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# Molecular Analysis of Microglial Activation and Macrophage Recruitment in Murine Models of Neuroinflammation.

A Dissertation submitted in partial satisfaction
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by

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# DEDICATION

To

My Family.

# ABSTRACT OF THE DISSERTATION

Molecular Analysis of Microglial Activation and Macrophage Recruitment in Murine Models of Neuroinflammation.

By

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Doctor of Philosophy, Graduate Program in Cell, Molecular and Developmental Biology
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Dr. Monica J. Carson, Chairperson

Microglial activation and the recruitment of peripheral macrophages are the hallmarks of CNS inflammation (reviewed in Carson et al. 2007). These two populations of effector macrophages cannot be reliably distinguished by histology, as CNS-resident microglia express most common macrophage markers. Although CNS-resident microglia and peripheral macrophages are characterized by similar expression of surface markers, a large body of research using irradiation bone marrow chimeras and flow cytometry assays have shown that these cells types are functionally distinct (Hickey and Kimura 1999, Schmid et al. 2009, Byram et al. 2004). This dissertation primarily focuses on the identification of distinct molecular pathways that regulate the process of microglial activation and macrophage recruitment in murine models of CNS inflammation.

Dysregulation of the polyamine biosynthetic pathway has been implicated in a variety of CNS neurodegenerative disorders like Alzheimer's disease, ischemia and amyotrophic lateral sclerosis (Clarkson et al. 2004, Kim et al. 2009, Morrison et al. 1995, Morrison et

al. 1998, Virgili et al. 2006). However, the CNS intrinsic functions of polyamine biosynthesis have been not completely defined due to the existence of conflicting data generated from in vitro versus in vivo models of neuroinflammation. (Tjandrawinata et al. 1994, Szabo et al. 1994, Gilad et al. 2004, Soulet and Rivest. 2003). Using a murine model of acute, self resolving CNS inflammation, we demonstrate polyamine biosynthesis plays a dual role in the maintaining tissue homeostasis and sustaining CNS inflammatory responses. In-situ hybridization analysis of brain tissue from mice sacrificed post intracerebral LPS injection revealed that both, neuronal as well as non-neuronal cells rapidly upregulated the expression of ornithine decarboxylase (ODC) mRNA expression, the rate limiting enzyme in polyamine biosynthesis. Interestingly, inhibition of polyamine biosynthesis by intracerebral injections of DFMO (a suicide inhibitor of ODC), resulted in decreased LPS-induced expression of the macrophage chemoattaractant CCL2 as well as macrophage influx into the inflamed CNS. However, polyamine inhibition had no effects of microglial activation in response to CNS inflammation.

In addition to dysregulated polyamine metabolism, a factor in the development of neurodegenerative diseases like Alzheimer's disease, is the propensity of the aged CNS to mount chronic inflammatory responses. Healthy aging has been long regarded as a key factor contributing to microglial immune responses (Bilbo S.D. 2009, Frank M.J et al. 2008). To understand the differential effects of aging on the immune responses of the resident microglia and the infiltrating macrophages, we used healthy 3 months (young

adult), 8 months (mid-aged adult) and 15 months (aged adult) mice to induce CNS inflammation. Using flow cytometry we showed that CNS resident microglia in the healthy, non-inflamed aged brain assumed a pro-inflammatory phenotype characterized by upregulated expression of MHC class II, CD40 and B7.2. In addition, the aged CNS displayed a modest increase in the infiltration by peripheral macrophages in the absence of any inflammation. Interestingly, the recruitment of peripheral macrophages to the CNS in response to CNS inflammation was not altered as a function of age. However, aging differentially affected the microglial immune responses upon LPS-induced neuroinflammation. CNS resident microglia from the aged CNS responded with a more robust upregulation of the classical pro-inflammatory activation markers, namely, MHC class II, CD40 and B7.2. In addition, we also observed an age associated increase in the upregulation of markers of alternative activation TREM2, but not mannose receptor (MMR). No such age-associated changes were seen in the immune response of the peripheral macrophages.

Lastly, we have also examined the regulation of a family of receptors, the TREM-like transcripts (TLTs) in response to inflammatory signals (LPS+IFNγ). The TLTs, which are members of the TREM family of receptors have been identified as putative receptors expressed by various cells of the myeloid lineage. Using in vitro and in vivo models of microglial and macrophage activation, we showed that TLT1, TLT2, TLT4 and TLT6 are differentially regulated by LPS+IFNγ in vitro and in vivo.

In summary, our data suggests that

1. The processes of microglial activation and recruitment of macrophages during CNS inflammation are regulated by distinct molecular mechanisms.
2. The CNS may be in active control of the immune responses mounted. Continuous, bi-directional communication between the neurons and the glial population may be critical in the shaping of glial immune responses.

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LIST OF ABBREVIATIONS.

AD: Alzheimer's disease

AMPA: α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate

APC: allophycocyanin

ApoD: apolipoprotein D

BBB: blood-brain-barrier

CCL2: chemokine (C-C motif) ligand 2

CNP: 2',3'-cyclic nucleotide 3'-phophodiesterase

CNS: central nervous system

COX: cyclo-oxygenase

Ct: comparative cycle threshold

DFMO: α-di-fluoro-methyl-ornithine

DMEM: Dubecco's modified Eagle's medium

DNA: deoxyribonucleic acid

EAE: experimentally induced autoimmune encephalomyelitis

FBS: fetal bovine serum

FITC: fluorescein isothiocyanate

FMN: facial motoneuron nucleus

GABA: gamma-aminobutyric acid

GFAP: glial fibrillary acidic protein

GFP: green fluorescent protein

HPRT: hypoxanthine phosphoribosyl transferase

Iba1: ionizing calcium binding protein 1

ICOS: inducible co-stimulator

IFN: interferon

IL: interleukin

INOS: inducible nitric oxide synthase

ITAM: immunoreceptor tyrosine based activation motif

ITIM: immunoreceptor tyrosine based inhibition motif

IP: intraperitoneal

KO: knock-out

LPS: lipopolysaccharide

MCP: monocyte chemoattractant protein

MG: microglia

MHC: major histocompatibility complex

MOG: myelin oligodendrocyte glycoprotein

MP: macrophages

NMDA: N-methyl-D-aspartic acid

MMR: macrophage mannose receptor

NO: nitric oxide

ODC: ornithine decarboxylase

PD: Parkinson's disease

PD-L1: programmed cell death ligand 1

PE: phycoerythrin

PLP: proteolipid protein

PMP: peritoneal macrophages

RNA: Ribonucleic acid

qPCR: quantitative real-time PCR

SPF: specific pathogen free

SSAT: N1-spermine-spermidine acetyl transferase

SV: splice variant

TCR: T cell receptor

TGF: transforming growth factor

TLR: toll-like receptor

TLT: Trem-like transcript

TNF: tumor necrosis factor

TREM: triggering receptor expressed on myeloid cells

# CHAPTER ONE

# INTRODUCTION

## 1.1 BACKGROUND

The mammalian central nervous system (CNS) is a complex integrated organ comprised of

1. Neurons (10% of total number of CNS cells and 50% of the total cell mass).
2. Glia (90% of the total cell number but only 50% of the total mass of the CNS) (Simons and Trajkovic. 2006, Verkhatsky et al. 2006, He et al. 2007)

Glia can be further divided into macroglia (oligodendrocytes and astrocytes) which are of neuroectodermal origin and microglia which are of mesenchymal origin. While neurons and microglia are endogenous cells of the CNS, microglia and/or their progenitors appear to invade CNS tissue very early during embryonic development (Carson et al. 1999, Carson et al. 2004, 2006).

The primary function of all glia is to maintain the optimal operation of the CNS. This involves : active regulation of the network's operations, ongoing maintenance to deal with normal wear and tear as well as active defense and repair following injury or pathogen attack (Carson et al.1999, Carson et al. 2004, 2006). Neurons, macroglia and microglia all coordinately and dynamically participate in these processes. All of these cells also interact with CNS-infiltrating immune cells as part of their regulation of inflammatory responses in the CNS. Until recently, the importance of microglia in homeostatic CNS function was not fully recognized.

### 1.1.1 What are Microglia?

Microglia express most common macrophage markers and are often referred to as the tissue macrophage of the brain. In the healthy brain, microglia have a stellate morphology and are found in all areas of the brain and the spinal cord. As commonly used in literature, the term microglia has been applied to at least three different types of myeloid cells : parenchymal microglia, perivascular microglia and acutely infiltrating blood-derived inflammatory macrophages that display a stellate morphology within the CNS. In this chapter, we will operationally define microglia as parenchymal cells that are largely from a self-renewing population or only rarely replenished from the adult bone marrow derived cells. We will operationally define macrophages as perivascular cells and myeloid cells which are acutely derived from the blood and which have the demonstrated potential to emigrate from the CNS shortly after entry (period of days to weeks. (Hickey et al.1998, Simard A.R. et a. 2006). In the healthy CNS, blood-derived macrophages are located in the perivascular regions and are termed perivascular macrophages (Reviewed in Carson et al. 2006)

As yet there are no cell surface markers able to distinguish acutely infiltrating macrophages from CNS-resident microglia in histological preparations. However, CNS-resident can be differentiated from CNS-infiltrating macrophages by quantifying surface expression of CD45 using flow cytometry. In the adult immune system, all nucleated cells of hematopoetic lineage express CD45 (also called leukocyte common antigen).

Microglia are a unique differentiated macrophage population in the mature adult. While all other immune cells express uniformly high levels of CD45, microglia continue to express the very low levels of CD45, characteristic of immature immune cells during early embryonic development of the hematopoetic system (Sedgwick et al. 1991, Ford et al. 1995, Carson et al. 1998). Thus by preparing single cell suspensions from healthy and inflamed CNS, flow cytometic analysis of CD45 levels can conclusively differentiate between microglia and CNS-infiltrating macrophages.

Differential CD45 expression is a useful method to purify and separate CNS-resident microglia (CD45-low) from CNS-infiltrating macrophages (CD45-high). However, the differential expression also indicates that these two populations are likely to be differentially regulated by interactions with CNS neurons. CD45 is an inhibitory receptor for CD22 (Mott et al. 2004, Han et al. 2005). CD22 was long recognized as being expressed by B cells. More recently, Tan and colleagues have demonstrated that CNS neurons secrete a soluble form of CD22 from axonal terminals (Mott et al. 2004). These authors also demonstrated that neuronally secreted CD22 was able to inhibit microglial production of TNFα. These data suggest that CNS neurons may be more effective at inhibiting the functions of macrophages that those of microglia because macrophages express more than 5-fold higher levels of CD45 than microglia.

To date, most research of microglial function has focused on their roles during injury, pathogen infection or chronic neurodegeneration (Bechmann et al. 2001, 2005). In part,

this focus is a consequence of the dramatic and rapid changes in the microglial morphology and gene expression observed immediately following CNS injury or infection. Conversely, microglia in the healthy CNS have often been presumed to be quiescent and largely inactive. However, important homeostatic functions of microglia are suggested by the human disease referred to as the Nasu-Hakola disease (Paloneva et al. 2000, 2001, Cella et al. 2003).

Nasu-Hakola disease is a genetic disorder leading to bone spurs, early onset cognitive dementia in the 20s and death in the 30s (Paloneva et al. 2000, 2001, Cella et al. 2003). Positional cloning identified mutations in the Triggering Receptor Expressed on Myeloid Cells- 2 (TREM2) or in the signaling pathway down-stream of the receptor as the genetic causes of the disease (Paloneva et al. 2000, 2001, Cella et al. 2003). Until the TREM2 pathway was identified as the cause, this cognitive dementia disease was assumed to be due to a genetic defect in neurons (Paloneva et al. 2000). However, TREM2 is not expressed by neurons. TREM2 mRNA can only be detected in microglia (Schmid et al. 2002, 2009, Thrash et al. 2009). These data, in conjunction with similar findings from other groups illustrate that a primary disease of microglia has the potential to lead to a disease with primary psychological manifestations, rather than primary inflammatory or autoimmune manifestations (Bouchon et al. 2001, Schmid et al. 2002, Daws et al. 2003, Melchoir et al. 2006).

### 1.1.2 **What do Microglia do in the normal CNS?**

Recently, a variety of relatively non-invasive techniques have been applied to explore microglial function in the healthy CNS, and to quantify changes in cellular activity upon acute injury (Davalos et al. 2005, Nimmerjahn et al. 2005). Using two-photon imaging of fluorescently labeled cells, these groups have monitored the extension of microglial processes and the motility of microglia, before and after introduction of a focal injury. In the healthy CNS, microglial cells had small, rod shaped cell bodies with numerous thin and highly ramified processes extending from the cell body in a symmetrical arrangement. Using time-lapse imaging, they observed that while the microglial cell bodies remained relatively fixed, their processes were remarkably motile. The processes underwent continuous cycles of de novo formation and withdrawal, apparently surveying all elements of their local environment every 6 hours.

As part of this analysis, the authors also noted that not all microglial processes were motile to the same degree (Nimmerjahn et al. 2005). A subset of microglia processes provided a stable scaffold, perhaps anchoring the microglia in place. These data suggest that the branching of the microglial processes may not be random and may serve to integrate homeostatic signals throughout the CNS. It is tempting to speculate that this may be a mechanism by which microglia help modulate the extensive network of neuronal synapses and functional plasticity of the healthy CNS.

Microglia are the resident immunocompetent cells of the CNS and provide the first line of defense in response to pathogens and neuronal injury. As such they can produce a wide variety of cytokines, proteases, reactive oxygen species as well as regulate CNS-infiltrating T cells in an antigen specific manner (reviewed in Carson et al. 2006). Microglia have been demonstrated to express basal levels of MHC class II, a molecule essential for presentation of antigens to T cells. This primary interaction between the antigenic peptide in the context of MHC class II and the T cell receptor (TCR) is the first signal that drives T cell activation (reviewed in Carson et al. 2006). Nimmerjahn et al. (2005) propose that the constant microglial surveying of the brain is necessary for microglia to fulfill their defense functions. Using targeted disruption of the blood-brain-barrier (BBB), Nimmerjahn et al. showed that microglial responses to the injury were rapid. Within minutes, their processes were spatially directed to the focal injury. Thus microglial activation can be triggered by tissue damage and not just by the presence of pathogens or foreign toxins.

Astrocytes are also an important part of the neuronal support system, and play a key role in CNS immune responses as well as in glutamate uptake in response to neuronal activity (Parpura et al. 2004, Volterra and Steinhauser, 2004). Somewhat surprisingly, Nimmerjahn et al. (2005) found that the basal motility of the microglial processes was much higher that that of the astrocytes. Notably, while microglial processes rapidly extended towards an acute focal injury, astrocytic processes did not. These data provided

a dramatic demonstration of the cell-type specific support provided by microglia and astrocytes.

### 1.1.3 What are microglia monitoring in the CNS?

Microglia are known to recognize pathogens using evolutionarily conserved pathogen-recognition receptors (PRPs), such as the Toll-like receptors (TLRs) (Lee and Lee .2002, Schiller et al. 2002). However, the obvious question is, how do microglia recognize changes in the CNS function and neuronal health? And can microglia directly detect neuronal activity? Over 10 years ago, Neumann and colleagues demonstrated that blocking neuronal activity in slice cultures with the sodium-channel blocker tetrodotoxin resulted in rapid microglial activation (Neumann et al. 1996, 1998). More recently, Nimmerjahn and colleagues reported that focal application of a GABA-blocker, bicucullin (which in turn results in upregulation of neuronal activity), dramatically increased the surveillance of the affected region by microglia in an otherwise un-injured region of the brain. (Nimmerjahn et al. 2005). How microglia directly detect neuronal activity is still a subject of debate. Microglia do express many molecules that potentially could allow them to detect neuronal activity. Specifically, microglia express inward rectifying potassium channels as well as receptors for many of the CNS neurotransmitters (Kettenmann et al. 1990, 1993, Schmidtamyer et al. 1994, Chung et al. 1999, Schilling et al. 2000). What is notable is that not all microglia simultaneously express all neurotransmitter receptors. Rather, CNS microglia are heterogeneous in their ability to

detect specific neurotransmitters (Kettenmann et al. 1990, 1993, Schmidtamyer et al. 1994, Chung et al. 1999, Schilling et al. 2000).

Neumann and his colleagues found in their studies that microglia were responding in part to the neurotrophins being secreted by the neurons in the cultures treated with tetrodotoxin (Neumann et al. 1996, 1998). Since then, several neuronally expressed cues able to regulate microglial function have been identified, including CD200, fractalkine, polyamines, CCL21 and ATP (reviewed in Carson et al. 2006, Melchior et al. 2006). Interestingly, these cues can be divided into those that are expressed by healthy neurons and suppress pro-inflammatory microglial responses, and those that are expressed by damaged and/or dying neurons. These latter cues by and large augment the pro-inflammatory responses of the microglia.

A dramatic demonstration of neuronally directed regulation of microglial responses is provided by the studies of Cardonna et al. examining the function of fractalkine (Cardonna et al. 2006). Fractalkine is a chemokine expressed as a transmembrane glycoprotein. It can be proteolytically cleaved from the membrane to generate a soluble fragment retaining the ability to bind to its receptor (Cook et al. 2001). Within the CNS the expression of fractalkine receptor (CX3CR1) is restricted to CNS-resident microglia and peripheral macrophages (both Iba1+). Neurons (NeuN+ cells), oligodendrocytes (NG2+ cells) and astrocytes (GFAP+ cells) do not express CX3CR1 (Cardonna et al. 2006).

By comparing microglial responses in $CX3CR1^{+/-}$ or $CX3CR1^{-/-}$ mice, Cardonna and colleagues were able to demonstrate the dual role of fractalkine in recruiting microglia to the sites of injury while simultaneously inhibiting their proinflammatory responses (Cardonna et al. 2006). Systemic inflammation induced by intraperitoneal administration of LPS in mice lacking the fractalkine receptor resulted in a significant increase in neuronal cell death in the hippocampus. To prove that this was indeed a consequence of dysregulated microglial function, microglia were isolated from the CNS of LPS-treated $CX3CR1^{+/-}$ and $CX3CR1^{-/-}$ mice. These cells were then transferred into the frontal cortex of wild-type littermates. Transferred, wild-type microglia were highly motile and trafficked along the white matter tracks of the wild-type recipient mice. Strikingly, microglia from the knock out mice failed to migrate and persisted at the site of injection for at least 36 hours following injection into the CNS. Moreover, apoptotic neurons were observed at the site of injection in animals receiving CX3CR1 KO microglia but not in those receiving wild-type microglia.

Cardonna and colleagues have identified IL1β as a key player in the dysregulated responses of the $CX3CR1^{-/-}$ microglia (Cardonna et al. 2006). Co-administration of an IL1 receptor antagonist at the same time as the injection of the $CX3CR1^{-/-}$ microglia into wild type recipients significantly reduced the number of apoptotic neurons. Transfer of these microglia into the CNS of IL1 receptor knockout mice only partially restored the migration ability of the $Cx3CR1^{-/-}$ microglia, but completely prevented the previously

observed neuronal apoptosis. Fractalkine is known to be a potent microglial chemoattractant (Cardonna et al. 2006). However, the partial restoration of migration in these studies suggests that fractalkine also serves to amplify microglial chemoattractant responses to other injury signals that are otherwise blocked by IL1 signaling.

Cardonna et. al. further demonstrated that fractalkine regulates more than just microglial responses to bacterial components such as LPS (Cardonna et al. 2006). They also examined microglial responses in the MPTP model of Parkinson's disease and in the transgenic $SOD^{G93A}$ model of ALS. In both of these models, neuronal loss was much greater in the $CX3CR1^{-/-}$ mice than in wild type or $CX3CR1^{+/-}$ animals.

### 1.1.4 Is it important to distinguish between CNS-resident microglia and acutely infiltrating macrophages?

Cardonna and colleagues (2006) observed heightened cytokine responses to LPS, in both peripheral macrophages in the peritoneum as well as in microglia from the $CX3CR1^{-/-}$ mice. Due to the experimental paradigm, they were unable to specifically examine the responses of the microglia (the CD45 low cells) as being distinct from those of the infiltrating macrophages from the periphery (the CD45 high cells). In many studies, microglia and macrophages are grouped together in one population and their differential functions are not examined. However, over the last 10 years several studies have

illustrated that these two populations are distinct from each other based on their functions as well as on the molecular level.

CNS-resident microglia are similar to most other macrophage populations in that they can be induced to express molecules such as MHC required to interact with T cells (reviewed by Carson et al. 2006). However as early as 1988, Hickey and Kimura used irradiation chimeric rodents to demonstrate that microglia and macrophages were phenotypically distinct. First, following whole body irradiation and supplementation with bone marrow, only peripheral macrophages were replaced by cells derived from donor bone marrow. Although the entire peripheral macrophage population was replaced by donor-derived cells within 3 months, all of the CNS-resident microglia remained of the host genotype for the one year period monitored. Second, Hickey and Kimura used these bone marrow chimeric rodents to differentiate between CNS-resident microglia and peripheral macrophages infiltrating the CNS. Using this model, they demonstrated that antigen-specific interactions between the CNS-microglia and myelin-specific T cells were not required to initiate or sustain destructive autoimmune responses during experimentally induced autoimmune encephalomyelitis (EAE), a rodent model of multiple sclerosis. This and other studies have subsequently demonstrated that perivascular macrophages, CNS-infiltrating macrophages and dendritic cells are by themselves, sufficient to trigger the onset and progression of EAE (Greter et al. 2005, McMohan et al. 2005). Conversely, in the absence of peripheral antigen-presenting cells (macrophages, dendritic cells), antigen-specific interactions between CNS-resident microglia and myelin-specific T cells were

insufficient to trigger and sustain EAE. In part, this inability may be due to the failure of resident microglia to traffic to draining lymph nodes (where naïve T cells are concentrated) at the same rates as perivascular macrophages or other CNS infiltrating immune cells (Carson et al. 1999).

These studies underscore the functional differences between peripheral macrophages and CNS microglia. These studies also imply that it is not simply the amount of microglia/macrophages that are present during neuroinflammation that determines pathology. Rather, the ratio of microglia to infiltrating macrophages can also determine the consequences of neuroinflammation in human disease.

### 1.1.5 Are microglia just an incomplete or redundant macrophage population?

From the types of studies mentioned above, it may be tempting to refer to microglia as partial macrophages and to assume that they merely play redundant functions in the CNS. Two studies, one using an EAE model and one using a facial axotomy model suggest otherwise.

In the first, Magnus and colleagues demonstrated that a B7-family member, B7 homologue-1 (also known as PD-L1) is abundantly expressed on the surface of CNS-resident microglia (Magnus et al. 2005). Microglial expression of PD-L1 is dramatically upregulated during the recovery phase of MOG and PLP forms of EAE and by direct IFNγ treatment. Several studies have revealed that PD-L1 acts in a negative feedback

loop, suppressing T-cell activation by decreasing IFNγ and IL2 production and by down regulating the expression of ICOS, a T cell activation marker. PD-L1 knock out mice develop more severe inflammation in the MOG-induced model of EAE consistent with PD-L1's anti-inflammatory function (Latchman et al. 2004).

CNS resident microglia can be induced to express MHC and co-stimulatory molecules such as B7.2 and CD40 and thus could act as antigen-presenting cells able to activate T cells (Carson M.J. et al. 1998). However, due to the low to intermediate level of expression of these molecules on CNS microglia with respect to macrophages CNS microglia are relatively inefficient at initiating or maintaining pro-inflammatory T cell responses (Carson M.J. et al. 1998*).* Consistent with these observations , ex-vivo T-cell proliferation assays show that while CNS resident microglia interact with T-cells, they cannot promote robust T-cell proliferation. (Carson M.J. et al.1999).

Microglial expression of the anti-inflammatory costimulatory factor PD-L1 suggests antigen-presentation by microglia may not have a fundamentally different function than other professional antigen-presenting cells. Rather, than promoting proinflammatory pathogen defense activation of T cells, microglia may help to protect neuronal function within the CNS by limiting the severity and spread of pro-inflammatory T cell responses. Consistent with this conclusion is the observation that CNS-resident microglia produce much higher levels of molecules such as prostaglandins and NO as compared to infiltrating macrophages. These inflammatory mediators repress proinflammatory

antigen-presentation by macrophages and dendritic cells and inhibit proinflammatory T cell activation (Carson et al. 1998, 1999a).

Using the facial axotomy model, Byram et. al. (2004) have demonstrated an essential and non-redundant neuroprotective function of the CNS-resident microglia. The cell body of the facial motoneuron resides within the CNS brainstem, while its axon transverses the cranium to innervate the vibrissae in the face. Slicing the axon at the point it exits the skull, causes the axon to withdraw and prevents it from subsequently regenerating and finding its natural target (Byram et al. 2004). Serpe et al. (1999) had previously shown that in this facial axotomy model, CD4(+) T cells limit the rate of facial motoneuron cell death. In the study by Byram and colleagues, the authors demonstrated that peripheral antigen-presenting cells (presumably macrophages and dendritic cells) were required to initiate a neuroprotective T cell response. While these peripheral immune cells could infiltrate the site of the facial motoneuron nucleus (FMN), they could not sustain the protective T cell response. CNS resident cells (presumably microglia) were absolutely essential to either evoke or sustain the protective lymphocyte responses.

### 1.1.6 Are CNS-infiltrating macrophages always bad for the CNS?

In both, the EAE and the facial axotomy models just discussed, microglia express high levels of MCP-1/ CCL2 (reviewed by Cardonna et al. 2006). Indeed, in many models of CNS injury and pathogen exposure, microglia are induced to express both MHC class II

(a pre-requisite to present antigen to CD4+ T cells) and CCL2 (a potent macrophage chemoattractant). Since macrophages are highly effective producers of free radicals and have demonstrated pro-inflammatory roles in EAE, microglial expression of a macrophage chemoattractant might be viewed as maladaptive for neuronal function. In contrast to this view, two studies using murine models of amyloid/Alzheimer's disease (AD) pathology suggest that microglial production of CCL2 and thus microglial recruitment of macrophages to the CNS may be an essential mechanism to impede the rate of AD pathogenesis (Simard et al . 2006, Khoury et al. 2007).

Microglia and macrophages surround the amyloid plaques in both, the human tissue and the murine models of AD (Simard et.al. 2006). However, the relative proportion of microglia to macrophages in these regions has usually not been addressed in analyses of these tissues. In one set of studies, Rivest et al. sought to identify the relative contribution of CNS-resident microglia versus hematogenously-derived macrophages in the cells surrounding the plaques (Simard et al. 2006). To this end, they created irradiation bone-marrow chimeric mice, in which the hematogenously derived macrophages expressed green-fluorescent protein (GFP) while the CNS-resident microglia did not. Not unexpectedly, the authors found that early in the formation of the amyloid plaques, peripheral macrophages were readily recruited into the CNS. Somewhat surprisingly, macrophage recruitment did not continue to increase with age and plaque distribution. Rather, the reverse trend was observed where the macrophage influx into the brain was reduced with increasing age and amyloid burden. The authors subsequently illustrated

that the peripheral macrophage population was more effective at phagocytosis of the amyloid than the resident cells. From these data, the authors concluded that the late stage failure to recruit peripheral macrophages contributed to the progression of AD pathogenesis.

El Khoury et al. recently confirmed and extended these studies (2007). The authors examined the amyloid responses in mice lacking CCL2 receptor ($CCR2^{-/-}$ mice). They demonstrated that in CCR2-/- animals, fewer peripheral macrophages were recruited to the CNS. As a result, AD pathogenesis developed more rapidly, deposition of amyloid within the vasculature was much more severe and lethality occurred at much earlier ages that in mice expressing normal levels of CCR2. Interestingly heterozygotes for the receptor expressed an intermediate phenotype, suggesting that the recruitment signal is carefully titrated in CNS immune responses.

### 1.1.7 Microglial activation gone awry – effects of peripheral infection and aging.

In the AD studies just discussed, it is speculated that the microglia are not effective in maintaining a sustained recruitment of peripheral macrophages. Cunningham and colleagues have since presented a reciprocal problem in which peripheral inflammation may prime microglia to respond in an overly aggressive fashion to neuronal insults (Perry et al. 2002, Cunningham et al. 2005).

For this study, the authors chose to examine the effects of peripheral inflammation in a mouse model for transmissible spongiform encephalopathy prion disease (ME7) (Perry et al. 2002, Cunningham et al. 2005). In the ME7 model, mice are injected within the hippocampus with brain homogenates from symptomatic prion-infected mice. .. In response to infection, mice develop vacuolation, loss of hippocampal CA1 neurons and extracellular deposition of an insoluble form of the prion protein termed $Prp^{Sc}$ . In contrast to many other neurodegenerative models (such as AD or Parkinson's disease) , microglial activation in the ME7 model is atypical and is mostly characterized by an over-expression of the anti-inflammatory cytokine, TGFβ. (Cunningham et al. 2005).

ME7 mice were subsequently challenged with LPS, either by injection into the CNS or into the peritoneal cavity. Microglia in LPS-injected ME7 mice were compared to those in ME7 mice injected with saline. Microglia had a very similar appearance in both conditions , but they displayed very different patterns of cytokine expression. Microglia from the CNS of mice challenged with LPS expressed much higher levels of IL1β and inducible nitric-oxide synthase (iNOS) (Cunningham et al. 2005). In addition, higher numbers of neutrophils were found in the CNS of mice receiving intracerebroventricular (icv) injections of LPS. Strikingly, the authors reported that intraperitoneal injection of LPS resulted in an exacerbated expression of IL-1β, COX-2 and TNF in the CNS. Furthermore, neuronal cell death was doubled in the ME7 mice receiving peripheral LPS injections. The precise mechanism underlying the observed exacerbation is as yet not fully defined. It is likely to be due to multiple mechanisms including vagal nerve

stimulation from the spleen to the hypothalamus which in turn induces BBB alterations and systemic increases in chemokine and cytokine levels.

Factors affecting the priming of the CNS resident immune cells have recently been investigated to address whether alterations in the CNS microenvironment and thus changes in the inflammatory milieu contribute to onset and progression of neurodegeneration. Healthy aging has been identified as a major factor contributing to altered glial phenotypes. CNS-resident microglia express low basal levels of MHC class II (Carson et al. 1998, 1999). The expression of MHC class II and co-stimulatory molecules such as B7.2, CIITA and IFNγ increase in the CNS with increasing age, but it is uncertain if the increase is a consequence of increased microglial activation or increased macrophage influx (Frank M.G. et al – 2005, Goodbout J.P. et al. 2009). Increased expression of GFAP indicative of astrocytic activation also increases with age (reviewed in Carson et al. 2006). Several hypotheses have been proposed to explain the appearance of reactive glia in the aged brain – increased oxidative stress and pathogen exposure over the lifetime of an individual (Goodbout J.P. et al. 2009, Bilbo S.D. et al. 2009, Dilger R.N et al. 2008). One can further speculate that glial phenotypes in the aging CNS may also be altered due to the altered environmental cues that these cells receive, particularly from aging neurons. Thus, the increase in glial reactivity associated with healthy CNS aging may be reflective of the efforts of the CNS glia in maintaining optimal CNS function.

### 1.1.8 So what goes right and what goes wrong with microglial activation?

Microglia are found in all mammalian brains and spinal cords. Conversely, there are no spontaneous animal models in which microglia are severely deficient or absent (reviewed in Carson et al. 2008). These two facts suggest an evolutionarily conserved function. However, like all myeloid cells, microglia are highly plastic and are able to summate cues from all aspects of their environment. Thus, at any point in time, the phenotype of an individual microglia is determined as a function of its environmental cues. This observation suggests two possible outcomes:

1. Microglial phenotypes are likely to be unstable and highly heterogeneous throughout the CNS and over the lifespan of the individual. This may be a direct consequence of the many different local CNS microenvironments.
2. Dysfunctional microglial responses may be a direct consequence of dysfunctional neurons and macroglia.

Lastly, in contrast to most peripheral macrophage populations, CNS-resident microglia are relatively long-lived. Thus, their dysfunction may have more long-lasting consequences than for other peripheral macrophage populations. Recently, Sierra et al. have demonstrated that with age, microglial pro-inflammatory responses become more robust (Sierra et al. 2007). These studies have been further confirmed and extended by Goudbout and colleagues, who demonstrated that induction of systemic inflammation in aged mice resulted in exaggerated glial reactivity in the CNS. Surprisingly, these studies also demonstrated that the exaggerated immune response in the CNS of aged mice was

characterized by increased production of both pro- and anti-inflammatory cytokines (Goudbout et al. 2009, Henry C.J. et al. 2009).

As yet it remains unexplained whether this altered immune response in the aged CNS is a primary dysfunction of the aged microglia or a consequence of the aging neuronal and microglial population providing inappropriate regulatory cues. In the end, it is apparent that for more of us, for most of our lives, microglial activation is either a benign or a beneficial event. However, for therapeutic interventions, it is important to discern whether the dysfunction apparent in many chronic neurodegenerative diseases is due to inherent deficits in microglia or whether inappropriate microglial activation is a consequence of a dysfunctional CNS micro-environment.

## 1.2 MATERIALS AND METHODS

The materials and methods used in the studies described in chapters 2-4 are presented below.

**Antibodies used:** Antibodies used for flow cytometry and immuno-histochemistry are as follows : from BiogenIdec - murine anti-TLT2 (0.1µg /mL), from Chemicon - murine anti-NeuN (biotinylated – 1mg/mL), murine anti – CNPase (1mg/mL), from Vector Labs - murine anti-tomato lectin (biotinylated – 1mg/mL), anti-mouse biotinylated IgG (1.5mg/mL), anti-rabbit biotinylated IgG (1.5mg/mL), from Wako - rabbit anti-Iba1 (0.5mg/mL), from BD biosciences - rat anti-mouse CD45-APC (0.2mg/mL), rat anti-mouseCD11b-FITC (0.5mg/mL), rat anti-mouse MHC class II-PE (0.2mg/mL), rat anti-mouse B7.2-PE (0.2mg/mL), rat anti-mouse CD40-PE (0.2mg/mL).

**1.2.2 Preparation of mixed glial cultures:** Mixed glial cultures were prepared as previously described (Carson et al. 1998, 1999). Briefly, brains from post-natal day 1-3 C57Bl/6 J mice were stripped of meninges, and the cortices mechanically dissociated, seeded into T-75 flasks and maintained in DMEM, supplemented with 10% FBS and insulin (5µg/ml). To isolate purified microglia, mixed glial cultures were trypsinized after being in culture for two weeks and single cell suspensions were incubated in DMEM media without phenol red for 30 minutes at $37^0$C to allow for the re-expression of trypsinized surface markers. Microglia were purified to >98% purity by flow cytometry using PE-conjugated antibodies directed against FcR/CD16/CD32 (BD

Bioscience, San Diego, CA). Cytoplasmic mRNA was prepared from isolated cells immediately after isolation as previously described (Schmid et al. 2002). Catabolic enzymes present in FBS rapidly convert putrescine, spermine and spermidine to acrolein, a highly cytotoxic compound (Lee and Sayre. 1998, Tanako et al. 2005). Therefore, to analyze the effects of polyamines, cells were cultured in serum-free media for 48 hours prior to polyamine addition.

**1.2.3 Preparation of peritoneal macrophage cultures:** Peritoneal macrophage cultures were prepared as previously described (Schmid et al. 2009, Carson et al. 1998,1999). Briefly, 3 mL of aged, sterile thioglycolate broth was injected into the peritoneum of C57BL/6J mice (Difco, Detroit, MI, USA). Mice were sacrificed 3 days post injection and peritoneal macrophages were harvested by rinsing the peritoneal cavity with two 5 mL washes of OM5 medium (Carson et al. 1998) supplemented with 5 U/mL heparin (Sigma, St. Louis, MO, USA). Exudate cells were plated and allowed to adhere. Any non-adherent cells were removed by rinsing the cultures. Greater than 90% of the remaining adherent cells were macrophages.

**1.2.4 RNA Extraction and Reverse Transcription:** RNA was extracted from isolated cells immediately after isolation as previously described (Schmid et al. 2002). RNA concentration was assessed by measuring the absorbance at 260 nm and was adjusted to 1 ug/ul. RNA integrity and absence of genomic DNA were verified by fractionating samples by electrophoresis in denaturing gels. Gels were stained with ethidium bromide

and the ratio of 28S to 18S RNA was quantified. Two micrograms of RNA were reverse transcribed using first strand cDNA synthesis kit according to manufacturers instructions (Amersham Biosciences, Buckinghamshire, UK).

**1.2.5 Quantitative Real time Polymerase Chain Reaction:** Primers were chosen to anneal with a DNA template at a temperature of 60°C and to encompass a coding fragment of 100-300 bp. Unless otherwise indicated, all the primers used were designed in the 3' UTR of the molecules tested so as to ensure specificity of amplification. Real time qPCR was performed as detailed previously (Schmid et al. 2009). In brief, a constant amount of 200 ng cDNA from each reverse transcription, or each dilution of the appropriate standard, was amplified in 25 µl of TaqMan PCR Core Reagent (Applied Biosystems) according to the manufacturer's instructions. The reaction mixture consisted of 0.5 U of AmpliTaq Gold polymerase, each of the 4dNTPs (0.2 mM), with dUTP replacing dTTP, each pair of primers (300 nM), and $MgCl_2$ (3 mM final concentration) in the above-described Tris buffer. Amplifications were performed in an ABI Prism 7700 Sequence Detector System (Applied Biosystems). The reaction was started with a step of 10 min at 50°C for the removal of uracyl residues incorporated into the cDNAs with 1 U of uracyl-N-glycosylase (AmpErase reagent). This was followed by an incubation of 10 min at 95°C, for AmpliTaq Gold activation, then 40 cycles each consisting of 15 sec at 95°C and 1 min at 60°C. At the end of each experiment, amplification products were fractionated by gel electrophoresis to verify that they migrated as a single band with the expected size. Each sample was analyzed in duplicate. For the quantitative analysis,

direct detection of PCR products was performed by measuring the progressive increase in fluorescence emitted by the binding of SYBR Green to double-stranded DNA. Data were normalized to endogenous expression of housekeeping gene hypoxanthine phosphoribosyl transferase (HPRT). Using the comparative cycle threshold (Ct) method, the amount RNA transcripts were expressed as a reference to unstimulated microglia.

**1.2.6 Induction of CNS inflammation and flow cytometric analysis of microglia :** Microglia were isolated as previously described (Carson et al. 1999) from the CNS of healthy adult C57Bl/6J mice or from mice injected intracerebrally with either LPS (100 ng/ml) or LPS (100 ng/ml) plus/minus IFNγ(10 U/ml) or LPS (100 ng/ml) plus/minus IFNγ(10 U/ml) plus-minus DFMO (0.25uM) in a total volume of 5 ul. In brief, mice were euthanized by halothane inhalation, and the brains of the mice rapidly removed and mechanically dissociated. The cell suspension was separated on a discontinuous 1.03/1.088 percoll gradient and microglia/macrophages were collected from the interface as well as from the 1.03 Percoll fraction. Microglia and CNS-infiltrating macrophages were purified by flow cytometry using APC-conjugated antibodies against pan-CD45 and phycoerythrin-conjugated antibodies against CD11b (BD Biosciences, San Diego, CA) and biotin conjugated antibodies against TLT2 (BiogenIdec., Cambridge, MA). Activated microglia were identified as CD11b-positive, CD45lo/intermediate and macrophages identified as CD11b-positive, CD45hi.

**1.2.8 In situ hybridization analysis:** *In situ* hybridization was performed on free-floating cryosections as described previously (Schmid et al. 2002). Briefly, coronal sections (25 µm) were hybridized at 55°C for 16 h with a $^{33}$P-labeled riboprobe ($10^7$ cpm/mL). Excess probe was removed by washing at room temperature (23°C) for 30 min in 0.03 M NaCl, 0.003 M sodium citrate (2 × SSC) containing 10 um β-mercaptoethanol, followed by a 1-h incubation with 4 µg/mL ribonuclease, 0.5 M NaCl, 0.5 M EDTA, 0.05 M Tris-HCl, pH 7.5, at 37°C. Sections were then washed under high-stringency conditions for 1.30 hours at 55°C in 0.5 × SSC, 50% formamide and 10 um β-mercaptoethanol, followed by a 1-h incubation at 68°C in 0.1 × SSC, 5 mmβ-mercaptoethanol and 0.1% N-lauryl sarcosine. Myeloid cells and blood vessels were identified by their ability to bind biotinylated tomato lectin (Sigma), while neuronal nuclei were identified by their labeling with biotinylated NeuN (Sigma). Bound biotinylated tomato lectin or NeuN was visualized by standard strepavidin–horseradish peroxidase methodology. Sections were mounted on to FisherBrand SuperFrost/plus slides (Fischer Scientific, Pittsburgh, PA, USA) and dehydrated with ethanol and chloroform. Slides were exposed for 3 days to Kodak X-AR film and dipped in Ilford K-5 emulsion (Polysciences, Warrington, PA, USA). After 3 weeks, slides were developed with Kodak D19 developer (Fischer Scientific), fixed and counterstained with Mayer's hematoxylin.

**1.2.9 In vitro stimulation of mixed glial cultures with polyamines:** Mixed glial cultures were grown on 8-welled chambered slides and treated with putrescine,

spermidine or spermine at final concentrations of 1μM, 100μM or 1000μM. Supernatant were removed 24 hours post treatment with polyamines. Cultures were fixed using ice cold 4% paraformaldehyde in 1X PBS. Immunohistochemistry was performed as described earlier.

**1.2.10 Microscopy :** Microscopic analysis of polyamine-treated mixed glial cultures and brain tissue sections stained with anti-NeuN or anti-Tomato lectin or anti-Iba1, was done using an Zeiss Axioscope 2 *mot plus* microscope.

**Quantification of $^{33}$P labeled riboprobe expression in tissue sections:** The degree of gene expression in each tissue section was expressed as a function of autoradiogram film exposure caused by tissue-bound $^{33}$P labeled riboprobe. To insure that equivalent regions were being quantified in tissue sections from different animals, quantification was performed in regions adjacent to the site of intracerebral injection. The site of injection was identified histologically based on needle induced tissue damage and microgliosis visualized by increased tomato lectin / Iba1 labeling. This area was then outlined on the corresponding autoradiogram using the Adobe Photoshop software and film exposure quantified using NIH image J software. Analysis was based on 16 tissue sections per condition in two replicate experiments.

**Detection of secreted cytokine with cytokine bead arrays:** Protein concentrations of interleukin-6 (IL-6), Interleukin-10 (IL-10), CCL2, interferon-γ (IFN-γ), tumor necrosis factor (TNF), and interleukin-12p70 (IL-12p70) in the supernatants of serum-free mixed

glial culture were measured using the BD$^{TM}$ cytokine bead array (CBA) mouse inflammation kit according to the manufacturer's protocol. In brief, 500ul of collected supernatants were incubated for 2 hours with 50ul of mixed capture beads and 50ul of the PE-detection reagent. The PE-detection reagent is a mixture of PE-conjugated anti-mouse IL-6, IL-10, CCL2, IFN-γ, TNFα and IL-12p70 antibodies. The samples were washed and resuspended in 300ul of wash buffer and analyzed immediately by flow cytometry using a BD FACs Calibur and BD Cell Quest software (version 5.2.1). A standard curve and individual capture beads for each cytokine are provided with the kit. Cytokine protein concentrations were determined using BD cytometric Bead Array Software per kit instructions.

**Generation of data graphs and statistical analysis**: All data graphs and statistical analysis was generated using the graphing software GraphPad Prism version 5.0a.

# CHAPTER TWO

## LPS – Induced CCL2 Expression And Macrophage Influx Into The Murine Central Nervous System Is Polyamine – Dependant.

## 2.1 Abstract

**Increased polyamine production is observed in a variety of chronic neuroinflammatory disorders, but in vitro and in vivo studies yield conflicting data on the immunomodulatory consequences of their production. Ornithine decarboxylase (ODC) is the rate-limiting enzyme in endogenous polyamine production. To identify the role of polyamine production in CNS-intrinsic inflammatory responses, we defined the CNS sites of ODC expression and the consequences of inhibiting ODC in response to intracerebral injection of LPS+/- IFNγ. In situ hybridization analysis revealed that both neurons and non-neuronal cells rapidly respond to LPS+/- IFNγ by increasing ODC expression. Inhibiting ODC by co-injecting an ODC suicide inhibitor decreased LPS-induced CCL2 expression and macrophage influx into the CNS, without altering LPS-induced microglial or macrophage activation. Consistent with these data, addition of putrescene and spermine, but not spermidine to mixed glial cultures increased levels of CCL2 and TNFα. Addition of all three polyamines to mixed glial cultures also decreased the numbers and percentages of oligodendrocytes present. However, inhibiting the basal levels of polyamine production was sufficient to induce expression of apolipoprotein D, a marker of oxidative stress, within white matter tracts. Considered together, our data suggest that: (1) elevating polyamine levels triggers pro-inflammatory responses within the CNS, (2) CNS-resident cells including neurons play active roles in recruiting peripheral macrophages into the CNS via polyamine-induced CCL2 expression (3) modulating polyamine production**

**in vivo may be a difficult strategy to limit inflammation and promote repair due to the dual homeostatic and pro-inflammatory roles played by polyamines.**

## 2.2 INTRODUCTION

Putrescine, spermidine and spermine belong to a class of small polycationic molecules termed polyamines (Bistulfi et al. 2009, Moinard et al. 2005, Pegg. 2009). Putrescine, spermidine and spermine can be synthesized by all nucleated eukaryotic cells and are sequentially produced following enzymatic decarboxylation of ornithine (figure 1a). In vivo, ornithine decarboxylase (ODC) is the rate-limiting enzyme in polyamine synthesis (Bistulfi et al. 2009, Moinard et al. 2005, Pegg. 2009). In the CNS, increased levels of ODC and polyamines are detected in animal models of several neurodegenerative disorders including amyotrophic lateral sclerosis, ischemia and Alzheimer's disease as well as in brain tissue from individuals with Alzheimer's disease (Clarkson et al. 2004, Kim et al. 2009, Morrison et al. 1995, Morrison et al. 1998, Virgili et al. 2006). Polyamines regulate a broad array of cellular functions in both neurons and inflammatory cells. However, conflicting in vitro and in vivo data have hindered determining the net neurotoxic versus neuroprotective consequences of elevated polyamine production within the CNS.

Within the CNS, polyamines have the potential to contribute to neurodegeneration by direct actions on neurotransmission. Studies using cultures of primary neurons and neuronal cell lines reveal their ability to bind and modulate nicotinic acetylcholine receptor, NMDA and AMPA-receptor mediated neurotransmission (Doyle et al. 1996, Mony et al. 2009, Rao et al. 1991, Shin et al. 2005). Polyamines also have the potential to contribute to neural repair and regeneration. In vitro, polyamines promote neurite

outgrowth and neurite fasciculation (Cai et al. 2002, Deng et al. 2009, Georgiev et al. 2008, Khaing et al. 2006, Schreiber et al. 2004). In vivo, polyamines stabilize synapse formation during development (Cai et al. 2002, Deng et al. 2009, Georgiev et al. 2008, Khaing et al. 2006). Furthermore, both in vitro and in vivo, axonal regeneration of dorsal root ganglions has been demonstrated to be dependent on spermidine synthesis (Cai et al. 2002, Deng et al. 2009, Gao et al. 2004, Spencer et al. 1994).

Polyamines also have the potential to play primary roles in regulating the inflammatory responses that are hypothesized to contribute to CNS neurodegenerative disease. In vitro studies using peripheral macrophages and macrophage cell lines suggests polyamines play primarily an anti-inflammatory role. For example, blocking polyamine export out of macrophages and thus increasing intracellular polyamine levels decreases activation-induced expression of INOS and pro-inflammatory cytokines (Tjandrawinata et al. 1994, Szabo et al. 1994). Treating LPS-activated macrophages and monocytes with spermine not only inhibits expression of INOS and the proinflammatory cytokines, IL12 and TNF, it increases expression of the anti-inflammatory cytokine, IL-10 (Hasko et al. 2000, Bussiere et al. 2005). Microglia are the tissue macrophage of the CNS. When α-di-fluoro-methyl-ornithine (DFMO), a suicide inhibitor of ODC is added to mixed glial cultures, both polyamine levels and microglial proliferation are reduced. (Gilad et al. 2007).

In contrast to these in vitro studies using cultured macrophages and microglia, Soulet and Rivest (2003) identified polyamines as pro-inflammatory mediators in murine models of

acute CNS neuroinflammation induced by intraperitoneal (IP) injection of LPS. IP injection of LPS is a standard model of murine sepsis and is associated with high systemic levels of proinflammatory cytokines. In their studies, Soulet and Rivest carefully documented that LPS was excluded from the CNS. Thus the CNS neuroinflammation observed in this model is a secondary response to systemic inflammation. Increases in ODC activity in brain homogenates and ODC expression throughout the murine CNS are observed in response to IP injections of LPS and precede the increases in TLR-2 and TNF expression. Conversely, the LPS-triggered increases in TNF and TLR2 expression in the CNS could be largely prevented when systemic and CNS ODC activity were inhibited by adding DFMO to the drinking water of IP-injected mice. Alone polyamines were insufficient to trigger inflammation; rather, polyamines exacerbated systemically-induced inflammation. Intracerebral injection of spermine did not induce TLR2 and TNF expression in the CNS. By contrast, intracerebral injection of spermine prior to IP injections of LPS increased the numbers of cells in the CNS expressing both TNF and TLR2. These data clearly identify a role promoting proinflammatory responses. However, ill-defined in these studies are the relative roles of polyamines on inflammatory responses initiated within and intrinsic to the CNS.

In the current studies, we test if polyamines play a necessary role in promoting or limiting pro-inflammatory responses initiated from within the CNS. Specifically, we initiate neuroinflammation from within the CNS by injecting LPS +/- IFNγ intracerebrally. When dissecting mechanisms contributing to CNS neuroinflammation and neurodegeneration, it

is important to note that two different macrophage populations participate: CNS-resident microglia and blood-derived, CNS-infiltrating macrophages (Carson et al.1998, Carson et al. 2007, Schmid et al. 2009, Simard et al. 2006). In contrast, to neuroinflammation caused as a secondary consequence to IP injections of LPS, intracerebral injection of LPS +/- IFNγ induces robust macrophage influx into the CNS (Carson et al. 1998, Schmid et al. 2009). Molecular and cellular assays have revealed that the CNS-resident microglia and CNS-infiltrating macrophages have distinct phenotypes and functional responses to pathogenic stimuli in vivo and ex vivo (Carson et al. 2009, Schmid et al. 2009, Simard et al. 2006). Therefore, in the current studies, we also test whether polyamines differentially regulate microglial and macrophage activation. In brief, we find that increased ODC expression by neurons and glia was a direct consequence of intracerebral injection of LPS +/- IFNγ and that inhibiting ODC within the CNS did not alter either microglial or macrophage activation. However, inhibiting polyamine production locally within the CNS did decrease glial production of macrophage chemoattractant, CCL2 and thus the numbers of peripheral macrophages entering the brain. In sum, our data further identify specific CNS-intrinsic contributions polyamines make toward promoting CNS neuroinflammation.

## 2.3 RESULTS

### 2.3.1 LPS/IFNγ induces cultured microglial expression of polyamine regulatory enzymes.

We and others have previously demonstrated that treating primary microglia derived from mixed glial cultures with LPS+/-IFNγ leads to their rapid activation, characterized by increased expression of pro-inflammatory molecules such as MHC class II, B7.2, and CD40 (Carson. 1998, Schmid et al. 2009). Treating peripheral (non-CNS) macrophages with pro-inflammatory stimuli such as LPS +/- IFNγ causes similar inductions of many pro-inflammatory molecules as well as increased expression of ODC, the rate limiting enzyme in polyamine production (figure 1a, reviewed in Salimudin 1999). Secondary CNS neuroinflammation caused by intraperitoneal LPS injection also causes increased ODC expression in limited numbers of CNS neurons and microglia (Soulet and Rivest ,2003). However, it was unexamined whether direct activation of microglia with pro-inflammatory stimuli would regulate microglial expression of polyamine biosynthetic enzymes.

Therefore using quantitative real-time PCR (qPCR) and cDNA templates prepared from mixed glial culture microglia, we quantified LPS/IFNγ regulated expression of three critical regulators of polyamine production: **(a)** ODC, **(b)** antizyme, an endogenous inhibitor of ODC that also promotes ODC degradation and **(c)** N1-spermine-spermidine acetyl transferase (SSAT) which catalyzes the regeneration of putrescine from spermine (figure 1a). In response to 24 hours of LPS/IFNγ treatment, ODC expression increased by

more than 5-fold over the basal level of expression ($p < 0.001$) (figure 1b). In addition, LPS/IFNγ treated increased expression of SSAT and antizyme, 1.89 ($p < 0.001$) and 3.50 ($p < 0.001$) fold over basal levels, respectively (figure 1b).

### 2.3.2 LPS/IFNγ induces ODC expression in both neurons and glia in the adult murine CNS.

We previously reported that cultured microglia are only in part predictive of microglial responses in vivo (Carson et al. 1998, Carson et al. 2009, Schmid et al. 2009). Therefore, we examined whether direct intracerebral injection of LPS/ IFNγ led to an increase in ODC expression in the CNS as revealed by $^{33}$P-labeled riboprobe exposure of autoradiograms. Basal levels of ODC expressed in the CNS of untreated specific pathogen-free (SPF) adult mice were at the border of detection by in situ hybridization analysis (figure 2a). However, as early as three hours post-injection of LPS/IFNγ, increased ODC expression was readily detected throughout the CNS, including sites distant from the site of injection (figure 2b). The highest level of ODC induction was observed in the meninges (downward arrow, figure 2b) and in the dentate gyrus (upward arrow, figure 2b). Within the dentate gyrus, the induction of ODC was always higher on the hemisphere receiving the intrastriatal injection of LPS/IFNγ (upward arrow, figure 2b). A similar pattern and level of ODC expression was detected at 6 hours post-injection of LPS+IFNγ (figure 2c). By 24 hours post-injection, ODC mRNA expression was still elevated over that of untreated controls but was much lower than the levels detected at 3 and 6 hours post-injection (figure 2d). No change in antizyme or SSAT mRNA

expression was detected by in situ hybridization following intracerebral injection of LPS/ IFNγ (data not shown).

To identify the cell types expressing ODC in brain sections from control and LPS/IFN-injected mice, microglia, macrophages and blood vessels were visualized using tomato lectin (figure 2e, f), while neurons were visualized using antibodies against NeuN (figure 2g, h). The global increase in riboprobe associated grain density throughout LPS/ IFNγ-injected brain sections suggests that ODC expression was increased in nearly all CNS cell types: neurons and glia (figure 2). However, the highest level of LPS/ IFNγ-induced ODC expression was observed in cells around the vasculature (figure 2e, f) and in hippocampal neurons (2g, h). The high level of ODC expression induced around the vasculature prevented conclusively distinguishing whether astrocytes, microglia or pericytes were the primary cells expression

### 2.3.3 Inhibiting ODC function in the CNS decreases LPS-induced influx of CD45hi macrophages into the CNS.

To identify the consequences of ODC expression in CNS neuroinflammation, we used α-di-fluoro-methyl-ornithine (DFMO) a suicide inhibitor of ODC that binds ODC and targets the molecule for rapid degradation (Wallace and Fraser. 2004). Intracerebral injection of LPS in the presence or absence of IFNγ induced similar kinetics and pattern of ODC expression in the CNS (data not shown). Therefore, for all subsequent studies only LPS+/- DFMO was injected intracerebrally into healthy adult mice.

Acutely infiltrating blood-derived macrophages cannot be distinguished histologically from CNS-resident microglia. However, these two types of myeloid cells can be distinguished in cell suspension by flow cytometric analysis of CD45 levels. CNS-resident microglia are CD45lo, while acutely infiltrating macrophages are CD45hi (reviewed in Carson et al 2008). Few CD45hi macrophages were detected by flow cytometry 24 hours after a sham intracerebral injection with vehicle only (figure 3a, 3e). Somewhat unexpectedly, intracerebral injection of DFMO alone caused a modest increase in the population of CD45hi macrophages present in the brain as compared to sham-injected brains (figure 3b, 3e, $*P < 0.05$). At low levels, polyamine production is known to play essential roles in many homeostatic functions (Bistulfi et al. 2009, Moinard et al. 2005). Thus inhibiting basal ODC activity may have generated modest cytotoxic/proinflammatory signals in the CNS. By contrast, intracerebral injection of LPS promoted accumulation of a large population of CD45hi macrophages within the CNS, 22 hours post-injection (figure 3c, 3e). Co-injection of DFMO with LPS decreased the LPS-induced macrophage accumulation in the CNS by 25% (figure 3d, 3e $*P < 0.05$). These data suggest that polyamines may have concentration dependent effects within the CNS. Basal levels of polyamines may be critical for maintaining tissue homeostasis in the CNS while high levels may promote and/or sustain CNS neuroinflammation. While co-injection of DFMO limited LPS-induced macrophage influx, it did not alter LPS-induced microglial or macrophage expression of MHC class II, B7.2 and CD40 (supplementary figure 1).

### 2.3.4 Putrescine and spermine induce production of pro-inflammatory cytokines TNFα and CCL2 in vitro.

To identify how polyamines promoted and/or sustained LPS-induced CNS neuroinflammation, we treated mixed glial cultures (comprised of astrocytes, microglia and oligodendrocytes) with putrescine, spermidine or spermine. Mixed glial cultures were maintained in serum free media for 48 hours prior to polyamine addition because polyamines are rapidly converted to the highly cytotoxic compound acrolein in the presence of serum (Lee and Sayre. 1998, Tanako et al. 2005). We then quantified chemokine and cytokine accumulation in culture supernatants using a flow cytometric bead assay following 24 hours treatment with individual polyamines. We found that at all concentrations tested (1uM-1000uM), none of the three polyamines induced detectable accumulation of the anti-inflammatory cytokine, IL-10 nor of pro-inflammatory cytokines IL12p70, IFNγ, IL10 and IL6 in culture supernatants (data not shown). By contrast, putrescine and spermine both had dose-dependent effects on the accumulation of TNF (figure 4a) and CCL2 (figure 4b) in culture supernatants. Low concentrations of these two polyamines (1uM) had no effect on the accumulation of either TNF or CCL2 in cultures supernatants. However, moderate (100uM) and high (1000uM) concentrations of putrescine induced a 2- and 4-fold increase respectively in TNF accumulation, while only the highest concentration of spermine were sufficient to induce a 3-fold increase in TNF (figure 4a, $P<0.001$). The effects of putrescine and spermine on CCL2 accumulation were more dramatic (figure 4b). While low

concentrations of each polyamine had no effect on the amount of CCL2 secreted into mixed glial culture supernatants, moderate (100uM) levels of both putrescine and spermine caused more than a 100-fold increase in CCL2. The intermediate polyamine synthetic product, spermidine had no effect on TNF or CCL2 production at all concentrations tested (figure 4b).

### 2.3.5 Polyamine treatment leads to depletion of oligodendrocytes from mixed glial cultures

To test whether polyamine associated cytokine induction was associated with cell toxicity, we quantified total cell numbers as a function of polyamine treatment in replicate experiments. At all polyamine concentrations tested, no effect on total cell number per culture was detected (figure 5a). However, all three polyamines including spermidine decreased the numbers of 2',3'-cyclic nucleotide 3'-phophodiesterase (CNP) positive oligodendrocytes present in these mixed glial cultures in a dose-dependent manner (figure 5b). The ability to promote TNF production did not correlate with oligodendrocyte depletion. Putrescine was the most potent polyamine in promoting TNF production, but it was the least potent in reducing oligodendrocytes numbers in mixed glial cultures.

### 2.3.6 DFMO inhibits LPS-induced expression of CCL2 in the adult CNS

Addition of polyamines to mixed glial cultures stimulated production of the macrophage chemoattractant CCL2, suggesting a role for polyamines in macrophage recruitment. We

therefore tested whether inhibiting ODC in vivo would reduce LPS-induced CCL2 expression in brain regions directly adjacent to the site of intracerebral injection (figure 6a). The region in which CCL2 expression was quantified is identified as the boxed regions in figures 6b, 6f. and 6j). Densitometric analysis of brain sections hybridized with $^{33}$P-labeled CCL2 riboprobes demonstrated that intracerebral injection of LPS induced a 2.5-fold higher level of CCL2 expression than DFMO alone (figure 6a, $P < 0.001$). Strikingly, co-injection of both LPS and DFMO dramatically limited LPS-induced CCL2 expression to nearly the same levels as observed in mice receiving DFMO-only injections (figure 6a, $P < 0.001$). Taken together, these in vitro and in vivo studies suggest that LPS-induced macrophage influx into the brain is in part a consequence of polyamine stimulated CCL2 production within the injected CNS.

Blood vessels, microglia and macrophages were visualized with tomato lectin in tissue sections hybridized with $^{33}$P-labeled CCL2 riboprobes to identify the spatial relationship of CCL2 expression with the vasculature (figure 6 b-m). Following intracerebral DFMO injection, CCL2 expression was detected in only a small number of both tomato lectin positive and negative cells, generally found closely adjacent to blood vessels (figure 6c-d). Following intracerebral injection of LPS, CCL2 expression was robustly induced in a large number of lectin positive and negative cells (figure 6g-i). However, induced CCL2 expression was not limited to cells directly adjacent to blood vessels. Co-injection of DFMO with LPS dramatically reduced the numbers of cells with detectable CCL2

expression, as well as the numbers of lectin-positive cells displaying an ameboid morphology (figure 6k-m).

### 2.3.7 Inhibiting basal ODC function induces apolipoprotein D expression in white matter tracts

Intracerebral injection of DFMO alone was sufficient to induce a small, transient accumulation of CD45hi macrophages within the CNS (figure 3), but did not lead to sustained micro- or astrogliosis (figure 5, and data not shown). Increased apolipoprotein D (ApoD) is an early stage response to tissue damage and inflammation and its expression is associated with oxidative stress (Gangfornia et al. 2008). We therefore, tested by in situ hybridization analysis whether elevated expression of ApoD would be detected in brains receiving intracerebral injections of DFMO alone (figure 7). ApoD expression was very low, but detectable in the meninges of brains receiving intracerebral injections of vehicle alone (figure 7a). Injection of DFMO alone, LPS alone or DFMO plus LPS, all led to similar increases in ApoD expression globally throughout the CNS, but most prominently within white matter tracts such as the corpus collosum (figures 7b-d). These data suggest that the observed influx of a small population of CD45hi cells into the CNS with DFMO only injections was in response to tissue stress and/ or damage caused by inadequate basal polyamine levels to maintain essential cell function. Our in vitro (figure 5) and in vivo (figure 7) data suggest that oligodendrocytes and white matter are highly sensitive to changes in normal polyamine metabolism.

## 2.4 DISCUSSION

The endogenous polyamines (putrescine, spermidine and spermine) regulate a diverse array of cellular functions and are essential for normal cell growth and survival (Moinard et al – 2005). Within the CNS, polyamines levels are high during early development and decrease to low steady state levels after birth (Khaing et al. 2006, Malaterre et al. 2004). Polyamines and/or expression of ODC increase dramatically in response to systemic inflammation (Soulet and Rivest. 2003) and during the pathogenesis of several CNS neurodegenerative diseases (Clarkson et al. 2004, Kim et al. 2009, Morrison et al. 1995, Morrison et al. 1998, Virgili et al. 2006). In our current studies, we demonstrate that increased ODC expression is an early response of nearly all CNS resident cells (neurons and glia) to neuroinflammation induced from within the CNS via intracerebral injection of LPS +/- IFNγ.

Previously published experiments aimed at determining the functional consequences of increased polyamine production during CNS injury and disease have yielded conflicting data. While very high levels of polyamines are toxic to neurons, in vitro and in vivo studies, reveal that polyamines can promote critical repair processes required to support axonal outgrowth and regeneration even in the presence of the inhibitory effect of intact myelin (Cai et al. 2004, Gao et al. 2004, Spencer et al. 2004). With respect to neuroinflammation, in vitro data examining the effects of polyamines on macrophage activation in vitro reveal an anti-inflammatory role of polyamines especially when these

molecules accumulate within macrophages (Tjandrawinata et al. 1994, Szabo et al. 1994, Hasko et al. 2000). Notably, LPS and IFNγ stimulated macrophage activation is associated with specific export of putrescine from intracellular stores, while pharmacologic inhibition of putrescine export inhibits LPS and IFNγ triggered proinflammatory cytokine production (Tjandrawinata et al. 1994, Szabo et al. 1994, Hasko et al. 2000). These data demonstrate the neuroprotective potential of increased polyamine levels during CNS injury and repair.

The potential contribution of polyamines toward pro-inflammatory responses has also been clearly demonstrated. In response to LPS-induced systemic inflammation (Soulet and Rivest. 2003), ODC activity transiently increases in the CNS concurrent with increased expression of two pro-markers of pro-inflammatory responses: TNF and TLR2. Adding DFMO, a suicide inhibitor of ODC to the drinking water of mice not only decreased ODC expression and activity in the CNS, it decreased CNS expression of both TNF and TLR2. One uncertainty from these experiments is whether the observed modulation in TNF and TLR2 expression in the CNS was an indirect consequence of modulating systemic inflammation. In this model, systemic inflammation was initiated by intraperitoneal injection of LPS and the authors carefully documented that LPS did not enter the CNS. In addition, the route of DFMO administration would have decreased systemic inflammation as well as CNS inflammation.

Our current studies focused on CNS-initiated and CNS-intrinsic regulation of neuroinflammation by polyamines. Here, we used intracerebral injection of LPS +/- IFNγ to trigger a robust, transient activation of CNS-resident microglia and influx of activated macrophages. Using this primary model of CNS neuroinflammation, we found that acute inhibition of ODC within the CNS via co-injection of the ODC suicide inhibitor, DFMO with LPS decreased LPS-triggered CNS expression of CCL2, a potent macrophage chemoattractant and accumulation of CD45hi blood derived macrophages within the CNS. Although the numbers of CNS-infiltrating macrophages were reduced, the activation states of microglia and macrophages were not altered when DFMO was co-injected with LPS into the CNS. Our data thus support the previous studies by Soulet and Rivest (2003) indicating a pro-inflammatory role for polyamines within the CNS. In addition, our studies identify a specific role for polyamines in facilitating immune cell recruitment into the CNS that is in part driven by neuronal expression of ODC. Our data also provide support for different polyamines playing different pro- and anti-inflammatory roles. For example, previous studies by Filbin and colleagues reveal that spermidine was the polyamine most effective at promoting axonal outgrowth and regeneration following injury (Deng et al. 2009). In our current studies, of the three endogenous polyamines, only spermidine failed to increase accumulation of CCL2 and TNF in mixed glial cultures.

The consequences of neuronal expression of ODC might at first glance be viewed as maladaptive if it ultimately results in chemokine-induced influx of macrophages into the

CNS. Chronic macrophage influx is associated with cytotoxicity. However, transient influx of macrophages into the CNS that leads to rapid removal of pathogens and/or damaged CNS tissue can be adaptive for CNS function. For example, decreased influx of blood-derived macrophages into the CNS has been correlated with increased amyloid plaque deposition in murine models of amyloid pathology (Simard et al. 2006). By contrast, in chronic neuroinflammatory conditions, it is likely that both repair and toxic functions present, but that chronic overproduction of polyamines could prevent resolution of inflammation and thus promote chronic neurodegeneration.

## 2.5 FIGURES AND LEGENDS

**Figure 2-1. Expression of key enzymes in polyamine biosynthesis is upregulated in primary microglia treated 24 hours with LPS/IFNγ. A:** Selected substrates and enzymes in the polyamine biosynthesis pathway. **B:** qRT-PCR quantification of enzyme expression over unstimulated control (ODC: ornithine decarboxylase, AZ: antizyme, SSAT: spermine/spermidine N1-acetyl transferase). Expression was calculated based on ddCTs normalized to the housekeeping gene, HPRT. Microglial expression of all mRNAs analyzed was increased in LPS/IFNγ stimulated cells as compared to unstimulated controls ($P < 0.0002$, two-tailed Student's t-test).

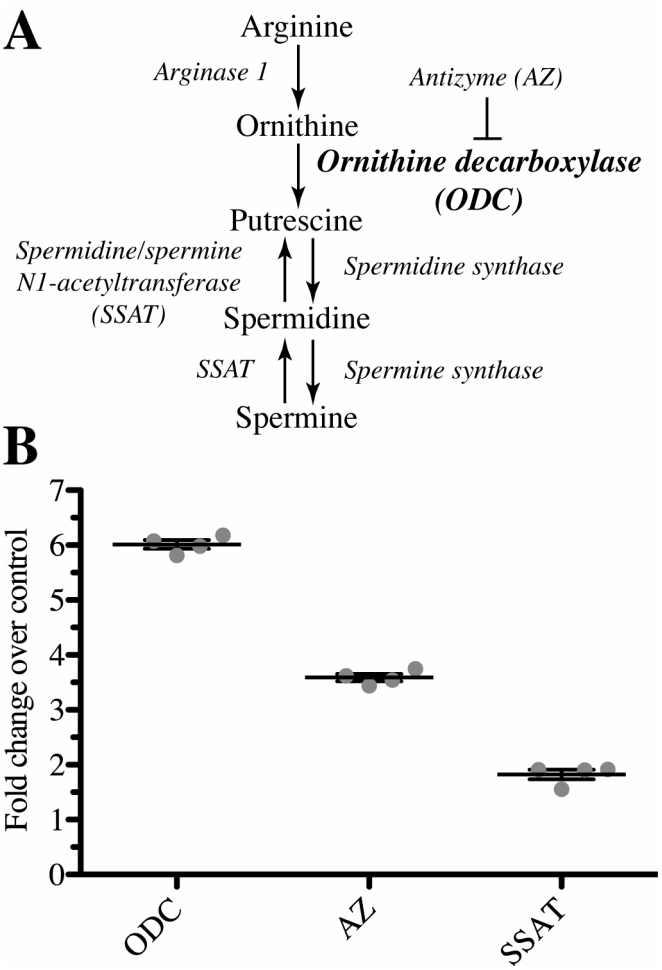

A
Arginine
Arginase 1
Antizyme (AZ)
Ornithine
Ornithine decarboxylase
(ODC)
Putrescine
Spermidine/spermine
N1-acetyltransferase
(SSAT)
Spermidine synthase
Spermidine
SSAT
Spermine synthase
Spermine
B
Fold change over control
0
1
2
3
4
5
6
7
ODC
AZ
SSAT

**Figure 2-2. ODC mRNA expression is robustly upregulated by perivascular cells and neurons following intracerebral injection of LPS/IFNγ.** ODC expression was detected by autoradiogram analysis of brain sections hybridized with $^{33}$P-labeled ODC riboprobes (A-D). Brain sections from uninjected control mice (A), from mice 3 hours (B), 6 hours (C) and 22 hours (D) post-LPS/IFNγ injection. Induced ODC expression is indicated in meninges by large downward arrow and in the dentate gyrus by small upward arrow panel in panel B. Panels E and F depict two high magnification focal planes from the region indicated by the asterick in panel B. Panels G and H depict two high magnification focal planes from region indicated by the small upward arrow in panel B. ODC expression is visualized by back grains in film emulsion (panels F and H). Blood vessels, microglia and macrophages are visualized in brown by tomato lectin (E and F). Neurons are visualized in brown by NeuN (G and H). Nuclei are visualized in blue with hematoxylin (E-H).

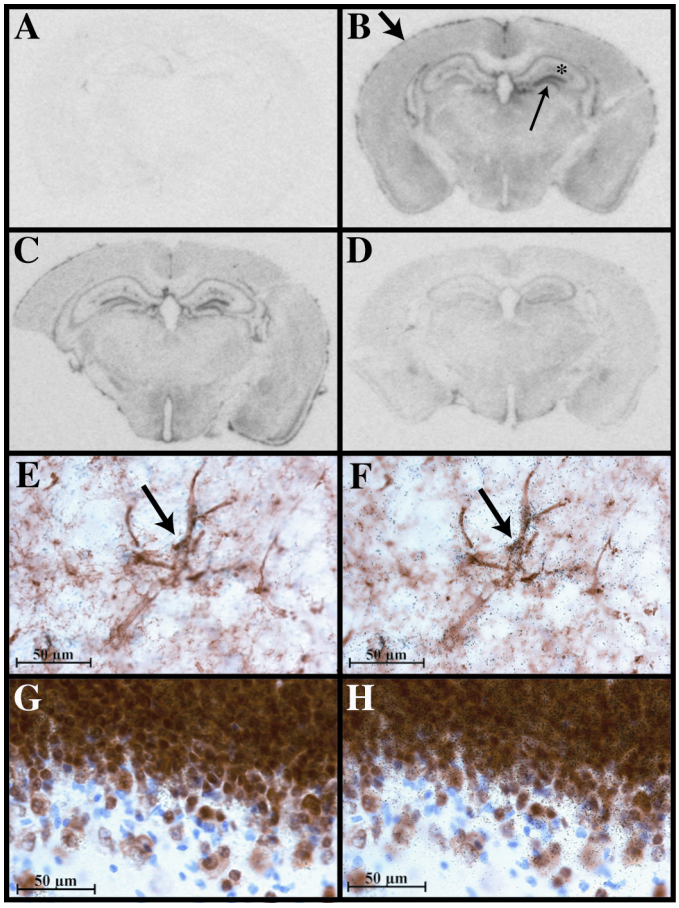

A
B
*
C
D
E
50 µm
F
50 µm
G
50 µm
H
50 µm

**Figure 2-3. LPS-induced accumulation of CD45hi macrophages in the adult CNS is decreased by co-injection of DFMO, an ODC inhibitor.** Panels A-D depict a representative experiment of the percentages of CD45lo microglia and CD45hi macrophages from the brains of mice 24 hours post-injection of A) vehicle control, B) DFMO, C) LPS, D) LPS+ DFMO. CD45 levels are gated on CD11b+ cells. E. Depicts the fold-increase in CD45hi macrophages as compared to the CNS of mice injected with vehicle (n=3 replicate experiments; ***$P < 0.05$).

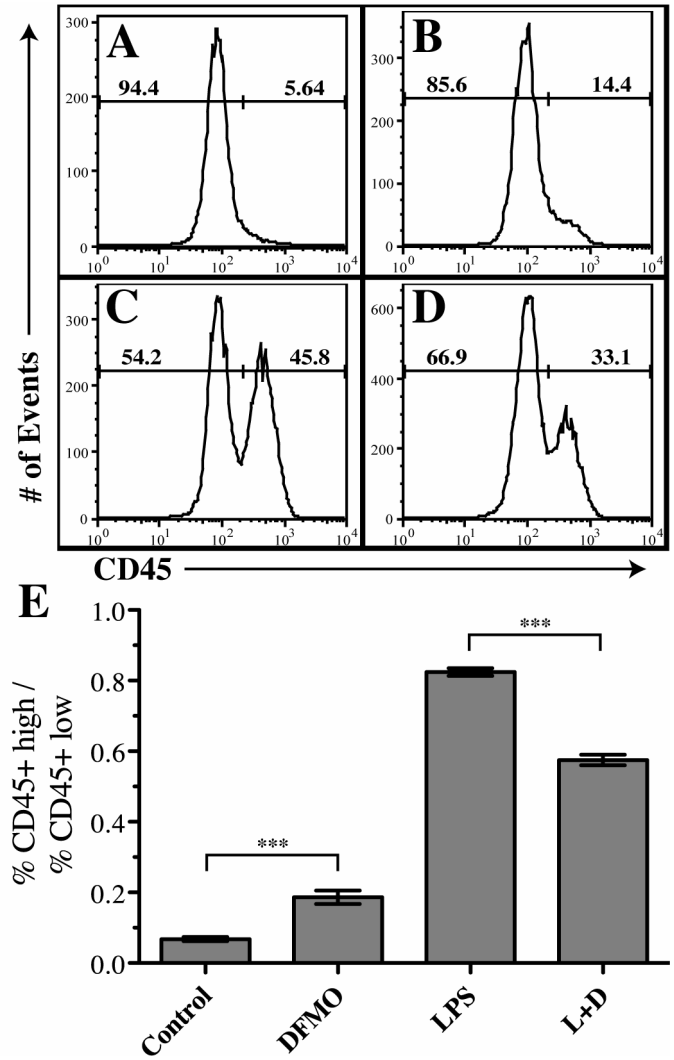

A
94.4
5.64
B
85.6
14.4
C
54.2
45.8
D
66.9
33.1
# of Events
CD45
E
% CD45+ high / % CD45+ low
***
***
Control
DFMO
LPS
L+D

**Figure 2-4. Mixed glial cultures robustly upregulate concentrations of secreted TNFα and CCL2 when exposed to putrescine and spermine, but not spermidine**. 0μM 1μM, 100μM, or 1000μM concentrations of putrescine, spermidine, or spermine were added to mixed glial cultures in the absence of FBS for 24 hours. Concentrations of TNF (A) and CCL2 (B) (**P < 0.01, ***P < 0.001, two-tailed Student's t-test).

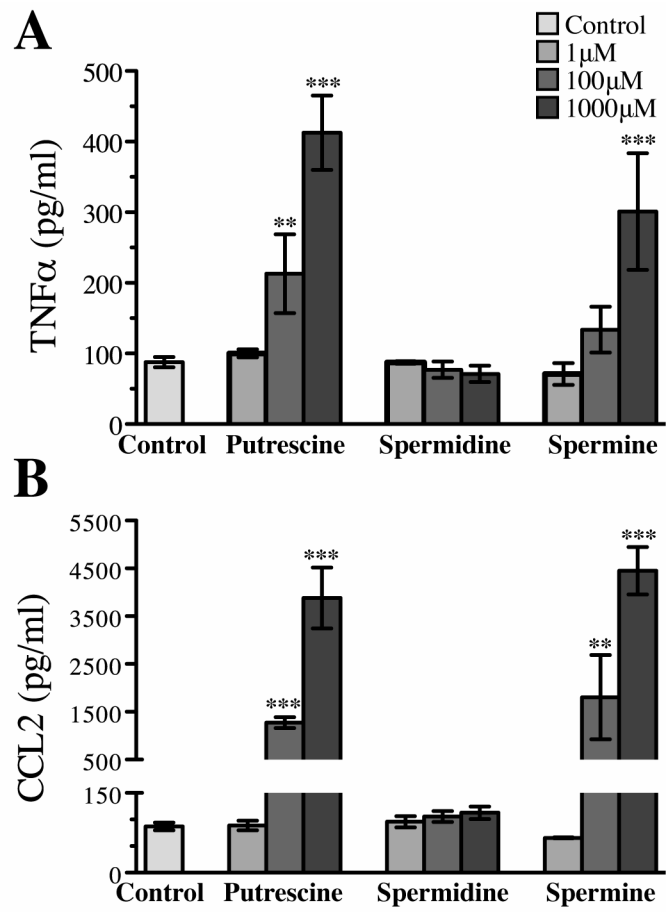

A
Control
1μM
100μM
1000μM
TNFα (pg/ml)
500
400
300
200
100
0
**
***
***
Control
Putrescine
Spermidine
Spermine
B
CCL2 (pg/ml)
5500
4500
3500
2500
1500
500
150
0
***
***
**
***
Control
Putrescine
Spermidine
Spermine

**Figure 2-5. LPS-induced expression of CCL2 in the adult CNS is decreased by co-injection of DFMO**.

CCL2 expression was quantified using densitometric analysis (A) of autoradiograms exposed to brain sections hybridized with $^{33}$P-labeled riboprobes 24 hours post-intracerebral injections of DFMO (B-E), LPS (F-I) or LPS+DFMO (J-M); (3 replicate experiments; ***$P < 0.001$, two-tailed Student's t-test). Boxed areas in panels B, F and J, represent the areas quantified in panel A. In panels C-E, G-I, K-M, microglia, macrophages and blood vessels are visualized in brown with tomato lectin. CCL2 expression is visualized by back grains in film emulsion. Arrows in panels C, G, K indicate the injection site. Panels D, H, L and E, F, H respectively depict two different focal planes of boxed areas in panels C,G, K. Small arrows identify CCL2 expressing, lectin positive cells. Large arrows identify CCL2 expressing lectin-negative cells.

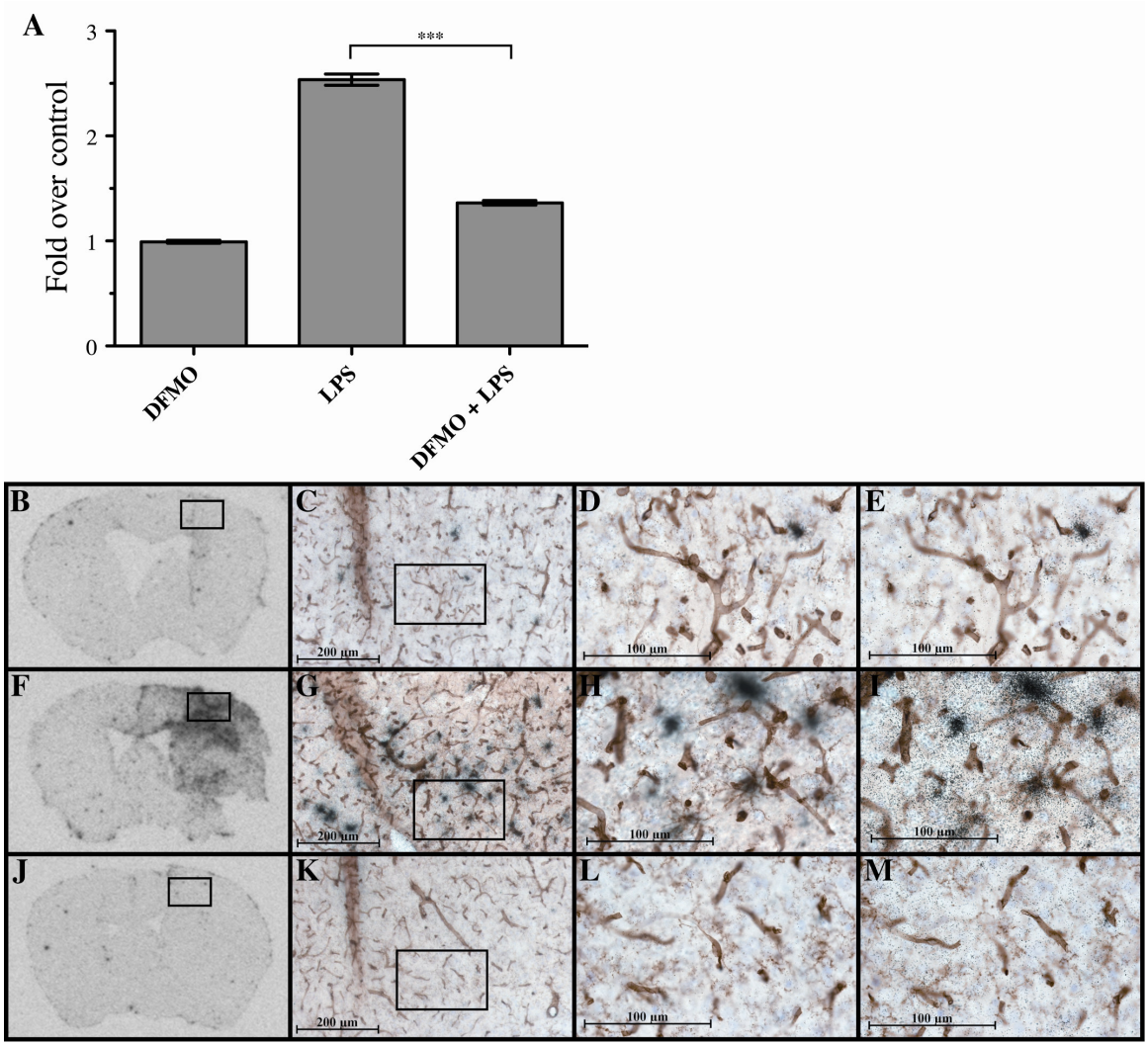

A
Fold over control
3
2
1
0
***
DFMO
LPS
DFMO + LPS
B
C
D
E
F
G
H
I
J
K
L
M
200 µm
100 µm

**Figure 2-6. High concentrations of all three polyamines decreased the percentage of oligodendrocytes in culture.** 0μM 1μM, 100μM, or 1000μM concentrations of putrescine, spermidine, or spermine were added to mixed glial cultures in the absence of FBS for 24 hours. Total number of cells per slide chamber (A) and percentage of CNP+ cells per slide chamber (B) (*$P < 0.05$, ***$P < 0.001$, two-tailed Student's t-test).

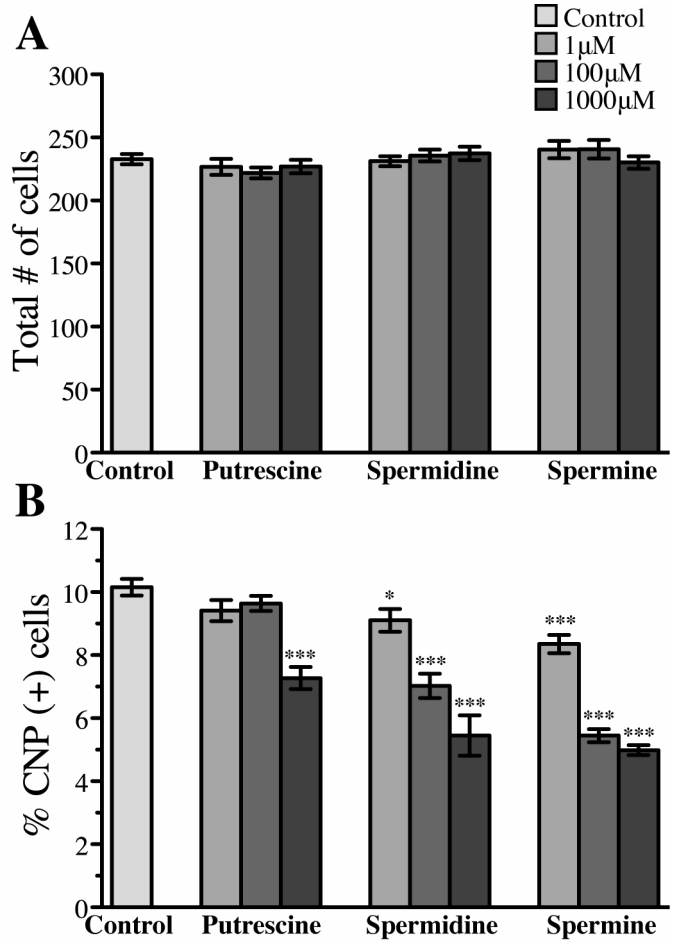
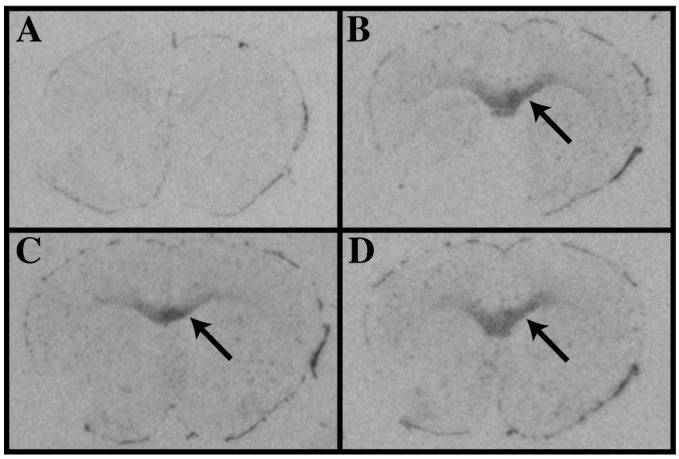

A
Control
1μM
100μM
1000μM
Total # of cells
300
250
200
150
100
50
0
Control
Putrescine
Spermidine
Spermine
B
% CNP (+) cells
12
10
8
6
4
2
0
*
***
Control
Putrescine
Spermidine
Spermine

Figure 2-7. **ApoD expression is induced in white matter tracts of mice receiving intracerebral injections of LPS, DFMO and LPS+DFMO.** ApoD expression was detected by autoradiogram analysis of brain sections hybridized with $^{33}$P-labeled riboprobes 24 hours post-intracerebral injection of A) vehicle, B) DFMO, C) LPS, D) LPS+DFMO.


A
B
C
D

# CHAPTER THREE

## Healthy aging results in altered microglial phenotypes and dysregulated CNS immune response.

## 3.1 Abstract

**Chronic inflammation with neurodegeneration is a prevalent feature of several age-associated CNS inflammatory diseases such as Alzheimer's disease and Parkinson's disease. Here we examine whether aging primes microglia to acquire phenotypes likely to promote chronic inflammation. Flow cytometric analysis revealed that aging is associated with increased microglial expression of markers of classical, pro-inflammatory activation, namely MHC class II, CD40 and B7.2 even in the absence of inflammatory stimuli. However, age-associated increases in the expression of TREM2, a receptor triggering robust anti-inflammatory responses was also observed. In addition, aging did have differential effects on the LPS-induced activation of CNS resident microglia versus CNS-infiltrating macrophages in LPS-induced inflammation. While microglial expression of MHC class II, CD40 and B7.2 increased in LPS-injected mice, macrophage expression of these same molecules did not change as a function of age. Age also did not alter the degree of LPS-induced macrophage influx into the CNS, nor the LPS-induced expression of ODC and CCL2. By contrast, sustained expression of the complement component C1qa was observed by microglia/macrophages at the site of injection in the older mice. Our data suggest that: (1) Healthy aging results in the 'priming' of the resident glial population. (2) Aging has differential effects on the immune responses of CNS-resident microglia versus acutely infiltrating macrophages. (3) Inflammatory responses in the aged CNS are not just hyper- or hypo-activation. Rather the ratio**

**of CNS inflammatory responses to the same inflammatory stimulus changes with age.**

## 3.2 INTRODUCTION

Understanding the molecular mechanisms underlying the onset, progression and resolution of CNS inflammation has been the focus of widespread research. Of the various factors that are known to regulate inflammatory responses in the CNS, the process of aging has been identified as having profound effects on neuroinflammation (Godbout J.P. et al. 2007, 2009).

The aged CNS is shown to be characterized by a reactive glial phenotype even in the absence of pathology. Studies have shown elevated mRNA expression of pivotal pro-inflammatory genes such as MHCII, CD86 and CD40 in the hippocampus of aged (24 months) rats (Frank M.G. et al. 2006). Similar investigations in the murine CNS show an age-associated induction in the expression of RNAs for scavenger receptors, Toll-like receptors and markers of astrocytic activation (Henry C.J. et al. 2009) (Goodbout J.P. et al. 2006, 2005). Based on these and similar results, many have hypothesized the microenvironment of the aged CNS is "primed" to respond to any CNS trauma or pathology with exaggerated proinflammatory immune responses.

By contrast, others have hypothesized that neurodegeneration is a consequence of microglia becoming senescent and non-responsive as a function of age (Streit 2009). For example, microglia in tissue sections from aged human CNS (>70 years of age) display increased morphological dystrophy and cytoplasmic degeneration indicative of lost cellular integrity and/or viability (Streit W.J. 2009). In this viewpoint, neurodegeneration

is a consequence of lost microglial neuroprotection. Similar dystrophic microglia are not observed in the rodent CNS, probably due to the much longer telomeres found in murine microglia as compared to human microglia (reviewed Carson et al. 2008). Consistent with this observation introduction of defects in telomere maintenance in mice, leads to rapid appearance of dystrophic microglia by 3 months of age. The absence of dystrophic microglia in the CNS of wild-type mice suggests that: (1) mice are poor models of neuroinflammation associated with catastrophic endstage neurodegeneration and (2) mice are better models of age-associated changes in CNS immune responses associated with early stages of neurodegeneration.

Direct examinations of microglial/macrophage response to pathogenic stimulus have not fully supported either the hypothesis that aging leads to increased propensity for pro-inflammatory responses or the hypothesis that aging leads to loss of microglial function. For example, secondary CNS inflammation elicited in response to intraperitoneal injection of LPS in aged mice is associated with microglial hyper-reactivity as seen by increased induction in the surface expression of MHC class II and TLR2. While these hyper-reactive microglia produced higher levels of pro-inflammatory IL1b, they also produced higher levels of the anti-inflammatory cytokine, IL10 (Henry C.J. et al. 2009).

A factor largely ignored in consideration of immune responses in the aging CNS is age-associated changes in macrophage infiltration. In part this is a consequence of the reliance on tissue sections for most aging studies. As yet, there are no markers that

distinguish CNS-resident microglia from CNS-infiltrating macrophages in tissues sections. Using relative levels of surface expression of CD45, it is possible to distinguish between CNS resident microglia (CD11b+CD45lo) and peripheral macrophages (CD11b+CD45hi) using flow cytometric analysis of brain cell suspensions.

Using flow cytometric approaches to distinguish resident microglia from CNS-infiltrating macrophages, we investigated cell-type specific immune consequences of aging. To focus on how aging affects CNS-regulated inflammatory responses, we have chosen to use a model of primary CNS inflammation in which inflammation is initiated from within the CNS by intracerebral injection of LPS (Fig 3-1).

In our studies, we have chosen to examine microglial activation and macrophage influx in three different ages: 3 months (young-adult), 8 months (mid-aged adult) and 15 months (aged-adult) mice. At 3 months of age, mice are sexually mature and neuronal and macroglial development is completed in the CNS (Suckow M.A, Denneman P., Brayton C, 2001). At 8 months,female mice are beginning to exhibit declining fertility. At this age, the initial signs of neurodegeneration can be detected in transgenic models over expressing amyloid and/or cytokines (Suckow M.A, Denneman P., Brayton C, 2001). These transgenic data implicate this age as a potential tipping point for changed immune responses to pathogenic signals. In the Carson mouse colony, murine lifespan extends to nearly 3 years. At 15 months, mice still appear healthy and mobile, but females are no

longer fertile. In addition, frank severe features of neurodegeneration and neurodysfunction are observed in transgenic models of amyloid pathology.

In brief, by comparing 3, 8 and 15 month old mice, we find that aging has differential effects on the inflammatory responses of CNS resident microglia relative to acutely infiltrating macrophages. Further, our data support the hypothesis that inflammation in the aged CNS is characterized by altered ratios of immune responses rather than a general hyper-or hypo-activation of all types of CNS immune responses.

## 3.3 RESULTS

### 3.3.1 Resident microglia from the aged CNS display an altered, pro-inflammatory phenotype.

To compare the phenotypic differences between resident microglia from the aged and the young CNS, we performed flow cytometry analysis of ex vivo microglia from healthy young adult (3 months), mid-aged adult (8 months) and aged adult (15 months) mice. We examined the age-associated differences in the surface expression of classical, pro-inflammatory markers of microglial activation: (Fig 1A) CD40, a co-stimulatory receptor for CD40L(also known as CD154) which is expressed on activated $CD4^{+}$Tcells, (Fig 1B) Major-histocompatibility complex II (MHC class II), a critical molecule in antigen-presentation, and (Fig 1C) B7.2, also known as CD86, a critical co-stimulatory molecule in eliciting a pro-inflammatory Th1 response. The process of healthy aging results in an

upregulation in microglial surface expression of all three of these proinflammatory markers: MHC class II, CD40 and B7.2 (Fig 1)..

### 3.3.2 Aging is associated with an increase in CNS-infiltrating macrophages in the absence of pathogenic stimulus.

Our data demonstrate that CNS resident microglia assume a pre-activated pro-inflammatory phenotype in response to healthy aging. We also examined if age alone in the absence of pathogenic signals was also associated with increased influx of macrophages into the CNS

Acutely infiltrating peripheral macrophages cannot be distinguished from CNS resident microglia in tissue sections.. These two populations of myeloid cells can be distinguished in brain cell suspensions using flow cytometric analysis of CD45 levels. Previous studies have characterized CNS resident microglia as CD45-low, while acutely infiltrating macrophages are CD45-high (Carson et al. –2009). Few CD45-high macrophages were detected in the CNS of healthy young (3 months – Fig 2A) and adult (8 months – Fig 2B) mice. However, an 8-fold induction in the recruitment of peripheral macrophages was detected in the CNS of healthy aged (15 months – Fig 2C) mice. These data suggest that the process of healthy aging triggers an immune response within the CNS comparable to the acute transient immune response triggered by systemic inflammation. Analysis of splenic immune cells from the same mice confirmed the absence of systemic inflammation (data not shown). Thus, aging by itself may be proinflammatory trigger.

### 3.3.3 Age does not alter the magnitude of LPS-triggered macrophage influx into the CNS.

To determine whether the net inflammatory response elicited within the CNS is altered as a function of age, we analyzed the influx and activation of CNS-infiltrating macrophages triggered by inflammation induced from within the CNS. For this purpose, we initiated inflammation by intracerebral injection of LPS. Intracerebral injection of LPS promoted accumulation of a large population of CD45-high macrophages within the CNS of the young adult (3 months) animals within 24 hours (Fig 3A). No differences were detected in the recruitment of peripheral macrophages to the CNS of LPS-injected mid-age adult (8 months) and aged adult (15 months) mice (Fig B – D). Our data indicate that although microglia in the aged CNS display a "primed" immune phenotype, initiation of primary neuroinflammation in the aged CNS does not increase influx of peripheral macrophages into the CNS.

### 3.3.4 Age does not alter the kinetics and pattern of LPS-induced CCL2expression in the CNS.

CCL2 (also known as monocyte chemoattractant protein 1: MCP1), is one of the primary chemokines critical for the recruitment of peripheral macrophages to the CNS during neuroinflammation (Huang D.R. et al. – 2001, Dogan R.E. et al. – 2008). To determine if aging causes alteration of the kinetics of macrophage recruitment during inflammation,

we used radiolabled in-situ hybridization to examine the kinetics and pattern of CCL2 expression in the inflamed CNS.

$^{33}$P labeled, anti-sense CCL2 riboprobes were hybridized to coronal brain sections from LPS injected young adult (3 months), mid-aged adult (8 months) and healthy aged adult (15 months) mice. Robust upregulation of CCL2 was observed at the site of injection in the CNS of young (3 months) mice as early as 3 hours post injection of LPS (Fig 4A). Expression peaked at 24 hours post LPS injection (Fig 4E) and was completely resolved at 72 hours post LPS injection (Fig 4I). CCL2 induction was regulated with similar kinetics in the inflamed 15 months old CNS (Fig 4M, 4Q, 4U). Densitometric analysis of CCL2 expression at the site of injection showed no statistical differences in the regulation of CCL2 expression in the young adult (3 months) and the aged adult (15 months) CNS upon inflammation (Fig 3E). To identify cell types in the CNS expressing CCL2, microglia and peripheral macrophages were visualized in tissue sections using antibodies against the myeloid marker Iba1. In CNS of 3 months old mice, an increase in grain density indicative of CCL2 expression was associated with Iba positive cells (Fig 4C, 4D; right-pointing arrow) and Iba1negative cells (Fig 4C, 4D; left-pointing arrow) in the vicinity of the site of injection (Fig 4B – box). A similar pattern of CCL2 expression was observed at 24 hours post injection of LPS, which corresponds to the peak of macrophage influx into the CNS. Very few Iba1 positive cells are associated with CCL2 expression at 72 hours post injection, the resolution phase of the inflammatory response.

The pattern of CCL2 expression in the 15 months old CNS was similar to that seen in the CNS of 3 months old animals (Fig 4N – 4X).

### 3.3.5 Aging affects the LPS-induced activation of CNS resident microglia but not CNS-infiltrating macrophages.

Using flow cytometry, we also examined if aging altered LPS-triggered activation of CNS-resident microglia and CNS-infiltrating macrophages isolated from the same inflamed microenvironment. 3 months, 8 months and 15 months old mice were sacrificed 24 hours post intra-cerebral injection of LPS. Ex-vivo microglia from the young adult (3 months) CNS expressed detectable levels of all proinflammatory activation markers: CD40 (Fig 5A), MHC class II (Fig 5C) and B7.2 (Fig 5E). In comparison, the expression levels of these markers increased with increasing on CD45lo microglia from the mid-aged adult (8 months) and aged adult (15 months) CNS (Fig 5A, 5C, 5E). Strikingly, the same age-associated increase in the expression of activation markers are not seen on CNS-infiltrating macrophages isolated from the same brains as CNS-resident microglia. The levels of CD40 (Fig 5B), MHC class II (Fig 5D), and B7.2 (Fig 5F)were identical on macrophages isolated from LPS-injected mice at all ages. . Our data suggests that aging specifically affects the pro-inflammatory activation state of CNS resident microglia in the absence of co-incident changes in other LPS triggered immune responses: CCL2 expression, peripheral macrophage recruitment and peripheral macrophage activation.

### 3.3.6 Alternative activation: Age-associated increases in TREM2 but not Mannose receptor in microglia and macrophages.

Data from our ageing study suggests that inflammatory response in the aged CNS are characterized by exaggerated upregulation of classical, pro-inflammatory markers. To examine if we observe a simultaneous upregulation of anti-inflammatory responses as a function of age, we performed flow cytometric analysis of the expression of TREM2 and mannose receptor (MMR). Both TREM2 and MMR are well characterized markers of alternative macrophage activation states (Turnbull I.R. et al. 2006, Stein M. et al. 1992) associated with tissue repair and resolution of proinflammatory immune responses.

At all ages examined, CNS resident microglia express detectable levels of TREM2 24 hours post-LPS injection (Fig 6 and data not shown). By contrast, most CNS-infiltrating macrophages were TREM2 negative. However, the level of TREM2 expression did increase on microglia (Fig 6A-C) as well as the small population of TREM-2 positive CNS-infiltrating macrophages as a function of age (figures 6 D-F). Similar analysis to examine MMR expression showed that MMR was not regulated as a function of age on either microglia or macrophages. (Fig 7).

### 3.3.7 The pattern of LPS-triggered Inflammatory responses changes as a function of age

The data just presented indicate that there is a general increased immune response to LPS as a function of aging. We therefore, examined whether aging was associated with a

global hyper-responsiveness –in CNS inflammatory responses. We analyzed the kinetics of induction of ornithine decarboxylase (ODC), complement component C1qa and monocyte chemoattractant CCL2 by in situ hybridization. Densitometric analysis of autoradiograms from each set of tissues revealed that neither the kinetics, nor the extent of ODC (Fig 6A) and CCL2 (Fig 3E) induction were altered as a function of age. In contrast, the kinetics as well as extent of C1qa expression was dramatically altered in an age-dependant manner. Expression of C1qa in response to acute CNS inflammation in the young brain peaked at 24 hours post LPS injection, and completely resolved at subsequent time points (Fig 6B ; black line). However, in the adult (8 months) and the old (15 months) CNS, the expression of C1qa peaked at 72 hours post injection of LPS (Fig 6B; grey, dashed lines), which is the resolution phase of the inflammatory response in this model of CNS inflammation. Thus, not all pro-inflammatory responses in the aged CNS are hyper activated. Rather, our data suggests that neuroinflammation in the aged CNS is primarily dysregulated.

### 3.3.8 Sustained C1qa expression is seen primarily on lectin(+) cells at the site of injection of 8 months and 15 months old mice.

To identify CNS specific cell types expressing C1qa, tissue sections hybridized to $^{33}$P-labeled antisense riboprobes against C1qa from un-injected control brains and LPS injected brains at 3 months, 8 months and 15 months of age were used to perform immunohistochemisty. Microglia, macrophages and blood vessels were visualized using biotinylated tomato lectin.

CNS resident microglia in the uninjected control brains expressed detectable basal levels of C1qa (data not shown). In the young CNS (3 months) peak expression of C1qa was observed at the site of injection at 24 hours post injection of LPS (Fig 6B). At this age, increased density of dark grains indicative of C1qa expression was seen associated with lectin(+) microglia/macrophages (Fig 7C, 7D) at the site of injection (Fig 7B – box). C1qa expression at the site of injection returned to basal levels at 72 hours post injection of LPS (Fig 7E – 7H). As seen in the young (3 months) CNS, up-regulated C1qa expression was associated with lectin(+) microglia/macrophages at the site of injection in the CNS of 8 months old mice (Fig 7J – 7L) and 15 months old mice (Fig 7R – 7T) at 24 hours post injection of LPS. However, peak expression of C1qa at the site of injection in the 8 months (Fig 7M) and 15 months (Fig 7U) old CNS was observed at 72 hours post LPS injection. This sustained C1qa expression at the site of injection in the 8 months (Fig 7N – 7P) and 15 months (Fig 7V – 7X) old CNS is associated with lectin(+) microglia/macrophages. As C1qa expression has been previously reported to be upregulated preferentially on activated CNS resident microglia, our data indicate that inflammatory responses in the adult and old CNS may be characterized by sustained and prolonged microglial activation.

### 3.4 DISCUSSION

Continuous and bi-directional communication between the CNS and the peripheral immune system plays a vital role in all aspects of CNS physiology, ranging from

appropriate development of the neonatal and adult CNS (Bilbo S.D – 2009) to the initiation of effective immune responses to pathology or infectious agents. Factors affecting this cross-talk have thus appropriately been the focus of widespread research. The process of healthy aging has been identified as a major contributor to altered CNS-immune system signaling (Frank M.J et al. 2008, Bilbo S.D. 2009, Godbout J.P et al. 2007, 2009, Henry C.J et al. 2009). While these studies have aptly demonstrated that the immune responses in the aged CNS are significantly altered, whether microglia (the resident population of effector macrophages of the CNS) and the infiltrating population of peripheral macrophages are differentially affected by age has not been investigated.

We report that inflammatory responses in the aged CNS differ from those in the young CNS primarily in terms of the immune responses of the CNS resident microglia. The responses of the peripheral macrophages that infiltrate the CNS are not affected by age, neither with respect to their recruitment to the inflamed CNS nor their activation. We hypothesize that although the basal immune status of the CNS is shifted towards a more pro-inflammatory phenotype, the senescent CNS may still be in active control of the immune responses mounted in response to pathology.

Previous studies on the age-associated alterations in the basal immune status of the CNS have shown that the aged CNS is characterized by a reactive glial phenotype. This is seen primarily as upregulated levels of MHC class II, CD86 (B7.2), CD40, CIITA, TLR2 and IFNg in total brain RNA samples. (Frank M.G. et al. 2006, Sparkman N.L.et al. 2008,

Henry C.J. et al. 2009, Morgan T.E. et al. 1999). Consistent with these studies, we report that ex-vivo microglia from the brain of aged mice display an upregulated surface expression of MHCII, B7.2 and CD40. Upregulation of functional surface expression of these classic, pro-inflammatory activation markers indicates that microglia in the aged CNS are 'primed' to polarize T cell responses within the CNS towards the pro-inflammatory Th1 axis. In addition to these observations, we report that the aged CNS shows and increase in the basal infiltration by peripheral macrophages. Thus the aged CNS does not only display an increasingly "primed" glial population as has been reported so far, but also an altered CNS microenvironment.

Previous studies by Henry and colleagues have demonstrated that upon systemic inflammation, the aged CNS responds with exaggerated microglial activation with induction of pro- as well as anti-inflammatory cytokine production (Henry C.J et al. 2009). Consistent with this data, we report that upon central challenge with LPS, the CNS resident microglia, as well as the infiltrating macrophages respond with an age-associated increase in the expression of TREM2. However, we do not observe any age-associated changes in MMR expression. We thus hypothesize that although the responding glial population in the aging brain does up-regulate anti-inflammatory activation markers, complete polarization to either a pro- or anti-inflammatory phenotype does not occur.

Various hypotheses are currently proposed to explain the appearance of reactive glia in the aged CNS. Repeated inflammatory cytokine exposure over the life-time of an animal

(Godbout J.P. 2009, Bilbo S.D. 2009), neonatal infection (Bilbo S.D. 2009, Adams-Chapman J. 2006), and increased oxidative stress and damage due to enhanced ROS production, impaired antioxidant defense mechanisms and scavenging capacity have been suggested as a few of the critical factors contributing to altered immune responses in the CNS (Godbout J.P. 2009). In addition, based on observations that aging neurons show down regulation of essential proteins such as a-synuclein (Mak S.K. et al. 2009) and CD200 (Frank M.G. et al. 2006), it is possible the appearance of microglia with altered phenotypes in the aged CNS may be a direct consequence of neuronal signaling. The altered microglial phenotype in the healthy aging brain may thus be reflective of the CNS resident microglia trying to maintain optimal functions in the aging CNS.

We show that while aged microglia respond with an exaggerated immune activation in response to CNS inflammation, peripheral macrophages display no such hyper-reactivity. CNS resident microglia and peripheral macrophages are two effector macrophages populations that respond to neuroinflammation. Our data suggests that aging has differential effects on the inflammatory responses of CNS resident microglia and infiltrating peripheral macrophages. It is tempting to speculate that the identification of specific genes that may be differentially upregulated in the aging macrophages may have important applications in 'resetting' the phenotypes of aging microglia.

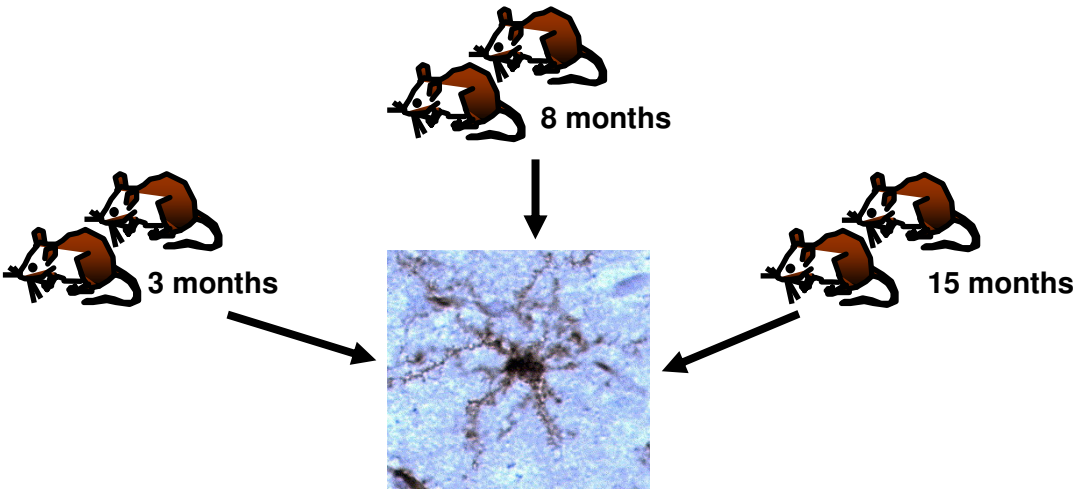
Previous studies by Streit and colleagues have identified the appearance of 'dystrophic' glial phenotypes which are associated with senescence in the aging brain (Streit W.J. et

al. 2006, 2009). Unlike their data, we do not observe any such alterations in microglial morphology with aging. However, it is important to note that our studies have been done using murine models of CNS inflammation in contrast to the human tissues used by Streit et al.

Our data suggests that the inflammatory responses in the aging brain are characterized by prolonged activation of CNS resident microglia. Thus, the age-associated alteration the CNS microenvironment may play important roles in the initiation and progression of chronic neuroinflammatory responses which are characteristic of neurodegenerative disorders.

## 3.5 FIGURES AND LEGENDS

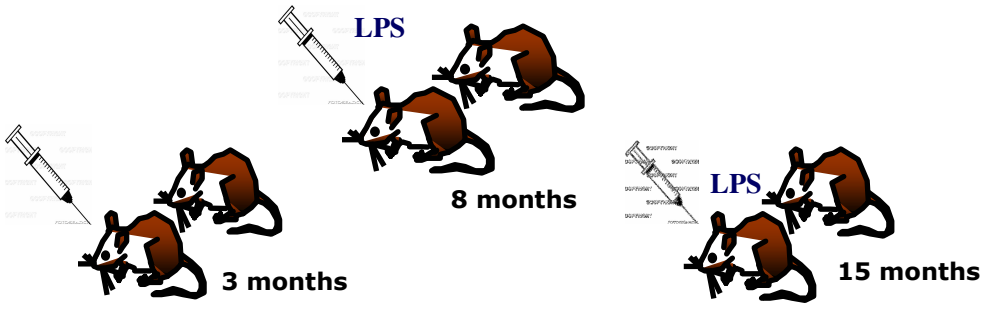
Figure 3-1. **Schematic representation of experimental design for the flow cytometric analysis of the microglial phenotypes in the young, adult and old CNS.**




**Isolate Microglia and any CNS inflammatory infiltrates**

**Perform flow cytometry to analyze activation of CNS resident microglia and CNS infiltration.**

Figure 3-2. **Schematic representation of the experimental design to analyze changes in gene-expression and the immune responses of CNS-resident microglia and infiltrating macrophages in response to LPS-induced CNS inflammation.**




**LPS = Acute, self resolving CNS inflammation; activation of microglia; infiltration of CNS by peripheral macrophages; no lymphocyte infiltration.**

**Sacrifice animals at 1 day post injection of LPS.**

**Isolate microglia and any infiltrating, peripheral immune cells from the brain**

**Perform Flow Cytometry analysis to analyze activation status of immune cells.**

**Dissect out the brain and cryosection into 25 uM thin sections.**

**Perform $^{33}$P labeled in-situ hybridization to analyze regulation of gene expression.**

Figure 3-3. **The phenotype of CNS-resident microglia becomes more pro-inflammatory in response to healthy aging.** Expression of markers of classical, activation by CNS-resident microglia from the brain tissue of 3 months, 8 months and 15 months old mice. Microglia from the CNS of healthy, aging mice were separated on a discontinuous percoll gradient. Surface expression of **(A)** CD40 ; **(B)** MHC class II and **(C)** B7.2 by CD45-lo microglia from the 3 months (blue histogram), 8 months (red histogram), and 15 months old (green histogram) was analyzed by flow cytometry. Microglial expression of all there activation markers was upregulated with increasing age. Data is representative of three replicate experiments.

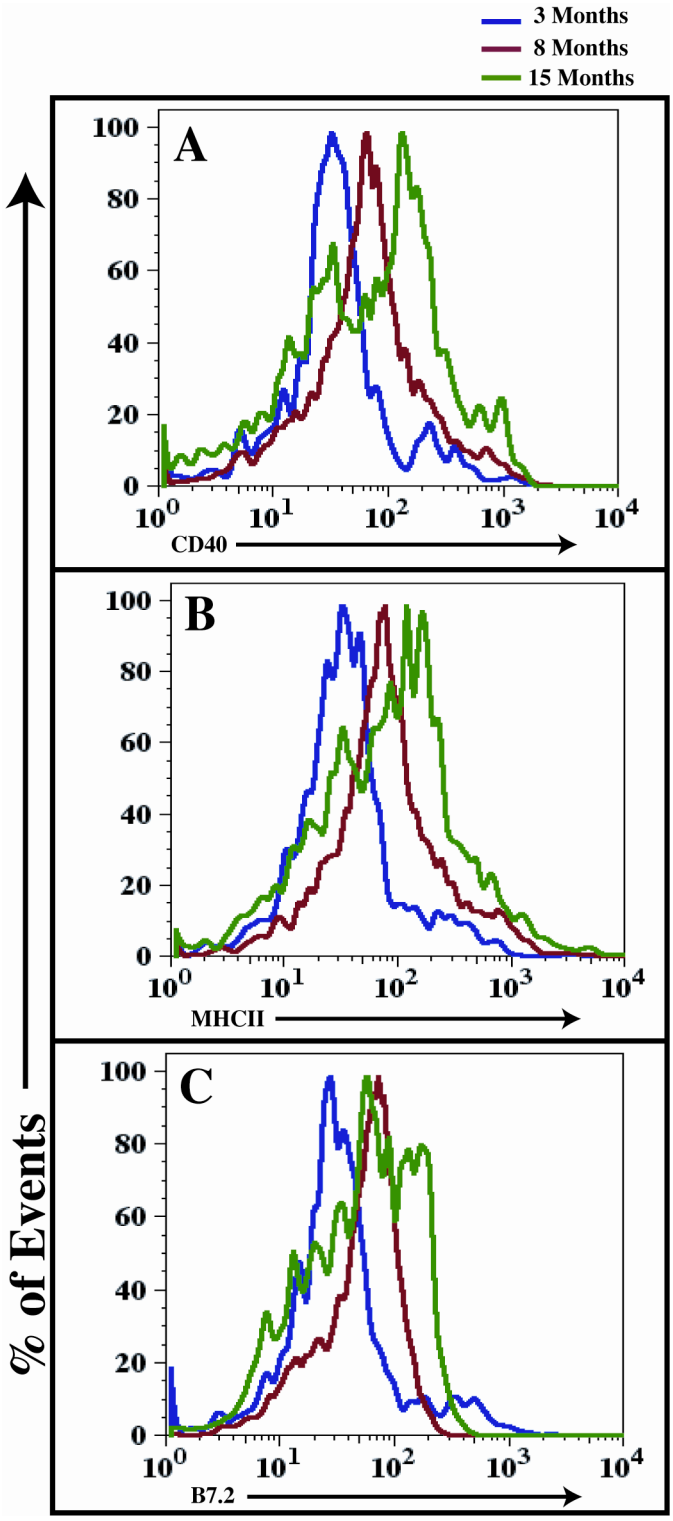

3 Months
8 Months
15 Months
A
B
C
% of Events
CD40
MHCII
B7.2
0
20
40
60
80
100
$10^0$
$10^1$
$10^2$
$10^3$
$10^4$

Figure 3-4. **The aged CNS (15 months) is characterized by a modest increase in the basal infiltration by peripheral macrophages in the absence of inflammation.** CD45-low vs. CD45-high profiles of the CNS of **(A)** 3 months, **(B)** 8 months, and **(C)** 15 months old mice. Microglia and any peripheral infiltrates were collected from a discontinuous percoll gradient. Microglia and peripheral macrophages were identified by analyzing the CD45 levels on CD11b(+) gated cells. Healthy aging of the CNS results in a modest influx of peripheral macrophages in the absence of infection. Data is representative of three replicate experiments.

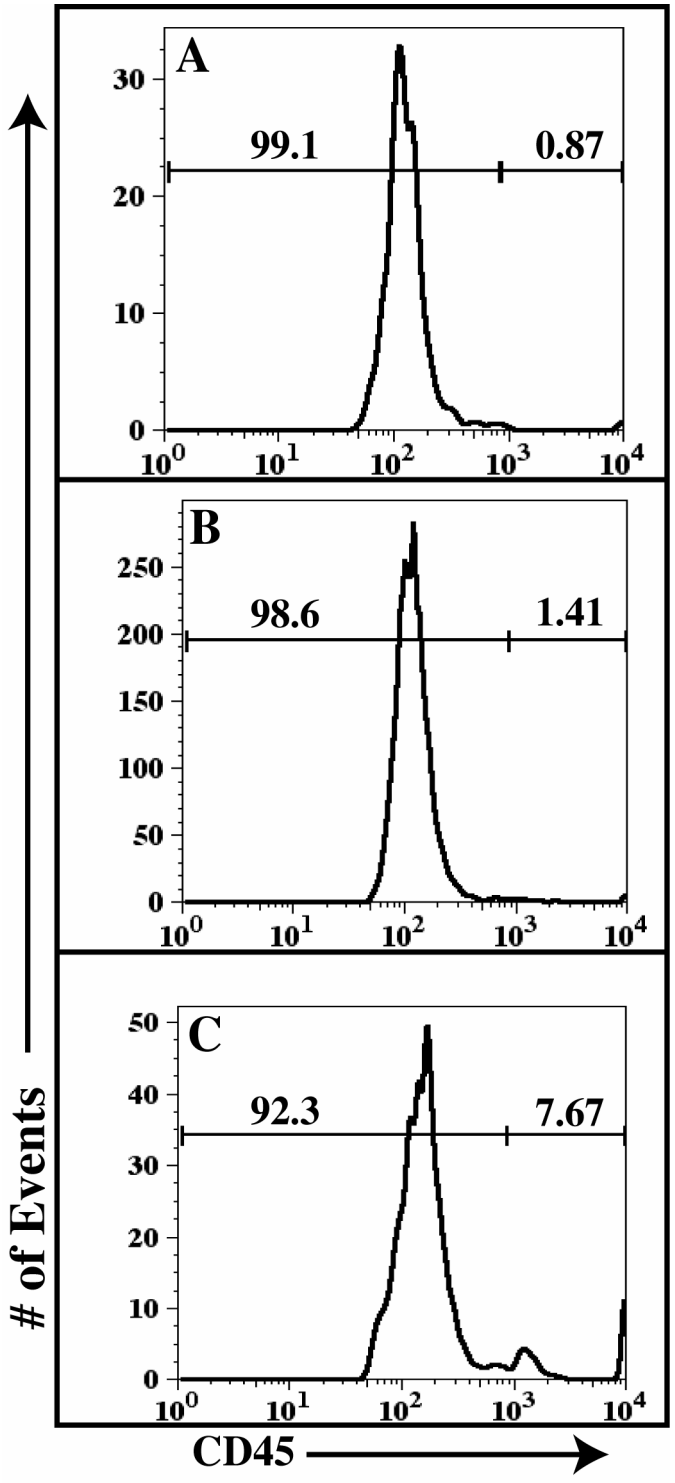

A
99.1
0.87
B
98.6
1.41
C
92.3
7.67
# of Events
CD45

Figure 3-5. **LPS-induced infiltration of CD45-high macrophages in the CNS is not altered by healthy aging.** Panels A – C depict the percentages of CD45-low microglia and CD45-high macrophages from the brain tissue of **(A)** 3 months, **(B)** 8 months, **(C)** 15 months mice, 24 hours post LPS injection. **(D)**: Quantification of the percent CD45-high macrophages infiltrating the CNS normalized to the percent CD45-low microglia at all ages. No significant differences are observed in the percent CD45-hi macrophages infiltrating the CNS at all ages analyzed ($P<0.05$, two tailed Student's t-test). **(E)**: Depicts the quantification of CCL2 expression at the site of injection at various time points post injection of LPS. No significant differences were observed in the kinetics and the extent of CCL2 upregulation in response to LPS-induced CNS inflammation in the 3 months (black) and 15 months (dashed) mice. ($P<0.05$, two tailed Student's t-test). All data are representative of three replicate experiments.

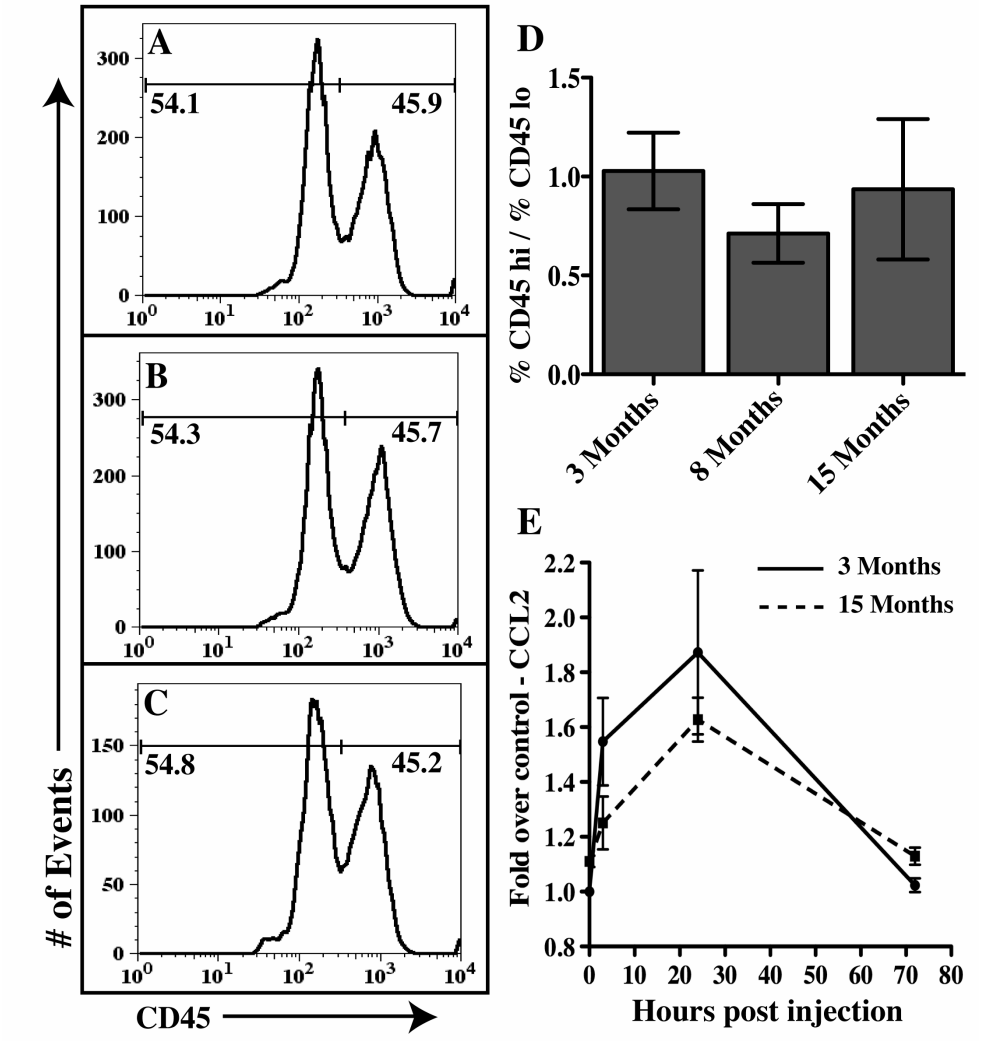

A
54.1
45.9
B
54.3
45.7
C
54.8
45.2
# of Events
CD45
D
% CD45 hi / % CD45 lo
3 Months
8 Months
15 Months
E
3 Months
15 Months
Fold over control - CCL2
Hours post injection

Figure 3-6. **CCL2 mRNA expression is robustly upregulated by Iba(+) and Iba1(-) cells at the site of injection following intracerebral injection of LPS.** CCL2 mRNA expression in the CNS of **(A-L)** 3 months and **(M-X)** was detected by in-situ hybridization of 33P-labeled riboprobes to brain tissue at 3 hours, 24 hours and 72 hours post injection. Robust upregulation of CCL2 expression of CCL2 was seen at the site of injection of 3 months old mice at **(A)** 3 hours and **(E)** 24 hours post injection. Panels B, F and J depict low magnification (10X) of the site of injection at 3 hours, 24 hours and 72 hours post injection respectively. Panels C – D, G – H and K – L depict two high-magnification focal planes from the boxed areas in B, F and J respectively. Similar upregulation of CCL2 mRNA is seen at the site of injection in 15 months old mice at **(M)** 3 months, **(Q)** 8 months and **(U)** 72 hours post injection. Panels N, R, and V depict low magnification (10X) of the site of injection at 3 hours, 24 hours and 72 hours post injection respectively. Panels O – P, S – T, and W – X depict two high-magnification focal planes from the boxed areas in N, R and V respectively. CCL2 expression is visualized by black grains in photographic emulsion. Microglia/macrophages are visualized in brown by Iba1. Data is representative of three replicate experiments.

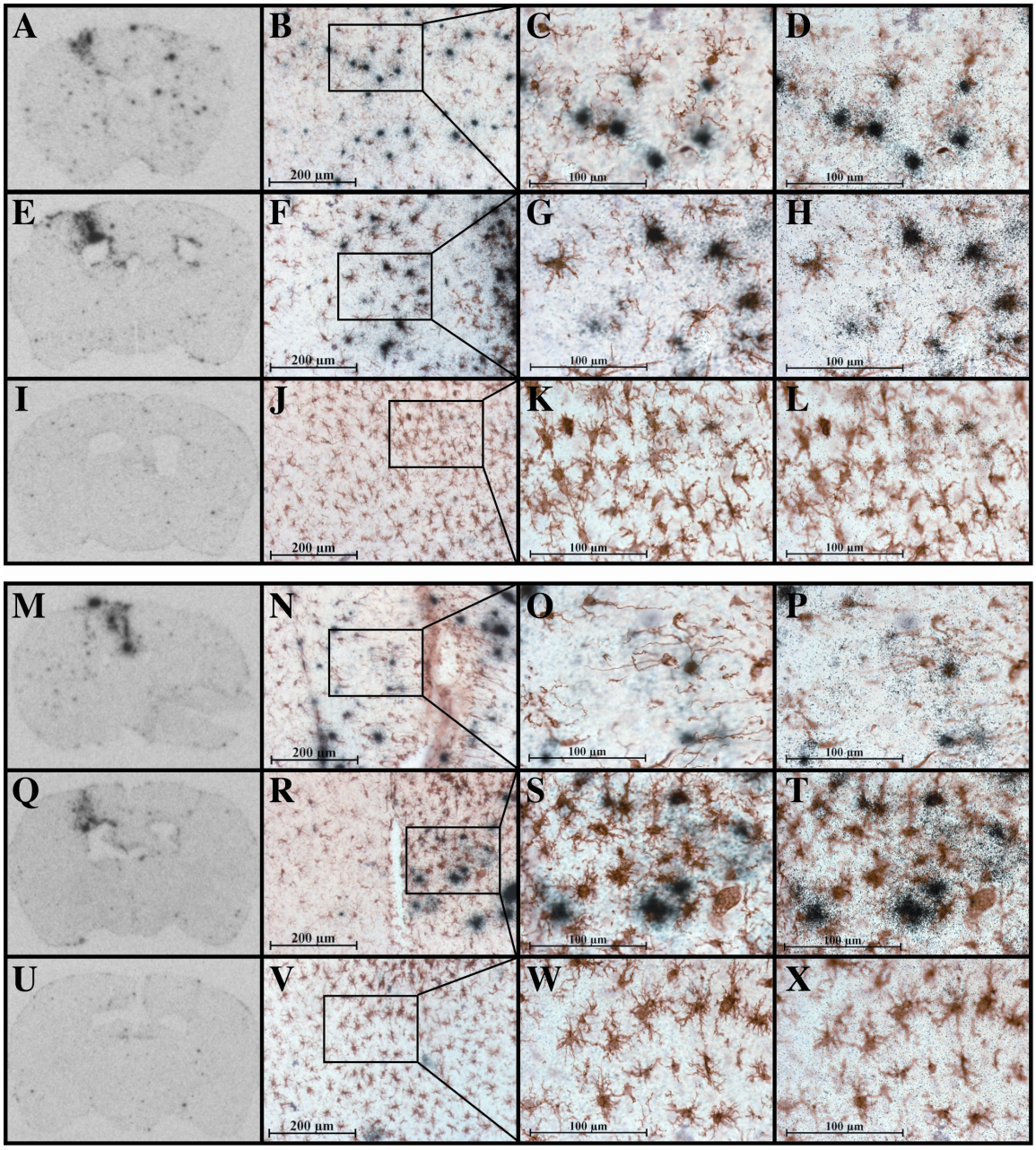

A
B
200 µm
C
100 µm
D
100 µm
E
F
200 µm
G
100 µm
H
100 µm
I
J
200 µm
K
100 µm
L
100 µm
M
N
200 µm
O
100 µm
P
100 µm
Q
R
200 µm
S
100 µm
T
100 µm
U
V
200 µm
W
100 µm
X
100 µm

Figure 3-7. **Aging differentially affects the upregulation of CD40, MHC CLASS II and B7.2 by CNS-resident microglia in response to LPS-induced inflammation.** Induction of surface expression of **(A – B)** CD40, **(C – D)** MHC class II and **(E – F)** B7.2 by CNS resident microglia and infiltrating macrophages 24 hours post LPS injection. Panels A, C, E depict the expression of CD40, MHC CLASS II and B7.2 by the CNS resident microglia respectively. The extent of upregulation of all three markers of classical activation shows an age-dependant increase in the 3 months (blue), 8 months (red) and 15 months (green) microglia. Panels B, D, F depict the regulation of CD40, MHC class II and B7.2 expression on infiltrating macrophages respectively. No age associated alteration was seen in the upregulation of CD40, MHC CLASS II and B7.2 in the macrophages at all ages tested. Data is representative to three replicate experiments.

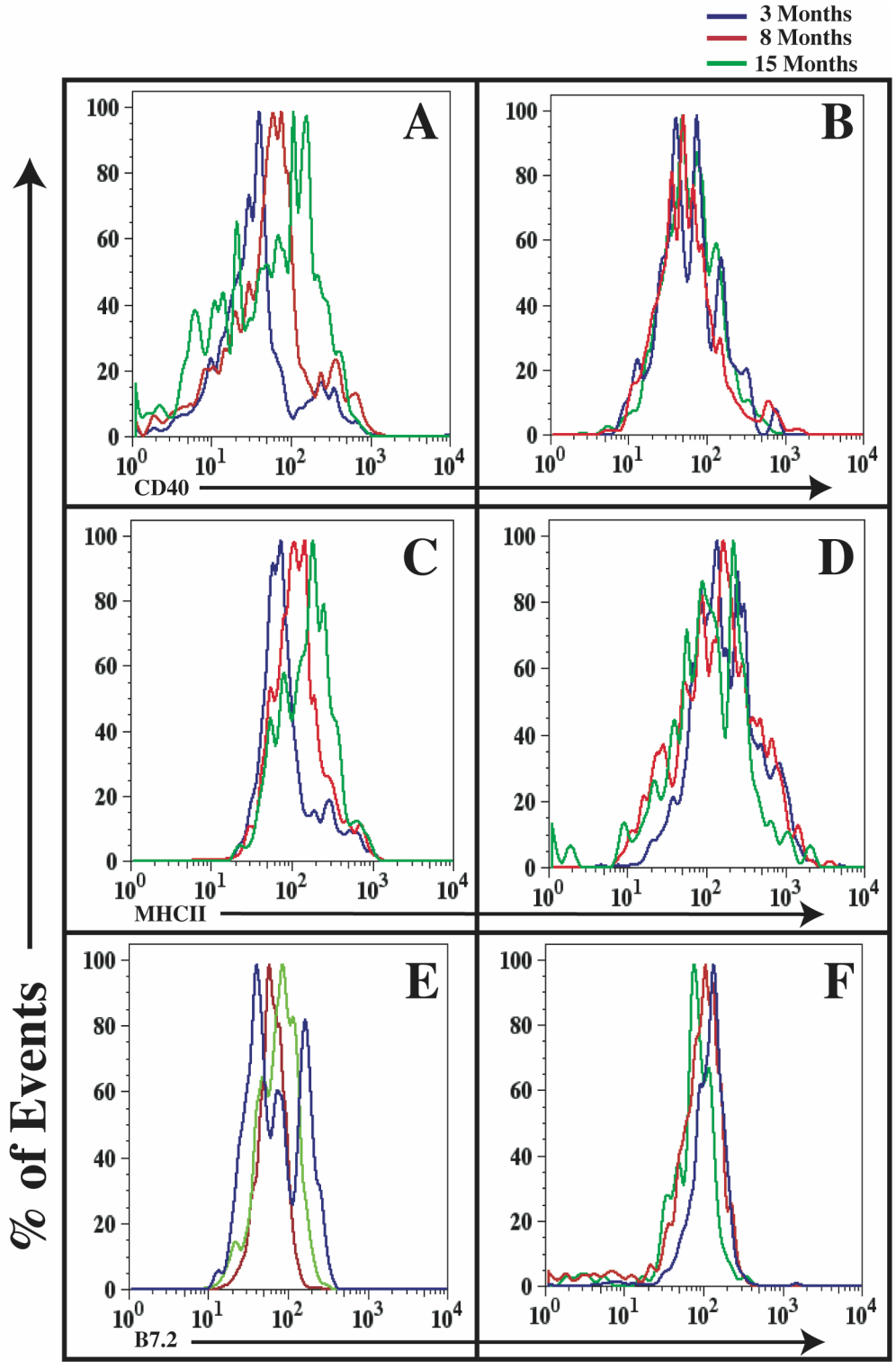

3 Months
8 Months
15 Months
A
B
C
D
E
F
CD40
MHCII
B7.2
% of Events
0
20
40
60
80
100
$10^0$
$10^1$
$10^2$
$10^3$
$10^4$

Figure 3-8. **TREM2 expression by CNS-resident microglia and infiltrating macrophages is upregulated in an age-dependant manner in response to LPS-induced CNS inflammation.** Induction of TREM2 expression by **(A, C, E)** CD45lo CNS resident microglia and **(B, D, F)** CD45-hi infiltrating macrophages in response to LPS-induced CNS inflammation at **(A – B)** 3 months, **(C – D)** 8 months and **(E – F)** 15 months of age. Activated CNS resident microglia (red histigrams – A,C,E) show upregulation of TREM2 beyond the background autoflouresence (blue histograms) at all ages tested. Activated Peripheral macrophages do no upregulate TREM2 expression (red histograms – B,D,F) at any of the ages tested. Data is representative of two replicate experiments.

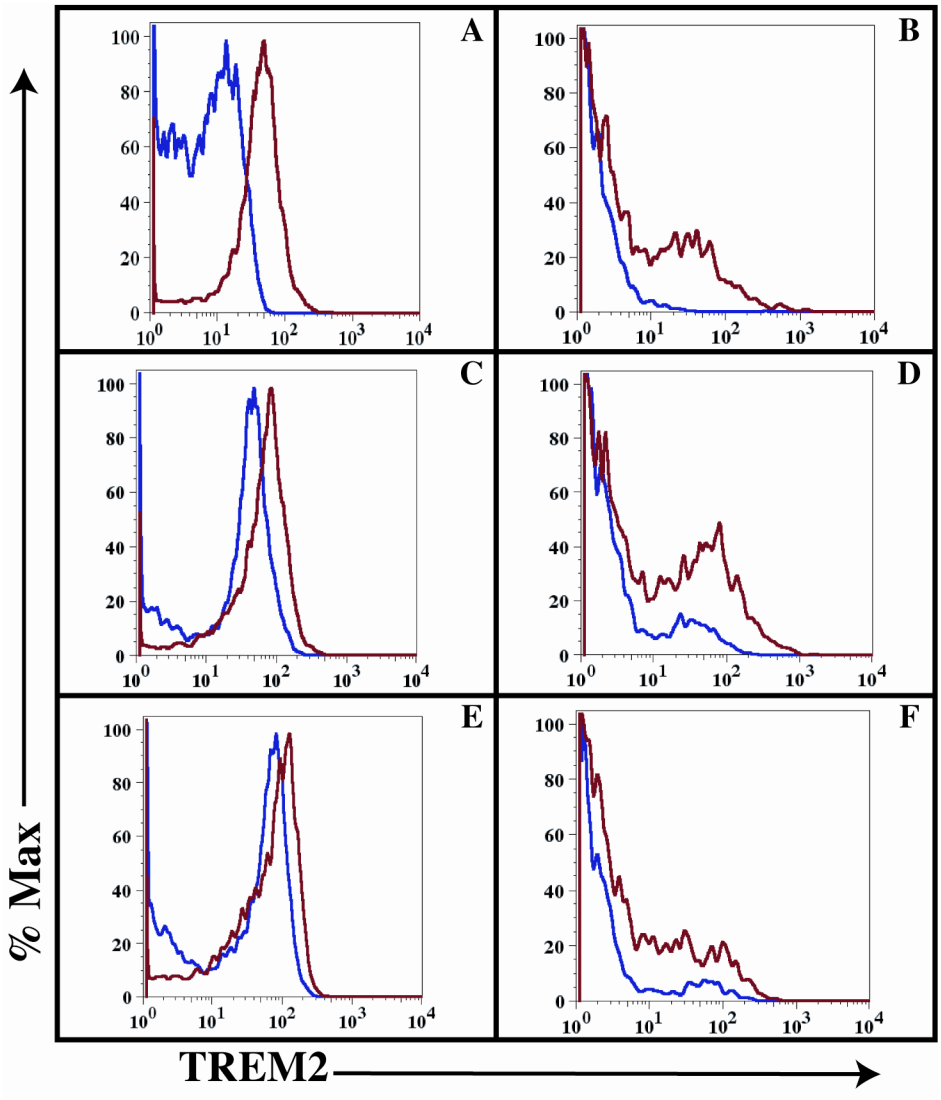

A
B
C
D
E
F
% Max
TREM2

Figure 3-9 **Expression of MMR by CNS-resident microglia and infiltrating macrophages is not affected by aging.** Regulation of MMR expression by **(A, C, E)** CD45lo CNS resident microglia and **(B, D, F)** CD45-hi infiltrating macrophages in response to LPS-induced CNS inflammation at **(A – B)** 3 months, **(C – D)** 8 months and **(E – F)** 15 months of age. Activated CNS resident microglia from **(A** - red histogram**)** 3 months and **(C** – red histogram**)** 8 months old mice do not show upregulation of MMR expression beyond the background autoflouresence (A,C - blue histograms). **(E)** Modest upregulation of MMR expression is seen by CNS resident microglia (E – red histogram) beyond the background (E – blue histogram). Activated Peripheral macrophages do not upregulate MMR expression (red histograms – B,D,F) at any of the ages tested. Data is representative of two replicate experiments.

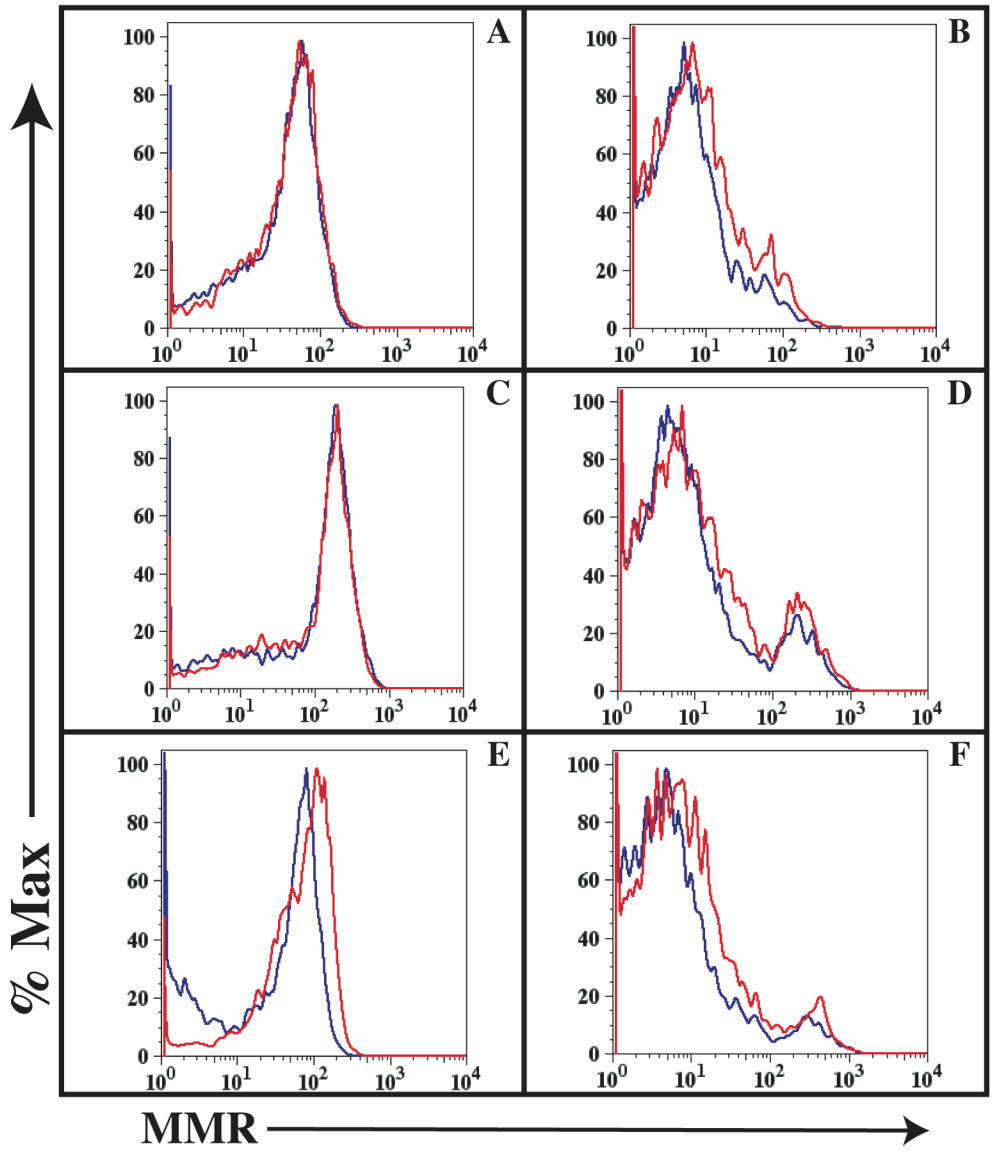

A
B
C
D
E
F
% Max
MMR
0
20
40
60
80
100
$10^0$
$10^1$
$10^2$
$10^3$
$10^4$

Figure 3-10. **The expression of a subset of genes associated with CNS inflammation is dysregulated in the aging CNS.** Quantification of expression of ODC, and C1qa at the site of injection from the CNS of 3 months, 8 months and 15 months old mice. Auto-radiograms from $^{33}$P-labeled insitu-hybridization studies were used to quantify gene expression at the site of injection. **(A)** ODC expression at various time-points post LPS injection. Expression peaked at 24 hours post injection and returned to basal levels at 72 hours post injection. No significant differences were seen in the 3 months (black), 8 months (grey) and the 15 months (dashed) CNS ($P > 0.05$). **(B)** C1qa expression shows age-associated alterations. Expression of C1qa peaked at 24 hours in the 3 months old mice (black) and was resolved at 72 hours post injection. Peak expression of C1qa was observed at 72 hours post injection in the 8 months (grey) and the 15 months (dashed) CNS. Data is representative of three replicate experiments. ( ***$P < 0.001$, two-tailed Student's t-test).

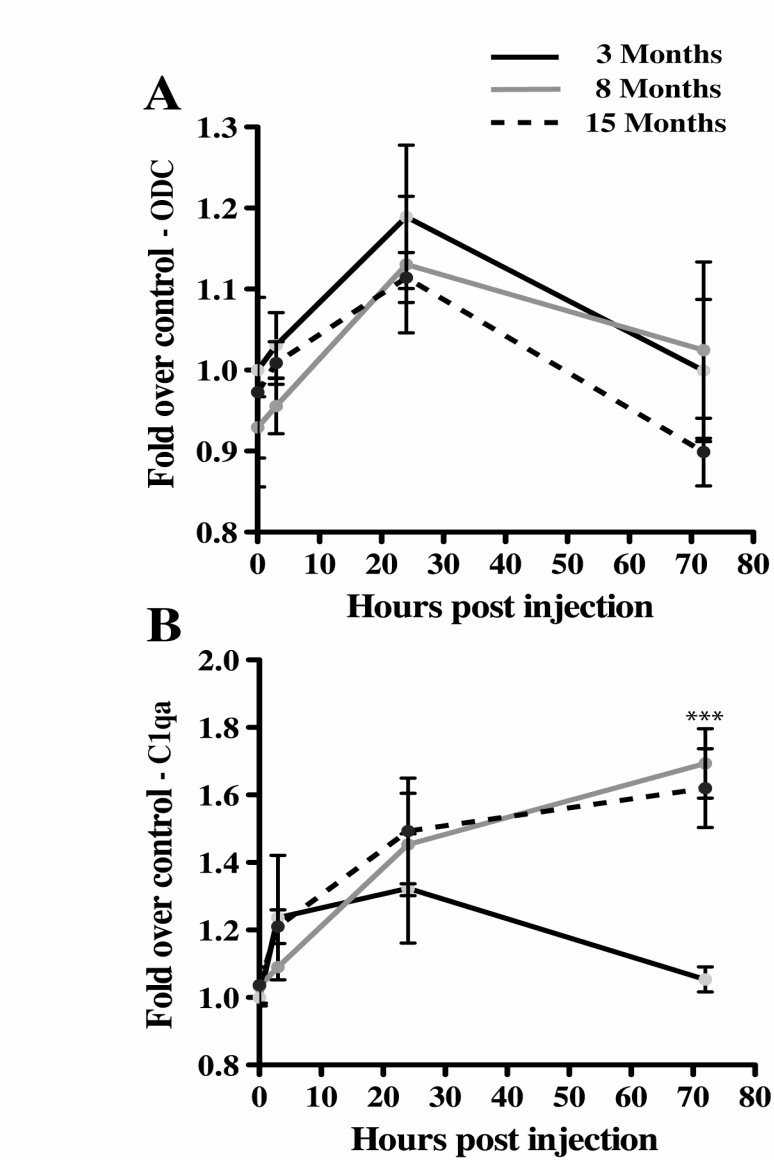

A
3 Months
8 Months
15 Months
Fold over control - ODC
1.3
1.2
1.1
1.0
0.9
0.8
0
10
20
30
40
50
60
70
80
Hours post injection
B
Fold over control - C1qa
2.0
1.8
1.6
1.4
1.2
1.0
0.8
***
0
10
20
30
40
50
60
70
80
Hours post injection

Figure 3-11. **C1qa mRNA expression is upregulated at the site of injection by lectin(+) microglia/macrophages.** C1qa mRNA expression in the CNS of **(A-L)** 3 months and **(M-X)** was detected by in-situ hybridization of 33P-labeled riboprobes to brain tissue at 3 hours, 24 hours and 72 hours post injection. Robust, sustained upregulation of C1qa expression of was seen at the site of injection of **(M)** 8 months and **(U)** 15 months old mice at 72 hours. Panels B and F depict low magnification (10X) images of the site of injection at 24 hours and 72 hours post injection respectively. Panels C – D and G – H depict two high-magnification (63X) focal planes from the boxed areas in B and F respectively. Similarly, panels J and N depict low magnification (10X) images of the site of injection at 24 hours and 72 hours post injection respectively in the 8 months old CNS. Panels K – L and O – P depict two high-magnification (63X) focal planes from the boxed areas in J and N respectively. Panels R and V depict low magnification (10X) images of the site of injection at 24 hours and 72 hours post injection respectively in the 15 months old CNS. Panels S – T and W – X depict two high-magnification (63X) focal planes from the boxed areas in R and V. C1qa expression is visualized by black grains in photographic emulsion. Microglia/macrophages are visualized in brown by tomato lectin. Data is representative of three replicate experiments.

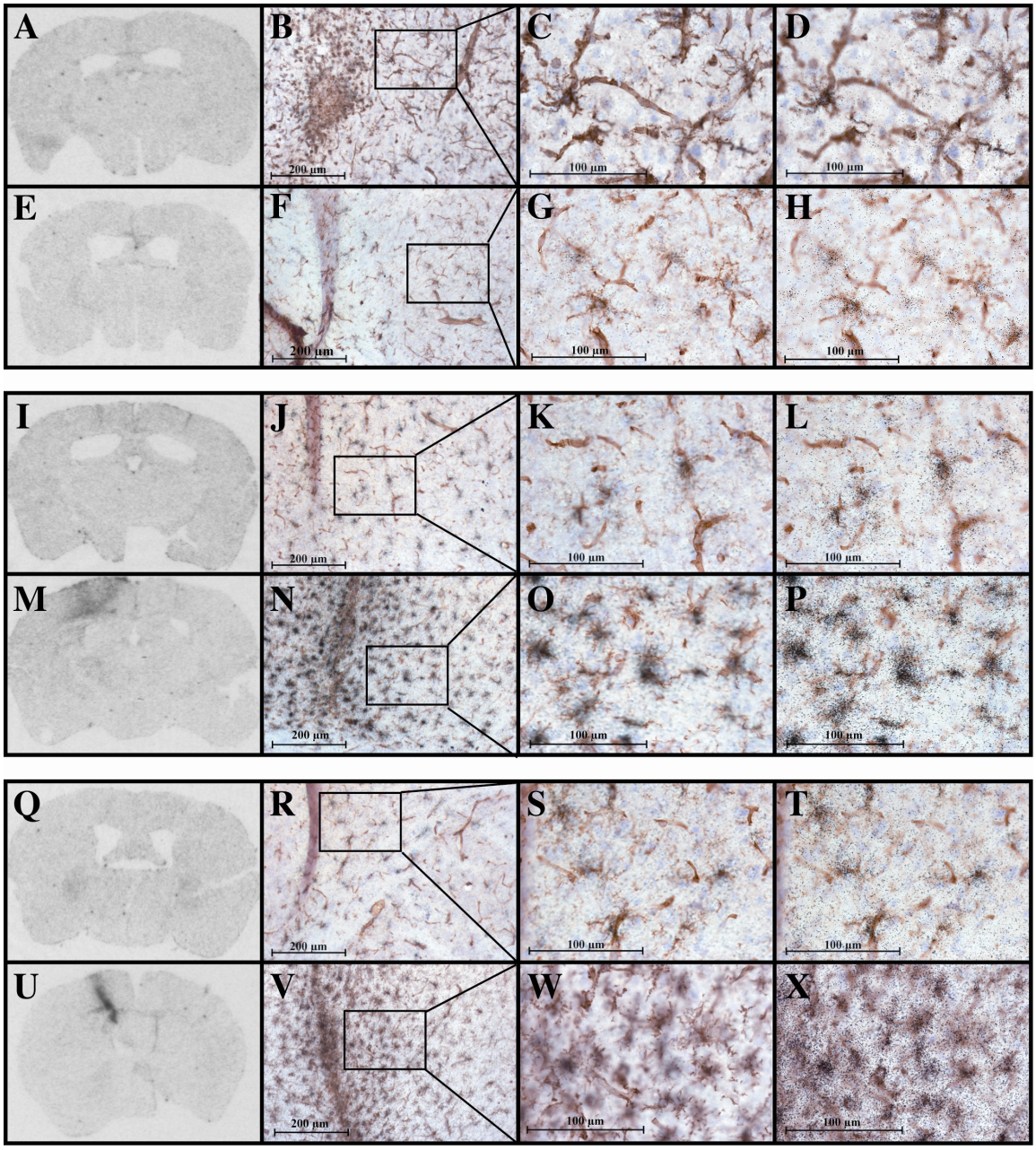
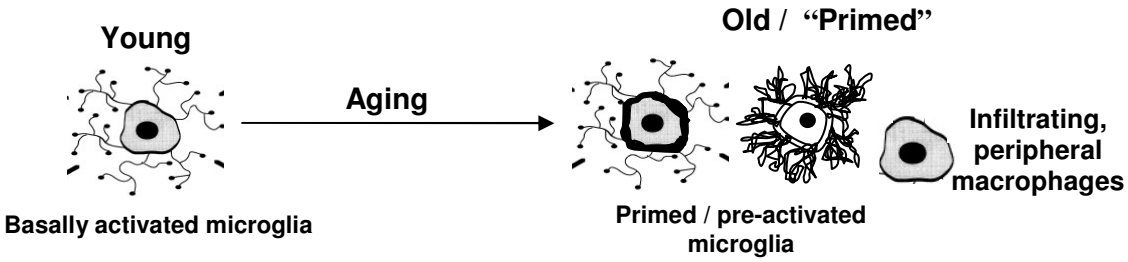

A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
Q
R
S
T
U
V
W
X
200 µm
100 µm

Figure 3-12. **The effect of aging on the immune phenotypes of CNS-resident microglia and on the basal immune status of the CNS.** CNS-resident microglia express basal levels of MHC class II, CD40 and B7.2. As the animal ages, the basal expression of MHC class II, CD40 and B7.2 by CNS-resident microglia increases and they assume a 'primed / pre-activated' phenotype. In addition, the old CNS shows increase in the basal infiltration by peripheral macrophages. These alterations in the aged CNS may result in dysregulated immune response and an increased propensity towards chronic neuro-inflammatory responses.

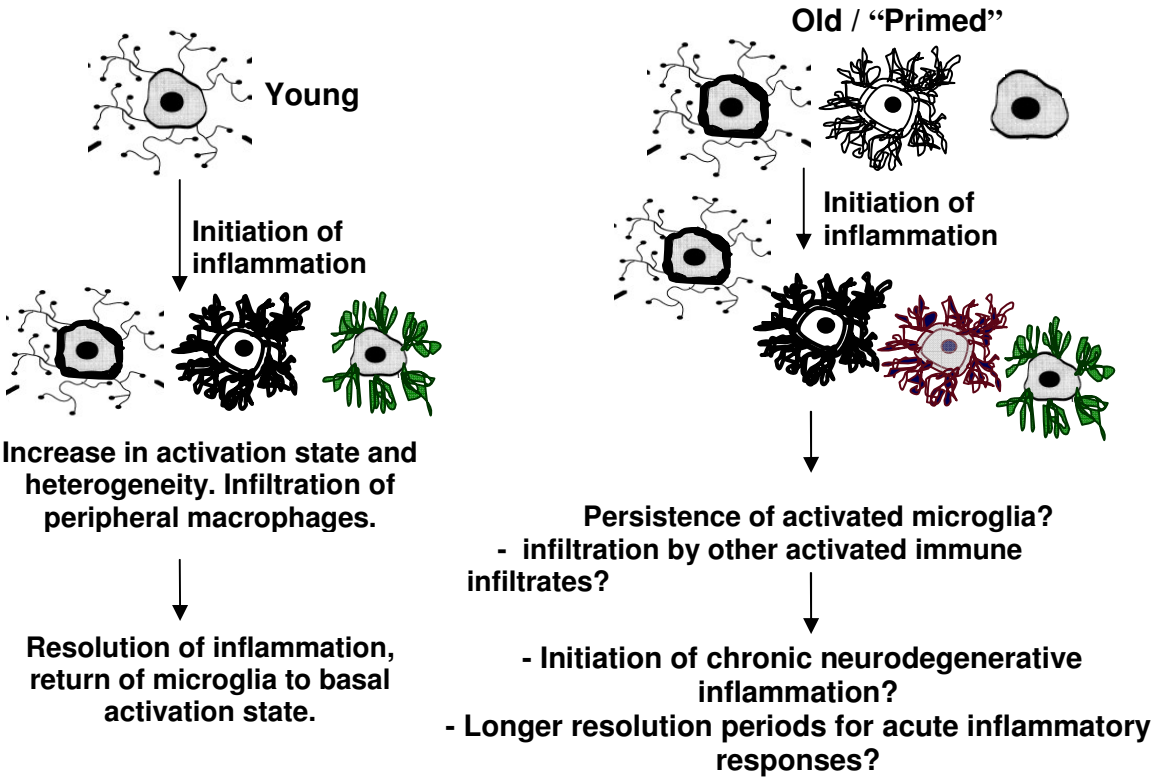



**- Increase in basal activation state and in microglial phenotype.**
**- Increase in infiltration by peripheral macrophages.**

**Does the increased basal activation state of the aged CNS lead to :**

- **an increased sensitivity to inflammation? / an increased propensity to chronic inflammatory responses?**
- **unnecessary / improper antigen presentation?**
- **dysregulated signaling from senescent neurons?**

Figure 3-13. **Inflammation in the aged CNS is characterized by persistence of activated microglia.** Inflammation in the young CNS is characterized by microglial activation and infiltration by peripheral macrophages. Upon resolution of inflammation, the microglia get reset to their basal activation state. In contrast, microglia in the aged CNS display a basal preactivated or a primed state. The induction of CNS inflammation results in further activation of CNS-resident microglia and infiltration by peripheral macrophages. These activated CNS-resident microglia persist in the aged CNS into the resolution phase of inflammation. This persistence of activated microglia in the aged CNS may result in initiation of chronic-neurodegeneration and inflammatory responses with longer resolution periods.


Young
Initiation of inflammation
Increase in activation state and heterogeneity. Infiltration of peripheral macrophages.
Resolution of inflammation, return of microglia to basal activation state.
Old / "Primed"
Initiation of inflammation
Persistence of activated microglia?
- infiltration by other activated immune infiltrates?
- Initiation of chronic neurodegenerative inflammation?
- Longer resolution periods for acute inflammatory responses?

# CHAPTER FOUR

## Differential Expression of Trem-like Transcripts (TLTs) by CNS – Resident Microglia and Peripheral Macrophages: Are in vitro Models Predictive of in vivo Inflammatory Responses?

## 4.1 Abstract

**Inflammatory responses in the CNS are mediated chiefly by CNS resident microglia and a population of peripheral macrophages that infiltrate the inflamed CNS. Although these two populations represent distinct antigen presenting cell types, studies focusing on the differential regulation of their inflammatory responses are limited. We therefore investigated the differential expression of the family of orphan receptors, the Trem-like transcripts (TLTs) by the CNS resident microglia and the peripheral macrophages in response to inflammatory activation. In addition, we also investigated the degree of commonly used in vitro models of microglial and macrophage activation to predict in vivo inflammatory responses of CNS resident microglia and infiltrating macrophages. qPCR analysis of LPS/IFNγ activated microglia from murine mixed glial cultures and in vitro activated peritoneal macrophages showed that the expression of TLT1, TLT2, TLT4 and TLT6 is regulated by inflammatory signals. In vitro, TLT2 and TLT6 expression is differentially regulated by activated microglia as compared to activated macrophages. The regulation of TLT gene expression by ex-vivo activated microglia and ex-vivo CNS infiltrating macrophages was distinct from that seen in vitro. In addition to inflammatory signals, in vivo expression of TLT2 is also regulated by developmental cues. Our data suggest that i) In vitro models of microglial and macrophage activation are only partially predictive of in vivo inflammatory responses. ii) Microglial phenotypes with respect to TLT expression are largely influenced by the cues received from the CNS microenvironment. iii) The**

**inflammatory responses of the CNS resident microglia and infiltrating macrophages may be actively controlled by the CNS micro-environment.**

## 4.2 INTRODUCTION

Inflammatory responses in the CNS are mediated by two distinct macrophage populations. A population of antigen presenting cells (APC) is seen to reside in the CNS parenchyma and is referred to as the CNS resident microglial population. (Aloisi F. 2001, Carson M.J. 2002, Schmid et al. 2009.) These cells are regarded as long term resident macrophages of the brain and are defined by a CD45-lo phenotype. On the other hand, a CD45-hi APC population, residing predominantly in the perivascular regions, meninges and the choroids plexus, is composed of hematogenously derived cells that are replenished every few weeks. (Carson et al. 2006,Schmid et al. 2009, Simard et al. 2006, Papenfuss et al. 2007). Despite the ability to distinguish these two populations in cell suspensions, as yet histologic markers able to conclusively distinguish them in tissue sections have not been identified. Consequently, a large body of research has referred to these populations simply as "microglia/macrophages", thus disregarding the possibility of differential regulation of their activation and effector functions during CNS inflammation.

Our research group has screened for differential gene expression between microglia and macrophages to address this issue. As part of these ongoing studies, we identified an orphan family of receptors called the 'triggering receptors expressed on myeloid cells (TREM)' as being expressed by microglia (Schmid C.D et al. 2009). The TREM family of receptors is divided into classes of receptors: the classic TREMs and the TREM-like Transcripts (TLTs). Both, the TREMs and the TLTs have a single extracellular V-set Ig

domain (Washington A.V. et al. 2002). These receptor sub-types differ primarily with respect to their cytoplasmic tails. The TREMs have a short cytoplasmic tail and a charged residue in their trasmembrane domain that allows them to associate and signal through the transmembrane adaptor molecule DAP12 (Washington A.V. et al. 2004). The TLTs have longer cytoplasmic tails and are capable of signaling without adaptor molecules (Washington A.V et al. 2004). TLT1, has a immunoreceptor tyrosine based inhibitory motif (ITIM) motif in its signaling tail and is predicted to be an inhibitory receptor .

Various members of the TREM family have been previously implicated in the regulation of innate as well as adaptive immune responses. TREM2 is preferentially expressed on microglia as compared to other myeloid populations (Schmid C.D. et al. 2002, 2009). Functionally, TREM2 promotes partial acquisition of antigen-presenting function but promotes anti-inflammatory T cell responses (Bouchon et al. 2001, Melchior et al. ms in submission) (Bouchon et al. 2001) TREM1 is expressed primarily on neutrophils, monocytes and CNS-infiltrating macrophages. TREM1 expression has been implicated in the mounting and regulation of inflammatory responses to bacterial infection and septic shock (Bouchon et al. 2001). Similar studies to characterize the function of the TLTs, have indicated that these receptor-subtypes also may play important roles in the regulation of peripheral immune responses. TLT2 expression has been detected on developing B cells, neutorphils and macrophages and can be increased by treatment with pro-inflammatory signals (King G.R. et al. 2006).

Studies by Gattis and colleagues have demonstrated the existence of a smaller putative soluble isoform of murine TLT1 comprised of just the extracellular domain(Gattis et al. 2006). This soluble form appears to be generated by cleavage of the mature receptor on the plasma membrane of activated platelets (Gattis et al. 2006) The regulation of such putative "soluble" splice variants of the TLTs is of great importance as these may act as decoy receptors limiting ligand access or may act to prevent TLT ligand degradation.

As yet, expression of TLTs in the CNS is unexamined. In this study, we examine the regulation of TLT1, TLT2, TLT4 and TLT6 in response to inflammatory stimuli. TLT3 and TLT5 are pseudogenes and were not studied. Using in vitro models, such as LPS/IFNγ activated microglia from mixed glial cultures and cultured thioglycolate elicited peritoneal macrophages, we compared the regulation of TLT expression to that of ex vivo activated microglia and infiltrating macrophages. Our data shows that these commonly used in vitro models are only partially predictive of the in vivo regulation of TLTs in response to inflammatory signals. We show that the regulation of TLT expression in vivo is not as dramatic as that seen in vitro. We demonstrate that putative splice variants of all the TLTs examined are expressed in vitro as well as in vivo. Our results thus indicate that TLT expression in vivo is not only monitored at the cellular level but may also be regulated by expression of so-called "decoy" receptors.

## 4.3 RESULTS

### 4.3.1 TLT1 mRNA expression is preferentially expressed in ex vivo adult microglia as compared to CNS-infiltrating macrophages. .

Previous literature has identified TLT1 as a putative, inhibitory receptor expressed by megakaryocytes and platelets, which are cells of the myeloid lineage. We thus sought to examine whether the expression of TLT1 would be regulated by other cells of the myeloid lineage in response to CNS inflammation, namely peripheral macrophages and CNS resident microglia.

We initially chose to compare gene expression in microglia from mixed glial cultures and thioglycolate-elicited peritoneal macrophages because large numbers of highly purified cells could be obtained from these preparations. Mixed glial cultures are prepared from the CNS of newborn pups and consist of ~70% astrocytes, ~5% oligodendrocytes and ~25% microglia (Carson et al. 1998, Schmid et al. 2002). Microglia differentiate in these cultures in the absence of functional neurons and in the presence of proliferating glia. Thioglycolate elicited peritoneal macrophages (PMPs) are considered models of tissue infiltrating macrophages because they have been induced to infiltrate the peritoneal cavity in response to the presence of thioglycolate broth (reviewed in Carson et al. 2006). Using quantitative real time PCR (qPCR) and cDNA templates from mixed glial culture microglia and cultured, peritoneal macrophages, we quantified LPS/IFNγ regulated expression of TLT1. In response to 24 hours of activation by LPS/IFNγ, in vitro activated microglia (Fig 2B MG+LPS: black bar) and peritoneal macrophages (Fig 2B PMP+LPS

: grey bar) showed an approximate 5 fold down regulation of the dominant form of TLT1 (Fig 2A) with respect to unstimulated microglial controls (Fig 2B MG-LPS: black bar). Thus, in vitro, activated microglia and activated peripheral macrophages express similar levels of TLT1. In contrast, ex-vivo macrophages that infiltrate the inflamed CNS in response to intra-cerebral injection of LPS/IFNγ showed an approximate 5-fold reduction in the expression of TLT1 (Fig 2C CD45-high: grey bar) as compared to the activated CNS resident microglia. (Fig 2C CD45-low: black bar).

### 4.3.2 Regulation of TLT4 mRNA expression in response to inflammatory signals is distinct from that of the TLT4 splice variant in vivo and in vitro.

To understand how inflammatory signals regulate TLT expression we generated primers that recognize two forms of these receptors. The dominant form is the full, functional TLT receptor (Fig 3A - dominant form). The splice variant lacks a trans-membrane domain, thus resulting in the loss of the membrane-anchoring capacity of the receptor (Fig 3A – sv). This may be a putative, soluble form of these receptors.

24 hours post activation with LPS/IFNγ, cultured, microglia (Fig 3B MG+LPS ; black bar) show an approximate 5 fold induction in the expression of the dominant form of TLT4 as compared to un-activated controls (Fig 3B MG-LPS ;black bar). In contrast activated peritoneal macrophages (Fig 3B PMP+LPS ; grey bar) showed an approximate 4 fold down-regulation as compared to un-stimulated microglia (Fig 3B MG-LPS ; black bar). Thus, in vitro activated macrophages show approximately 25-fold reduced

expression of TLT4 as compared to activated microglia (Fig 3B). In comparison, the expression of the TLT4 splice variant was more modestly regulated. Cultured, activated microglia (Fig 3D MG+LPS : black bar) respond with an approximate 2.5 fold induction in the expression of the splice variant as compared to un-activated controls (Fig 3D MG-LPS ; black bar). While the un-stimulated peritoneal macrophages (Fig 3D PMP-LPS ; grey bar) show a robust basal expression of TLT4 splice variant, the activated macrophages (Fig 3D PMP+LPS ; grey bar) down-regulated their expression to levels comparable to that of the un-stimulated microglia (Fig 3D MG-LPS ; black bar). Thus, in vitro activated macrophages show an approximately 3 fold reduced expression of the TLT4 splice variant as compared to activated microglia.

The ex-vivo regulation of the dominant as well as the splice variant of TLT4 was distinct from that seen in vitro. The ex-vivo infiltrating macrophages (Fig 3C CD45-high: grey bar) showed a modest 2.5 fold reduction in the expression of the dominant form of TLT4 with respect to activated microglia (Fig 3C - CD45-low: black bar). However, the regulation of the expression of the TLT4 splice variant was more dramatic, with the infiltrating macrophages (Fig 3E – CD45-high: grey bar) showing a 10 fold down-regulation as compared to the activated microglia (Fig 3E - CD45-low: black bar). Thus, our in vitro model is again only partially predictive of the in vivo regulation of TLT4 (both, dominant and splice variant) expression.

### 4.3.3 In vitro and ex-vivo activated microglia and peripheral macrophages show similar regulation of TLT6 mRNA in response to inflammatory signals.

Using cDNA templates generated from microglia isolated 24 hours post treatment of mixed glial cultures with LPS/IFNγ, we examined the regulation of TLT6 expression in vitro. Cultured microglia responded with a robust 25 fold induction in the expression of both, the dominant (4A-dominant form, 4B MG+LPS ; black bar) as well as the splice variant of TLT6 (4A-sv, 4D MG+LPS ; black bar) in response to LPS/IFNγ. In contrast, activated, peritoneal macrophages show a relatively modest, 3 fold induction in the expression of dominant TLT6 (4B PMP+LPS ; grey bar) as well as its splice variant (4D PMP+LPS ; grey bar) as compared to the un-treated control microglia.

To compare the in vitro regulation pattern of TLT6 to in vitro responses, we performed qPCR using cDNA templates generated from activated CNS resident microglia and infiltrating macrophages isolated 24 hours post intra-cerebral LPS/IFNγ injection. Ex-vivo, infiltrating macrophages respond to neuroinflammation with a similar 3 fold down-regulation in the expression of the dominant variant of TLT6 (4C – CD45-high: grey bar), and a 1.25 fold down-regulation in the expression of the splice variant (4E – CD45-high: grey bar) as compared to the activated CNS resident microglia (4C, 4E – CD45-low: black bars).

### 4.3.4 Differential regulation of TLT2 mRNA expression is observed in response to inflammatory signals in vivo but not in vitro.

We quantified LPS/IFNγ regulated expression of TLT2 by qPCR using templates generated either from microglia isolated from mixed glial cultures and cultured peritoneal macrophages, or CNS resident microglia and infiltrating macrophages directly isolated from the inflamed CNS.

In vitro stimulated microglia (Fig 5B MG+LPS ; black bar) respond with a 6 fold induction in the expression of the dominant TLT2 variant (Fig 5A-dominant form) as compared to the un-stimulated controls (Fig 5B MG-LPS ; black bar). In comparison, the cultured peritoneal macrophages (Fig 5B PMP+LPS ; grey bar) showed a robust, approximate 17 fold induction in TLT2 expression as compared to the un-activated microglia. Similar analysis of the regulation of the TLT2 splice variant (Fig 5A-sv) showed that cultured microglia responded with a robust 17 fold induction in the expression of the splice variant (Fig 5D MG+LPS ; black bar) as compared to un-stimulated controls (Fig 5D MG-LPS ; black bar), while activated peritoneal macrophages showed a robust 42 fold induction (Fig 5D PMP+LPS ; grey bar) compared to un-activated microglial controls (Fig 5D MG+LPS ; black bar). Thus, as compared to activated microglia, peritoneal macrophages responded to in vitro inflammatory stimuli with a 2.8 fold induction in the expression of the dominant variant of TLT2 and a 2.5 fold

induction in the TLT2 splice variant expression. In contrast to the in vitro data, the expression of the TLT2 variants was differentially regulated in vivo. Infiltrating macrophages showed a modest 1.2 fold down-regulation of the expression of dominant TLT2 (Fig 5C – CD45-high: grey bar), and a 2.5 fold induction in the expression of the splice variant (Fig 5E – CD45-high: grey bar) as compared to the activated CNS resident microglia (Fig 5C, 5E – CD45-low: black bars).

### 4.3.5 TLT2 protein expression by CNS resident microglia is regulated by inflammatory signals in vivo.

In collaboration with BiogenIdec, we generated antibodies against the TLTs. BiogenIdec performed all analysis of the specificity of the antibodies. Therefore, the data characterizing the TLT antibodies will not be presented in this thesis. Using this reagent, we sought to examine if expression of the functional TLT receptors could be detected on CNS resident microglia and infiltrating macrophages and whether this expression would be regulated by inflammatory signals in vivo.

We performed flow cytometric analysis of expression of TLT1, TLT2, TLT4 and TLT6 on CNS resident microglia and infiltrating macrophages 24 hours post intracerebral injection with LPS. We could not detect the expression of TLT1, TLT4 and TLT6 on either the microglia or the infiltrating macrophages(data not shown). . However, CD45-low microglia from the CNS of 3 months old mice showed detectable expression of TLT2 in TLT2 expression above background fluorescence (Fig 5A). By contrast, no TLT2

expression was detectable on the cell surface of CD45-hi infiltrating macrophages (Fig 5B). Thus, our in vivo data confirmed data generated from our in vitro models that TLT2 expression is differentially upregulated by CNS resident microglia in response to neuroinflammation

## 4.4 DISCUSSION

The understanding of the mechanisms of CNS inflammation has been the critical focus of research for development of therapeutic interventions for various CNS inflammatory disorders. However, these studies have been limited by the fact that distinct populations of effector macrophages, namely the CNS resident microglia and the infiltrating peripheral macrophages, respond to and contribute to CNS inflammation.

Due to the technical difficulty in isolating large numbers of microglia and macrophages from the rodent CNS, various in vitro models have been widely used to identify functional differences between microglia and macrophages (Reviewed by Carson et al. 2008). Although microglial and macrophage cell lines are commonly used, these oncogene-transformed cells are obviously not representative of healthy microglia and macrophages in vivo. Thus, in our studies we have used primary microglia isolated from mixed glial cultures. These microglia are differentiated in the presence of oligodendrocytes and astrocytes and thus mature in the presence of normal, developmental cues as in vivo microglia (Carson et al. 1998, 1999). The cultured, peritoneal macrophages are thioglycolate elicited, and thus are activated in vivo to induce

expression of pattern-recognition receptors (Schmid C.D. et al. 2009). As both of these in vitro models are derived from primary microglia and macrophages, a valid comparison of their inflammatory responses can be made with those of in vivo CNS resident microglia and CNS infiltrating macrophages.

Our data demonstrate that not only are TLTs expressed by microglia and macrophages, but that each member of the TLT family is regulated in a distinct manner. While, in vitro activated microglia and macrophages express similar levels of TLT1, in vitro activated peritoneal macrophages express approximately 3-fold lower levels of TLT4 and 8-fold reduced expression of TLT6 as compared to in vitro activated microglia. On the other hand, the expression of TLT2 on activated macrophages is approximately 2 fold higher relative to activated microglia.

In comparison, TLT expression in vivo shows a distinct regulation in response to inflammatory stimuli than that predicted by out in vitro models. Infiltrating macrophages express 5 fold lower levels of TLT1 as compared to activated CNS resident microglia. Expression of TLT6 and TLT2 is 3 fold and 1.2 fold lower on activated macrophages as compared to microglia. In contrast, activated macrophages show a 2.5 fold induction in the expression of TLT4 as compared to activated microglia.

The existence of soluble isoforms of members of the TREM and the TLT family of receptors has been described in previous literature (Gattis et al. 2006). It has been

suggested that owing to their structural similarity with the extacellular domain of their respective functional receptors, these may act as "decoy" receptors and may result in down-stream signaling independent of their cytoplasmic signaling motifs (Gattis et al. 2006, Gibot S. et al. 2004). Our data suggests that the regulation of the expression of soluble forms of TLT2 and TLT6 that are distinct from that of the full functional forms of these receptors. Our studies thus highlight the possibility CNS inflammatory responses can be regulated, not only due to active signaling though surface receptors. Soluble isoforms of the TLTs may also lead to active regulation of inflammatory responses by either acting as ligands to surface receptors or by acting as decoy receptors for putative soluble ligands.

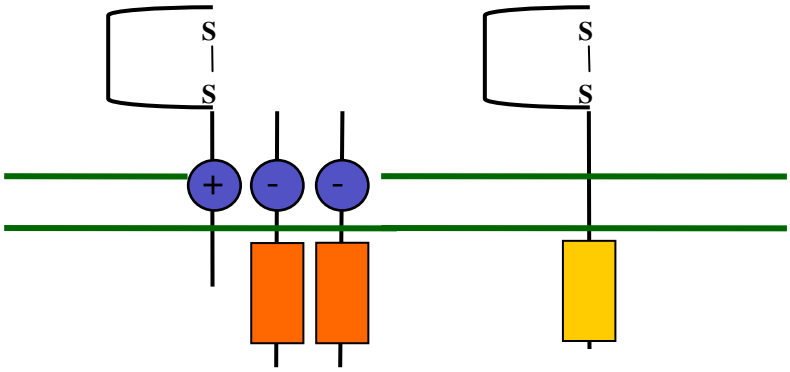
An additional point of interest demonstrated by our in vitro data is the robust LPS-induced upregulation of TLT6 expression, in vitro and in vivo. TLT6 is an exclusive murine isoform, with no human isoform being identified to date. Although the function of TLT6 in murine inflammatory responses is not known, the exclusive expression of TLT6 in mice is indicative of the fact that murine models of CNS inflammation may not be completely predictive of human inflammatory responses. It is possible that TLT6 signaling results in an additional level of control that is not seen in humans.

Using TLT2-fusion proteins, which have the extracellular Ig domain of TLT2 fused with the human Fc region, Carson et al. have demonstrated that the binding of these proteins can be detected on neurons. This suggests that neurons may be sources of the putative

ligand for TLT2. Our data thus adds to the growing literature that interactions between microglia and neurons may be important in the regulation of microglial immune responses. Microglia cultured without all the necessary environmental cues that are provided by a functional CNS are not predictive of in vivo microglia and their responses. Our ex-vivo real time PCR data shows that not only are certain genes such as that for TLT1, TLT2 and TLT4 differentially regulated, their regulation is more modest in vivo as compared to in vitro cultured microglia. Previous studies have identified similar differences in gene expression, where despite robust expression of C1qa, GPR84, CXCL14 etc. on in vitro activated microglia relative to activated macrophages, no such differential expression is observed in vivo (Schmid C.D. et al. 2009). It is known that within an intact CNS, continuous interaction between the glia and the neurons is an important determinant of microglial phenotypes. Thus, neurons, through expression of surface molecules such as CD200 and fractalkine . actively control glial reactivity. In vitro cultured microglia are known to display a pre-activated phenotype due to the absence of such neuronal cues (reviewed by Carson – 2007). The absence of regulatory cues provided by functional neurons may explain in part the differences observed between microglia differentiating in mixed glial cultures and those differentiating within the intact CNS.

## 4.5 FIGURE LEGENDS

**Figure 4-1. Schematic representation of the extracellular and the cytoplasmic signaling domains for TREM- and TLT receptors.** The TREMs and the TLTs have similar extracellular domains consisting of a V-set Ig domain. The TREMs are characterized by the presence of a charged residue in the transmembrane domain and a short cytoplasmic tail. This enables them to associate with adaptor molecule DAP12. The TREMs signal through immunoreceptor tyrosine based activation motif (ITAM). The TLTs, have longer cytoplasmic tails and thus are able to signal without association with adaptor molecules. The TLTs signal via putative immunoreceptor tyrosine based inhibition motif (ITIM).




**(DAP-12)**

**Figure 4-2. Expression of TLT1 by microglia and macrophages is regulated by LPS+IFNγ in vitro and in vivo.** qRT-PCR quantification of the regulation of TLT1 by microglia and macrophages in response to LPS+IFNγ - induced activation in vitro and in vivo. **(A)** Schematic representation of introns (black box) and exons of the TLT1 gene **(B)** Cultured microglia activated with LPS+IFNγ in vitro (MG+LPS – black bars) down regulated expression of TLT1 as compared to unstimulated controls (MG-LPS – black bars). Cultured peritoneal macrophages responded to LPS-induced activation by similar down-regulation of TLT1 expression (PMP+LPS – grey bars). **(C)** Infiltrating macrophages isolated from the CNS 24 hours post intra-cerebral injection of LPS down-regulated expression of TLT1 (CD45-high – grey bar) as compared to activated CNS resident microglia (CD45-low – black bar).

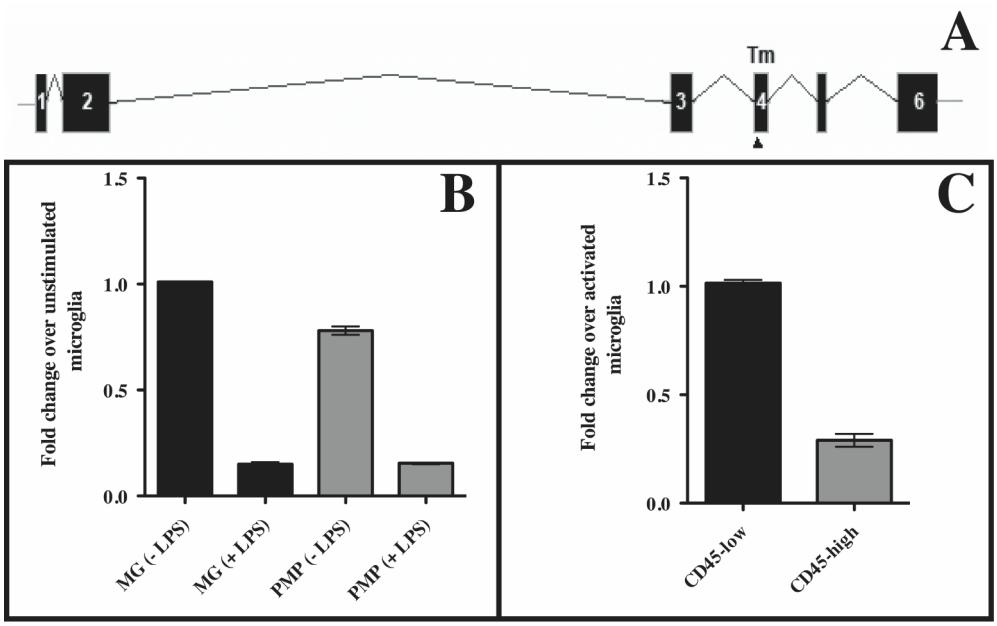

A
Tm
1
2
3
4
6
B
Fold change over unstimulated microglia
1.5
1.0
0.5
0.0
MG (- LPS)
MG (+ LPS)
PMP (- LPS)
PMP (+ LPS)
C
Fold change over activated microglia
1.5
1.0
0.5
0.0
CD45-low
CD45-high

**Figure 4-3. LPS+IFNγ differentially regulates the expression of TLT4 by microglia and macrophages in vitro and in vivo.** qRT-PCR quantification of the expression of the dominant-form and the splice-variant of TLT4 by activated microglia and macrophages in vitro and in vivo. **(A)** Schematic representation of introns (black box) and exons of the donimant form and the splice variant (sv) of TLT2. Panels **B , D** depict the regulation of expression of the **(B)** dominant-form of TLT4 and the **(D)** splice-variant of TLT4 by cultured microglia (MG) and peritoneal macrophages (PMP) in response to LPS+IFNγ. Both, the dominant and the splice variant forms of TLT4 are upregulated by in vitro activated microglia (MG+LPS – black bars) as compared to un-stimulated controls (MG-LPS – black bars). Cultured peritoneal macrophages (PMP+LPS – grey bars) down-regulated expression of the dominant form and the splice variant of TLT4 in response to in vitro activation with LPS+IFNγ. **(C)** Infiltrating macrophages (CD45-high – grey bar) isolated from the CNS, 24 hours post injection with LPS+IFNγ responded with a down regulation of the TLT4 dominant form as compared to activated CNS-resident microglia (CD45-low – black bar)). **(E)** Infiltrating macrophages (CD45-high – grey bar) isolated from the CNS, 24 hours post injection with LPS+IFNγ responded with a down regulation of the TLT4 splice variant as compared to activated CNS-resident microglia (CD45-low – black bar).

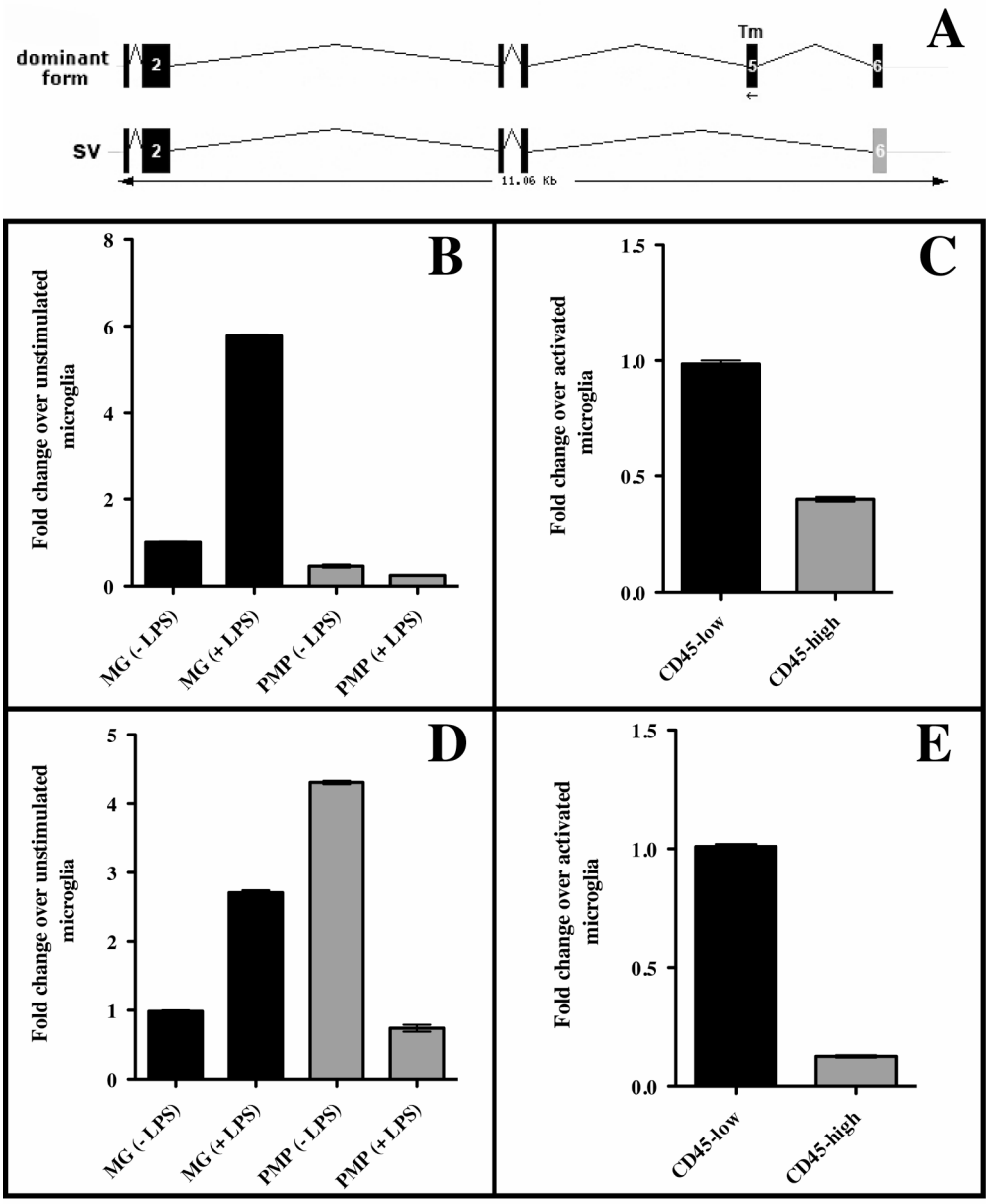

A
dominant form
Tm
SV
11.06 Kb
B
Fold change over unstimulated microglia
MG (- LPS)
MG (+ LPS)
PMP (- LPS)
PMP (+ LPS)
C
Fold change over activated microglia
CD45-low
CD45-high
D
Fold change over unstimulated microglia
MG (- LPS)
MG (+ LPS)
PMP (- LPS)
PMP (+ LPS)
E
Fold change over activated microglia
CD45-low
CD45-high

**Figure 4-4. TLT6 expression is upregulated on microglia and macrophages in response to LPS+IFNγ in vitro and in vivo.** qRT-PCR quantification of the expression of TLT6 by microglia and macrophages in response to LPS+IFNγ induced activation. **(A)** Schematic representation of introns (black box) and exons of the dominant form and splice variant (sv) of TLT6. **Panels B, D** depict the regulation of the **(B)** dominant form and **(D)** the splice variant of TLT6 in response to in vitro activation of microglia (MG+LPS – black bars) and peritoneal macrophages (PMP+LPS – grey bars). Cultured microglia and peritoneal macrophages show upregulation in the expression of TLT6 in response to LPS+IFNγ relative to unstimulated controls. **Panels C, E** depict the regulation of TLT6 expression in response to intracerebral injection of LPS+IFNγ. Activated macrophages isolated 24 hours post LPS+IFNγ (CD45-high – grey bar) injection showed a down-regulation in the expression of **(C)** the dominant form as well as **(E)** the splice variant of TLT6, as compared to activated CNS-resident microglia (CD45-low – black bar).

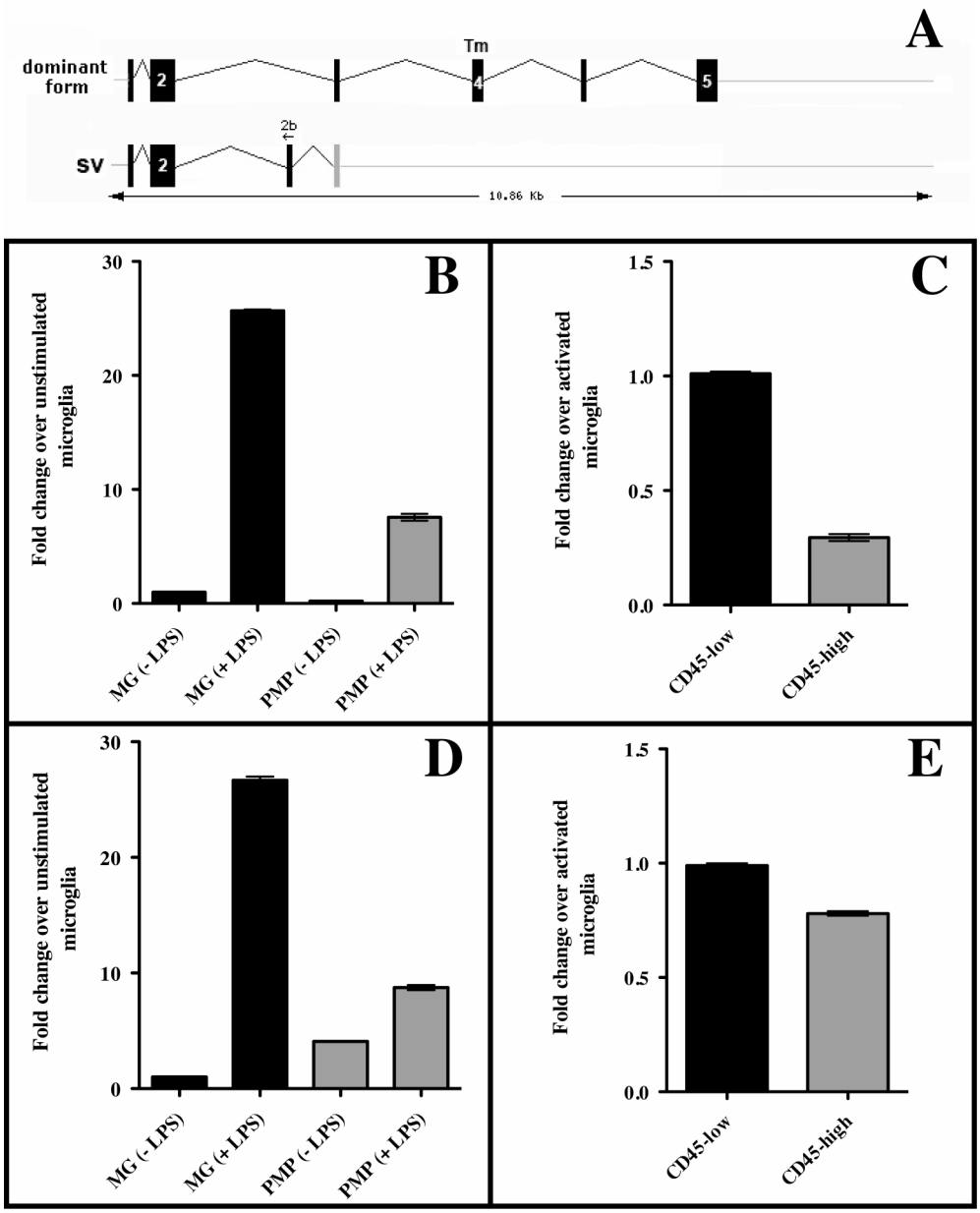

A
dominant form
Tm
2
4
5
sv
2b
10.86 Kb
B
Fold change over unstimulated microglia
30
20
10
0
MG (- LPS)
MG (+ LPS)
PMP (- LPS)
PMP (+ LPS)
C
Fold change over activated microglia
1.5
1.0
0.5
0.0
CD45-low
CD45-high
D
Fold change over unstimulated microglia
30
20
10
0
MG (- LPS)
MG (+ LPS)
PMP (- LPS)
PMP (+ LPS)
E
Fold change over activated microglia
1.5
1.0
0.5
0.0
CD45-low
CD45-high

**Figure 4-5. Expression of TLT2 by LPS+IFNγ activated microglia and macrophages is differentially regulated in vivo but not in vitro.** qRT-PCR quantification of the expression of TLT2 by microglia and macrophages in response to LPS+IFNγ induced activation. **(A)** Schematic representation of introns (black box) and exons for the dominar form and splice variant (sv) of the TLT2 gene. **Panels B, D** depict the relative expression of TLT2 by cultured microglia (black bars) and peritoneal macrophages (grey bars) in response to in vitro activation with LPS+IFNγ. **(B)** Expression of the dominant form of TLT2 is upregulated by culture activated microglia (MG+LPS – black bar) and cultured activated peritoneal macrophages (PMP+LPS – grey bar) as compared to respective un-stimulated controls. **(D)** Expression of the splice variant of TLT2 is upregulated by culture activated microglia (MG+LPS – black bar) and cultured activated peritoneal macrophages (PMP+LPS – grey bar) as compared to respective un-stimulated controls. **Panels C , E** depict the regulation of TLT2 expression in response to intra-cerebral injection of LPS+IFNγ. **(C)** Ex-vivo infiltrating macrophages (CD45-high: grey bar) isolated from the CNS 24 hours post injection of LPS+IFNγ show a modest down regulation in the expression of the dominant from of TLT2 as compared to activated microglia (CD45-low: black bar). **(D)** Ex-vivo infiltrating macrophages (CD45-high: grey bar) isolated from the CNS 24 hours post injection of LPS+IFNγ show a modest increased expression of the splice variant of TLT2 as compared to activated microglia (CD45-low: black bar).

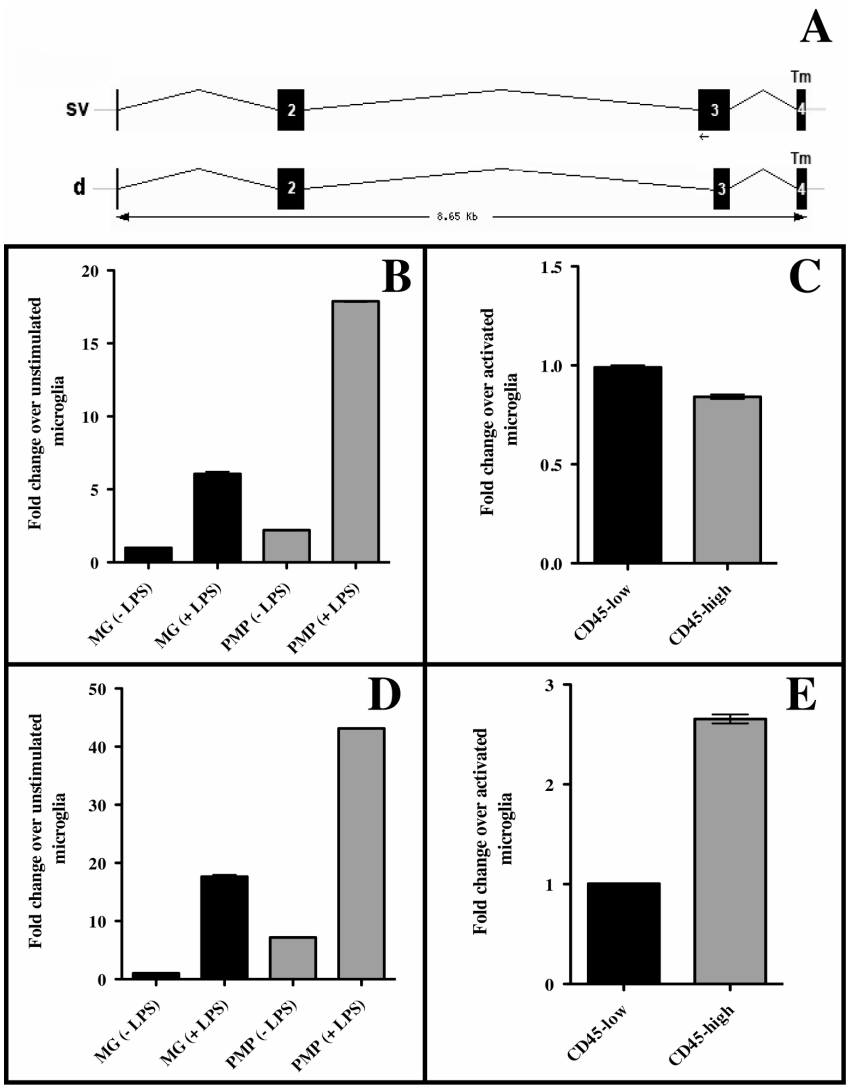
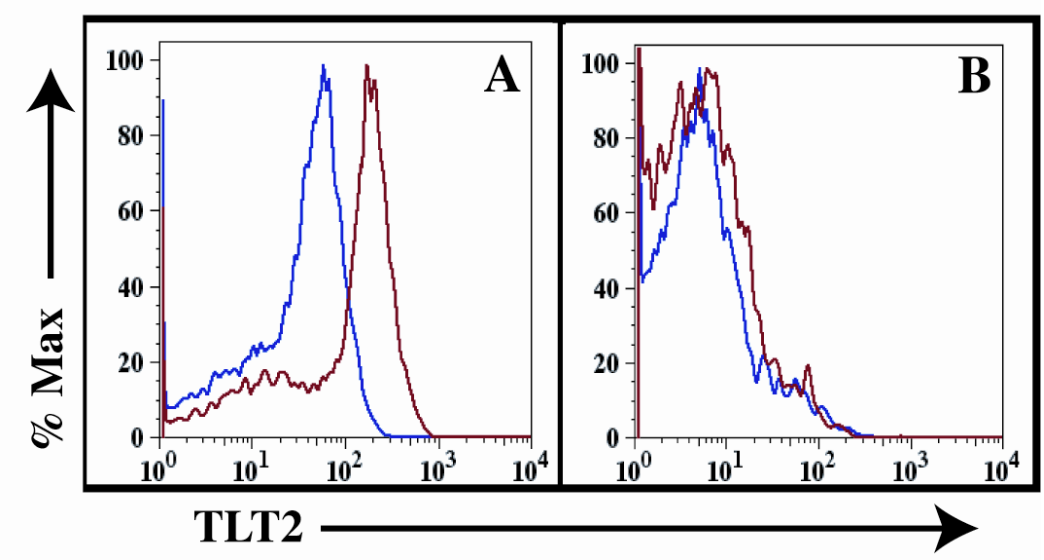

A
sv
d
2
3
4
Tm
8.65 Kb
B
Fold change over unstimulated microglia
MG (- LPS)
MG (+ LPS)
PMP (- LPS)
PMP (+ LPS)
C
Fold change over activated microglia
CD45-low
CD45-high
D
E

**Figure 4-6. Microglial expression of TLT2 in the CNS is regulated by inflammatory signals in vivo.** Flow cytometric analysis of TLT2 expression by CNS-resident microglia and infiltrating macrophages. **(A)** Expression of TLT2 by activated CNS resident microglia (CD11b+CD45-low gated) cells is upregulated (red histogram) as compared to unstained controls (blue histograms). **(B)** Infiltrating macrophages (CD11b+CD45-high gated cells) do not show upregulation in TLT2 expression (red histogram) as compared to unstained controls (blue histogram)


A
B
100
80
60
40
20
0
$10^0$
$10^1$
$10^2$
$10^3$
$10^4$
% Max
TLT2

# CHAPTER FIVE

## Conclusions

CNS inflammation is a prevalent feature of many CNS pathologies such as Alzheimer's disease (AD) (Simard et al. 2008, Schmid et al. 2009), CNS-trauma and Parkinson's disease (PD). Understanding the molecular mechanisms that play critical roles in the onset and progression of neuroinflammation has thus been essential in the development of therapeutic interventions for classic neurodegenerative disorders and CNS injury.

The appearance of activated CNS-resident microglia is one of the primary events that occurs during CNS inflammation (Carson et al. 1999, 2009). Thus, many therapies for the alleviation of symptoms have been directed towards modulation of microglial responses. However, these approaches have not been completely successful primarily because the net outcome of an inflammatory response in the CNS is a combined contribution of multiple cell populations. Two distinct populations of effector macrophages, namely the CNS-resident microglia and the peripheral macrophages that infiltrate the inflamed CNS play crucial roles in the onset and progression of CNS inflammation. Due to lack of cell type specific markers to distinguish these two populations from each other by histology, much of the current literature refers to these as "macrophages".

In this dissertation, we focus on the various aspects of regulation of CNS inflammatory responses, namely –

1. Regulation of the immune activation of CNS-resident microglia and how they can be differentially regulated as compared to infiltrating macrophages.
2. Regulation of the recruitment of peripheral macrophages to the inflamed CNS.

Our data suggests that these two events, that are characteristic of early inflammatory responses during neuroinflammation, are under the regulation of distinct molecular mechanisms.

Dysregulation of polyamine metabolism within the CNS has been identified in animal models of several neurodegenerative disorders including amyotrophic lateral sclerosis, ischemia and Alzheimer's disease as well as in brain tissue from individuals with Alzheimer's disease (Clarkson et al. 2004, Kim et al. 2009, Morrison et al. 1995, Morrison et al. 1998, Virgili et al. 2006). Our data shows that polyamine metabolism in the CNS may have dual functions in CNS physiology and inflammatory responses. Basal levels of polyamines may be critical for maintaining tissue homeostasis in the CNS by restricting the access of the healthy CNS to peripheral macrophages. Elevated levels, as seen during neuroinflammation, may promote and/or sustain CNS neuroinflammation by regulating CCL2 dependant macrophage influx. However, we propose that the biology of polyamines in the CNS may be more complicated than what is outlined in this dissertation. While we have focused on a model of acute and self-resolving CNS inflammation to dissect out the functional role of polyamine induction, inflammatory response in chronic neuroinflammation are completely different. Thus, we hypothesize that the regulation of polyamine biosynthesis and its down-stream effects on neuroinflammation are context dependant. It remains to be investigated whether the effects of polyamine metabolism within the CNS vary depending on concentrations of individual polyamines at each stage of CNS inflammation. A major difficulty is the

understanding the complex regulation of the polyamine metabolism pathway. Thus, while polyamine metabolism does not regulate microglial activation in a murine model of acute CNS inflammation, it is important for the regulation of macrophage recruitment.

Healthy aging has been identified as another factor that affects immune responses in the CNS. Current literature has abundantly highlighted the spontaneous appearance of reactive glia in the aged CNS (Goodbout J.P. et al. 2009, Bilbo S.D. et al. 2009). These studies have also focused on the altered immune activation of CNS-resident glia in response secondary CNS inflammation following systemic LPS administration (Goodbout J.P et al. 2009, Frank M.G. et al. 2006, Dilger R.M. et al. 2008). In this dissertation we try to extend these studies to look at whether induction of CNS inflammation by direct, intracerebral LPS injections results in altered immune responses in the aged brain. Our data has identified aging as an important factor that differentially regulates the immune responses of CNS-resident microglia. However, aging does not have any effect of the recruitment of macrophages to the inflamed CNS. Consistent with this observation, we do not see any alteration in the regulation of ODC responses in the aged CNS. Thus, while polyamine metabolism differentially affects recruitment of peripheral macrophages to the inflamed CNS, aging has differential effects on the immune activation of CNS resident microglia.

This data leads to further speculation that this differential behavior of microglia stems from the fact that these cells are permanent residents of the CNS, in that, they are

terminally differentiated and are never replenished over the life-time of an individual. This is unlike peripheral macrophages, which are hematogenously derived cells that are replenished every few weeks (Carson et al. 2006, Simard et al.2006, Papenfuss et al. 2007). Thus it is possible that the CNS-resident microglia from the aged mice are more reflective in their phenotypes and their immune-response of repeated incidences of activation over the lifetime of an individual. Our data further hypothesizes that although the phenotypes of the CNS-resident microglia become more reactive, inflammation in the aged CNS may be characterized by a more subtle dysregulation of immune responses. Our data suggests that the CNS by itself may be intrinsically in control of immune responses mounted, and is not a mere by-stander to inflammatory reactions.

The understanding of molecular mechanisms and factors that affect the mounting of an effective immune response is critical to the identification of "molecular tipping points" to enhance the neuroregenerative capacity of the CNS, while minimizing the neurodegenerative responses. Through this dissertation, we have tried to gain further understanding of the differential contribution of CNS-resident microglia and infiltrating macrophages to the CNS inflammatory response. A description of distinct molecular pathways that are either activated or 'switched off ' in these cell types is important for the design of effective therapeutics and for the future identification of targets in the treatment of CNS inflammatory diseases.

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