. Other studies have used pre-treatment with IL-10 (Qian et al., 2006; Sawada et al., 1999) or used IL-10 in mixed glial cultures (Ledeboer et al., 2000; Lodge and Sriram, 1996). Nonetheless, IL-10 is an important inflammatory cytokine in the brain. For instance, icv injection of IL-10 reversed LPS-induced sickness behavior (Bluthé et al., 1999) and IL-10 deficient mice had a prolonged LPS-induced sickness response (Richwine et al., 2009). Our results indicate that IL-10R1 expression remained low on microglia and that activated microglia in culture did not respond to the anti-inflammatory promoting effects of IL-10. Thus, it is possible that IL-10 has indirect anti-inflammatory effects on microglia through astrocytes.
In support of this notion, cultured astrocytes expressed higher levels of IL-10R1 protein compared to microglia (Ledeboer et al., 2002) and IL-10 treatment of mixed glial cultures reduced LPS-induced inflammatory cytokine production (Ledeboer et al., 2000).

A novel finding of this study was that LPS did not increase IL-4Rα protein expression on the surface of microglia from aged mice and that this was associated with a reduced sensitivity to the M2 promoting-effects of IL-4. Reduced microglial sensitivity to IL-4 may have significant implications in neurological disease, aging, and CNS injury. For example, IL-4 production within the brain during EAE (Begolka et al., 1998) and multiple sclerosis (Benveniste, 1997) was positively correlated with remission of the disease. In addition, increased IL-4 concentrations in the brains of APP+PS1 mice improved NMDA-receptor function and reversed deficits in spatial learning and memory (Kiyota et al., 2010). In aging, reduced IL-4 levels in the brain of aged rats corresponded with increased neuroinflammation and reduced long-term potentiation (LTP) (Maher et al., 2005; Nolan et al., 2005). In addition, induction of hippocampal IL-4 successfully restored LTP in these aged rats (Clarke et al., 2008). We did not detect age-associated reductions in IL-4 mRNA expression in the current study, but this was likely because of species differences in immunity (BALB/c mice versus Wistar rats) (Haley, 2003). Nonetheless, a deficit in IL-4Rα expression and IL-4 sensitivity in aged microglia following an inflammatory stimulus support the idea that IL-4 regulation of microglia is impaired in the aged. Thus, a functional consequence of reduced IL-4-mediated microglial regulation may be reduced cognitive ability and worsened outcome in CNS injury. For example, working memory and long-term memory were reduced in aged mice (Abraham and Johnson, 2009b; Chen et al., 2008) and rats (Barrientos et al., 2006) following a peripheral immune challenge. In addition, IL-4 and IL-4Rα were strongly upregulated within 24 h following spinal cord (Lee et al., 2010) and brain injury (Xiong et
al., 2011). In these traumatic CNS injuries IL-4 deficient mice or mice treated with anti-IL-4 had exaggerated monocyte activation, CNS damage, and had worsened neurological scores. Similar to these mice, cortical impact injury in aged C57BL/6 mice caused exaggerated glial activation (i.e., astrocytes and microglia) and CNS damage compared to injured adults (Onyszchuk et al., 2008; Sandhir et al., 2008). Therefore, it is plausible that worsened injury outcome in aged mice was associated with an impaired microglial response to the M2 and repair promoting effects of IL-4.

In conclusion the present study provides novel evidence that impaired expression of IL-4Rα on microglia from aged mice was associated with reduced sensitivity to the M2-promoting cytokine IL-4. In addition, we propose an important interaction between age-related neuroinflammation and microglial activity where an exaggerated M1 response causes these cells to be refractory to anti-inflammatory feedback by IL-4. These findings support previous work by our lab and others that impaired regulation of microglia in aged mice leads to amplified and prolonged microglial activation. Furthermore, these IL-4 data add to the growing body of literature the glial regulatory systems including CD200-CD200R (Lyons et al., 2007a) and CX3CL1-CX3CR1 (Bachstetter et al., 2011; Lyons et al., 2009a; Wynne et al., 2010) are impaired in the aged brain.
Chapter 4: Role of IL-4 Signaling and M2a Responses in the Inflammatory CNS: Consequences of Age-related Deficits in IL-4Rα

Abstract

Alternative activation of microglia/macrophages (M2a) by interleukin (IL)-4 is a critical immune signal purported to support intrinsic growth and repair processes after traumatic injury to the central nervous system. Nonetheless, alternative activation of microglia is poorly understood in vivo, particularly in the context of inflammation, injury, and aging. Here, we show that aged mice had reduced functional recovery after spinal cord injury (SCI) associated with impaired induction of IL-4 receptor alpha (IL-4Rα) on resident microglia, attenuated arginase expression, and reduced recruitment of IL-4Rα+ macrophages to the injured cord. A similar deficit in arginase induction was evident in microglia/macrophages of IL-4RαKO mice after SCI. Next, immune-based studies showed that inflammatory-induced IL-4Rα expression on microglia was necessary for IL-4-dependent arginase expression in vivo and promotion of axon growth ex vivo. In addition, IL-4-mediated re-direction of activated microglia increased expression of IL-1β and CCL2 associated with myeloid cell recruitment. Consistent with these results, IL-4-mediated alternative activation of microglia resulted in increased presence of peripheral CCR2+/IL-4Rα+/arginase+ myeloid cells in the brain. Collectively, our data indicate that alternative activation of microglia promotes functional recovery after SCI while recruiting peripheral myeloid cells to the CNS, and that this phenotype is impaired in aged mice.
Introduction

Alternative activation of microglia/macrophages (M2a) is purported to play a protective role after spinal cord injury (SCI) participating in intrinsic survival, growth, and repair processes (David and Kroner, 2011). Nonetheless, alternative activation of microglia is poorly understood in vivo and may be impaired with normal aging. Several studies indicate that microglia in the aged central nervous system (CNS) become more inflammatory and less responsive to anti-inflammatory (Henry et al., 2009; Lee et al., 2013) or regulatory signals (Fenn et al., 2012; Jurgens and Johnson, 2012; Kumar et al., 2012; Norden and Godbout, 2013). For example, aged microglia were less responsive to the anti-inflammatory promoting effects of fractalkine (CX3CL1) corresponding with exaggerated inflammation after an immune challenge (Henry et al., 2009; Wynne et al., 2010). Moreover, we reported that microglia from the brain of aged mice failed to increase surface expression of interleukin (IL)-4Rα after an inflammatory stimulus and, in turn, were less responsive to anti-inflammatory feedback signals provided by IL-4 (Fenn et al., 2012).

Impaired microglia regulation may explain why older individuals (60+ years of age) have impaired functional recovery and high mortality rates after a traumatic injury to the brain or spinal cord (Chen et al., 1997; Fassett et al., 2007; Injury-Statistical-Center, 2013). In rodent models of traumatic brain injury, worse functional outcome in the aged corresponded with reduced alternative activation markers (Kumar et al., 2012), reduced neurotrophin production (Anderson et al., 2009) and increased CNS inflammation (Sandhir et al., 2008). Few studies, however, have investigated a mechanism for age-associated increases in morbidity and mortality after SCI. Impaired locomotor recovery was observed in middle-aged rats compared to young adults for both hemisection (Gwak et al., 2004a) and spinal cord contusion injuries (Siegenthaler et al., 2008).
Studies using aged mice (modeling age 60+ in humans), however, are lacking and mechanisms for increased morbidity and mortality are unknown. This is a significant problem because the incidence of SCI in aged individuals has increased 5-fold in the last 30 years and currently comprises 15% of SCI cases each year (Fassett et al., 2007). Thus, a better understanding of how different microglia/macrophage phenotypes affect injury and recovery in the aged is required.

Understanding of microglia/macrophage biology has expanded within the last decade. Mononuclear cells take on distinct phenotypes (i.e., M1, M2a, M2b, or M2c) in response to different inflammatory signals. Unfortunately, these phenotypes have been primarily characterized \textit{in vitro} and whether or not these distinct microglia/macrophage phenotypes develop \textit{in vivo} and what functions they carry out are poorly defined (Mosser and Edwards, 2008). Relevant to CNS trauma, the M1 (classically activated) and IL-4/IL-13-driven M2a (alternatively activated) phenotypes have received the most attention.

In the context of CNS injury and disease, the M1 profile is considered destructive by contributing to impaired neurite growth and increased secondary degeneration (David and Kroner, 2011; Gensel et al., 2009; Horn et al., 2008; Liao et al., 2012). The M1 phenotype is induced early after SCI and persists in the spinal cord lesion creating a microenvironment that no longer supports the differentiation or maintenance of M2a macrophages by 14 dpi (Kigerl et al., 2009). Indeed, the M2a profile peaks within the first week after SCI but is scarce within the lesion after 14 d (Kigerl et al., 2009). The M2a profile is thought to promote a level of endogenous repair during injury or CNS disease (David and Kroner, 2011; Liao et al., 2012). In models of nerve and spinal cord injury, increased expression of arginase and growth factors (e.g., BDNF) (Derecki et al., 2010), consistent with an M2a response, was associated with prolonged axonal protection (Barrette et al., 2010) and improved neuronal growth and survival (Cao et al., 2005).
vitro, enhanced neurite outgrowth was achieved when neurons were cultured with media conditioned by IL-4-treated macrophages (Kigerl et al., 2009). In a model of myocardial infarction, Ly6C\textsuperscript{lo} monocytes with an M2 skew (i.e., high vascular endothelial growth factor, low TNF-\(\alpha\)) were necessary to promote collagen deposition and angiogenesis (Nahrendorf et al., 2007), to processes that are important for repair after SCI. Thus, early induction of an M2a phenotype after SCI may be required for efficient endogenous repair. An inability to respond to IL-4/IL-13 in the aged and a failure to induce an M2a myeloid phenotype after SCI may underlie worse functional recovery.

Therefore, the aim of this study was to test the hypothesis that reduced development of an M2a phenotype in microglia of aged mice results in an attenuated response to injury and worse functional outcome following SCI. We show novel data that aged mice had significantly reduced functional recovery after SCI and that this behavioral deficit was associated with failed induction of IL-4R\(\alpha\) on microglia. An age-related deficit in IL-4R\(\alpha\) expression corresponded with attenuated M2a responses in microglia (reduced arginase) and impaired inflammatory cytokine (IL-1\(\beta\)) and chemokine (CCL2) expression necessary to recruit IL-4R\(\alpha\)^+ monocytes/macrophages to the injured spinal cord. Moreover, evidence from immune-based studies show that induction of IL-4R\(\alpha\) on microglia was required for IL-4-dependent arginase expression \textit{in vivo} and axon outgrowth \textit{ex vivo}. Finally, inflammatory activated microglia redirected with IL-4 \textit{in vivo} did not exhibit an anti-inflammatory phenotype. Instead, they increased expression of IL-1\(\beta\) mRNA associated with heightened CCL2 in the brain and increased recruitment of CCR2^+/IL-4R\(\alpha\)^+/Arg^+ myeloid cells to the CNS.
Materials and Methods

Animals

Adult BALB/c mice were obtained from a breeding colony kept in barrier-reared conditions in a specific-pathogen-free facility at the Ohio State University. A breeding colony was established for adult IL-4Rα deficient (IL-4RαKO) mice maintained on a BALB/c background (BALB/c-Il4ratm1Sz/J) (#003514) originally purchased from Jackson Laboratories (Bar Harbor, ME). Adult C57BL/6 mice were purchased from Charles River Breeding Laboratories (Wilmington, MA), adult C57BL/6-Tg(CAG-EGFP) 131/leysopJ mice (#006567) were purchased from Jackson Laboratories, and aged BALB/c mice (18-22 mo) were purchased from the National Institute on Aging specific-pathogen-free colony (maintained at Charles River Laboratories, Inc., MA). 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.

Spinal Cord injury

SCI was performed as previously described (Jakeman et al., 2000). In brief, mice were deeply anesthetized using ketamine and xylazine (100 mg and 10 mg/kg BW i.p., respectively) and received a laminectomy at vertebral level T9 or a laminectomy followed by a spinal cord contusion using the Infinite Horizons device (75 kdyn; Precision Systems Instrumentation). Mice were returned to a slide warmed cage (35-37°C) with 3-4 mice per cage and administered 2 mL sterile saline, and 100 μL gentamicin (5 mL/kg) subcutaneously (s.c.). Mice were maintained on the slide warmed cage over night. Once
a day for 5 d mice received a s.c. injection of 1-2 mL sterile saline and 100 μL gentamicin (5 mL/kg). Mice receiving a spinal cord contusion underwent bladder expression twice daily for the duration of the experiment.

**Motor Recovery**

Basso Mouse Scale (BMS) scoring was completed as previously described (Basso et al., 2006). In brief, mice were acclimated to a plastic bottomed open field (100 cm diameter, 21 cm wall height) every other day for one week before injury. Because BALB/c mice freeze in an open field under bright light, dim light was used for all BMS testing. Under dim light BALB/c mice moved readily throughout the open field. After injury mice were placed individually in the open field 1, 3, 5, 7, 14, 21, and 28 dpi. During testing mice were scored by two observers blinded to age or genotype for 4 min. Mice were rated by on a scale of 0-9. Raters discussed scoring during testing and discrepancies were scored in favor of the deficit. Testing was completed during the light phase at the same time each day.

**Intracerebroventricular (i.c.v.) cannulation and injections**

I.c.v. cannulation was performed as previously described (Huang et al., 2008). In brief, mice were deeply anesthetized using ketamine and xylazine (100 mg and 10 mg/kg BW i.p., respectively). Mice were positioned in a stereotaxic instrument and a 1.5 cm incision was made on the cranium to reveal the bregma. A 26-guage stainless-steel guide cannula was placed in the lateral cerebral ventricle using the following stereotaxic coordinates from bregma: Lat 1.2 mm; A-P 0.5 mm; and Hor -2 mm from the dura mater. Two anchoring cranial screws were inserted adjacent to the cannula and the cannula was secured with cranioplastic cement. A dummy cannula was inserted in the guide
cannula to prevent occlusion and infection. Mice were injected subcutaneously with Buprinex (111 μg/kg BW) after surgery and 12 h later. Mice were provided a minimum of 7 d to recover before treatment.

After at least 7 d post-i.c.v. cannulation, mice were given an intraperitoneal (i.p.) injection of saline or LPS (0.33 mg/kg – BALB/c; 0.5 mg/kg – C57BL/6). One hour after mice then received an i.c.v. injection at a rate of 2 μl / min of vehicle (0.1% BSA) or IL-4 (25 ng/μl). Twenty-four hours after LPS injection mice were euthanized or perfused and brains were collected for flow cytometric, RNA, or immunohistochemical analyses.

**DRG neuronal cultures**

Cervical, thoracic, and lumbar DRG neurons were isolated from the spinal cord as previously described (Gensel et al., 2009). In brief, DRGs were isolated from an adult mouse spinal cord and plated at a density of 800 cells/well in a 24-well plate and maintained in fresh media (Neurobasal A with 2% B27 supplement (Invitrogen), 1% Glutamax, and 1% penicillin/streptomycin) at 37° C/5% CO₂ for 3 d before treatment. After 3 d, primary microglia from adult mice were added to 0.4 μm transwell inserts situated directly above the DRG neurons and cultures were treated with vehicle (0.1% BSA) or IL-4 (20 ng/mL) for 24 h.

**GFP⁺ Bone Marrow (BM)-Chimera**

BM chimeras were established using chemical ablation with busulfan and reconstitution of the bone marrow with donor cells obtained from C57BL/6-Tg(CAG-EGFP) mice as previously described (Wohleb et al., 2013). In brief, recipient C57BL/6 male mice (6 weeks old) were injected intraperitoneally (i.p.) once daily for two consecutive days with busulfan in a 1:1 solution of DMSO and deionized H₂O (30 mg/kg/100μl).
Donor C57BL/6-Tg(CAG-EGFP) BM-derived cells were isolated from the femur, passed through a 70 µm cell strainer, and total number of cells was determined with a BD Coulter Particle Count and Size Analyzer (Beckman Coulter, Inc., Indianapolis, IN). Donor BM-derived cells (1x10^6) were transferred to recipient mice by tail vein injection (100μl) 48 h after the second dose of busulfan. Mice were left undisturbed for 4 weeks to allow engraftment. Engraftment was verified by determining the percentage of GFP^+ cells in the BM and the blood. Mice that had less than 30% BM engraftment were excluded from the study.

**Microglial/Macrophage isolation from brain and spinal cord**

Microglia and macrophages were isolated from brain (Fenn et al., 2012; Henry et al., 2009) and spinal cord homogenates (Donnelly et al., 2011) as previously described using a 70/50/35/0% Percoll gradient for brain and 70/35/0% Percoll gradient for spinal cord. For the brain, microglia/macrophages were collected from the 70%/50% Percoll interphase. For the spinal cord, microglia/macrophages were collected from the 70%/35% Percoll interphase. The brain extraction yielded approximately 3 x 10^5 viable cells whereas the spinal cord extraction yielded approximately 5 x 10^4 viable cells. Cells collected from the brain are referred to as enriched brain CD11b^+ cells based on previous studies demonstrating greater than 90% CD11b^+ cell yield (Wohleb et al., 2012).

**RNA isolation and RT-PCR**

RNA was isolated from a 1 mm coronal brain section or two, 1 mm spinal cord sections immediately adjacent to the injury epicenter using the using the Tri-Reagent protocol (Sigma, MO) and subjected to the DNA-free™ RNA clean up procedure (Ambion, TX) according to manufacturer instructions. RNA was isolated from Percoll
enriched CD11b+ cells using the PrepEase kit (Affymetrix, CA) according to manufacturer instructions. Real-time (RT)-PCR was performed using the Applied Biosystems (Foster, CA) Taqman® Assays-on-Demand™ Gene Expression protocol as previously described (Wohleb et al., 2012; Wohleb et al., 2011). In brief, experimental cDNA was amplified by real-time PCR where a target cDNA (e.g., IL-1β, Arginase, 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, Foster, CA). Data were analyzed using the comparative threshold cycle (Ct) method and results are expressed as fold difference from GAPDH.

Flow cytometry

Cells were assayed for surface antigens by flow cytometry as previously described (Fenn et al., 2014b; Henry et al., 2009). Percoll Enriched cells were incubated with rat anti-mouse CX3CR1-FITC or CD14-FITC, CD11b-PE, CD45-PerCP-Cy5.5, and CCR2-APC antibodies (eBioscience, CA). Expression of these surface receptors was determined using a Becton-Dickinson FACSCaliber four color Cytometer. Ten thousand events characterized as microglia/macrophages were recorded. Microglia and macrophages were identified by CD11b+/CD45lo and CD11b+/CD45high expression, respectively (Wohleb et al., 2011). For each antibody, gating was determined based on appropriate negative isotype stained controls. Flow data were analyzed using FlowJo software (Tree Star, CA).
Immunohistochemical Staining

Mice were euthanized by CO₂ and transcardially perfused with 0.1 M PBS followed by 4% formaldehyde. Tissues were cryoprotected with 30% sucrose for 72 h following post-fixation. For all tissues three representative images from each sample were taken for analysis. Fluorescent images were taken with a Zeiss 510 Meta confocal microscope and analyzed using Metamorph (Molecular Devices) Analysis software. DAB/EC images were taken with a Carl Zeiss Axioplan 2 Imaging microscope and analyzed using ImageJ NIH software.

Spinal cord: spinal cords were rapidly frozen in OCT compound. Serial coronal sections (10 μm) were cut using a Microm HM550 cryostat (Mikron Instruments) and collected every 100 μm throughout a 5 mm segment with the lesion epicenter at the middle. Sections were stained with antibodies against arginase I (1:200; Santa Cruz Biotechnology) and Tomato lectin (1:200, Sigma) (Fenn et al., 2012) for fluorescent staining, or neurofilament (1:1000, Aves Labs), and erichrome cyanine (Sigma) solution for DAB staining.

Brain: brains were frozen in dry-ice cooled isopentane and stored at -80°C until sectioned. Coronal sections (10 μm) were cut using a Microm HM550 cryostat and collected every 20 μm throughout the i.c.v. injection site. Sections were stained with antibodies against arginase I (1:200; Santa Cruz Biotechnology), Iba-1 (1:1000; Wako Chemicals), and IL-4Rα (1:100; Santa Cruz Biotechnology) for fluorescent staining. Images were taken from the region adjacent to the icv injection site and >40 μm distal to the icv injection site.

DRG cultures: DRG neurons were fixed with 2% formaldehyde for 20 min and stored in 0.1 M PBS at 4°C until staining. Cells were stained with β-tubulin III (1:2000, Sigma) and images were taken every 1500 μm with a Carl Zeiss Axioplan 2 Imaging
microscope and analyzed using Metamorph Analysis software. Only images that contained a DRG neuronal cell body were analyzed. DRG cell bodies were not used in analysis of β-tubulin III threshold staining.

**Statistical Analysis**

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), or three-way (i.e. Pretreatment x Treatment x Time) ANOVA using the General Linear Model procedures of SAS. Analysis of dependent variables was subjected to covariate analysis to eliminate dependency. 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 < 0.05 and a tendency at p ≤ 0.1.

**Results**

*Age-related impairment in the induction of IL-4Rα surface expression on microglia after SCI*

Our previous work indicates that peripheral challenge with lipopolysaccharide (LPS), a potent activator of the innate immune system, markedly increased surface expression of IL-4Rα on microglia of adult, but not aged mice (Fenn et al., 2012). Impaired upregulation of IL-4Rα on microglia from aged mice corresponded with reduced sensitivity to *ex vivo* IL-4 stimulation. These data are relevant because several studies
indicate that IL-4 re-directs active microglia towards an M2a or alternatively activated phenotype that supports intrinsic repair processes after traumatic CNS injury (Gordon, 2003; Kigerl et al., 2009; Liao et al., 2012). Accordingly, we hypothesize that the functional consequence of impaired IL-4Rα induction in microglia of aged mice is an attenuated M2a response and worse functional recovery after traumatic CNS injury.

To test this hypothesis, adult (3-4 mo) and aged (18-19 mo) mice were subjected to a moderate (75 kilodyne) mid-thoracic (T₉) laminectomy or spinal cord contusion injury (SCI). Flow cytometry was used to quantify IL-4Rα expression on spinal cord microglia (CD11b⁺/CD45lo) enriched by Percoll-gradient separation at 1 or 3 days post injury (dpi). Representative bivariate dot plots of IL-4α labeling on adult (Fig.4.1A) and aged (Fig.4.1B) microglia are shown. Adult and aged microglia were analyzed using separate isotypes controls because of higher auto-fluorescence in aged microglia (Fig.4.1B). Fig.4.1A-C show that moderate SCI increased the expression of IL-4Rα on the surface of microglia (F(1, 33)= 9.21, P<0.006), but this increase was dependent on age (Age×SCI: F(2,33)=10.39, p<0.008). For example, IL-4Rα protein expression was increased on microglia from the spinal cord of adult mice 1 dpi (P<0.006) and 3 dpi (P=0.08), but IL-4Rα was not increased on microglia of aged mice at either time point (Fig.4.1C). Thus, SCI induced increase in microglial IL-4Rα surface expression was impaired in aged mice.

Next, a 2 mm section of the spinal cord adjacent to the injury epicenter was collected 1 and 3 dpi from adult and aged mice to measure SCI-induced changes in mRNA expression of IL-4 and IL-13, the ligands for IL-4Rα. IL-4 mRNA was increased 3 dpi independent of age (P < 0.02, Fig.4.1D). In contrast, IL-13 mRNA was reduced after SCI, but only in adult mice (Age×SCI: F(2,23)=6.11, P < 0.01). Although aged mice had lower IL-13 levels at baseline (P < 0.04), IL-13 mRNA was maintained after injury in the
Figure 4.1. Age-related impairment in the induction of IL-4Rα surface expression on microglia after SCI. Adult (3-4 mo) and aged (18-22 mo) female BALB/c mice were subjected to a mid-thoracic (T9) laminectomy (Lam) or spinal cord contusion injury (SCI). After 1 or 3 dpi, enriched CD11b+ cells from the spinal cord were collected by Percoll gradient separation. Representative bivariate dot plots of CD11b/IL-4Rα labeling in microglia from (A) adult and (B) aged mice. Gating was based on age-matched isotype controls. (C) Average percentage of IL-4Rα+ microglia (n=4-5). (D) IL-4 and (E) IL-13 mRNA expression was determined from two 1 mm sections of spinal cord collected adjacent to the lesion epicenter 1 and 3 dpi (n=4-5). Bars represent the mean ± SEM. (ANOVA: *, P < 0.05, +, P = 0.1 from Adult-Lam controls).

-aged and was significantly higher at 3 dpi in aged mice compared to adult mice (P < 0.05). Therefore, microglial IL-4Rα induction was impaired with age, whereas IL-4 and IL-13 levels were maintained or increased in the aged compared to adults after SCI.
Diminished presence of IL-4Rα+ myeloid cells in the spinal cord of aged mice after injury

A hallmark of SCI is the active recruitment of peripheral myeloid cells (i.e., granulocytes, monocytes) to the injury site (Popovich et al., 1997; Shechter et al., 2009; Shechter et al., 2013; Sroga et al., 2003). As these cells may also express IL-4Rα, IL-4Rα expression was determined on Percoll-enriched granulocytes (CD11b+/CD45hi/GR-1hi) and monocytes/macrophages (CD11b+/CD45hi/GR-1lo) collected from the spinal cord 1 and 3 dpi. The flow cytometric gating strategy used to determine myeloid cell types is illustrated in Fig.4.2A. As monocytes and macrophages were not differentiated from one another, they are described here as macrophages. Fig.4.2B shows that the relative number of GR1hi granulocytes was increased 1 dpi (P < 0.0001) and 3 dpi (P < 0.0001) independent of age. Moreover, GR-1hi granulocytes did not express high levels of IL-4Rα (Fig.4.2C). Indeed, >95% of the IL-4Rα+, peripherally-derived cells recovered from the spinal cord were GR-1lo (macrophages) (data not shown). Evaluation of the total relative number of IL-4Rα+ myeloid cells in the spinal cord (granulocytes, macrophages, and microglia) revealed a significant interaction between age and SCI (F(2,33)=4.83, P < 0.02). For example, there was a robust increase in the number of IL-4Rα+ cells in the injured spinal cord of adult mice 1 dpi (p<0.01) and 3 dpi (p=0.09), but the number of IL-4Rα+ cells was unchanged in aged mice at either time point (Fig.4.2C). Of the IL-4Rα+ cells after SCI, ~70-75% were microglia and ~25-30% were macrophages (Fig.4.2C).

Next, we determined if the proportion of macrophages recruited or maintained in the injured spinal cord differed with age. At 1 dpi the total relative number of macrophages (both IL-4Rα- and IL-4Rα+) in the spinal cord of aged mice was increased compared to adult mice (P < 0.04, Fig.4.2D). Despite this increase in macrophage number, the proportion of IL-4Rα+ macrophages was reduced in aged mice. For example, the percentage of IL-4Rα+ macrophages to total macrophages increased in the
injured spinal cord of adults from ~18% at baseline, to >40% at 1 and 3 dpi (P < 0.0002). In contrast, the percentage of IL-4Rα+ macrophages was reduced in aged mice after SCI from 35% at baseline down to <15% by 3 dpi (P < 0.01, Fig.4.2E). Collectively, these data indicate that impaired induction of IL-4Rα after SCI in aged mice occurred in both microglia and peripherally-derived macrophages.
Figure 4.2. Recruitment of IL-4Rα+ cells to the injured spinal cord was impaired in aged mice. Adult (3-4 mo) and aged (18-22 mo) female BALB/c mice were subjected to a T9 Lam or SCI. After 1 or 3 dpi, enriched CD11b+ cells from the spinal cord were collected by Percoll gradient separation. (A) Representative dot plots of CD11b/CD45 and CD11b/GR-1 labeling for microglia (CD11b+/CD45lo) and peripherally-derived granulocytes (CD11b+/CD45hi/GR-1hi) and macrophages (CD11b+/CD45hi/GR-1lo). (B) Relative number of granulocytes associated with the spinal cord out of 10,000 live cells (n=4-5). (C) Relative number of IL-4Rα+ granulocytes, microglia, and monocytes associated with the spinal cord out of 20,000 live cells (n=4-5). (D) Relative number of IL-4Rα+ and IL-4Rα- macrophages associated with the spinal cord out of 10,000 live cells (n=4-5). (E) Percentage of IL-4Rα+ macrophages of the total monocyte/macrophage population associated with the spinal cord (n=4-5). Bars represent the mean ± SEM. (ANOVA: *, P < 0.01 compared to Lam controls, #, P < 0.04 compared to SCI-Adult 1 dpi, +, P = 0.09 compared to age-matched Lam control).
Enhanced IL-1β and CCL2 mRNA expression in adult mice after SCI

Because myeloid cell recruitment may be affected by age and SCI, the mRNA expression of two key mediators of myeloid cell recruitment (CCL2 and IL-1β) were measured in the spinal cord proximal to the injury epicenter. In these experiments, adult WT, aged WT, and adult IL-4RαKO BALB/c mice were used. Fig.4.3A&B show there was a main effect of SCI on the induction of CCR2 and IL-1β mRNA (P < 0.05 for each). These increases in CCR2 and IL-1β after SCI were time and age dependent. For example, IL-1β mRNA was induced in the spinal cord 1 dpi (P < 0.0001), but was higher in the spinal cords of adult mice compared to aged mice (Age×SCI: F(1,23)=14.95, P < 0.001, Fig.4.3A). Similarly, adult mice had exaggerated expression of CCL2 mRNA 1 dpi compared to aged mice (Age×SCI: F(1,23)=6.03, P < 0.03, Fig.4.3B). By 3 dpi, however, IL-1β and CCL2 mRNA expression levels were not different between age groups. A similar profile of IL-1β and CCL2 mRNA expression was detected in IL-4RαKO mice compared to WT controls after SCI. For instance, IL-1β mRNA expression was reduced in IL-4RαKO mice at 1 dpi compared to WT mice (P = 0.09, Fig.4.3C) and CCL2 mRNA was significantly reduced in IL-4RαKO mice compared to WT mice by 3 dpi (KO×SCI: F(1,23)=29.39, P < 0.0001, Fig.4.3D). Taken together, increased expression of IL-1β and CCL2 in the injured spinal cord was associated with functional IL-4Rα signaling.
Arginase protein expression is reduced in the injured spinal cord of aged mice

Here we show that IL-4Rα induction on microglia was impaired in aged mice after SCI. Our previous studies indicate that a lack of IL-4Rα induction after an inflammatory
response results in reduced IL-4-mediated arginase production (Fenn et al., 2012). Therefore, arginase, a key marker of M2a or alternative-activated microglia/macrophages was determined in adult WT, aged WT, and adult IL-4RαKO mice 7 dpi. This time point (7 dpi) represents the peak of arginase expression after SCI in adult mice (Kigerl et al., 2009). Fig.4.4A&B show representative labeling of arginase and Tomato-lectin (TomL) in adult WT, aged WT and adult IL-4RαKO mice after SCI. Consistent with previous work (Kigerl et al., 2009; Sroga et al., 2003), a dense accumulation of TomL+ microglia/macrophages was evident in the epicenter of injured adult and aged spinal cord and that arginase protein co-localized specifically with TomL+ microglia/macrophages (Fig.4.4B).

While arginase protein expression was increased in microglia/macrophages after SCI, this increase was dependent on age (tendency for SCI×Age: F(1,15)=3.40, P = 0.09). Arginase expression was reduced in the spinal cord of Aged-SCI mice compared to Adult-SCI mice (P < 0.009). A similar pattern of reduced arginase expression was evident after SCI in IL-4RαKO mice compared to WT mice (P < 0.02, Fig.4.4D). It is important to note that there was appreciable arginase induction in microglia/macrophages in the injured cord of IL-4RαKO mice. Thus, SCI induces both IL-4Rα-dependent and IL-4Rα-independent arginase expression. Nonetheless, these data indicate that IL-4Rα-dependent arginase induction was significantly reduced in myeloid cells of aged mice 7 dpi.
Aged mice have impaired functional recovery and extended lesion length after SCI

Reduced arginase expression in the injured spinal cord of aged mice may be associated with deficiencies in endogenous repair and worse functional recovery after SCI. To test this hypothesis, adult and aged mice were subjected to a laminectomy or SCI and functional recovery was assessed over a 28 day period using the Basso Mouse.
Scale (BMS) (Basso et al., 2006). Fig.4.5A shows the BMS results of sham and SCI mice at baseline and 1, 3, 5, 7, 14, 21 and 28 days later. Age alone did not influence the BMS scoring parameters of the uninjured mice as Adult-Lam and Aged-Lam controls had similar scores over the 28 day testing period. SCI, however, caused a significant reduction in locomotor function (F(1,153)=2870, P < 0.0001) that was dependent on time (SCI×Time: F(6,153)=19.57, P < 0.0001) and age (Age×SCI: F(1,153)=12.96, P < 0.0001). For example, both Adult-SCI and Aged-SCI mice had improved motor function over the 28 day time period compared to 1 dpi. Nonetheless, spontaneous recovery was significantly reduced in aged mice compared to adult mice at 1, 3, 5, 7, 21, and 28 dpi (P < 0.03 for each).

A significant functional milestone in recovery from SCI is the ability to achieve frequent or consistent bilateral weight-supported stepping. Mice that achieved a BMS score =5 have achieved this recovery milestone. Fig.4.5B shows that ~75% of the Adult-SCI mice achieved a BMS score of 5 by 28 dpi, but none of the Aged-SCI mice reached this level of recovery (P < 0.0001). Aged mice averaged a BMS score of 3 associated with hind limb plantar placement, but without stepping.

After the completion of locomotor function testing at 28 dpi, the percentage of tissue sparing and total lesion length was determined using EC/neurofilament staining (Donnelly et al., 2011). Although tissue sparing at the lesion epicenter was reduced by 10% in aged spinal cords, this was not significantly different from adults (Fig.4.5C). Lesion length, however, was 38% longer in Aged-SCI mice compared to Adult-SCI mice (P = 0.06, Fig.4.5D&E). Indeed, 1.2 mm rostral and caudal of the epicenter Aged-SCI tissue still showed evidence of a lesion, whereas the lesion was not present in Adult-SCI mice at this distance (Fig.4.5D). Taken together, aged mice had reduced functional recovery and an extended lesion size compared to adult mice following SCI.
IL-4Rα induction on activated microglia is required for IL-4-dependent arginase expression

Our data indicate that aged mice have reduced functional recovery after SCI associated with impaired microglial induction of IL-4Rα. This age-related deficit in IL-4Rα...
after SCI corresponded with attenuated arginase expression, differential cytokine and chemokine expression, and reduced presence of IL-4Rα+ macrophages in the injured spinal cord. Nonetheless, it is difficult to understand the significance of enhanced or impaired IL-4/IL-4Rα signaling in microglia after SCI in vivo because the onset and nature of the inflammatory stimulus is not known and various cross-signaling mechanisms likely control the magnitude and effect of inflammation. Therefore, we moved to an immune-based model in which the inflammatory stimulus (LPS) and delivery of IL-4 could be better controlled.

Mice were surgically implanted with an indwelling cannula in the lateral ventricle and allowed to recover for at least 7 d. After 7 d, mice were given an intraperitoneal (i.p.) injection of saline or LPS. After 1 h, mice were then given an intracerebroventricular (i.c.v.) injection of vehicle or IL-4 and arginase expression was assessed after 24 h. Fig.4.6A shows that IL-4Rα expression was upregulated on microglia 24 h after LPS in both the spinal cord (P < 0.05) and the brain (P < 0.05) to a similar extent. Because cannulas for central IL-4 infusion can be better established in the brain compared to the spinal cord, we investigated IL-4/IL-4Rα-induced M2a responses in brain resident microglia. Fig.4.6B shows that i.c.v administration of IL-4 increased mRNA expression of arginase (F(1, 32)= 4.5, P < 0.05) and this induction was augmented over 160-fold in mice that were pre-treated with LPS (LPS×IL-4: F(1, 32)=4.4, P < 0.05). Peripheral injection of LPS alone, however, did not increase arginase mRNA expression.

To confirm these mRNA results and to determine the extent to which the induction of arginase was microglia specific, arginase protein was determined in brain-resident microglia (Iba-1+) proximal to the i.c.v injection site (Fig.4.6C). Representative images of Iba-1 (i., green) and arginase (ii., Arg, red) labeling are shown from the four experimental groups (Fig.4.6D). Arginase protein was not present in the brain of the
control mice (Saline-Vehicle) or mice injected with just LPS (LPS-Vehicle) (Fig.4.6 D&F). Although a low level of arginase was induced by IL-4 (Saline-IL-4) consistent with the mRNA data (Fig.4.6B), this was not significantly different from control mice. Arginase protein, however, was markedly increased in the brain of mice that received both peripheral LPS and i.c.v. IL-4 (LPS×IL-4: F(1,14)=23.72, P < 0.0005, Fig.4.6D&F). Moreover, this robust induction of arginase was detected specifically in Iba-1+ microglia (Fig.4.6D-iv., Fig.4.6E). These data support the hypothesis that the upregulation of IL-4Rα on the surface of active microglia was key for their ability to respond IL-4 and induce arginase expression.

In the SCI model, we showed that Aged-SCI mice had deficits in microglial IL-4Rα induction with corresponding impairments in arginase protein expression. Therefore, the same immune-based protocol as above was also performed in a subset of adult and aged mice to assess arginase protein expression. Representative images of the Adult-LPS-IL-4 group and Aged-LPS-IL-4 group are shown (Fig.4.6G). Because we observed higher baseline arginase labeling in Aged-Saline-IL-4 controls compared to Adult-Saline-IL-4 controls (data not shown), changes in arginase labeling in LPS-IL-4 mice were compared to age-matched Saline-IL-4 controls. Fig.4.6H shows that IL-4 mediated re-direction of active microglia to an arginase+ phenotype was attenuated by 79% in the brain of aged mice compared to adults. These data indicate that redirection of activated microglia by IL-4 was impaired with age. Thus, a robust induction of arginase in vivo required 1) an inflammatory/activating signal and 2) responsiveness to an M2a re-direction signal.
Figure 4.6. IL-4Rα induction on activated microglia was required for IL-4-dependent arginase expression. Adult (3 mo) male BALB/c mice received an i.p. injection of saline or LPS and 1 h later received an i.c.v. injection of vehicle or IL-4. Brains and spinal cords were collected 24 h after LPS. (A) Average percent of IL-4Rα+ microglia 24 h after LPS in the brain and spinal cord by flow cytometry (n=2-3). (B) mRNA expression of arginase (Arg) from a 1 mm coronal section at the i.c.v. injection site (n=8-18). (C) Schematic of the area where images were collected for Iba-1/Arg labeling. (D) Representative images of Iba-1 (i.), Arg (ii.), and Iba-1/Arginse (iii.) labeling. White bars, 100 μm. White arrow indicates microglia used for higher magnification in (iv.). White bar, 25 μm. (E) Representative image for orthogonal analysis of colocalization from a z-stack image for Iba-1 (green)/Arg (red) labeling. (F) Percent Iba-1+/Arg+ microglia (n=3-4). (G) Adult (3 mo) and aged (20 mo) male BALB/c mice were given Sal/LPS and Vehicle/IL-4 injections as above. Brains were collected 24 h after LPS. Representative images for Arg staining. White bar, 50 μm. (H) Percent increase in Arg staining for LPS-IL-4 mice compared to age-matched Saline-IL-4 controls (n=3). Bars represent the mean ± SEM. (ANOVA: *, P < 0.05 compared to Saline control, **, P < 0.01 compared to Saline-Vehicle, ***, P < 0.0001 compared to Saline-Vehicle, #, P < 0.01 compared to Saline-IL-4).
Figure 4.6

A. % IL-4Rα+ Microglia

B. Arg Brain mRNA (Fold Change)

C. % IL-4Rα+ Microglia

D. Immunofluorescence images showing Iba-1, Arginase, and Iba-1/Arg staining in different groups:
   - Saline
   - LPS
   - IL-4

E. % Arg+ Microglia

F. % Increase Arg (vs SI)

G. Adult vs Aged

H. % Increase Arg (vs SI)
IL-4 mediated re-direction of activated microglia supports neurite outgrowth

Previous studies show that M2a microglia/macrophages (arginase+) promote axon growth in vitro and enhance remyelination in vivo (Kigerl et al., 2009). Whether or not a similar repair potential is elicited in microglia primed by an inflammatory stimulus has not been tested. Therefore, axon growth was determined in adult dorsal root ganglion (DRG) neurons co-cultured with inflammatory-activated microglia, with or without reprogramming with IL-4. Microglia from LPS- or saline-injected adult mice were collected from the brain 4 h after injection and cultured ex vivo in transwell inserts placed over adult DRG neurons. Co-cultures were treated with either vehicle or IL-4 and neurite outgrowth was measured 24 h later. Fig.4.7A shows representative images from the four different experimental groups. There was a significant interaction between microglia activation and IL-4 stimulation on DRG neurite outgrowth (LPS×IL-4, F(1,20)=4.77, P < 0.05). For instance, DRG neurite threshold was reduced by IL-4 when neurons were co-cultured with microglia from saline-injected mice (Saline-IL-4, P < 0.03). DRG axon was unaffected by the addition of active microglia alone (LPS-Vehicle). Neurite threshold staining was highest, however, when DRG neurons were co-cultured with active microglia re-directed with IL-4 compared to all other treatment groups (Fig.4.7B, P = 0.1). We interpret these data to indicate that IL-4 mediated re-direction of activated microglia represented a profile that supported neurite outgrowth and complexity.

L.c.v. Infusion of IL-4 following microglial activation augments mRNA expression of IL-1β and CCL2

We show that there is differential distribution of macrophages in the aged after SCI that was associated with impaired IL-4Rα induction on microglia, reduced arginase expression, and reduced mRNA expression of IL-1β and CCL2. The LPS model
confirmed that IL-4-mediated reprogramming of inflammatory microglia induces robust arginase expression similar to that observed after SCI. Therefore, IL-1β and CCL2 mRNA were measured in a coronal brain section 24 h after LPS and IL-4 treatments. Consistent with data from the SCI model, mRNA expression of IL-1β was increased 24 h after LPS compared to saline controls (P = 0.08, Fig.4.8A). IL-1β mRNA expression, however, was further enhanced by i.c.v. IL-4 (LPS×IL-4, F(1,44)=5.40, P < 0.03). A similar pattern was evident for brain CCL2 in that injection with peripheral LPS and i.c.v. IL-4 resulted in enhanced mRNA expression (LPS×IL-4: F(1,35)=11.17, P < 0.003, Fig.4.8B). Taken together, IL-4 mediated re-direction of activated microglia enhanced expression of IL-1β and CCL2 compared to LPS or IL-4 alone.
IL-4 mediated re-direction of activated microglia increases the recruitment of CCR2+ macrophages to the brain

We show there is increased mRNA expression of IL-1β and CCL2 1 dpi in the spinal cord of adult mice compared to aged mice (Fig.4.3). This is important because these are two critical factors associated with recruitment of macrophages to the CNS. Increased IL-1β and CCL2 1 dpi in the spinal cord of adult mice corresponded to increased numbers of IL-4Rα+ macrophages compared to aged mice (Fig.4.2). In the LPS model, IL-4 re-direction of activated microglia also augmented mRNA expression of IL-1β and CCL2 (Fig.4.8). Therefore, we sought to determine the extent to which IL-4 mediated re-direction of activated microglia increased the recruitment of macrophages to...
the brain. First, we confirmed that IL-4Rα was induced on Percoll-enriched microglia in WT C57BL/6 mice after LPS and was not affected by i.c.v. IL-4 injection (Fig.4.9). Second, the relative proportion of microglia and brain-associated macrophages was determined after i.p. LPS injection and i.c.v. IL-4 injection. Representative bivariate dot plots of CD11b and CD45 labeling of Percoll-isolated microglia and macrophages collected from the brain of each treatment group are shown (Fig.4.10A). Fig.4.10B shows that the highest percentage of brain-associated macrophages occurred in mice with activated microglia re-directed by i.c.v. IL-4 (LPS-IL-4, P < 0.04). Next, the phenotype of recruited macrophages was investigated. Fig.4.10C shows that treatment with LPS and i.c.v. IL-4 enhanced trafficking of CCR2+ macrophages to the brain (P < 0.03). These CCR2+ cells showed an increase in IL-4Rα expression after LPS treatment from ~5% at baseline to over 20% IL-4Rα+ expression (Fig.4.10D). In fact, ~60% of all IL-4Rα+ macrophages were also CCR2+ (data not shown).

To confirm that these macrophages were BM-derived and to determine their neuro-anatomical distribution, GFP BM chimera mice on a C57BL/6 background were established (Wohleb et al., 2013). The presence of BM-derived cells in the brain was determined 24 h after microglia activation and IL-4 re-direction. First, flow cytometric analysis confirmed that the increased population of CD45+ cells after LPS were peripherally derived (Fig.4.11). Next, the neuro-anatomical distribution and M2a phenotype (arginase+) was examined in close proximity to the icv injection site. Fig.4.10E shows representative images of GFP+ cells in the injection site 24 after LPS in LPS-Vehicle and LPS-IL-4 treatment groups. These sections were also labeled to identify Iba-1+ parenchymal microglia/macrophages and arginase+ cells. Histological analysis confirmed our flow cytometric studies and revealed that trafficking of BM-derived cells to the brain was significantly increased in LPS-IL-4 treated mice in the
Figure 4.9. IL-4Rα was upregulated 24 h after LPS on microglia of C57BL/6 mice. Adult (3 mo) male C57BL/6 mice were given an i.p. injection of saline or LPS and 1 h later received an i.c.v. injection of vehicle or IL-4. Enriched CD11b+ cells were isolated from the brain 24 h after LPS by Percoll gradient separation. Cells were stained for CD11b/CD45/IL-4Rα for flow cytometry. Percent of IL-4Rα+ microglia (n=3-6). Bars represent the mean ± SEM. (ANOVA: *, P < 0.03 compared to saline-Vehicle).
Figure 4.10. IL-4 mediated re-direction of activated microglia increased the recruitment of CCR2+ macrophages to the brain. Adult (3 mo) male C57BL/6 mice were given an i.p. injection of saline or LPS and 1 h later received an i.c.v. injection of vehicle or IL-4. Enriched CD11b+ cells were isolated from the brain 24 h after LPS by Percoll gradient separation. (A) Representative dot plots for CD11b/CD45 staining. Oval indicates peripherally-derived macrophages (CD11b+/CD45hi). (B) Percentage of CD11b+/CD45hi macrophages associated with the brain (n=4-7). (C) Relative number of CCR2+ macrophages associated with the brain out of 10,000 live cells (n=4-7). (D) Percentage of CCR2+ macrophages that were IL-4Rα+ (n=3). (E) Partial GFP-bone marrow (BM) chimera mice were established on the C57BL/6 background. GFP-BM chimera mice were injected with i.p. LPS and i.c.v. IL-4 as above and perfused 24 h later with 0.1 M PBS and 4% formaldehyde. Representative images showing GFP (i.), GFP/Arg (ii.), and GFP/Arg/Iba-1 (iii.) staining. White bars, 100 μm. White arrows indicate GFP+ or GFP+/Iba-1+ cells used for insets in (ii., iv., v.), and white arrowheads indicate Arg+ colocalization with GFP+, Iba-1+, or GFP+/Iba1+ cells used for insets in (ii., iv., v.). White bars, 25 μm. (F) Number of GFP+ cells per section at the i.c.v. injection site and nearby choroid plexus (n=4-5). (G) Percent Arg+ cells that were GFP+, Iba-1+, and GFP/Iba-1+ in the LPS-IL-4 group. Bars represent the mean ± SEM. (ANOVA: *, P < 0.04 compared to Saline-Vehicle, #, P < 0.04 compared to LPS-Vehicle, +, P = 0.07 compared to Saline-Vehicle, ‡, P = 0.10 compared to Saline-IL-4).
Figure 4.10

A. 
Saline - Vehicle  Saline - IL-4  LPS - Vehicle  LPS - IL-4

B. 
% Macrophages

C. 
Relative Number of CCR2+ Macrophages

D. 
% CCR2+ = IL-4Rα

E. 
GFP  GFP/Arg  GFP/Arg/Iba1

LPS Vehicle

i.  ii.  iii.

LPS IL-4

iv.  v.

F. 
GFP Cells (# / section)

G. 
% Arg+ Cells
Figure 4.11. CD11b+/CD45high cells were peripherally derived. Partial GFP-bone marrow chimera mice were established on the C57BL/6 background. Mice achieved an average of 60-80% engraftment. After 4 weeks mice were injected with saline or LPS. Brains were collected after 24 h and enriched CD11b+ cells were isolated. Representative dot plot of CD11b/CD45 staining used for percent GFP+ analysis. Over 80% of the cells designated as macrophages were GFP+ indicating they derived from a peripheral source.

Discussion

There is significant interest in understanding the specific immune profiles of microglia and macrophages, especially in the context of traumatic CNS injury and aging. The primary aim of this study was to test the hypothesis that impaired M2a regulation of microglia in the aged CNS results in an attenuated microglial response and worse functional outcome after SCI. A secondary aim was to better understand the role of alternative activated microglia/macrophages \textit{in vivo} in the context of injury and aging. Our previous work elucidated that the receptor for IL-4, IL-4Rα, was markedly upregulated on microglia of adult, but not aged mice following a peripheral LPS challenge (Fenn et al., 2012). Of consequence, little to no IL-4 or IL-13 was present in the CNS under these conditions (Fenn et al., 2012; Lovett-Racke et al., 2000) and no arginase/M2a microglia phenotype was observed. Nonetheless, LPS-activated microglia from adult mice were more responsive to IL-4 treatment \textit{ex vivo} (Fenn et al., 2012). Therefore, we extended these findings into two models in which elevated IL-4 is present in the inflammatory CNS: SCI and i.p LPS/i.c.v. IL-4 injection. We show that after SCI,
IL-4Rα was strongly upregulated on microglia of adult, but not aged mice. Upregulated IL-4Rα corresponded with increased association of IL-4Rα+ macrophages with the spinal cord, increased arginase expression 7 dpi, and improved functional recovery compared to aged mice. Our in vivo i.p. LPS/i.c.v. IL-4 injection studies further showed that inflammatory-induced IL-4Rα expression was required for IL-4-driven arginase responses in microglia. Moreover, i.c.v. IL-4 injection into the inflammatory CNS enhanced expression of inflammatory mediators (IL-1β, CCL2) creating a unique microglia/macrophage phenotype (arginase+/IL-1β+) that supported neurite growth and enhanced the recruitment of peripheral monocytes to the CNS.

One novel aspect of this study was that aged mice had significantly worse functional recovery at both early (1-7 dpi) and later (21-28 dpi) time points after a moderate spinal cord contusion injury compared to adult mice. For example, ~75% of adult mice had frequent or consistent stepping with no coordination (average BMS score = 5) by 28 dpi, whereas 0% of aged mice achieved this score. Instead, ~75% of aged mice achieved an average BMS score of only 3 by 28 dpi (plantar placing the paw with or without weight support OR dorsal stepping with no plantar stepping). Consistent with a worse BMS score, lesion length was longer 28 dpi in aged mice compared to adults. These data are clinically relevant because aged persons (60+ years of age) have increased morbidity/mortality after SCI (Chen et al., 1997; Fassett et al., 2007; Injury-Statistical-Center, 2013). Our data are consistent with previous studies demonstrating increased pathology and demyelination in middle-aged (12 mo) rats after a spinal cord contusion injury (Siegenthaler et al., 2008) and worse functional recovery in middle-aged (12 mo) rats after hemisection (Gwak et al., 2004b). We further extended these studies into evaluation of a mouse model of advanced age (18-19 mo).
A potential mechanism for increased morbidity after SCI in the aged is reduced responsiveness to IL-4 by microglia/macrophages. We focused on IL-4 rather than IL-13 because both adult and aged mice had increased IL-4 mRNA expression after SCI, whereas IL-13 was reduced acutely after injury. In support of an IL-4-dependent mechanism, there was a failure to induce microglial IL-4Rα expression in the spinal cord of aged mice compared to adults (1 – 3 dpi). This corresponded with significantly altered distribution of IL-4Rα on macrophages 3 dpi and reduced percentage of arginase+ myeloid cells in aged mice 7 dpi. Based on our previous studies, we interpret the increase of IL-4Rα on microglia as an indication that these cells will show higher responsiveness to the two primary ligands of IL-4Rα, IL-4 and IL-13. IL-4 mRNA expression was increased acutely after injury consistent with other studies (Guerrero et al., 2012; Lee et al., 2010). Although we detected increased IL-4 mRNA at 3 dpi, other studies detected IL-4 protein from microglia as early as 1 dpi (Guerrero et al., 2012) corresponding with increased IL-4Rα expression. Importantly, there was no difference in IL-4 mRNA induction between adult and aged mice. Thus, a reduction in IL-4Rα expression, rather than reduced levels of IL-4, corresponded with less arginase+ myeloid cells in the spinal cord 7 dpi in aged mice. This is consistent with previous results showing that aged mice had reduced IL-4 and IL-13-mediated signaling in vivo and were unable to upregulate IL-4/IL-13 response genes to the same degree as adult mice (Lee et al., 2013). Reduced arginase expression after SCI was paralleled in adult IL-4Rα knockout (IL-4RαKO) mice which had significantly less arginase in the spinal cord 7 dpi compared to adult wild type (WT) mice. It is important to mention that while arginase expression was reduced in the spinal cords of aged and IL-4RαKO mice, it was not ablated. Thus, SCI induced both IL-4Rα-dependent and IL-4Rα-independent arginase expression. Nonetheless, reduced arginase in the spinal cord of aged and IL-4RαKO mice
indicated an impaired ability to promote an IL-4Rα-dependent M2a phenotype. This M2a profile is relevant because an IL-4 or IL-13-directed M2a response (Mantovani et al., 2004) is associated with the support of intrinsic wound healing, angiogenesis, and tissue repair processes (Derecki et al., 2011; Fukushi et al., 2000; Gordon, 2003; Kigerl et al., 2009; Liao et al., 2012; Nahrendorf et al., 2007).

Another important aspect of this study is that we extended investigations of the IL-4-driven M2a phenotype in vivo. Most studies have investigated IL-4-driven M2a responses using in vitro culture systems and reported that IL-4 promotes arginase and growth factor expression while reducing inflammatory mediators including IL-1β and TNF-α (Butovsky et al., 2006; Kitamura et al., 2000). These studies, however, used pre-treatment of microglia and macrophages with IL-4 or IL-13. In the context of in vivo CNS injury, however, IL-4-mediated signaling would occur after inflammation had been established. Therefore, we modeled this with immune based studies in which microglia were first activated by a peripheral LPS injection, and then treated with IL-4 by i.c.v. injection. In both the SCI and LPS model, IL-4-mediated redirection of activated microglia resulted in increased expression of arginase, but also increased expression of the inflammatory mediators IL-1β and CCL2 in microglia or brain/spinal cord samples. In contrast, reduced IL-4Rα expression (i.e., aged, IL-4RαKO mice) corresponded with less arginase, IL-1β, and CCL2. Thus, these studies may represent a true “alternative activation” phenotype that exists in vivo. Although this phenotype is more inflammatory, it appears critical for restricting pathology. For example, intact IL-4 signaling in Adult-SCI mice corresponded to reduced tissue lesion length compared to Aged-SCI. Furthermore, in our DRG neuronal culture model, neurite growth and complexity was promoted by co-culture with LPS-activated microglia treated with IL-4. This result is consistent with the only other study using post-treatment with IL-4 in which LPS-activated microglia post-
treated with IL-4 showed marked reductions in nitric oxide levels and enhanced survival of motor neurons compared to pre-treatment with IL-4 (Zhao et al., 2006). Although here IL-4 alone reduced total neurite threshold, this does not necessarily contradict previous studies showing that conditioned media from IL-4-treated bone marrow-derived macrophages resulted in productive neurite growth (Gensel et al., 2009; Kigerl et al., 2009). Here we only looked at total neurite threshold levels (growth + complexity), whereas previous studies used Scholl analysis to investigate total neurite length. Therefore, IL-4 alone could have still promoted neurite growth without increasing the complexity. Nonetheless, the combined LPS-IL-4 treatments created an environment permissive to the development of neurite growth and complexity.

Increased neurite growth in the presence of increased inflammation was consistent with recent studies examining the role of TLR2-mediated activation, and minocycline-induced deactivation, of microglia/macrophages in regulating neurite development and axonal dieback. Indeed, when DRG neurons were cultured with conditioned media from bone marrow derived macrophages treated with zymosan (TLR-2/dectin-1 agonist), neurite growth was significantly increased over baseline (Gensel et al., 2009). In a model of laser-induced SCI, stimulating the TLR2 pathway with a specific agonist (Pam2CSK4) resulted in exaggerated production of inflammatory mediators (IL-1β, CCL2, iNOS) and protected against axonal dieback (Stirling et al., 2013). Moreover, TLR2KO mice had attenuated inflammatory responses and showed worse functional recovery after a contusive SCI (Kigerl et al., 2007; Stirling et al., 2013). In contrast, minocycline-induced deactivation of macrophages resulted in reduced inflammation after sciatic nerve injury and impaired axon regeneration (Kwon et al., 2013). Thus, an intact inflammatory response by microglia may be protective in the context of SCI.
Increased inflammation following an intact IL-4 response may be beneficial in recruiting the correct macrophage population to the spinal cord after injury. Indeed, functional IL-4/IL-4Rα signaling corresponded with increased association of IL-4Rα+ monocytes to the spinal cord in Adult-SCI mice. In contrast, the injured spinal cord of aged mice was dominated by IL-4Rα− macrophages. This phenomenon was further explored in our LPS model where IL-4-mediated redirection of activated microglia increased expression of CCL2 corresponding with increased recruitment of CCR2+/IL-4Rα+ macrophages to the brain. This phenotype may be more beneficial for repair as CCR2+/CX3CR1− monocytes were characterized as iNOSlo and associated with less pathology after injury (Donnelly et al., 2011). Moreover, depletion of CCL2 after SCI is associated with impaired recovery (Shechter et al., 2009). A possible explanation for increased recruitment of CCR2+ macrophages to the CNS after IL-4 treatment is elevated arginase expression. Arginase is the rate-limiting enzyme for the production of polyamines, a necessary factor for CCL2 induction and macrophage recruitment to the brain (Puntambekar et al., 2011).

Critically, in the present study peripherally-derived GFP+ macrophages had the potential to migrate into the brain parenchyma and switch to an arginase+ phenotype in the presence of IL-4. The ability of peripherally derived macrophages to adopt this arginase+ phenotype after LPS-IL-4 treatment is important in the context of repair after SCI because other models demonstrate that IL-4Rα+/arginase+ myeloid cells function as myeloid derived suppressor cells (MDSCs) (Bronte et al., 2003; Kohanbash et al., 2013; Pesce et al., 2009). Although intact IL-4 signaling was initially associated with increased inflammatory markers, it is possible that the presence of these arginase+ myeloid cells would promote a micro-environment permissive to repair. Support for this idea is evident in a recent study that eliminated the trafficking of MDSCs after SCI and observed worse
functional recovery corresponding with reduced levels of inflammatory (IL-1β), anti-inflammatory (IL-10), and alternative activation (arginase) genes (Saiwai et al., 2013). Moreover, these MDSCs may prevent a robust T-cell response to myelin antigens, a response that is detrimental to SCI and may allow chronic inflammation to persist (Jones et al., 2002). Further studies will be needed to determine if IL-4Rα+ myeloid cells functionally suppress T-cell responses after SCI.

In summary, these results propose a novel cellular mechanism by which inflammatory-induced IL-4Rα promotes increased sensitivity to IL-4 resulting in elevated arginase, IL-1β, and CCL2 expression and the recruitment of CCR2+/IL-4Rα+ cells to the CNS to benefit recovery after SCI. Taken together, our results demonstrate a novel role for IL-4 signaling in the CNS and provide insight into the function of alternative activated microglia in neuroprotection and macrophage recruitment. These data provide an innovative cellular perspective on the pathophysiology of age-related deficits after CNS trauma.
Chapter 5: Immune activation promotes depression one month after diffuse brain injury: a role for primed microglia

Abstract

Traumatic brain injury (TBI) is associated with a higher incidence of depression. The majority of individuals who suffer a TBI are juveniles and young adults and thus, the risk of a lifetime of depressive complications is a significant concern. The etiology of increased TBI-associated depression is unclear, but may be inflammatory-related with increased brain sensitivity to secondary inflammatory challenges (e.g., stressors, infection, and injury). Adult male BALB/c mice received a sham (n=52) or midline fluid percussion injury (TBI) (n=57). Neuroinflammation, motor coordination (rotarod), and depressive behaviors (social withdrawal, immobility in the tail suspension test, and anhedonia) were assessed 4 h, 24 h, 72 h, 7 d, or 30 d later. Moreover, 30 d after surgery, sham and TBI mice received a peripheral injection of saline or lipopolysaccharide (LPS) and microglia activation and behavior were determined. Diffuse TBI caused inflammation, peripheral cell recruitment, and microglia activation immediately after injury coinciding with motor coordination deficits. These transient events resolved within 7 d. Nonetheless, 30 days post-TBI a population of de-ramified and major histocompatibility complex (MHC)II+ (primed) microglia were detected. After a peripheral LPS challenge, the inflammatory cytokine response in primed microglia of TBI mice was exaggerated compared to microglia of controls. Furthermore, this LPS-induced microglia reactivity 30 d after TBI was associated with the onset of depressive-like
behavior. These results implicate a primed and immune-reactive microglial population as a possible triggering mechanism for the development of depressive complications after TBI.

Introduction

Traumatic brain injury (TBI) elicits immediate neuroinflammatory events that contribute to acute cognitive, motor, and behavioral disturbances (Lifshitz et al., 2007b; McCrea et al., 2003; Tang et al., 1997; Woodcock and Morganti-Kossmann, 2013). Despite resolution of these acute complications, depression can develop and persist years after TBI (Fleminger, 2008; Jorge et al., 1993; Kreutzer et al., 2001). Indeed, individuals who suffer a TBI are 5-10 times more likely to develop symptoms of depression compared to the general population (Gualtieri and Cox, 1991). Depressive symptoms are diagnosed in 30-40% of individuals within the first year of TBI (Jorge et al., 1993; Jorge et al., 2004), in 60% of individuals within 8 years of TBI (Hibbard et al., 1998), and 50 years after TBI patients continue to report higher rates of depression (Holsinger et al., 2002). Moreover, most (69%; CDC, 2002-2006) brain injuries occur in juveniles (34.7%, 0-14 years) and young adults (34.3%, 15-34 years) implicating an increased risk for a lifetime of depressive complications that negatively affect quality of life and life-span (Holsinger et al., 2002; Teasdale and Engberg, 2001). Furthermore, the limited number studies on anti-depressant therapies (e.g., amitriptyline, sertraline) after TBI show reduced efficacy in TBI patients (Ashman et al., 2009; Saran, 1988). We propose that TBI-associated depression is inflammatory-based and associated with increased brain sensitivity to acute immune challenges.

In support of this premise, clinical and experimental data indicate a cause/effect relationship between inflammation and depression (Miller and Raison, 2008; Raison et
Patients with higher inflammatory cytokine levels in circulation and within the central nervous system (CNS) report a higher incidence of treatment-resistant depression (Miller and Raison, 2008). These patients have elevated levels of the inflammatory cytokine interleukin (IL)-6 in circulation, and anti-depressant therapies fail to reduce tumor necrosis factor (TNF)α (Lanquillon et al., 2000). Critically, TBI patients have increased levels of IL-6, IL-1β, and TNFα in cerebrospinal fluid (Fassbender et al., 2000; Maier et al., 2001) and serum (Kossmann et al., 1995) immediately after injury. Moreover, markers of neuroinflammation (e.g., CD68, CR3/43) persist in the brain parenchyma up to 16 years after TBI (Gentleman et al., 2004). Although several studies report increased neuroinflammation after TBI and others report increased depression after TBI, the extent to which prolonged brain inflammation contributes to neurobehavioral complications after TBI is unclear.

One potential consequence of heightened and prolonged brain inflammation after TBI is increased sensitivity to secondary challenges including subsequent injuries, stressors, and infections (Barrientos et al., 2010). In models of aging, stress, early life infection, sterile CNS injury, and preclinical neurologic disease increased sensitivity to inflammatory challenges corresponds with the development of a primed and more inflammatory microglia phenotype (e.g., increased major histocompatibility complex II [MHCII], IL-1β, CD68, complement receptor [CR]3) (Bland et al., 2010; Cunningham et al., 2009; Henry et al., 2009; Palin et al., 2008; Wohleb et al., 2012). Microglia are the innate immune cells of the CNS and responsible for interpreting and propagating inflammatory signals that affect neuronal function (Jurgens and Johnson, 2012; Tremblay et al., 2011). Thus, enhanced microglial activation and amplified inflammatory cytokine production can impair normal neurologic function. In support of this idea, primed and MHCII+ microglia in the aged brain become hyper-reactive to a systemic
injection of lipopolysaccharide (LPS) and produce exaggerated levels of IL-1β (Henry et al., 2009) corresponding to impaired cognitive performance (Barrientos et al., 2009a; Barrientos et al., 2010; Chen et al., 2008), protracted sickness behavior (Godbout et al., 2005b; Huang et al., 2008), and depressive-like behavior (Godbout et al., 2008). Relevant to the axonal and neuronal damage done during TBI, models of optic nerve crush also demonstrate microglia priming (CD68+) and exaggerated IL-1β, TNFα, and IL-6 expression after LPS challenge 28 days post injury (dpi) (Palin et al., 2008). Although not discussed in the context of microglial priming, increased MHCII (OX6) expression has also been detected in the brain 16 dpi in a rat model of cerebral contusion (Holmin et al., 1995). Thus, a primed microglia phenotype after TBI may set the stage for exaggerated responses to acute challenges, precipitating the development of chronic neuropsychiatric disorders.

Based on these data, we hypothesize that a diffuse TBI induces microglial priming, and that an acute immune challenge weeks to months after injury results in a hyper-inflammatory microglia response triggering the development of depressive-like behavior. To test this hypothesis, the midline fluid percussion injury (FPI) model of TBI was used in mice. Midline FPI causes mild neuronal pathology including diffuse axonal injury (Lifshitz et al., 2007a) and transient neurological deficits (Morales et al., 2005) that recapitulate complications after mild to moderate concussive head injuries in humans (Lifshitz, 2009). Here, we show that TBI caused immediate, but transient, neuroinflammation and behavioral impairments. Nonetheless, evidence of microglial priming was detected in the brain 30 dpi. Furthermore, activation of the peripheral immune system 30 days after TBI caused exaggerated microglial expression of IL-1β and TNFα corresponding with induction of depressive-like behavior. Collectively, these
data support the premise that a diffuse TBI sensitizes the brain to secondary inflammatory challenges that may precipitate depression.

**Materials and Methods**

*Mice and LPS injections*

Adult (3 mo) male BALB/c mice were obtained from a breeding colony at The Ohio State University (OSU). Mice were individually housed and maintained at 25°C under a 12 h light/12 h dark cycle with *ad libitum* access to food and water. For injections, mice were intraperitoneally (i.p.) injected 30 dpi with saline or LPS (0.33 mg/kg; serotype 0127:B8, Sigma) 1-2 h before the start of the dark phase (between 1700 and 1900) (Fenn et al., 2012; Godbout et al., 2005b). All procedures were in accordance with the NIH Guidelines for the Care and Use of Laboratory Animals and were approved by the OSU Institutional Laboratory Animal Care and Use Committee.

*Midline fluid percussion injury*

Mice received a midline and diffuse TBI using a fluid percussion injury (FPI) apparatus (Custom Design & Fabrication) as previously described (Witgen et al., 2005) and detailed in Supplemental Materials and Methods. This diffuse injury is well characterized and occurs in the absence of contusion, tissue cavitation, or gross neuronal loss, and causes diffuse axonal injury in the neocortex, hippocampus, and dorsolateral thalamus (Bachstetter et al., 2013; Kelley et al., 2006; Kelley et al., 2007). Immediately after sham injury or TBI the injury hub was removed, dural integrity was confirmed, and mice were evaluated for injury severity using the self-righting test (Lifshitz et al., 2007b). Based on previous studies in FPI (Witgen et al., 2006), self-righting inclusion criteria was modified for BALB/c mice as follows: sham≤60 s; 60
s_mild<200 s; 200 s_moderate<540 s; severe>_540 s. Only mice with a moderate TBI were used.

*Motor function and depressive-like behavior:*

*Motor coordination* was assessed using rotarod (Rotamex) as previously described (Dunham and Miya, 1957) and detailed in the *Supplementary Materials and Methods*.

*Activity* was determined using an activity box paradigm (Open Field and Fusion software; AccuScan Instruments). Mice were placed into independent 8 x 8 inch chambers and automated software packaging was configured to determine the total distance traveled and total movement time for 10 min. A subset of mice was tested for 30 min and values were recorded in 10 min increments.

*Sickness and depressive-like behavior* were determined through un-motivated (locomotor) (Godbout et al., 2005b) and motivated (social exploratory behavior (Corona et al., 2010; Godbout et al., 2005b; Wohleb et al., 2012), TST (Corona et al., 2013; Godbout et al., 2008), sucrose preference (Henry et al., 2008)) behavioral tests as described in the *Supplemental Materials and Methods*. For the TST, the same mice were tested at both 7 dpi and 30 dpi, with another subset of mice tested only at 30 dpi. At 30 dpi, immobility between the two subsets was not significantly different and data were collapsed.

*Isolation of enriched brain CD11b*<sup>+</sup> *cells*

Enriched CD11b<sup>+</sup> cells (microglia/peripheral myeloid cells) were isolated from whole brain homogenates as previously described (Fenn et al., 2012). In brief, brains were homogenized and re-suspended in a discontinuous, isotonic Percoll gradient.
Microglia were collected from the interphase of the 70% and 50% Percoll layers. We have previously characterized these cells as approximately 90% CD11b+/CD45+ “enriched CD11b+” cells (Henry et al., 2009; Wohleb et al., 2012).

**RNA isolation and RT-PCR**

RNA was isolated from individual brain regions or enriched brain CD11b+ cells using the Tri-Reagent protocol (Sigma), or the PrepEase kit (USB), respectively. RNA concentration was determined and RNA was reverse transcribed to cDNA. Real time PCR (RT-PCR) was performed using the Applied Biosystems Taqman® Gene Expression assay using an ABI PRISM 7300-sequence detection system as previously described (Godbout et al., 2005b). Data were analyzed using the comparative threshold cycle (ddCt) method and results are expressed as fold difference from controls.

**Flow cytometry**

Enriched brain CD11b+ cells were assayed for surface antigens by flow cytometry as described (Fenn et al., 2012; Henry et al., 2009). In brief, cells were incubated with rat anti-mouse antibodies (eBioscience; CD11b-APC, CD45-PerCP-Cy5.5, and CD14-PE). Surface expression was determined using a Becton-Dickinson FACSCaliber four color Cytometer. Twenty thousand events were recorded and microglia (CD11b+/CD45low) and peripheral myeloid cells (PMCs) (CD11b+/CD45high) were identified by CD11b/CD45 expression (Wohleb et al., 2011). Gating was determined based on appropriate negative isotype controls. Data were analyzed using FlowJo software (Tree Star).
**Immunohistochemistry**

Fluorescent staining for glial fibrillary acidic protein (GFAP) and ionized binding association protein (Iba)-1 was performed as described in Supplemental Materials and Methods. Threshold staining was determined using NIH ImageJ and quantification was assessed for each image using digital image analysis (DIA) (Donnelly et al., 2009). Cell counts and cell body size were assessed using Metamorph Image (Metamorph Offline, 6.1) analysis. Results are reported as the average percent area for GFAP+ and Iba-1+ staining, number of cells / image, and average cell body size (μm²).

**Statistical Analysis**

Data were subjected to Shapiro-Wilk test using Statistical Analysis Systems (SAS) software. To determine significant main effects and interactions between main factors, data were analyzed using one-, two-, or three-way ANOVA. Body mass data were also assessed using repeated measures ANOVA. When appropriate, differences between treatment means were evaluated by Student’s t-test. All data are expressed as treatment means ± standard error of the mean (SEM).

**Supplemental Methods**

**Midline fluid percussion injury**

Mice received a midline and diffuse TBI using a fluid percussion injury (FPI) apparatus (Custom Design & Fabrication, Richmond) as previously described (Witgen et al., 2005). This diffuse injury occurs in the absence of a compression or contusion, does not induce tissue cavitation or gross neuronal loss, and causes diffuse axonal injury, particularly in the neocortex, hippocampus, and dorsolateral thalamus (Bachstetter et al., 2013; Kelley et al., 2006; Kelley et al., 2007). In brief, a midline craniectomy was
performed with a 3 mm outer diameter trephine and a rigid Luer-loc needle hub was secured over the craniectomy and capped. After 4-6 h, injury was induced by imposing a 10-20 ms pulse of saline (1.2-1.5 atmospheres) onto the dura through the injury hub (Lifshitz et al., 2007b; Witgen et al., 2006). All sham controls received the same procedure without the fluid pulse. Immediately after injury the injury hub was removed, dural integrity was confirmed, and mice were evaluated for injury severity using the self-righting test (Lifshitz et al., 2007b). Only mice with a moderate TBI were used. Based on previous studies in FPI (Witgen et al., 2006), self-righting inclusion criteria was modified for BALB/c mice as follows: sham≤60 s; 60 s<mild≤200 s; 200 s<moderate≤540 s; severe>540 s. In the current study righting reflex times were 27.8 ± 3.5 s for sham injury and 360.5 ± 15.4 s for moderate TBI. Overall, 36 mice were excluded from the study for 1) an inappropriate righting time for sham mice (>60 s; n=6), 2) mild (n=7) or severe (n=7) TBI, 3) compromised dura (n=11), or 4) fatalities after injury (n=5).

Motor function and depressive-like behavior

Motor function consisted of one day of acclimation and seven days of testing. Acclimation (4 d before injury) and testing (3-1 d before injury; 1 h, 1, 4, and 7 dpi) were performed 2-3 h prior to the dark phase of the photoperiod. Testing consisted of 3 trials in which mice were placed on the rod at a speed of 10 rpm with 0.2 rpm/s acceleration. Trials 1 and 2 were done back-to-back to prevent associations between falling and returning to the home cage, and mice were given 10 min between Trials 2 and 3. The average time spent on the rod before falling for the best two trials is reported. Baseline motor coordination was established as time spent on the rotarod 1 d before injury.

Sickness and depressive-like behavior was conducted during the dark phase of the photoperiod under red light. Behavior was scored by a trained observer who was
blinded to experimental treatments. For *social withdrawal* (Corona et al., 2010; Godbout et al., 2005b; Wohleb et al., 2012), a novel juvenile mouse was introduced into the home cage of an experimental mouse and behavior was video recorded for 10 min. Results are expressed as total time spent in social exploration. For *locomotor activity* (Godbout et al., 2005b), mice were maintained in their home cage and activity was video recorded for 3 min. Cages were divided into six identical virtual squares and the number of lines crossed was determined. For the *tail suspension test (TST)* (Corona et al., 2013; Godbout et al., 2008), mice were suspended by their tail and immobility was video recorded for 10 min. Results are expressed as time spent immobile. Two subsets of mice were used for the TST, one in which mice were tested both 7 dpi and 30 dpi, and a second in which mice were only tested 30 dpi. Immobility between the two subsets was not significantly different at 30 dpi and data for this time point were collapsed. For the *sucrose preference test* (Henry et al., 2008), mice were acclimated to a two water bottle system for two days. On the third day, mice were water deprived for two hours and then provided water or 1% sucrose solution. Sucrose preference was determined (Sucrose consumed / (Sucrose + Water consumed) * 100 = % Sucrose preference) 15 h later.

*Immunohistochemistry*

Fluorescent staining for glial fibrillary acidic protein (GFAP) and ionized binding association protein (Iba)-1 was performed as previously described (Wohleb et al., 2013). Mice were perfused with 4% formaldehyde. Brains were post-fixed in 4% formaldehyde for 24 h, cryoprotected in 20% sucrose for 72 h, frozen using dry-ice cooled isopentane (-165°C), and sectioned (25 µm) using a Microm HM550 cryostat (Mikron Instruments). Sections were blocked (5% normal goat serum, 1% bovine serum albumin, 0.3% Triton x-100) for 1 h at room temperature and stained for glial fibrillary acidic protein (GFAP)
(1:500; Dako) or ionized binding association protein (Iba)-1 (1:1000; Wako Chemicals) at 4° C over night. Fluorochrome-conjugated secondary antibodies (Alexa Fluor 488 and 546) were then applied for 1 h at RT. Sections were mounted and cover-slipped with Fluoro-Gel (Electron-Microscopy Sciences, Hatfield). Images were taken with a Zeiss 510 Meta confocal microscope at 20x and analyzed using LSM Image Browser Zhen and Metamorph Analysis software (Metamorph Offline, 6.1) or NIH ImageJ software. Threshold staining was determined using ImageJ software and quantification was assessed for each image using digital image analysis (DIA). Cell counts and cell body size were assessed using Metamorph Image analysis. Cell counts represent the number of cells per 20x image. All analyses were done using 3 representative images collected for each CSN region. Results are reported as the average percent area for GFAP⁺ and Iba-1⁺ staining, number of cells / image, and cell body size (μm²) / region.

**Results**

*Diffuse TBI promoted neuroinflammation in mice in a time-dependent manner*

Coinciding with previous studies using midline FPI, moderate and diffuse TBI caused mild hematoma, limited brain edema, and transient blood brain barrier permeability in the absence of overt tissue cavitation or neurodegeneration (data not shown) (Hamm, 2001; Kelley et al., 2007; McIntosh et al., 1987; Morales et al., 2005; Schmidt and Grady, 1993). In addition, Fig.5.1A-C show that the mRNA expression of several key inflammatory genes associated with microglia activation including IL-1β, CD14, and TNFα were increased in the cortex (CX) and hippocampus (HPC) 4 h after TBI (p<0.0006, for each). In the sham mice, inflammatory gene expression was not significantly different between the 4 and 72 h time points so the data were combined and presented as a single “sham” group. By 72 h after TBI, all markers were reduced in the
CX, but CD14 remained elevated in the HPC compared to controls \((p<0.0002)\). Other inflammatory genes had a similar expression pattern following TBI (Table 5.1). For example, chemokine ligand 2 (CCL2) mRNA was increased 4 h after TBI in the CX \((p<0.009)\) and HPC \((p<0.0008)\), and remained elevated 72 h after TBI in the CX \((p<0.02)\). GFAP, a marker of astrocyte activation, tended to be increased 4 h after injury in the HPC \((p=0.1)\) and was significantly increased 72 h after TBI in both the CX and HPC \((p<0.02\text{ for each})\). TBI also increased the expression of several anti-inflammatory genes 4 h after injury in the CX (arginase and IGF-1, \(p<0.05\text{ for each}\)), as well as 4 and 72 h after TBI in the HPC (IL-10, \(p<0.05\)) (Table 5.1). Overall, a moderate TBI elicited brain region-dependent inflammatory responses that were transient.

Next, PMC (neutrophil, monocyte, macrophage) trafficking after TBI was determined. PMCs \((\text{CD11b}^+/\text{CD45}^{\text{high}})\) were differentiated from microglia \((\text{CD11b}^+/\text{CD45}^{\text{low}})\) based on CD45 expression (Wohleb et al., 2012). Fig. 5.1D&E show that the number of PMCs associated with the brain increased 2-3 fold 4 and 72 h after TBI \((p<0.006)\). In the same set of samples, CD14 surface expression was determined on microglia \((\text{CD11b}^+/\text{CD45}^{\text{low}})\). Consistent with increased inflammatory markers after injury (Fig. 5.1; Table 5.1), the percentage of CD14\text{high} microglia was increased 4 and 72 h after TBI (Fig. 5.1F&G, \(p<0.01\)). Collectively these data indicate that TBI increased the number of brain associated PMCs and enhanced microglia activation for at least 72 h after injury.
### Cortex mRNA levels

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sham</th>
<th>TBI (4h)</th>
<th>TBI (72h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GFAP</td>
<td>1.14 ± 0.17</td>
<td>1.38 ± 0.23</td>
<td>2.47 ± 0.63*</td>
</tr>
<tr>
<td>CCL2</td>
<td>1.37 ± 0.42</td>
<td>12.1 ± 0.27*</td>
<td>6.46 ± 1.78*</td>
</tr>
<tr>
<td>IFNy</td>
<td>1.19 ± 0.28</td>
<td>1.90 ± 0.40</td>
<td>2.27 ± 0.39*</td>
</tr>
<tr>
<td>iNOS</td>
<td>1.06 ± 0.13</td>
<td>0.74 ± 0.05*</td>
<td>1.65 ± 0.14*</td>
</tr>
<tr>
<td>IL-4</td>
<td>1.03 ± 0.08</td>
<td>0.73 ± 0.15*</td>
<td>1.44 ± 0.22*</td>
</tr>
<tr>
<td>Arg</td>
<td>1.04 ± 0.12</td>
<td>2.26 ± 0.51*</td>
<td>0.93 ± 0.11</td>
</tr>
<tr>
<td>IL-10</td>
<td>1.24 ± 0.27</td>
<td>1.26 ± 0.39</td>
<td>1.22 ± 0.39</td>
</tr>
<tr>
<td>IGF-1</td>
<td>1.02 ± 0.08</td>
<td>1.40 ± 0.19 +</td>
<td>2.05 ± 0.24*</td>
</tr>
</tbody>
</table>

### Hippocampus mRNA levels

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sham</th>
<th>TBI (4h)</th>
<th>TBI (72h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GFAP</td>
<td>1.02 ± 0.08</td>
<td>1.40 ± 0.19*</td>
<td>2.05 ± 0.24*</td>
</tr>
<tr>
<td>CCL2</td>
<td>1.03 ± 0.12</td>
<td>7.57 ± 5.43*</td>
<td>1.41 ± 0.36</td>
</tr>
<tr>
<td>IFNy</td>
<td>1.14 ± 0.21</td>
<td>0.91 ± 0.14</td>
<td>2.15 ± 0.74</td>
</tr>
<tr>
<td>iNOS</td>
<td>1.09 ± 0.14</td>
<td>0.78 ± 0.07*</td>
<td>1.59 ± 0.11*</td>
</tr>
<tr>
<td>IL-4</td>
<td>1.20 ± 0.22</td>
<td>0.59 ± 0.08*</td>
<td>2.04 ± 0.60*</td>
</tr>
<tr>
<td>Arg</td>
<td>1.06 ± 0.12</td>
<td>1.19 ± 0.21</td>
<td>1.08 ± 0.07</td>
</tr>
<tr>
<td>IL-10</td>
<td>1.06 ± 0.14</td>
<td>4.57 ± 1.41*</td>
<td>1.97 ± 0.38*</td>
</tr>
<tr>
<td>IGF-1</td>
<td>1.02 ± 0.07</td>
<td>1.17 ± 0.17</td>
<td>1.17 ± 0.09</td>
</tr>
</tbody>
</table>

**Table 5.1.** TBI results in robust mRNA expression of several immunomodulatory genes. Adult mice were subjected to a sham injury (sham) or a moderate midline fluid percussion injury (TBI) (n=6-9). After 4 or 72 h mRNA levels of inflammatory (grey) and anti-inflammatory (white) genes were determined in the CX and HPC. Values represent the mean ± SEM. Means with (*) are significantly different (p<0.05) and means with (+) tend to be different (p<0.1) from sham controls.
Figure 5.1. Diffuse TBI promoted neuroinflammation in mice in a time-dependent manner. Four or 72 h after sham operation or TBI, mRNA levels of IL-1β, CD14, and TNFα were determined in the CX and underlying HPC (n=6-9), or enriched brain CD11b+ cells were collected and CD11b, CD45, and CD14 surface expression was determined by flow cytometry (n=3-5). A) There was a main effect of TBI on IL-1β expression in both the CX (F(2,21)=11.01, p<0.0007) and HPC (F(2,22)=28.22, p<0.0006). B) There was a main effect of TBI on CD14 expression in both the CX (F(2,20)=33.23, p<0.0001) and HPC (F(2,19)=22.22, p<0.0001) and CD14 remained elevated in the HPC 72 h after TBI (p<0.0002). C) There was a main effect of TBI on TNFα in both the CX (F(2,20)=32.00, p<0.0001) and HPC (F(2,21)=284.89, p<0.0001). D) Representative bivariate dot plots of CD11b/CD45 staining of enriched brain CD11b+ cells. E) TBI increased the relative number of PMCs (CD11b+/CD45high) associated with the brain both 4 and 72 h after injury (F(2,12)=34.37, p<0.006). F) Representative bivariate dot plots of CD11b/CD45 staining on microglia (CD11b+/CD45low). G) TBI increased the percent of CD14high microglia present 4 and 72 h after injury (F(2,10)=28.23, p<0.01). Bars represent the mean ± SEM. Means with (*) are significantly different (p<0.05) from sham controls. Means with (#) are significantly different (p<0.05) from sham control and TBI-4h groups. CX = cortex; HPC = hippocampus.
Diffuse TBI promoted transient deficits in body mass, motor coordination, and depressive-like behavior

To determine the degree to which diffuse TBI caused motor and behavior deficits, body weight, motor coordination, activity, and depressive-like behavior were determined at several time-points after TBI. Fig. 5.2A shows that TBI mice had exaggerated weight loss 1-3 dpi ($p<0.004$), but recovered to sham levels by 7 dpi. Fig. 5.2B shows that motor coordination was reduced following TBI ($p<0.02$) in time dependent manner ($p<0.001$). For example, TBI mice had impaired motor coordination 1 h and 1-4 dpi compared to baseline and sham levels, but recovered to baseline motor coordination by 7 dpi (Fig. 5.2B). Moreover, activity levels were equivalent in sham and TBI mice 7 dpi (Fig. 5.2C).

Because depression can develop and persist after TBI (Hibbard et al., 1998; Holsinger et al., 2002), behavioral resignation was determined by immobility in the TST 7 dpi (El Yacoubi et al., 2003), a time when weight, motor coordination, and activity were not disrupted in TBI mice (Fig. 5.2A-C). TBI mice spent more time immobile in the TST 7 dpi compared to controls (Fig. 5.2D, $p<0.04$). By 30 dpi, however, immobility was similar between the groups (Fig. 5.2E). Increased immobility 7 dpi was not associated with exhaustion in TBI mice as activity levels were equivalent between groups over a 30 min testing period (data not shown). Taken together, TBI caused transient weight loss, motor dysfunction, and depressive-like behavior.
Figure 5.2. Diffuse TBI promotes transient deficits in body mass, motor coordination, and depressive-like behavior. TBI caused A) reduced body weight 1-3 and 6 dpi ($F(1,44)=3.41, p<0.004$), but body weight recovered to sham levels by 7 dpi ($n=22$). B) Motor coordination was reduced by TBI ($F(1,111)=23.46, p<0.0001$) in a time manner ($F(6,111)=5.40, p<0.0001$) ($n=8$). The dashed, horizontal line indicates baseline motor function. C) Total movement time in the activity box did not differ between groups at either 7 or 30 dpi ($n=7$). D) TBI mice had increased immobility in the TST compared to sham mice 7 dpi ($F(1,13)=5.4, p<0.04$) ($n=7$). E) There was no significant difference in immobility in the TST at 30 dpi ($n=16$). Bars represent the mean ± SEM. Means with (*) are significantly different ($p<0.05$) from sham controls. Means with (‡) and (+) are significantly different ($p<0.01$) and tend to be different ($p=0.09$) from baseline levels, respectively.
Our data indicate that moderate TBI causes a transient increase in neuroinflammation corresponding with acute motor and behavioral impairments. We predict, however, that a degree of glial activation persists months to years after TBI. Thus, the relative level of astrocyte (GFAP\(^+\)) activation was determined 30 dpi. GFAP immunoreactivity was increased in the corpus callosum (CC) (Fig.5.3A&D, \(p<0.0001\)) and parietal cortex (PCX) (Fig.5.3B&E, \(p<0.002\)). This increase in astrocyte activation occurred in the absence of tissue cavitation (data not shown). GFAP immunoreactivity was primarily restricted to white matter and gray matter directly beneath the injury site (Fig.5.3G&H). Indeed, in brain regions more distal to the impact including the prefrontal cortex (PFC) (Fig.5.3C&F), paraventricular nucleus (PVN), medial amygdala (MeA), and hippocampus (CA1 and DG), GFAP immunoreactivity was not increased (Table 5.2). Collectively, evidence of astrocyte activation remained 30 dpi, but was restricted to areas proximal to the injury site.
**Figure 5.3. TBI-associated astrogliosis 30 dpi.** Brains were collected 30 d after sham operation or TBI and proportional area of GFAP expression was determined (n=11-13). Three images/sample at 20x were collected. Representative images of GFAP staining in the A) CC, B) PCX, and C) PFC of sham and TBI mice. Inset includes enlarged image of GFAP+ cell indicated by white arrow. Proportional area for GFAP staining was increased in TBI mice compared to sham mice in the D) CC (F(1,23)=25.05, *p*<0.0001) and E) PCX (F(1,24)=12.60, *p*<0.002), but was not different between groups in the F) PFC. Schematics taken from the High Resolution Mouse Brain Atlas (Sidman et al.: [http://www.hms.harvard.edu/research/brain/index.html](http://www.hms.harvard.edu/research/brain/index.html)) depicting where images were collected for the G) CC, H) PCX, and I) PFC. Bars represent the mean ± SEM. Means with (*) are significantly different (*p*<0.05) from sham controls. CC = corpus callosum; PCX = parietal cortex; PFC = prefrontal cortex

**TBI-associated microglia priming 30 dpi**

Next, the degree to which microglia retained a primed phenotype 30 dpi was determined. Representative images of Iba-1 labeling in the A) DG, B) PCX, and C) PFC...
of sham and TBI mice are shown. Iba-1 immunoreactivity was markedly increased 30 dpi in the DG ($p<0.04$, Fig.5.4D) and PCX ($p<0.0007$, Fig.5.4E), but was not different in the PFC (Fig.5.4F). Iba-1 immunoreactivity tended to be increased in the PVN, but was unchanged in the CA1, or MeA (Table 5.2). In addition, the number of Iba-1$^+$ cells was unchanged in brain regions examined with the exception of the PCX ($p<0.02$, Fig.5.4J). Moreover, increased cell soma size was only detected in the DG ($p=0.1$) and PCX ($p<0.03$, Fig.5.4K).

To further investigate this primed phenotype of microglia, MHCII mRNA and protein expression was determined in microglia from sham and TBI mice 30 dpi. Fig.5.4L shows that MHCII mRNA expression was increased ~2.5 fold in enriched CD11b$^+$ cells isolated from TBI mice compared to shams ($p=0.09$). Moreover, TBI increased the percentage of microglia (CD11b$^+$/CD45$^{low}$) that were MHCII$^+$ ($p<0.002$, Fig.5.4M), and increased the level of MHCII expression on a per cell basis by ~15% ($p<0.009$, Fig.5.4N&O). Taken together, diffuse TBI induced a population of MHCII$^+$ primed microglia 30 dpi.
Figure 5.4. TBI-associated microglia priming 30 dpi. Brains were collected 30 d after sham operation or TBI. In one cohort protein expression of Iba-1 was determined (n=10-11). In a separate cohort, CD11b+ cells were collected and mRNA and protein expression of MHCII was determined (n=9). A) Iba-1 staining in the DG of the HPC, B) PCX, and C) PFC of sham and TBI mice. Three images / sample at 20x were collected. Inset includes enlarged image of Iba-1+ cell indicated by white arrow. Proportional area for Iba-1 staining was increased in TBI mice compared to sham mice in the D) DG (F(1,19)=5.48, p<0.04) and E) PCX (F(1,18)=17.05, p<0.0007), but was not different between groups in the F) PFC. Schematics taken from the High Resolution Mouse Brain Atlas (Sidman et al.: http://www.hms.harvard.edu/research/brain/index.html) depicting where images were collected for the G) DG, H) PCX, and I) PFC. J) Average cell counts per section were only significantly different in the PCX (F(1,19)=6.81, p<0.02) and K) average cell soma size was increased in the DG (F(1,18)=2.51, p=0.1) and PCX (F(1,19)=5.61, p<0.03). Enriched CD11b+ cells from TBI mice had L) increased MHCII mRNA expression (F(1,15)= 3.4, p=0.09), M) and increased percentage of MHCII+ microglia (CD11b+/CD45low) (F(1,15)=16.18, p<0.002). N) Representative histogram of mean fluorescence intensity (MFI) for MHCII in microglia (CD11b+/CD45low). O) The average MFI for MHCII was increased in microglia (CD11b+/CD45low) of TBI mice (F(1,16)=9.13, p<0.009). Bars represent the mean ± SEM. Means with (*) are significantly different (p<0.05) and means with (+) tend to be different (p<0.1) from sham controls. DG = dentate gyrus; HPC = hippocampus; PCX = parietal cortex; PVN = paraventricular nucleus; MeA = medial amygdala; PFC = prefrontal cortex.
Figure 5.4

A. Sham (30d)

DG

Iba-1

PCX

Iba-1

PFC

Iba-1

B. 30d

MHCII mRNA (Fold Change)

0 1 2 3 4

C. Sha

m TBI

MHCII MFI

30d

D. 10

G. 30d

MHCII -FITC

0 20 40 60 80 100 % of Max

E. 30d

Sham TBI

PCX Iba-1 (% area)

F. 5

Sham TBI

PFC Iba-1 (% area)

G. 10

H. 4

I. 6

J. 120

K. 60

Cell Number (count / section)

CA1 DG PCX PVN MeA PFC

L. 4

M. 3

MHCI mRNA (Fold Change)

0 1 2 3 4

N. 30d

% MHCII+ Microglia

0 1 2 3

O. 6

MHCI MFI

0 1 2 3 4 5 6

Sham TBI 30d
Table 5.2. GFAP and Iba-1 Threshold area 1 m after TBI. Adult mice were subjected to a sham injury (sham) or a moderate midline fluid percussion injury (TBI) (n=10-13). Brains were collected 30 d later and proportional area of GFAP or Iba-1 expression was determined. Results are presented as proportional area for GFAP or Iba-1 threshold staining. Values represent the mean ± SEM. Means with (*) are significantly different (p<0.05) and means with (+) tend to be different (p=0.06) from sham controls.

Peripheral LPS injection caused exaggerated microglial cytokine expression associated with protracted social withdrawal and depressive-like behavior in TBI mice.

In several models, microglial priming or sensitization is associated with an exaggerated inflammatory cytokine response associated with cognitive impairment and the development of depressive-like behaviors (Godbout et al., 2008; Williamson et al., 2011; Wohleb et al., 2012). Therefore, we examined the degree to which primed microglia from TBI mice had an exaggerated response to peripheral LPS challenge 30
Fig. 5.5A shows that LPS injection reduced social exploratory behavior \((p<0.0001)\) in a time dependent manner \((p<0.001)\). The return to baseline social behavior 24 h after LPS was impaired in TBI-LPS mice compared to sham-LPS mice. For example, post-hoc analysis revealed that TBI mice injected with LPS had the lowest level of social exploration 24 h after injection compared to all other groups \((p<0.02)\). After completing behavioral testing, enriched CD11b\(^+\) cells were isolated from whole brain homogenates and mRNA expression of IL-1\(\beta\) and TNF\(\alpha\) was determined. IL-1\(\beta\) mRNA expression was increased in microglia 24 h after LPS in both sham and TBI mice \((p<0.005)\), but was exaggerated in microglia of TBI mice injected with LPS (TBI-LPS) compared to all other groups \((p<0.04, \text{ Fig. } 5.5\text{B})\). A similar pattern of TNF\(\alpha\) expression was evident 24 h after LPS injection \((p<0.02)\). Indeed post-hoc analysis showed that the highest expression of TNF\(\alpha\) was in microglia from TBI-LPS mice \((p<0.02, \text{ Fig. } 5.5\text{C})\). mRNA expression of iNOS, arginase, and IL-4R\(\alpha\) was also determined in enriched CD11b\(^+\) cells, but no significant differences were detected (Table 5.3).

In a related study, locomotor and depressive-like behaviors were evaluated in sham and TBI mice after LPS. Because generalized lethargy and malaise can confound behavioral tests of depression (Godbout et al., 2008), locomotor activity was determined 24, 48, and 72 h after LPS. Fig. 5.5D shows that TBI mice had a ~60% reduction in locomotor activity 24 h after LPS indicating a maintained sickness response, but recovered to baseline activity by 72 h after LPS. Next, two major behavioral components of depression, resignation (TST) and anhedonia (sucrose preference), were determined 72 h after LPS. Fig. 5.5E shows that immobility in the TST was increased by both LPS \((p<0.03)\) and TBI \((p<0.1)\), and that TBI-LPS mice had the highest immobility compared to all other groups (post-hoc: \(p<0.05\) from sham-saline and TBI-saline; \(p<0.1\) from sham-LPS). Consistent with these results, sham-LPS mice preferred a 1% sucrose solution
over water, but TBI-LPS mice did not ($p<0.03$, Fig.5.5F). Taken together, LPS caused exaggerated IL-1β and TNFα expression in microglia of TBI mice (30 dpi) corresponding with prolonged social withdrawal, resignation, and anhedonia.
A. shows that the TBI-LPS group was significantly elevated compared to all other groups (behavior was determined 72 h after LPS by time spent immobile in the TST (n=9-12). There was a main

Locomotor activity was determined at 0 (baseline), 24, and 72 h after injection (n=5-6). E) Depressive-like

C) mRNA expression of TNFα was increased after LPS (F(1,32)=10.1, p<0.004) and post-hoc analysis shows that the TBI-LPS group was significantly elevated compared to all other groups (p<0.02). D) Locomotor activity was determined at 0 (baseline), 24, and 72 h after injection (n=5-6). E) Depressive-like behavior was determined 72 h after LPS by time spent immobile in the TST (n=9-12). There was a main effect of TBI (F(1,41)=2.87, p<0.1) and LPS (F(1,41)=5.31, p<0.03) and post-hoc analysis shows that the TBI-LPS group significantly differed from both sham-saline and TBI-saline groups (p<0.05 for both) and tended to be different from the sham-LPS group (p<0.1). F) Depressive-like behavior was also assessed with a sucrose preference test for anhedonia starting 72 h after LPS. Whereas sham-LPS mice showed a strong preference for a 1% sucrose solution (77.0% ± 5.9%) (F(1,7)=8.98, p<0.03), TBI-LPS mice showed no preference (50.0% ± 6.8%). Bars represent the mean ± SEM. Means with (*) are significantly different (p<0.05) from sham-saline controls. Means with (#) are significantly different (p<0.05) from sham-saline and sham-LPS groups. Means with (+) tend to be different from sham-LPS groups.

Figure 5.5. Peripheral LPS injection caused exaggerated microglial cytokine expression associated with protracted social withdrawal and depressive-like behavior in TBI mice. Mice were injected i.p. with saline or LPS (0.33 mg/kg) 30 d after sham operation or TBI. A) Social exploration was reduced by LPS (F(1,37)=87.88, p<0.0008) in a time dependent manner (F(4,196)=13.01, p<0.0001) (n=11-13). At 24 h after LPS, post-hoc analysis revealed that TBI-LPS mice had significantly reduced social exploration compared to all other groups (p<0.02). The dashed, horizontal line indicates baseline social exploratory behavior. B) Following the completion of the behavior tests (24 h), enriched brain CD11b+ cells were collected (n=9). mRNA expression of IL-1β was increased after LPS (F(1,32)=15.8, p<0.005), with the highest levels observed in TBI-LPS mice (TBI x LPS interaction: F(1,32)=4.96, p<0.03). The dashed, horizontal line indicates baseline social exploratory behavior. C) mRNA expression of TNFα was increased after LPS (F(1,27)=10.1, p<0.03) and post-hoc analysis shows that the TBI-LPS group was significantly elevated compared to all other groups (p<0.02). D) Locomotor activity was determined at 0 (baseline), 24, and 72 h after injection (n=5-6). E) Depressive-like behavior was determined 72 h after LPS by time spent immobile in the TST (n=9-12). There was a main effect of TBI (F(1,41)=2.87, p<0.1) and LPS (F(1,41)=5.31, p<0.03) and post-hoc analysis shows that the TBI-LPS group significantly differed from both sham-saline and TBI-saline groups (p<0.05 for both) and tended to be different from the sham-LPS group (p<0.1). F) Depressive-like behavior was also assessed with a sucrose preference test for anhedonia starting 72 h after LPS. Whereas sham-LPS mice showed a strong preference for a 1% sucrose solution (77.0% ± 5.9%) (F(1,7)=8.98, p<0.03), TBI-LPS mice showed no preference (50.0% ± 6.8%). Bars represent the mean ± SEM. Means with (*) are significantly different (p<0.05) from sham-saline controls. Means with (#) are significantly different (p<0.05) from sham-saline and sham-LPS groups. Means with (+) tend to be different from sham-LPS groups.
Table 5.3. Gene expression in enriched CD11b+ cells 72 h after LPS. Adult mice were subjected to a sham injury (sham) or moderate midline fluid percussion injury (TBI) (n=8-10). After 30 d, mice were injected i.p. with saline or LPS (0.33 mg/kg). Following the completion of the behavior tests (24 h), enriched brain CD11b+ cells were collected. The mRNA expression of inflammatory-associated genes (iNOS) and anti-inflammatory-associated genes (Arg, IL-4Rα) were determined. Values represent the mean ± SEM. Arg = arginase 1.

Discussion

Previous studies in rodents indicate that early life infection, aging, sterile CNS injury, and pre-clinical neurodegenerative disease leads to the development of a primed and immune-reactive population of microglia (Bilbo and Schwarz, 2009; Cunningham et al., 2005; Henry et al., 2009; Murray et al., 2012; Palin et al., 2008). Here we defined primed microglia by increased Iba-1 labeling, increased cell soma size, increased MHCII mRNA and protein expression, and a hyperactive inflammatory response after acute immune challenge. We show that in a diffuse model of TBI (i.e., midline FPI), a population of primed microglia develops and persists in the brain for at least 30 dpi. Indeed, microglia from TBI mice (30 dpi) were hyperactive following an acute immune stimulus (i.p. LPS injection) associated with prolonged social withdrawal, resignation, and anhedonia. These are novel data that indicate diffuse TBI sensitizes microglia to an acute inflammatory challenge promoting depressive-like complications weeks after the initial injury.
An important element of this study was that diffuse TBI caused transient neuroinflammation, deficits in motor coordination, and depressive-like behavior. For example, TBI caused acute neuroinflammation (4-72 h) with increased mRNA expression of inflammatory mediators (IL-1β, TNFα, etc.) in the CX and HPC. Moreover, TBI resulted in a 2-3 fold increase in the number of PMCs that were associated with the brain. In focal models of CNS injury (e.g., spinal cord injury, axonal crush) these cells are thought to traffic to the site of injury and aid in clearance of debris or promote repair, but may also contribute to secondary damage (David and Kroner, 2011). It is unclear what the roles of these cells are after a diffuse TBI, but they may contribute to increased neuroinflammation after injury. In the context of behavioral recovery, TBI mice were impaired in motor coordination on the rotarod immediately after injury and for at least 4 dpi, but recovered to baseline levels within 7 dpi. Although sham mice continued to have improved performance compared to TBI mice, this was likely an effect of training. A return to baseline motor coordination and body mass within 7 days after TBI is relevant because cognitive, balance, and motor impairments associated with mild-to-moderate TBI in humans are typically resolved within 7 dpi (McCrea et al., 2003). Despite the recovery to baseline motor coordination and activity, TBI mice had increased resignation behavior 7 dpi. This is relevant because 30-40% of human TBI patients develop symptoms of depression acutely after injury (Jorge et al., 1993; Jorge et al., 2004). Increased depressive-like behavior in TBI mice, however, was no longer detected 30 dpi. Consistent with this result, TBI-related depression in humans is associated with periods of remission (Hibbard et al., 1998; Jorge et al., 1993) and re-establishment (Holsinger et al., 2002). Therefore, the remission/re-occurrence of depression may point to secondary stimuli that can trigger the onset of these depressive-like symptoms.
Another key finding of the study was that primed microglia were detected in the brain of TBI mice 30 dpi. Indeed, expression of MHCII (mRNA and protein) was increased in microglia of TBI mice compared to sham controls. In addition, Iba-1 immunoreactivity and corresponding de-ramified morphology were increased in microglia of the HPC and PCX 30 dpi, consistent with previous studies investigating microglia activation 7 dpi (Bachstetter et al., 2013). This hypertrophic (increased cell soma size) and increased Iba-1 profile in the DG and PCX is consistent with a primed and more inflammatory microglial phenotype (Chen et al., 2012; Corona et al., 2010). Notably, microglia activation and astrocyte activation did not occur in all of the same regions. This may suggest a larger role for astrocytes in responding to the primary injury whereas microglia may play a larger role in propagating the secondary injury. This microglial profile (increased Iba-1 and MHCII) in the brain of adult TBI mice is consistent with a primed phenotype detected in models of aging, early life infection, optic nerve crush, and pre-neurodegenerative disease (Bilbo and Schwarz, 2009; Bland et al., 2010; Cunningham et al., 2005; Frank et al., 2006; Palin et al., 2008; Streit, 2004). It is likely that these microglia will remain in a primed and hyper-reactive state for months to years after the injury. In support of this notion, positron emission tomography (PET) imaging studies on humans with a moderate to severe TBI show increased microglia activation by ligand [11C](R)PK11195 (PK) up to 17 years after injury (Ramlackhansingh et al., 2011). We interpret our data to indicate that the initial diffuse brain injury caused significant inflammation (cytokine expression, peripheral cell recruitment, etc.) preventing the resolution of microglia activation within the HPC. In support of this notion, increased CD14 mRNA expression persisted in the HPC 72 h after TBI coinciding with persistent microglia activation (CD14high). This is relevant because the HPC has a high proportion of inflammatory-associated receptors, glutamatergic neurons, and undergoes
rapid remodeling making it more sensitive to inflammatory damage (Ban et al., 1991; Cunningham et al., 1992; Williamson and Bilbo, 2013). Indeed, several models including aging, early life infection, pre-neurodegenerative disease, and social stress show that inflammatory-associated microglia priming is detected in the HPC (Bland et al., 2010; Chen et al., 2008; Cunningham et al., 2009; Cunningham et al., 2005; Wohleb et al., 2011). It is also relevant to highlight that regional specific priming may explain why we only observed a modest increase in MHCII mRNA and protein in microglia collected from whole brain homogenates. Taken together, these data provide increased evidence of the development of region-specific, primed microglial populations that persist after a diffuse TBI.

Related to the notion of microglia priming discussed above, acute activation of the immune system caused exaggerated expression of two key inflammatory cytokines, IL-1β and TNFα, in microglia of TBI mice. Microglia have an active role in the interpretation and propagation of cytokine signals that are initiated in the periphery. The production of these inflammatory cytokines by microglia normally results in an evolutionarily adaptive behavioral and physiological sickness response (Corona et al., 2012; Godbout et al., 2005b; Godbout and Johnson, 2009). Exaggerated or prolonged expression of these inflammatory mediators, however, can lead to increased neuronal damage and maladaptive behavioral responses including depression and delirium (Cunningham et al., 2009; Godbout et al., 2008; Murray et al., 2012; Palin et al., 2008). For instance, exaggerated microglial activation in TBI mice after a peripheral LPS injection was associated with prolonged social withdrawal (24 h), and behavioral resignation and anhedonia (72 h). These behaviors are components of depressive-like behavior (Beck et al., 1961; El Yacoubi et al., 2003) and were undetectable in shams injected with LPS. Notably, TBI-associated microglia priming and the exaggerated
inflammatory response to LPS were evident 30 dpi, which was well after these mice had returned to baseline activity and behavior. Thus, an acute systemic immune challenge unrelated to the initial head injury induced the re-occurrence of depression in TBI mice. The underlying mechanism of these inflammatory-associated cognitive and depressive complications is likely reduced long-term potentiation and neuronal firing within the HPC (Dantzer et al., 2008; Frenois et al., 2007; Maher et al., 2005) in conjunction with increased metabolism of tryptophan to the neuroactive excitotoxin, quinolinic acid (Dantzer et al., 2011; Steiner et al., 2011b). This inflammatory-related depression may explain the higher percentage of TBI patients with resistance to conventional anti-depressant treatment (Ashman et al., 2009; Saran, 1988). Thus, it will be important for future studies to investigate potential therapies that either prevent TBI-associated microglial priming or reverse priming once it has been established.

In conclusion, we provide the first evidence that an exaggerated response by primed microglia to a secondary inflammatory challenge is a potential trigger for the development/reoccurrence of neuropsychiatric complications. The critical component of this exaggerated microglia reactivity was the establishment of depressive-like behavior following an acute immune stimulus in TBI mice. Based on these data, we postulate that individuals who have suffered a TBI are more sensitive to inflammation caused by secondary stimuli (e.g., immune activation, stress, etc) resulting in the promotion of neuropsychiatric complications.
Chapter 6: Discussion and Conclusions

The studies presented here demonstrate three novel components of microglial dysregulation with age: 1) Age-associated upregulation of miR-29a/b-1 inhibits the production of CX3CL1 and IGF-1 associated with increased microglial priming and reactivity. 2) Age-associated microglia priming corresponds with impaired upregulation of IL-4Rα and a reduced IL-4-mediated alternative activation phenotype (arginase+/IL-1β+) resulting in worsened functional recovery after SCI. 3) Microglial priming can be accelerated by a single, moderate TBI resulting in a hyper-inflammatory response after a secondary immune challenge and the development of depressive-like behavior. Although these findings reveal several novel mechanisms for microglial priming and age-associated functional and behavioral deficits, several questions remain regarding how these pathways are integrated.

Amalgamation of neuronal-, immune-, and miR-mediated regulation

While it is tempting to address neuronal-, immune-, and miR-mediated microglial regulation as separate entities, the truth is these pathways probably influence and rely on one another. So rather than the loss of one regulatory system being compensated for by the presence of multiple others, with the aged brain it appears that the loss of one pathway can actually perpetuate or cause the reduction of several others.

Here I presented evidence that exaggerated levels of miR-29a/b-1 inhibit the production of CX3CL1 and IGF-1, two important factors for microglia regulation.
Importantly, an increase in miR-29a/b-1 with age indicates that this regulatory pathway remains intact. Indeed, miR-29a/b is induced by inflammation (Smith et al., 2012; Ugalde et al., 2011), and functions to repress these inflammatory signals (Ma et al., 2011; Smith et al., 2012; Steiner et al., 2011a). Within the CNS, however, this has a negative influence on other regulatory pathways that may perpetuate an increase in inflammation. Intriguingly, IGF-1 is also induced by IL-4 (Zhao et al., 2006). Although my studies show equivalent levels of IL-4 mRNA in adult and aged mice, other researchers demonstrate reduced IL-4 protein within the hippocampus of aged rats (Nolan et al., 2005). Thus, reduced IGF-1 expression could also be caused by impaired IL-4 signaling.

IL-4 signaling could also influence miR expression. For example, macrophages isolated from the bone marrow (BM) or peritoneal cavity and treated ex vivo with IL-4 and IL-13 strongly upregulate miR-124 expression (Veremeyko et al., 2013). Moreover, miR-124 expression is also significantly blunted in a model of allergic inflammation in the absence of IL-4Rα (Veremeyko et al., 2013). Thus, age-associated reductions in IL-4 may also mediate the observed reductions in miR-124. Reduced miR-124, as mentioned previously, would then promote more of an M1 monocyte phenotype capable of enhanced responses to inflammatory stimuli (Ponomarev et al., 2011). Whether these results would also hold true in microglia is unclear. As mentioned previously the ontological origins of BM-derived macrophages and brain-resident microglia are separate (Ginhoux et al., 2009). Nonetheless, evidence that peritoneal macrophages respond similarly to BM-derived macrophages in regards to IL-4 induction of miR-124 (Veremeyko et al., 2013) suggests that microglia would also have this same response as these populations both derived from the embryonic yolk sac (Yona et al., 2013). In addition to miR-124 regulation, IL-4 is also associated with functional CD200 expression on neurons. In the absence of IL-4, CD200 is markedly reduced in the CNS
corresponding to elevated microglial reactivity and higher IL-1β protein (Lyons et al., 2009b). Because CD200 and CD200R expression within the CNS is an exclusive regulatory mechanism for neurons to microglia, disruption of this signaling cascade can profoundly effect microglia reactivity and inflammation (Lyons et al., 2009b). Indeed, recent evidence suggests that loss of CD200-CD200R signaling contributes to microglia-induced neurotoxicity in Parkinson’s disease (Wang et al., 2011). Moreover, intrahippocampal injection of a CD200 fusion protein to activate CD200R on microglia of aged mice significantly reduces microglia priming (i.e., reduced MHC II), and prevents exaggerated inflammatory cytokine expression in aged microglia following an immune challenge with LPS (Cox et al., 2012). Whether enhanced CD200 protein could restore miR-124 expression in aged microglia is unknown.

Work presented here also demonstrates that even if IL-4 levels were maintained in the aged CNS, responsiveness to IL-4 would still be diminished due to impaired expression of its receptor, IL-4Rα. Other studies have also supported an impaired ability for the aged CNS to respond to alternative activation signals (i.e., IL-4, IL-13) (Lee et al., 2013). This could derive from impairments in other immune modulatory systems. Recent evidence suggests that IL-10-induced TGFβ expression by astrocytes can strongly induce IL-4Rα expression on microglia (Norden et al., 2014). In support of reduced TGFβ as a mechanism for age-associated impairments in IL-4Rα expression, TGFβ mRNA is significantly reduced in the brain of aged mice following an inflammatory challenge compared to adults (Wynne et al., 2010). Although in my studies TGFβ mRNA was induced to the same extent in adult and aged mice after SCI (data not shown), these results do not eliminate deficient TGFβ signaling as a cause for impaired IL-4Rα expression. TGFβ undergoes extensive post-translational modifications to induce activation and even with similar levels of mRNA, protein concentrations of active TGFβ
can vary significantly (Annes et al., 2003). Intriguingly, TGFβ can also modify CX3CR1 expression in microglia. For example, BV-2 microglia-like cells treated with TGFβ protein markedly increase surface expression of CX3CR1 (Wynne et al., 2010). Deficits in CX3CR1 expression are also observed in aged microglia corresponding to a primed and more reactive phenotype (Corona et al., 2010; Wynne et al., 2010).

In summary, neuronal (CD200-CD200R, CX3CL1-CX3CR1), immune (IL-4, IL-10, TGFβ, IGF-1), and miR (miR-29, miR-124) signals that regulate microglial priming or activation are not isolated or distinct, but instead dynamically influence the expression and/or function of one another. Which factor is influenced first or if several are concomitantly affected by age is unclear. Nonetheless, integration of these signals is an exciting prospect because pharmacologically, the ability to influence one component has the potential to affect several downstream targets and restore functional microglial regulation.

Site specific microglia priming

It is important to note that my studies failed to show decreased miR-124 expression in microglia of aged mice. While this may have resulted from a lower sample size or because small changes in miR-124 expression may be difficult to detect considering its high expression within the CNS (Lagos-Quintana et al., 2002), this discrepancy may also be attributed to site-specific microglia priming or microglia heterogeneity. Indeed, aging is only associated with a 10-25% increase in the percentage of MHC II⁺ microglia (Henry et al., 2009). Thus, a change in miR-124 expression may only occur in this small primed microglia subpopulation and would not be detected during my gross analysis of whole brain. In support of a heterogeneous microglia population, the results presented here show that only 30-40% of microglia
became IL-4Rα+ following a peripheral, and thus systemic, LPS challenge. Similar effects were seen in the SCI model in which only ~20% of microglia became IL-4Rα+ 24 h after SCI. These results are intriguing because they indicate that even though microglia derive from the same precursor population, within the CNS they take on distinct phenotypes potentially as a result of their location.

Further support for region specific microglial heterogeneity comes from studies of psychosocial stress, LPS challenge, and TBI. In a repeated social defeat model of psychosocial stress, microglia are activated and release high levels of inflammatory mediators, including IL-1β, associated with recruitment of monocytes to the CNS and the development of anxiety (Wohleb et al., 2011; Wohleb et al., 2013). Microglia are not activated across the entire CNS, however. Instead, particular brain regions associated with the stress response including the pre-frontal cortex, amygdala, and the CA3 and dentate gyrus of the hippocampus have increased microglial activation (Wohleb et al., 2011; Wohleb et al., 2013). In a model of systemic LPS challenge, researchers note early induction of IL-1β protein within a distinct subset of microglia in close proximity to areas of high peripheral cytokine exposure (e.g., circumventricular organs, choroid plexus) (Quan et al., 1998). These results are consistent with my studies investigating microglial activation 30 days after a moderate TBI. Increased staining for Iba-1 was not noted in each brain region examined, but instead was confined to the parietal cortex (proximal to the fluid impact site), dentate gyrus of the hippocampus (near choroid plexus), and paraventricular nucleus (adjacent to the third ventricle and receives strong input from the circumventricular organs OVLT and SFO). Thus, exposure to increased levels of inflammatory signals from the periphery or activation signals from neurons may be the driving factor in site-specific microglia priming.
Further support for microglial heterogeneity is provided in my models of SCI and i.p. LPS/i.c.v IL-4. I show that arginase expression was not comprehensive in TomL⁺ or Iba-1⁺ immune cells but rather expressed in only 20-30% of immune cells and in a punctated pattern. Intriguingly, the punctate pattern of arginase expression after SCI and i.c.v. IL-4 suggests more complicated microglial heterogeneity than just region specific differences. Indeed, microglia adjacent to one another seem to take on different phenotypes in response to the same signals. Studies of TBI also demonstrate that microglia adjacent to one another can take on distinct phenotypes. Histological analysis of Ym-1 and CD68 co-labeling demonstrated that microglia within the same region were double negative, positive for one or the other, or double positive for these “M1” and “M2a”-associated markers (Kumar et al., 2012). How microglia within the same region would take on distinct phenotypes and the degree to which they differ in gene regulation is unknown. Future studies are required to determine the extent of microglial heterogeneity within each brain region and the ontogeny of these distinct populations.

Site specific microglial priming has also been observed in humans using live imaging studies. These studies indicate that microglia within distinct brain regions become more activated with age or neurodegenerative disease. For example, using carbon-11 staining of PK11195, a receptor for peripheral benzodiazepine that is upregulated on microglia after activation, PET scans show a steady increase in PK11195 labeling particularly within the temporal and thalamic regions of healthy aged individuals (Cagnin et al., 2001). Thus, region-specific microglial priming also occurs in aged humans. Moreover, because increased microglial activity is observed in the temporal region which contains the hippocampus, these studies further support a role for microglial priming and hyper-activity in the development of age-associated cognitive decline and depression. It is also important to mention that these studies further
demonstrated that region-specific PK11195 labeling was exaggerated in patients with Alzheimer's disease indicating a heightened inflammatory state in active neurodegenerative disease compared to aging alone (Cagnin et al., 2001).

In summary, these studies indicate a region-specific and even cell-specific microglial heterogeneity further complicating the issue of age-associated microglia priming and susceptibility to neurodegenerative disease. Future studies to determine which microglial phenotype is more susceptible to priming or M2α-mediated regulation would be of great interest.

*What is the true M2α phenotype in vivo?*

The M2α phenotype is historically characterized as anti-inflammatory and reparative with high arginase, mannose receptor, and scavenger receptor expression, and production of IL-10, IL-1RA, and chemokines to recruit eosinophils, basophils, and Th2 T-cells (Mantovani et al., 2004). Moreover, *in vitro* treatment of monocytes with the M2α-driving factor IL-4 prior to or at the same time as LPS reduces LPS-induced IL-1β expression (Allen et al., 1993; Hart et al., 1989). Even post-treatment with IL-4 promotes improved neuronal survival in culture (Zhao et al., 2006), and I present data here showing that post-treatment with IL-4 attenuated LPS-induced IL-1β expression in BV-2 microglia-like cells. But do these culture experiments truly represent the M2α phenotype that develops *in vivo*? Is there an M2α phenotype that develops *in vivo*? In regards to the latter question, it is clear that the application of IL-4 *in vivo* does induce a distinct response separate from LPS or other activating stimuli. Indeed, I show that a peripheral injection of LPS reduced arginase expression and increased expression of inflammatory-associated factors including TNF-α and IL-1β. In contrast, IL-4 strongly promoted arginase expression. Contrary to *in vitro* studies, however, IL-4 also promoted mRNA
expression of several inflammatory mediators. For example, IL-4 treatment after LPS exaggerated expression of IL-1β and CCL2 compared to LPS alone. Discrepancies between the anti- and pro-inflammatory actions of IL-4 may result from a lack of co-staining sections for both arginase and inflammatory mediators, including IL-1β. Studies investigating CNS trauma and the M2a vs. M1 phenotype show increased arginase with concomitant increases in inflammatory-associated factors (e.g., CD86, iNOS), but claim that these are separate cellular populations even though staining was performed on different tissue sections (Guerrero et al., 2012; Kigerl et al., 2009). Examination of the Guerrero et al., 2012 paper actually reveals that at least 50% of the arginase+ cells would also be iNOS+ if co-labeled images were provided. Studies that have done co-labeling on the same tissue section use markers that could be associated with either an M2a or M1 phenotype like CD68 (ED-1) (Kumar et al., 2012). Although CD68 is branded as an “M1” marker, it is associated with phagocytosis which can be attributed to both M1 and M2 phenotypes (David and Kroner, 2011; Leidi et al., 2009; Nahrendorf et al., 2007; Takahashi et al., 2005; Turnbull et al., 2006).

Further support for a fuzzy distinction in M1 versus M2a profiles derives from studies in atherosclerosis and myocardial infarction. Early after myocardial infarction an inflammatory Ly6C^hi population of monocytes traffics to the cardiac tissue followed by an anti-inflammatory wave of Ly6C^lo monocytes (Nahrendorf et al., 2007). This characterization has helped to drive the M1 vs. M2 characterization for monocytes. Recently, however, a study using a model of atherosclerosis demonstrated that scavenger receptor-A (SR-A)^+ monocytes, associated with an M2a phenotype (Fairweather and Cihakova, 2009; Mantovani et al., 2004) were actually derived from the more inflammatory Ly6C^hi monocytes (Robbins et al., 2013). Whether these monocytes would also be producing high levels of inflammatory cytokines is unknown, but certainly
deriving from the Ly6C\textsuperscript{hi} population suggests that M2a phenotypes can also be inflammatory. In further support of this notion, studies using a glioma cancer model show that IL-4R\(\alpha\) expression on monocytes was restricted to the Ly6C\textsuperscript{hi} lineage (Kohanbash et al., 2013). Interestingly, Ly6C\textsuperscript{hi} monocytes are typically CCR2\textsuperscript{+} (Geissmann et al., 2010; Saederup et al., 2010) coinciding with my studies here showing IL-4R\(\alpha\) expression occurs predominantly on CCR2\textsuperscript{+} monocytes. Of note, these CCR2\textsuperscript{+} monocytes are considered the inflammatory monocyte in humans (Geissmann et al., 2010).

Issues in M1 vs. M2 phenotyping may also arise if these phenotypes differ between microglia and infiltrating monocytes/macrophages. Although microglia are considered the “CNS macrophage” they originate from a different progenitor population than BM-derived monocytes (Ginhoux et al., 2010; Yona et al., 2013). IL-4 induces separate signaling cascades in B-cells compared to T-cells compared to monocytes, and may also produce a separate signaling cascades in microglia. Indeed, monocytes with an IL-4R\(\alpha^{+/}\)arginase\textsuperscript{+} phenotype are classified as myeloid derived suppressor cells (MDSCs) within the cancer literature and have anti-proliferative effects on CD4\textsuperscript{+} T-cells (Kohanbash et al., 2013). Whether microglia that become IL-4R\(\alpha^{+/}\)arginase\textsuperscript{+} can also function as MDSCs is unknown. Moreover, it is unclear which cell type is responsible for the enhanced IL-1\(\beta\) production following IL-4 stimulation after LPS. It is plausible that microglia may respond differently to IL-4 and promote IL-1\(\beta\) and CCL2, whereas infiltrating peripheral-derived macrophages could maintain an arginase\textsuperscript{+}/IL-1\(\beta^{-}\) phenotype associated with a classical M2a response. In support of this notion, conditioned media from IL-4 treated BM-derived macrophages promoted axonal growth (Kigerl et al., 2009) whereas I show data that IL-4 treated microglia significantly inhibited axonal growth/complexity. Alternatively, a previous study suggests that it is the monocytes that take on a dual phenotype in response to IL-4. In a model of experimental
autoimmune encephalomyelitis microglia stimulated with IL-4 became Ym-1+/NOlo consistent with a classical M2a phenotype. In contrast, infiltrating monocytes were Ym-1+/NOhi suggesting a mixed phenotypic state (Ponomarev et al., 2007). IL-4 is abundantly expressed in the periphery as it is associated with adaptive immune responses. Within the CNS, however, IL-4 is scarce and usually undetectable in adults (Lovett-Racke et al., 2000). Thus, it is not entirely preposterous to claim that a sudden increase in IL-4 expression within the CNS would have differential effects on CNS-resident microglia compared to peripherally-derived monocytes/macrophages. Further investigation into these distinct phenotypes in vivo is certainly warranted.

Characterizations of microglia versus peripheral monocytes/macrophages were previously difficult to execute resulting from a lack of markers to distinguish between them, particularly after a strong activating stimulus like CNS injury (David and Kroner, 2011). Recently, however, a transgenic mouse was established that specifically targets microglia in vivo (Goldmann et al., 2013; Parkhurst et al., 2013). It takes advantage of high microglial CX3CR1 expression, no CX3CR1 expression in other tissue-resident macrophages or BM progenitors, and moderate CX3CR1 expression in circulating monocytes. This mouse contains a Cre recombinase gene in the place of one CX3CR1 gene copy and is fused to a mutant estrogen binding domain that is sensitive to tamoxifen. As a result, recombination occurs only after the administration of tamoxifen. If these mice are bred with ROSA26-yfp-floxed-stop-floxed transgenic mice, tamoxifen would induce recombination in cells expressing CX3CR1 fluorescently tagging them. Using this method microglia along with circulating monocytes would be targeted. Ingeniously, however, the circulating monocytes will progressively turn over and lose their fluorescent labeling (within 4 weeks) allowing for selective targeting of microglia (Goldmann et al., 2013; Parkhurst et al., 2013). Therefore, future studies using this
mouse strain can investigate microglia and infiltrating monocyte phenotypes specifically, without the confounds of creating GFP-chimera mice (Ajami et al., 2007; Wohleb et al., 2013). Genes can also be selectively knocked-out of microglia using floxed/floxed mice creating unlimited possibilities for future studies investigating microglia physiology.

The role of IL-4 in the CNS

To discuss IL-4 signaling in the CNS we must first address the source of IL-4. It is well known that Th2 T-cells are the primary source of IL-4 within the periphery, but IL-4 is also produced by mast cells, eosinophils, and basophils during parasitic infections (Gessner et al., 2005). The source of IL-4 in the CNS is far less clear. One of the first studies to address IL-4 expression in the CNS found that CNS IL-4 protein was still detected in RAG-2 deficient mice (lacked T-cells) suggesting IL-4 protein derives from an intrinsic CNS source (Lovett-Racke et al., 2000). Nonetheless, this paper did not take into account production from mast cells, basophils, and eosinophils which can associate with the CNS (Campbell and Kernan, 1966; Ransohoff and Tani, 1998). The issue of a CNS-specific source of IL-4 was more directly addressed in a study that created BM-chimera mice in which wild type (WT) or IL-4 deficient mice were reconstituted with BM from wild-type mice. Thus, chimeras were only deficient in CNS IL-4. Following induction of EAE, mice deficient in CNS IL-4 (WT → IL-4⁻) exhibited more severe EAE concordant with increased microglial activation compared to WT controls (Ponomarev et al., 2007). These studies suggest IL-4 production within the CNS is critical in mediating the response to EAE. The cellular source of IL-4, however, was not defined. In 2002 it was suggested that astrocytes are high producers of IL-4 in active brain lesions strengthening support for CNS-specific IL-4 production (Hulshof et al., 2002). These data were corroborated in a 2012 study using a model of SCI demonstrating that GR-1⁻
CD45lo microglia are high producers of IL-4 after SCI by intracellular flow. Moreover, GR-1+/CD45hi neutrophils were very low producers or negative for IL-4 (Guerrero et al., 2012). In contrast to these studies, a separate group found that polynuclear cells expressing high levels of myeloperoxidase (i.e., neutrophils) are the primary source of IL-4 after SCI (Lee et al., 2010). Thus, it appears that a cellular source for CNS IL-4 is inconclusive. The source of CNS IL-4 is an important issue because the lag time between a CNS injury and IL-4 signaling will vary greatly depending on whether IL-4 is produced locally or infiltrates from the periphery. Because the microenvironment becomes progressively more inflammatory after SCI, signaling cascades initiated by central or peripheral IL-4 could vary greatly depending on timing. Understanding the microenvironment for IL-4 signaling is imperative to discern how a lack of IL-4 signaling in the aged affects recovery from CNS trauma.

The second point that naturally arises from the topic of IL-4 signaling in the CNS is whether it is a beneficial or detrimental phenomenon. My research indicates that acute IL-4 signaling after CNS trauma is likely beneficial. In support of this notion, I show that intact IL-4 signaling in adult WT mice promoted arginase expression and better recovery after a moderate SCI compared to aged mice. Although not presented here, I have preliminary data demonstrating that adult mice deficient in IL-4Rα also had worse recovery after SCI (data not shown). Therefore, IL-4 is required for a low level of endogenous repair.

Intriguingly, better recovery after SCI and upregulation of IL-4Rα was associated with enhanced inflammatory gene expression including IL-1β and CCL2. Although elevated IL-1β is typically associated with increased pathology in disease, in the context of injury its acute expression may be beneficial. In support of this notion, increased microglial IL-1β gene expression corresponded with enhanced DRG neuron axonal 176
growth in culture. Increased IL-1β and CCL2 gene expression also corresponded with increased recruitment of CCR2+/IL-4Rα+ myeloid-derived cells to the CNS. Previous studies show that CCR2+ monocytes are iNOS+ and contribute to improved recovery after SCI (Donnelly et al., 2011). Moreover, my research shows these peripherally-derived monocytes were capable of infiltrating the CNS and developing an arginase+ phenotype. Development of an arginase+ profile is important because studies in cancer models characterize IL-4Rα+/arginase+ myeloid cells as myeloid-derived suppressor cells (MDSCs) (Kohanbash et al., 2013). Critically, depletion of MDSCs prior to SCI worsens functional outcome (Saiwai et al., 2013). I also demonstrate that aged mice have reduced arginsa expression after SCI and i.c.v. IL-4 administration. Thus, aged mice may have an impaired ability to recruit MDSC to a site of injury. An inability for aged mice to recruit MDSCs to the CNS is unexpected considering aging is associated with an increased number of MDSCs in circulation (Corona et al., 2012; Enioutina et al., 2011). Although, these studies used GR-1+/CD11b+ labeling to classify MDSCs which is also the phenotype for activated monocytes (Bruhl et al., 2007; Serbina and Pamer, 2006), and neutrophils (Daley et al., 2008). Because aging is also associated with a defined increase in circulating monocytes and neutrophils (Corona et al., 2012; Fenn et al., 2014a), whether or not the increased CD11b+/GR-1+ populations observed in the aged are truly MDSCs is unclear. Nonetheless, cells collected from aged mice do have increased suppressive capabilities towards T-cell proliferation compared to cells from adults (Enioutina et al., 2011). Therefore, it appears that aged mice are unable to adequately recruit this suppressive population despite it being more numerous. In support of this idea, I show that even though aged mice had increased monocyte numbers compared to adult mice 1 day after SCI, the ratio of IL-4Rα+ to IL-4Rα- monocytes was significantly less in the aged. However, I did not demonstrate that the
arginase+ myeloid cells in my studies are capable of suppressing T-cell functions or inflammation after SCI and this should be a future point of investigation.

While failed recruitment of M2a monocytes in the aged is an intriguing notion, recent studies suggest that rather than impaired recruitment, the injured spinal cord of aged mice may be less supportive of M2a monocyte proliferation. Indeed, in a nematode infection model with *Litomosoides sigmodontis*, increased numbers of M2a-associated macrophages derived from *in situ* proliferation rather than recruitment of Ly6C^hi^ monocytes from the blood (Jenkins et al., 2011; Jenkins et al., 2013). Moreover, in a model of atherosclerosis, atherosclerotic progression coincides with gradual macrophage accumulation deriving from local proliferation of SR-A^+^ (M2) macrophages rather than recruitment of new macrophages to the plaque (Robbins et al., 2013). The extent to which proliferation of M2a macrophages, compared to recruitment of CCR2^+/IL-4Rα^+^ macrophages, contributes to the differential profiling observed in adult versus aged mice after SCI is unknown, and further studies using BrdU pulsing, Ki67 analysis, and phenotypic profiling should be performed.

Further support for a beneficial role for IL-4 in the CNS is the connection between IL-4 and triggering receptor expressed on myeloid cells (TREM2). IL-4 signaling through the JAK1,3/STAT6 pathway can upregulate TREM2 expression on myeloid cells (Turnbull et al., 2006). This is important as lentiviral-induced over-expression of TREM2 on primary microglia significantly improves phagocytic uptake of microsphere beads (Takahashi et al., 2005) and amyloid-beta (1-40) (Melchior et al., 2010). Phagocytic uptake of cellular and myelin debris after SCI is critical in the reparative response and necessary to promote axonal growth through the injury site (David and Kroner, 2011; Neumann et al., 2009). Further investigations are required to determine whether reduced
IL-4 signaling in the aged results in impaired TREM2 upregulation and reduced debris clearance.

The evidence presented above demonstrates that although IL-4 signaling in the CNS can promote a heightened state of inflammation, its acute induction is beneficial following CNS injury. Prolonged IL-4 signaling within the CNS, however, may do more harm than good. Indeed, chronic IL-4 induction in the periphery results in tissue fibrosis and scarring (Huaux et al., 2003; Kanellakis et al., 2012). Increased fibrosis within the spinal cord after injury would prevent axonal growth through the lesion and may restrict long-term recovery. For example, degrading the fibrotic network naturally formed by astrocytes and chondroitin sulfate proteoglycans promotes axonal growth and improved recovery after SCI (Houle et al., 2006; Kanno et al., 2014).

Chronic IL-4 expression within the CNS may also result in a B-cell response similar to that observed in allergies/asthma. IL-4-induced B-cell class switching to an IgE or IgG4 plasma cell promotes exaggerated airway inflammation in response to innocuous antigens that are acquired through ingestion or respiration (Gascan et al., 1991). It is possible that prolonged IL-4 expression would induce a strong B-cell response to CNS antigens resulting in chronic inflammation and increased morbidity. In support of this notion, IL-4 promotes the survival and maintenance of B-cells (Gascan et al., 1991) and mice deficient in B-cells have improved recovery after SCI (Ankeny et al., 2009). In contrast, extended IL-4 may promote increased levels of regulatory T-cells that would prevent T-cell-induced activation of B-cells (Homann et al., 1999). While this is an intriguing possibility, more recent studies indicate that IL-4 can also inhibit the development of regulatory T-cells in the presence of TGFβ (Dardalhon et al., 2008). This study showed that the combination of IL-4 and TGFβ generated CD45hi CD4+ T-cells that produced high levels of IL-9 and IL-10 but had no suppressive capabilities (Dardalhon et
al., 2008). Although not presented here, I have preliminary data that TGFβ levels are increased after SCI in concert with elevations in IL-4 (data not shown). Whether the TGFβ signal would persist until 7 dpi when T-cells and B-cells infiltrate the spinal cord lesion is unknown, but this prospect significantly reduces the likelihood that prolonged IL-4 would be beneficial in the context of an adaptive immune response after SCI.

Finally, chronic IL-4 signaling may promote extended recruitment or proliferation of CCR2+ myeloid cells in the CNS. Whereas I show these CCR2+ cells are also IL-4Rα+ and can take on a reparative arginase+ phenotype, others report that CCR2 expression on myeloid cells promotes neurodegeneration and inflammation detrimental to the CNS. For example, mice deficient in CCR2 show resistance to experimental autoimmune encephalomyelitis and reduced pathology (Izikson et al., 2000). In models of atherosclerosis, mice deficient in CCL2 show reduced atherosclerosis progression and attenuated pathology (Boring et al., 1998; Combadiere et al., 2008). Moreover, accumulation of CCR2+ cells in the CNS is associated with exaggerated inflammation and the development of neurobehavioral complications (Wohleb et al., 2013). Therefore, early recruitment of these cells may promote axonal growth and eventually inhibit inflammation through MDSC functions, whereas prolonged accumulation of these cells may potentiate inflammation and lead to exacerbated pathology.

**IL-4 induced polyamines – potential for inflammatory regulation**

The previous discussion highlighted several elements of IL-4 signaling, but the underlying mechanism of how IL-4 signaling results in these outcomes is unclear. Studies investigating the IL-4 signaling pathway demonstrate that IL-4 can induce NF-κB signaling through the IRS-1/2-PI3K-Akt pathway (Nelms et al., 1999) and activation of Iκκβ (Oh et al., 2010). Activation of IRS-1/2, however, is blocked during periods of high
TNF-α expression through the PI3K-Akt-mTOR pathway (Ozes et al., 2001). Therefore, IL-4Rα may not be able to signal through IRS-1/2 in the context of elevated inflammation. Moreover, in a model of ostoclastogenesis, IL-4 inhibited NF-κB signaling through a STAT-6-dependent mechanism (Abu-Amer, 2001). As IL-4 invariably results in STAT-6 phosphorylation and activation, but only occasionally promotes the IRS-1/2/NF-κB pathway (Nelms et al., 1999), it seems more likely that IL-4 would promote an anti-inflammatory state in vitro and in vivo. Why then, is IL-4 signaling associated with heightened levels of inflammatory mediators? The answer may lie in the downstream signaling cascade induced by STAT-6. A consistent theme in IL-4 signaling is the promotion of arginase expression. Arginase converts L-arginine to ornithine and urea and is best known for its role in nitrogen elimination in the liver. Outside the liver, more focused is placed on the enzymatic product ornithine. Ornithine can be further metabolized into putrescine, one of three polyamines. As arginase is the rate-limiting enzyme for the production of polyamines (Cai et al., 2002; Curran et al., 2006), an increase in arginase usually confers an increase in polyamine production. Critically, polyamine expression may be further exaggerated the context of an inflammatory response. Indeed, peripheral injection with LPS promotes neuronal and microglial expression of ornithine decarboxylase (ODC) (Soulet and Rivest, 2003), the enzyme responsible for metabolism of ornithine into putrescine. Although not reported here, my research demonstrates that peripheral LPS increased ODC levels within 24 h after injection (data not shown). Therefore, inflammatory induced IL-4Rα upregulation and exaggerated arginase expression coupled with increased ODC expression would robustly increase putrescine concentrations. This is important because treatment with the ODC inhibitor D,L-α-difluoromethylornithine (DFMO), prevented the LPS-induced increase in TLR2 expression within the CNS (Soulet and Rivest, 2003). TLR2 expression
is associated with a heightened inflammatory response, but may also be important in the context of SCI. Indeed, stimulation of TLR2 with a specific agonist prevents laser-induced axonal dieback (Stirling et al., 2013), and mice deficient in TLR2 have a reduced inflammatory response and impaired functional recovery after SCI (Kigerl et al., 2007). Thus, the production of putrescine and increased expression of TLR2 may be one way IL-4 potentiates inflammation and promotes improved recovery.

Increased CCL2 production and recruitment of peripheral monocytes to the CNS after IL-4 treatment may also arise from increased polyamines. Indeed, treatment with DFMO also prevented LPS-induced CCL2 expression in the CNS resulting in reduced trafficking of peripherally-derived monocytes to the CNS (Puntambekar et al., 2011). Polyamine production may also explain elevated axonal growth in vitro after IL-4 treatment. Research in 2002 demonstrated that arginase-induced polyamines were sufficient to inhibit myelin-associated glycoprotein (MAG) in culture and promote axonal growth (Cai et al., 2002). These studies demonstrated that axonal growth was significantly stunted in culture following treatment with DFMO. Importantly, improved axonal growth is one mechanism that may underlie improved functional recovery in adult mice compared to aged mice after SCI.

Metabolism of putrescine into the higher polyamines spermidine and spermine could also confer increased anti-inflammatory potential over time in the adult spinal cord. From my studies it is unknown how long adult mice maintain a heightened inflammatory response compared to aged mice after SCI, or whether the aged spinal cord would become more inflammatory over time corresponding to increased pathology. Evidence from peripheral immunological studies, however, indicates that spermine can inhibit macrophage production of TNF-α and IL-1β (reviewed in (Zhang et al., 2000)). In addition, several studies indicate that injured tissues, or tissues undergoing active
regeneration, produce high levels of spermine (Zhang et al., 2000). Thus, spermine likely plays a role in recovery after SCI. Increased expression of polyamines in the adult spinal cord may initially promote an inflammatory profile while allowing for potent anti-inflammatory regulation over time. It is unknown, however, whether polyamine concentrations are different between adult and aged mice after SCI.

In summary, the studies discussed above and my data presented here indicate that IL-4 signaling following a SCI results in multiple pleiotropic responses culminating in improved recovery (Fig. 6.1). From these data I hypothesize that a moderate SCI results in the acute induction of IL-4Rα and increased responsiveness to IL-4. I propose that increased IL-4 signaling results in enhanced TREM2 expression promoting clearance of cellular and myelin debris and also promotes increased arginase expression.

Figure 6.1. Proposed mechanism for IL-4 signaling after SCI.
Concordantly, SCI-induced inflammation increases ODC expression that, coupled with increased arginase, produces exaggerated levels of polyamines. The initial polyamine, putrescine, induces TLR2 upregulation resulting in reduced axonal dieback and increased IL-1β. The polyamines putrescine, spermidine, and spermine along with heightened IL-1β induce CCL2 expression allowing for increased recruitment of CCR2+/IL-4Rα+/arginase+ MDSCs. Moreover, heightened levels of polyamines also inhibit MAG. Reduced MAG expression along with reduced cellular debris then allows for increased axonal growth and improved functional recovery following SCI (Fig.6.1). In sum, I hypothesize that IL-4Rα upregulation after SCI is impaired in the aged preventing IL-4-induced arginase, TREM2, and polyamine expression resulting in reduced MDSC recruitment, limited axonal growth, reduced debris clearance, heightened chronic inflammation, increased pathology, and reduced functional recovery.

Differentiation between aging, TBI, and neurodegenerative disease

The results of Chapter 5 indicate that TBI may represent a form of accelerated brain aging. Indeed, microglia from TBI mice took on an increased MHC II+ phenotype and showed a hyper-inflammatory response to a peripheral immune challenge similar to that observed in aged mice (Henry et al., 2009; Wynne et al., 2010). Moreover, this heightened brain inflammation resulted in prolonged sickness behavior and the development of depressive-like behavior similar to the behavioral phenotype observed in aged mice (Godbout et al., 2005b; Godbout et al., 2008; Wynne et al., 2010). These results bring up 3 primary questions: 1) Does TBI recapitulate the aged brain in other aspects (e.g., reduced CD200, CX3CL1, IL-4Rα)? 2) Is TBI-associated microglia priming more closely related to normal brain aging or neurodegenerative disease? 3) Is a TBI
affiliated with a distinct brain profile separate from aging or age-associated neurodegenerative disease?

To first answer these questions we first need to identify the difference between normal brain aging and neurodegenerative brain aging (e.g., Alzheimer’s disease). Microglia dysfunction occurs as a component of normal brain aging (Frank et al., 2006; Henry et al., 2009; Letiembre et al., 2007; Sheffield, 1998; Simard et al., 2006), but is more severe or more apparent in Alzheimer’s disease. For example, normal brain aging is associated with increased MHC II expression, enhanced inflammation, and in the context of brain injury, reduced motility and reparative potential (Damani et al., 2011; Kumar et al., 2012; Sandhir et al., 2008). In Alzheimer’s disease, however, MHC II expression and inflammatory cytokine production is amplified and more closely associated with amyloid beta fibrils and plaques. Moreover, these deficits are compounded by the inability of microglia in the Alzheimer’s diseased brain to phagocytose and process amyloid beta (Simard et al., 2006). Moreover, despite an increase in inflammatory potential, normal brain aging is not associated with gross neuronal loss whereas Alzheimer’s disease is characterized by phosphorylated tau buildup and neuronal death (Fenn et al., 2014a). An interesting distinction between aging and neurodegenerative disease was addressed in the present studies of miR-29a/b-1 expression. I show that miR-29a/b-1 was increased in the brain and in microglia, specifically, of aged mice and humans. This was in accordance with previous studies showing increased miR-29a/b-1 expression in the liver and muscle in a mouse model of accelerated aging (Ugalde et al., 2011) and reduced miR-29 in a model of delayed aging (Bates et al., 2010). In Alzheimer’s disease, however, miR-29a/b-1 is reportedly reduced associated with increased expression of the enzyme BACE1 responsible for cleaving amyloid precursor protein into Aβ fibrils (Hebert et al., 2008). This discrepancy in miR-
29a/b-1 expression indicates that the aged brain and the Alzheimer’s brain have differential responses to inflammation and are truly distinct from one another.

Unfortunately, relatively little research has been done investigating TBI-associated brain aging. Acutely after severe TBI, CX3CL1 and CX3CR1 are increased in the human brain associated with a better clinical score (Gaetani et al., 2013). These results indicate that TBI could be affiliated with normal brain aging in that those who had reduced levels of CX3CL1 and CX3CR1 had increased pathology and worse prognosis. Nonetheless, it is unknown how long CX3CL1 is increased after TBI, whether patients with reduced CX3CL1/CX3CR1 would go on to develop neurodegenerative disease, or whether CX3CL1 would precipitously decline over time mimicking an aged brain. Similarly, miR-124 is increased in the plasma of TBI patients acutely after injury (Laterza et al., 2009). Increased CX3CL1 and miR-124 indicates an active reparative or damage control response that occurs after TBI. Nonetheless, it is unclear whether these factors would decline over time and eventually drop below baseline as a result of chronic secondary inflammation resulting in an aged brain phenotype.

Evidence that TBI may better resemble a neurodegenerative disease condition rather than accelerated brain aging is the development of chronic traumatic encephalopathy (CTE). TBI is the only known cause of CTE which consists of the accumulation of hyper-phosphorylated tau in neurons and eventual neuronal death (McKee et al., 2013). CTE is distinct from Alzheimer’s disease because of the lack of amyloid beta fibrils (McKee et al., 2013). Nonetheless, TBI is one of the primary risk factors for the development of Alzheimer’s disease (Mannix and Whalen, 2012; Mortimer et al., 1991) suggesting that amyloid-beta deposition may be merely delayed in these brains and that CTE represents an alternative form of Alzheimer’s disease. More evidence that TBI may be linked to neurodegenerative brain aging was not presented
here, but ongoing studies in our lab indicate that treatment of TBI with the anti-oxidant compound methylene blue (MB) limits immediate TBI-associated complications (i.e., edema, neuroinflammation) and ameliorates the development of TBI-associated depression (data not shown). These studies are significant because MB is currently undergoing clinical trial as a treatment to halt the progression of Alzheimer’s disease (Oz et al., 2011; Oz et al., 2009). Some of the same properties that benefit Alzheimer’s patients including reduced reactive oxygen species burden and reduced amyloid plaque deposition could also be important in the treatment of TBI. We will be continuing these studies to evaluate the degree to which early intervention with MB can limit long-term microglial priming and reactivity as a result of TBI.

In summary, TBI appears to resemble normal brain aging and neurodegenerative disease, as well as develop a distinct phenotype separate from both. Further research into how glia change with age after a TBI and the extent to which patients with CTE go on to develop Alzheimer’s disease are necessary to determine how to approach long-term treatment of TBI.

Examples of successful aging

It is also important to highlight that there are examples of successful aging without the development of immunological or neurological deficits. Indeed, centenarians (persons aged 100 years +) rarely have evidence of neurological disease or cognitive decline (Sansoni et al., 1993). Critically, improved mental health in centenarians is associated with maintained immune function similar to that observed in adults. Indeed, centenarians exhibit maintained lymphopoiesis and T-cell cytotoxicity compared to healthy adults (aged 19-36 years) whereas the majority of aged individuals (65-98 years) have markedly reduced lymphopoiesis and T-cell cytotoxicity (Sansoni et al., 1993). The
microglial profile of centenarians has not been examined, but would likely show a more ramified phenotype that is not hyper-reactive to an inflammatory response. How these individuals maintain adequate immune function in advanced age is unclear, but there is a noticeable connection between a healthy immune system and longevity free from mental illness.

Aging is a complex process that affects nearly every bodily system. Nonetheless, it is evident that a healthy and functional immune system may underlie the majority of age-associated complications, particularly in the context of the brain. Thus, future studies to restore basic immune competence in the brain and periphery may prevent microglial priming and impaired M2a-mediated regulation allowing for successful and healthy aging free from depression, delirium, and increased morbidity following CNS trauma.
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203


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