UNIVERSITY OF CALIFORNIA
RIVERSIDE

CNS Immunity is Developmentally Regulated

A Dissertation submitted in partial satisfaction
for the requirement of the degree of

Doctor of Philosophy

in

Biomedical Sciences

by

Deirdre Shawna Davis

August 2011

Dissertation Committee:
Dr. Monica J. Carson, Chairperson
Dr. David Lo
Dr. Emma Wilson

The Dissertation of Deirdre Shawna Davis is approved:

_____

_____

_____

Committee Chairperson

University of California, Riverside

ABSTRACT OF THE DISSERTATION

CNS Immunity is Developmentally Regulated

by

Deirdre Shawna Davis

Doctor of Philosophy, Graduate Program in Biomedical Sciences
University of California, Riverside, August 2011
Dr. Monica J. Carson, Chairperson

Specialized glial cells called microglia serve as the resident immunocompetent cells of the central nervous system (CNS). Though many describe microglia as having a "resting" state in the healthy brain, their processes are highly mobile and constantly survey their microenvironment (Nimmerjahn et al. 2005). In response to trauma, inflammation or infection, microglia become activated and peripheral macrophages are recruited to the CNS. Both microglial morphology and the collection of receptors that they express changes with their activation state. In the adult CNS, the type and magnitude of microglial activation and macrophage influx triggered by inflammation are well characterized. However, little is known about if and how CNS immunity changes during critical periods of postnatal brain development associated with synaptogenesis and oligodendrocyte maturation. This dissertation primarily focuses on understanding how microglial phenotype changes in the healthy brain during the early postnatal period and how their phenotype and the degree of macrophage influx changes following systemic inflammation.

A variety of receptors throughout the body have roles in mediating immune responses. Recently, a family of receptors called the Triggering Receptors Expressed on Myeloid cells (TREMs) have been implicated in regulating both innate and adaptive immune responses. TREM1 expression has been shown to amplify TLR mediated pro-inflammatory immune responses (Bouchon et al. 2001). Studies have shown that TREM2 is expressed by various cell types throughout the body including osteoclasts, dendritic cells, macrophages and microglia, the tissue macrophages of the brain (Paloneva et al. 2003, Bouchon et al. 2001, Schmid et al. 2002). Lack of a functional TREM2 signaling pathway has been shown to cause a rare genetic disorder called Nasu-Hakola disease (Madry et al. 2007). This disease is characterized by bone cysts, presenile cognitive dementia and death in the 40's or 50's (Madry et al. 2007).

Using dual in situ hybridization/immunohistochemistry, quantitative RT-PCR and flow cytometric studies, we show that several TREMs are expressed in the brain in a cell type specific manner and that they are regulated differently at the RNA and protein level. In situ hybridization/immunohistochemistry analysis of 3 month old unmanipulated murine brain illustrates that TREM4 is expressed by subsets of neurons and is not regulated by inflammation. qRT-PCR studies of 3 month old mice sacrificed 24 hours post intracerebroventricular (ICV) injection of lipopolysaccharide (LPS) demonstrate that TREM1 mRNA is higher in CD45lo microglia than CD45hi CNS infiltrating macrophages, TREM2 mRNA is higher in microglia and TREM3 mRNA is higher in infiltrating macrophages. At the protein level, we have found that microglia upregulate TREM2 following an ICV LPS injection, while CNS infiltrating macrophages express

TREM1 and a small population of macrophages express TREM3. Together, these data demonstrate that TREMs are differentially expressed by cells within the brain and along with their roles in innate and adaptive immune responses, TREMs may also play roles in healthy brain and cognitive development as well as in CNS diseases.

Inflammation that is experienced during the perinatal period has been implicated in playing a role in the development and/or pathogenesis of several neurodevelopmental disorders (Chew et al., 2006; Doorduin et al., 2008; Pardo et al., 2005). Though microglial activation has been observed in the brains of patients with cerebral palsy, schizophrenia and autism, the potential roles of these cells in the pathogenesis of these disorders are unknown. Using flow cytometry, we show that during the early postnatal period, microglia are in a pre-activated state that is non-polarized. This pre-activated state is lost as the brain matures. We also examined microglial phenotypes and responses to systemic inflammation and the degree of macrophage influx as a function of normal development. Here we demonstrate that systemic inflammation induced by an intraperitoneal injection of LPS leads to widespread activation of microglia and the transient influx of peripheral macrophages into the brain in an age specific manner. Interestingly, systemic inflammation led to the induction of TREM2, PD-L1 and CX3CR1 which are known to promote anti-inflammatory microglial activation. This data contributes to the literature illustrating that the brain may display age specific susceptibilities to insults.

Interestingly, the aged brain is also characterized by changes in microglial phenotype. By examining morphology and the expression of activation markers, several

studies have shown that microglia become activated in the brain with healthy aging. Moreover, many have shown that microglia display exaggerated immune responses following inflammatory events. Though several studies have found that both the CNS microenvironment becomes more pro-inflammatory in the aged brain and that microglia produce the pro-inflammatory molecules CD40, B7.2 and IL-1β in during this period, many have only begun to examine whether anti-inflammatory molecules are produced as well (Carson lab unpublished data, Henry et al. 2009). Here we confirm that following intracerebroventricular injection of LPS, the anti-inflammatory molecule TREM2 is more highly induced by microglia isolated from the young adult murine brain than those isolated from the aged murine brain. We also show that in the 3 month old murine brain, the anti-inflammatory molecules CX3CR1 and TREM2 binding activity are present and induced following inflammation. This is not observed in the 15 month old murine brain.

Overall these studies suggest that (1) TREM receptors are expressed on a variety of cell types in the brain and their contribution to immune responses depends on the compartment (CNS intrinsic versus CNS extrinsic) in which they are expressed and (2) CNS immune responses in the healthy and inflamed brain are age-specific.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

APC: Antigen Presenting Cell

Arg1: Arginase 1

BBB: Blood Brain Barrier

BMDM: Bone Marrow Derived Macrophages

CD200R: CD200 Receptor

CNS: Central Nervous System

COX-2: Cyclooxygenase-2

CP: Cerebral Palsy

CVO: Circumventricular Organs

DAMPS: Danger Associated Molecular Pattern

DAP12: DNAX activating protein of 12 kDa

DC: Dendritic Cell

EAE: Experimental Autoimmune Encephalomyelitis

Fizz1: Found in inflammatory zone 1

Hox: Homeobox

ICV: Intracerebroventricular

IFNγ: Interferon-gamma

IHC: Immunohistochemistry

iNOS: Inducible Nitric Oxide Synthase

IP: Intraperitoneal

ISH: In situ Hybridization

ITAM: Immuno-receptor Tyrosine based Activation Motifs

ITIM: Immuno-receptor Tyrosine based Inhibitory Motifs

IV: Intravenous

KO: Knockout

LPS: Lipopolysaccharide

M-CSF: Macrophage-Colony Stimulating Factor

MHC Class II: Major Histocompatibility Complex Class II

MMR: Macrophage Mannose Receptor

mTNFα: murine TNFα

PAMP: Pathogen Associated Molecular Pattern

PBMC: Peripheral Blood Mononuclear Cell

PD-L1: Programmed cell Death-Ligand 1

qRT-PCR: Quantitative Real Time-Polymerase Chain Reaction

RANKL: Receptor Activator of Nuclear factor κB Ligand

Th1: T helper 1 cell

Th2: T helper 2 cell

TIR: Toll/IL-1 Receptor

TNFα: Tumor Necrosis Factor alpha

TLR: Toll Like Receptor

TLT2: Triggering Receptor Expressed on Myeloid cells Like Transcript-2

TREM1: Triggering Receptor Expressed on Myeloid cells-1

TREM2: Triggering Receptor Expressed on Myeloid cells-2

TREM2KO: Triggering Receptor Expressed on Myeloid cells-2 Knock Out

TREM3: Triggering Receptor Expressed on Myeloid cells-3

TREM4: Triggering Receptor Expressed on Myeloid cells-4

TREM5: Triggering Receptor Expressed on Myeloid cells-5

WT: Wild Type

CHAPTER 1

Introduction

Specialized glial cells called microglia serve as the resident immunocompetent cells of the central nervous system (CNS). Though many describe microglia as having a "resting" state in the healthy brain, their processes are highly mobile and constantly survey their microenvironment (Nimmerjahn et al. 2005). In response to trauma, inflammation or infection, microglia become activated and peripheral immune cells are recruited to the CNS by microglia and other CNS resident cells. Both microglial morphology and the collection of receptors that they express change with their activation state. In the adult CNS, the type and magnitude of microglial activation and macrophage influx triggered by inflammation are better characterized than in the developing CNS. However, many researchers use in vitro methods, including microglia from mixed glial cultures or immortalized microglial cell lines to study microglia in the "adult" brain. Microglial phenotype in the intact brain is highly dependent on the summation of cues provided by cells in the microenvironment. Though in vitro methods help us understand how these cells may react to a specific stimulus in isolation, experimental findings from in vitro studies may differ significantly from what occurs in vivo.

Currently, little is known about if and how CNS immunity changes during critical periods of postnatal brain development associated with synaptogenesis and oligodendrocyte maturation. This dissertation primarily focuses on understanding how microglial phenotype changes in the healthy brain during the early postnatal period and how microglial phenotype and the degree of macrophage influx changes during different

periods of brain maturation following systemic inflammation. With these studies we hope to gain a better understanding of microglial physiology in the intact brain during critical periods of healthy postnatal CNS development. These studies will also shed light on the potential roles of microglia in the development of numerous neurological disorders currently hypothesized to result from perinatal inflammation.

1.1.1. CNS Environment: Neurons and Glia

The CNS is a complex organ system consisting of several cell types. The main cell types of the brain and spinal cord are the neurons and glia. Glia can be further divided into the macroglia including astrocytes and oligodendrocytes which are of neuroectodermal origin, and microglia which are of mesenchymal origin.

Though neurons form the major communication networks that allow the brain to control all other organ systems in the body, all glia perform a diverse array of functions that serve to maintain homeostasis in the CNS. Specifically, glia provide 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, Carson et al. 2006). To perform the appropriate functions, glial cells constantly communicate with neurons. Specifically, neurons, macroglia and microglia are equipped with molecules that allow them to communicate with each other and with immune cells from the periphery that are recruited to the CNS following inflammation (Biber et al. 2007, Carson et al. 2007, D'Mello et al. 2009). Though neuroimmunologists are becoming increasingly aware of how peripheral immune cells affect the mature CNS,

we have yet to fully explore whether these cells exert distinct effects on the developing CNS.

1.1.2 What are microglia?

Microglia are commonly referred to as the tissue macrophage of the CNS. As these cells express most macrophage markers, their origin has served as the source of debate for some time. To date, the commonly accepted hypothesis is that microglia populate the CNS in two waves (Andjelkovic et al. 1998, Lichanska et al. 1999, Soulet et al. 2008). The first wave of microglia appear in the CNS in the nonvascularized embryonic period, and these cells originate from progenitors in the yolk sac (Lichanska et al. 1999, Soulet et al 2008). The second wave of microglia is hypothesized to originate from circulating monocytes that populate the brain during the postnatal period (Andjelkovic et al. 1998, Lichanska et al. 1999, Soulet et al. 2008). Using several microglia/macrophage markers, some studies have also established that during the developmental period microglia exist in specific subpopulations (Hutchins et al. 1990, Schmid et al. 2002). It is currently unclear how the functions of these specific subpopulations differ.

A recent report using flow cytometric and lineage studies illustrates that embryonic and adult mouse microglia arise from primitive myeloid precursors in the extra embryonic yolk sac (Ginhoux et al. 2010). Using parabiotic mice, Ginhoux et al. demonstrated that though 30% of monocytes and tissue macrophages were donor derived at 1 and 12 months following parabiosis, only 5% of microglia were donor derived at

these time points. These findings illustrate that CNS resident microglia are not routinely replenished by blood derived cells. This finding also illustrates that experiments using bone marrow chimeric mice and sub-lethal irradiation may present inaccurate results as Ginhoux et al. 2010 also observed that 10-20% of parenchymal microglia in the recipient mouse were of donor origin in their own bone marrow chimeric studies. Irradiation is known to cause changes to the BBB, and thus the higher percentage of donor derived microglia in bone marrow chimeric recipients may occur as a result of BBB damage (Carson et al. 2008, Ginhoux et al. 2010). Ginhoux and colleagues also used lineage tracing studies to depict that primitive myeloid progenitors that arise before embryonic day 7.5 contribute significantly to adult microglial homeostasis in the healthy brain. These same progenitors gave rise to very few circulating leukocytes in the adult (Ginhoux et al. 2010). Contrary to what previous studies have found, Ginhoux et al. reported that myeloid progenitors require blood vessels to migrate from the yolk sac to the brain between embryonic days 8.5 and 9.5.

As glial cells, microglia perform various functions that facilitate proper function of the brain and spinal cord. However microglia are also immune cells and they are able to detect when CNS immunity has been compromised and respond appropriately by changing their morphology and the collection of receptors and molecules that they express. Though their origin has served as a major debate since their discovery, many now agree that microglia are myeloid derived cells that can be detected in the CNS during early prenatal development (Ransohoff et al. 2010, Carson et al. 2009). In the healthy brain, microglia have a stellate morphology and can be found throughout the

brain and spinal cord (Carson et al. 2009). When microglia become fully activated, they resemble blood derived inflammatory macrophages as their processes shorten and their cell bodies become more round.

1.1.3 How do we study microglia?

Microglia are highly plastic cells and their phenotype depends greatly on the summation of their external cues provided by cells within their CNS microenvironment (Carson et al. 2007, Carson et al. 2008). In order to effectively study microglia in the lab, in vitro and in vivo methods have been devised. However, as we are not yet able to study microglia in the fully intact and living brain, both types of experimental methods have pros and cons that must be carefully considered before studying these cells.

In vitro studies

In the brain microglia are surrounded by cells and a variety of components that reside within the intact CNS. In order to study microglia and their response to specific stimuli, in vitro methods are used. However, when performing in vitro studies, one must remember that in the intact brain microglia are not isolated from surrounding cells. Instead, microglia are very active in the healthy state and constantly survey their microenvironment by extending their processes. Moreover, microglia depend on cues from their environment to establish when problems arise in the CNS.

The in vitro methods that are most often used to study microglia are (1) immortalized microglial cell lines including the BV2 and N9 cell lines, (2) primary

microglia generated from embryonic and neonatal mixed glial cultures, (3) primary microglia isolated from CNS tissue and analyzed ex vivo, and (4) organotypic slice cultures isolated from the living and intact brain (Carson et al. 2008). As mentioned above, microglial phenotype is highly dependent on cues from their environment (Carson et al. 2008). When these cues are removed and microglia are cultured in isolation, studies have shown that microglial physiology may differ greatly from their in vivo physiology.

CD45, also called leukocyte common antigen, is a type I transmembrane protein tyrosine phosphatase that is expressed by all nucleated hematopoietic cells and their precursors (Thomas et al. 1989, Tan et al. 2000). Studies have shown that CD45 is involved in the negative regulation of cellular activation (Tan et al. 2000). While all other cells of hematopoietic origin increase their expression of CD45 from the neonatal period through adulthood, microglia do not (Carson et al. 1998, Sedgwick et al. 1991, Ford et al. 1995). In the intact brain, activated microglia upregulate CD45 (Sedgwick et al. 1991, Carson et al. 1998, Carson et al. 2007). However, the CD45 levels of activated microglia remain lower than that expressed by peripheral macrophage populations (Sedgwick et al. 1991, Carson et al. 1998, Carson et al. 2007). Curiously, unactivated microglia cultured in the absence of neurons express higher CD45 levels than that expressed by activated microglia in vivo (Carson et al. 1998, Carson et al. 2007, Carson et al. 2008). This clearly depicts that microglia cultured in the absence of CNS cells do not necessarily mirror microglial phenotype and/or physiology in the intact brain.

Organotypic brain slice cultures are also a widely used setup that can be used to study microglia. In many ways, slice cultures may prove to be more beneficial to specific studies because, unlike immortalized cell lines or primary microglial cultures, slice cultures allow researchers to study microglia in a brain slice that not only retains the cellular diversity of the CNS, but also the tissue structure (Carson et al. 2008, Kovacs et al. 2011). Slice cultures also allow researchers to treat the slice with pharmacological agents, toxins, pathogens and cells to observe how cells within the slice react (Carson et al. 2008). Though slice cultures can be beneficial for studies, there are several reasons why they may be problematic in microglial studies. When the slice itself is cut from the whole brain, "wounds" are made leading to microglial and astrocyte activation (Carson et al. 2008). Moreover, extensive and ongoing cell death occurs in these slices, potentially skewing results and observations (Kovacs et al. 2011). Depending on the type of experiment planned, slice cultures do not allow for the infiltration of blood derived immune cells following pathology.

Isolation and analysis of microglia (without culture) from the intact murine brain can also serve as a useful method to study microglia. These cells develop and mature in the intact murine brain and thus are more likely to resemble in vivo microglia than cultured cells. However, depending on the types of studies planned, a flow cytometer or a real time PCR (RT-PCR) machine may be necessary. Though the numbers of microglia isolated from the murine brain may be sufficient for RT-PCR analysis, it is often difficult to collect adequate numbers for high-throughput assays (Carson et al. 2008).

Though the various in vitro methods used to study microglia can be readily helpful in understanding their physiology, the results should be analyzed with extreme caution. More specifically, in order to truly validate results using these in vitro methods, researchers may want to run studies using several of these methods to create valid conclusions. Not only will this help to solidify the results, but it will also provide researchers with confidence in the usefulness of these methods.

In vivo studies

In vivo studies are useful because they allow us to observe microglia in their intact microenvironment. Though informative methods have been created to study these cells in the living brain, methods have not been devised that allow for a clear understanding of their physiology using non-invasive techniques. The main methods used to study these cells in vivo are (1) bone marrow chimeric studies, though the cells are analyzed in postmortem tissues, and (2) imaging techniques (Carson et al. 2008).

In bone marrow chimeric studies, mice generally receive sublethal irradiation to kill off their bone marrow, and then they receive bone marrow from a donor mouse to replenish their peripheral immune compartment (Carson et al. 2008). Interestingly, these studies show that though recipient mice receive donor bone marrow, the majority of microglia remain recipient derived (Ginjoux et al. 2010). Generally, these studies involve genetically altering the donor bone marrow in order to trace the cells in the recipient (Carson et al. 2008). Bone marrow chimeric studies can be incredibly useful in studies looking at the degree of influx of peripheral immune cells into the brain, and also the role

that these peripheral cells have in several CNS pathologies. However, studies have shown that the irradiation itself can damage the vasculature and blood brain barrier (BBB), indicating that the numbers of peripheral immune cells detected in the CNS may occur as a result of the irradiation and not the healthy or pathological physiology (Carson et al. 2008, Ginhoux et al. 2010). Moreover, these studies are typically analyzed in postmortem tissue, making it difficult to observe the interactions between the donor and recipient cells in the live brain.

Recently, powerful imaging techniques have been used to observe microglia in the intact brain. Two-photon microscopy is a powerful tool that has been used by researchers studying microglial physiology and motility in the healthy and inflamed brain (Nimmerjahn et al. 2005, Davalos et l. 2008). Typically, those using two-photon microscopy use explants of the tissue of interest and remove the organ entirely from the animal, or intravital methods where the animal is immobilized and the tissue is exposed (Germain et al. 2006). Most reported microglial studies have used preparations in which the skull is removed or thinned (Germain et al. 2006, Carson et al. 2008). Moreover, to visualize microglia, transgenic mice with fluorescent microglia are typically used (Nimmerjahn et al. 2005, Davalos et l. 2008). Two major studies performed by Nimmerjahn and colleagues and Davalos and colleagues used this powerful technique and found that microglia are not "resting" in the healthy brain. Instead, in the healthy brain microglia constantly extend their processes to survey their microenvironment (Nimmerjahn et al. 2005). Not only did Nimmerjahn and colleagues demonstrate that

microglia do not rest in the healthy brain, but their finding suggest that microglia collectively survey the entire brain every few hours.

Though two-photon microscopy has become a very powerful tool to visualize these cells in intact tissue, this technique also has limitations. A major caveat for intravital setups of two-photon microscopy is that motion artifacts can occur because of movements generated from breathing or involuntary muscle contractions (Germain et al. 2006).

1.1.4 What are microglial functions in the healthy CNS?

For some time, microglial functions have been studied in the context of pathology caused by trauma or a specific disease. This focus is potentially due to the fact that microglia change their morphology and the types of genes that are expressed when they become activated. Because of the visible changes microglia undergo during their activation, many have regarded microglia in the healthy brain to be in a "resting" state. Recently, several labs have used two photon imaging of the cerebral cortex of live mice and show that in the healthy brain, microglial processes are highly motile and constantly sample their environment (Nimmerjahn et al. 2005). Because microglial processes monitor their microenvironment extensively, Nimmerjahn et al. suggest that the entire brain parenchyma is surveyed by these "homeostatic" microglia once every few hours. In these studies, it was observed that microglia also phagocytose tissue samples which are then transported to the soma (Nimmerjahn et al. 2005).

It seems we are just beginning to understand the roles that microglia play in the healthy CNS. Specifically, studies have shown that a receptor expressed by microglia may directly or indirectly affect cognitive and other important functions in the healthy CNS. Nasu-Hakola disease is a rare recessive human genetic disorder that causes early onset cognitive dementia, bone cysts and death in the 40's (Verloes et al. 1997, Thrash et al. 2009). Studies have shown that this disease is caused by loss of function mutations in the Triggering Receptor Expressed on Myeloid cells-2 (TREM2) or in the TREM2 signaling pathway regulated by the adaptor molecule DNAX-activating protein of molecular mass 12 kDa (DAP12) (Paloneva et al. 2000, Thrash et al. 2009). DAP12 is a transmembrane adaptor molecule expressed by several immune cells that signals via an immunoreceptor tyrosine based activation motif (ITAM) (Paloneva et al. 2002, Thrash et al. 2008). Though DAP12 is required for TREM2 signaling, because Nasu-Hakola disease has obvious affects on cognition, a process most often thought to be regulated by neurons, many believed that the cognitive symptoms were caused by a defect in neurons. Interestingly, we and others have found that TREM2 is expressed by subsets of microglia in the healthy murine brain (Schmid et al. 2002, Thrash et al. 2009).

Overexpression and knockdown studies have shown that TREM2 expression promotes microglial phagocytosis of cellular debris and apoptotic cells, decreases microglial motility, inhibits microglial and macrophage cytokine expression and limits the severity of a mouse model of multiple sclerosis (Takahashi et al. 2007, Melchior et al. 2010, Bouchon et al. 2001, Piccio et al. 2007). Though there is much that is not

understood, these data demonstrate that lack of a microglial protein can lead to the development of a disease with cognitive manifestations.

1.1.5 What are microglial roles in the developing CNS?

While there is a current lack of understanding of the roles microglia have in the healthy mature brain, there is perhaps an equal lack in knowledge regarding microglial functions in the developing brain. It has been well documented by several labs that microglial morphology and markers of microglial activation change as the brain develops (Hristova et al. 2010). Hristova and colleagues performed informative immunohistochemical studies depicting that periventricular microglia express high levels of several integrin subunits and B7.2, a co-stimulatory molecule, at birth that decreased linearly by the second postnatal week. This clearly depicts that microglial activation state changes during the first postnatal week in rodents, corresponding to the last trimester in humans. A curious observation from this study is that though both cortical and subcortical microglia would presumably be exposed to cellular apoptosis during this postnatal period, which could influence microglial activation status, only subcortical microglia displayed an activated phenotype. Are the microglia in the subcortical regions more active or performing different functions than the cortical microglia in the developing brain?

The developing brain experiences a great deal of proliferation followed by programmed cell death in order to shape the final form and connections of the mature CNS (Blaschke et al. 1996, Cheng et al. 2009). A readily accepted hypothesis is that

microglia are involved in the removal of apoptotic neurons in the developing brain. Using time lapse recordings of cultured ex vivo cells and immunohistochemistry, Parnaik et al. demonstrate that professional phagocytes of the brain can indeed engulf apoptotic cells in the postnatal developing brain. Though Parnaik et al. clearly demonstrate that microglia from brain cultures can phagocytose experimentally induced apoptotic neurons, they fail to clearly explain how microglia are capable of clearing such substantial numbers of apoptotic neurons during this critical postnatal period of brain development. Another study using silver impregnated tissues and immunohistochemistry also demonstrated that apoptotic cells in the cerebral cortex were engulfed by phagocytes in the brain during the first postnatal week of life (Ferrer et al. 1990). It was not clear from this paper whether the transitory phagocytes were CNS resident microglia or blood derived macrophages.

1.1.6 What are the types of microglial/macrophage activation?

For years microglia were presumed to be in a "quiescent" state in the healthy brain and were thought to become activated following infection and/or inflammation. As previously mentioned, it is now understood that microglia are not quiescent in the healthy brain and instead are incredibly active and perform a variety of "surveillance" functions. Researchers have also discovered that microglial activation is not as simple as it was thought to be. Though microglial activation has mostly been discussed in the context of CNS pathology, microglia are highly plastic and can display a range of activation states that are not always associated with pathogen defense functions (Stein et al. 1992, Mosser

et al. 2008). Now that it has become common knowledge that microglial activation is not synonymous with cytotoxicity, researchers must take a closer look at the activation states of these cells in order to accurately define their roles in the developing, damaged, diseased or aging CNS.

The activation state that most researchers are familiar with is the classical activation of microglia and macrophages. Very detailed studies have shown that cytokines that are produced by activated T-helper 1 (Th1) cells of the immune system, namely TNFα and/or IFNγ, cause microglia to convert from "homeostatic" cells into classically activated cells that are able to defend the host from invading pathogens (Buchmeier et al. 1985, Nathan et al. 1983, Mosser et al. 2008, Martinez et al. 2009). These signals in turn cause the microglia to produce factors such as IL-1 and IL-6 and reactive oxygen species that primarily serve to destroy foreign pathogens by amplifying the immune response, recruiting immune cells to the site of inflammation, and activating immune and non-immune cells (Agarwal et al. 1995, Mosser et al. 2008). However, if excess pro-inflammatory molecules are produced and/or not cleared in a timely fashion, these molecules can also damage host tissues (Agarwal et al. 1995, Mosser et al. 2008). Because of the potential cytotoxic responses, the functions of classically activated macrophages must be carefully regulated.

As mentioned, classically activated phagocytes will increase their production of inducible nitric oxide synthase (iNOS) in order to produce reactive oxygen species to defend the host from pathogens. Normally, classically activated phagocytes will also

increase their expression of molecules associated with antigen presentation, including MHC Class II, and molecules that will allow them to effectively activate T cells, namely co-stimulatory molecules such as B7.2 and CD40.

Though the removal of invading pathogens and/or debris is an essential function performed by microglia and macrophages, at times they are required to repair tissue that has been damaged by a foreign organism or by an immune response. To perform these functions, microglia and macrophages must become alternatively activated. IL-4 and IL-13 can be produced by cells associated with innate and/or adaptive immunity, including basophils, mast cells, eosinophils, NKT cells, CD4+ T helper 2 (Th2) cells and CD8+ T cells (Seder et al. 1991, Moqbel et al. 1995, Yoshimoto et al. 1995, Demeur et al. 1995, Seder et al. 1992). These factors have been shown to promote arginase activity in microglia and macrophages which can then convert arginine into ornithine (Guaoyao et al.1998). Ornithine is then converted by ornithine amino transferase into proline, which is important in collagen production (Guayao et al. 1998, Kreider et al. 2007, Mosser et al. 2008). Ornithine can also be converted by ornithine decarboxylase into polyamines which are involved in cellular proliferation (Guayao et al. 1998, Kreider et al. 2007, Mosser et al. 2008). Both products allow alternatively activated cells to promote wound healing and tissue repair in part by building up the extracellular matrix.

When phagocytes become alternatively activated, it is generally accepted that these cells upregulate a number of specific molecules that serve to resolve a helminth infection and build up the extracellular matrix. First, as discussed before, arginase 1 is an

enzyme that is consistently induced by alternatively activated microglia and macrophages and it competes with iNOS for L-arginine in order to produce factors that promote tissue repair (Guayao et al. 1998, Kreider et al. 2007, Mosser et al. 2008). There is a vast amount of literature that also states that alternatively activated microglia and macrophages induce mannose receptor (CD206), Ym1 and found in inflammatory zone 1 (Fizz1) (Stein et al. 1992, Nair et al. 2003). Mannose receptor is a phagocytic receptor that mediates the binding and ingestion of micro-organisms containing surface mannose residues (Stein et. al. 1992). The functions of Ym1 and Fizz1 are not completely understood and their roles in alternative activation have not been discerned. However, these two molecules are often upregulated by alternatively activated phagocytes in vitro, by IL-4 stimulation and in vivo during Th2 mediated immune responses.

Ym1 is a member of the chitinase family that was originally described as a chemotactic factor for eosinophils that was produced by CD8+T cells (Owhashi et al. 1998, Owhashi et al. 2000). Fizz1 is a member of a family of secreted cysteine-rich molecules (Steppan et al. 2001, Nair et al. 2003). It is known that Fizz1 expression is induced in alternatively activated microglia and macrophages. Studies have suggested that Fizz1 promotes wound healing and fibrosis in Th2 mediated immune responses (Morat et al. 2006). Interestingly, a recent study using Fizz1 knockout mice and *Schistosoma mansoni* infection showed that Fizz1 acts as a negative regulator of helminth induced Th2 mediated immune responses (Pesce et al. 2009). In this paper they demonstrated that Fizz1 knockout mice infected with *S.* mansoni had increased inflammation in the lung and accelerated fibrosis in the liver (Pesce et al. 2009). Though

these studies were focused on peripheral organs, their findings would suggest that though Fizz1 is induced following IL-4 stimulation, it functions to regulate the degree of the immune response.

Though an important first step has been made by recognizing that microglia and macrophages have the potential to adopt a variety of activation states, we must also acknowledge that these cells very rarely will adopt a single phenotype. However, the ability to characterize the activation states of microglial or macrophage populations will allow researchers to begin to understand the roles of these cells in CNS pathologies.

1.1.7 Can microglia be distinguished from blood macrophages?

There are a variety of macrophage like cells that can be found within the CNS. These include parenchymal microglia, perivascular microglia and acutely infiltrating blood derived macrophages. Though these populations have been recognized for some time, no conclusive method that will allow one to distinguish microglia from CNS infiltrating macrophages in tissue sections has been identified. However, methods have been created that allow researchers to separate the two populations using single cell brain suspensions and flow cytometry (Ford et al. 1995). Moreover, recent studies may have identified markers that may one day be used to detect bone marrow derived macrophages in the brain (Chen et al. 2010).

In the brain, microglia and macrophages express CD11b, an integrin that promotes migration, adhesion and phagocytosis (Akiyama et al. 1990). CD45 is expressed by all nucleated hematopoietic cells and their precursors (Thomas et al. 1989).

Using single cell brain suspensions and flow cytometry, Sedgwick et al. demonstrated that microglia can be distinguished from CNS infiltrating leukocytes based on the levels of CD45 that are expressed by each population. Specifically, if one were to label myeloid cells with antibodies against CD11b and CD45, microglia would be the CD11b+ CD45lo population while CNS infiltrating blood derived macrophages would be the CD11b+ CD45hi population (Sedgwick et al. 1991).

For some time, it was thought that the main role of Homeobox (Hox) genes was to establish body plans by providing positional information along the anteroposterior axis of the embryo (Greer et al. 2002, Chen et al. 2010). Recent studies, however, demonstrate that Hox genes may also play roles in the development of specific organs, such as the kidney (Wellik 2011). Thorough investigations performed by Chen et al. demonstrate that Hoxb8 mutant mice show behavioral deficits including excessive and pathological grooming that is characteristic of human patients with the obsessive compulsive disorder trichotillomania (Chen et al. 2010). Instead of finding Hoxb8 expressed by cells in a neural circuit controlling grooming, Chen et al. established that Hoxb8 was expressed by bone marrow derived microglia in the brain. They came to this conclusion because 1) only a subpopulation of microglia in the brain, specifically 40%, were Hoxb8 + and 2) in newborn mice these Hoxb8 + cells were found in the choroid plexus, meninges and ventricular lining eventually populating cortical and forebrain regions by postnatal day 14 (Chen et al. 2010). This migration strongly suggests that the Hoxb8 + microglia are bone marrow derived. Though these studies were performed in the naïve brain, it would be interesting to determine whether blood derived macrophages that enter the CNS during

pathology were also Hoxb8+. This would help to elucidate whether Hoxb8 could be used as a marker to distinguish yolk sac derived microglia from bone marrow derived macrophages in the brain tissue sections.

1.1.8 Do microglia have different functions than macrophages?

Microglia and macrophages express many of the same markers including F4/80, Iba-1 and CD11b (Carson et al, 2006). During an immune response, both cell types can induce molecules such as MHC Class II, B7.2 and CD40 that allow them to communicate with and activate T cells in the presence of antigen (Carson et al. 2006). Because of these similarities, many have believed that these cell types are very similar, if not the same cell. However, studies such as those performed by Chen and colleagues (discussed in the previous section) in relation to Hoxb8 expression, and also studies by Ford et al. showing that microglia express lower levels of CD45 than peripheral macrophages, clearly demonstrate that in the CNS, microglia may exhibit different functions than bone marrow derived macrophages.

In seminal studies performed by Hickey and Kimura, bone marrow chimeric rats were used to demonstrate that antigen specific interactions between CNS resident microglia and myelin specific T cells were not required to initiate or sustain autoimmune responses. In these studies, experimental autoimmune encephalomyelitis (EAE), an experimental model of multiple sclerosis in rodents was used. Specifically, these studies and others have nicely demonstrated that perivascular macrophages and dendritic cells are capable of initiating and sustaining EAE (Hickey et al. 1988, McMahon et al 2005).

Moreover, bone marrow chimeras lacking antigen presenting cells (APC) in the periphery (macrophages, dendritic cells) were not able to induce EAE, indicating that CNS resident microglia were in some manner insufficient to induce this autoimmune response (McMahon et al. 2005).

Differences between CNS microglia and macrophages have also been established in a model of facial axotomy. Used as a model to study the mechanisms of survival of the facial motoneuron cell body and regeneration of the axon, the facial axotomy model involves transecting the axon of the facial motoneuron at the site where it exits the CNS to the periphery (Sanders et al. 2006). This transaction permanently prevents reconnection of the axon to the cell body while the neuron cell body survives (Sanders et al. 2006). Previous studies using the facial axotomy model showed that activated CD4+ T cells promote facial motoneuron survival (Serpe et al. 2003). Studies by Byram and colleagues support this study by demonstrating that bone marrow derived peripheral APC's (macrophages, dendritic cells) are required to initiate CD4+ T cell activation, but cannot sufficiently sustain a protective T cell response (Byram et al. 2004). Furthermore, CNS resident microglia are required to reactivate CD4+ T cells in order to sustain survival of the facial motoneuron (Byram et al. 2004).

Together this data demonstrates that microglia and macrophages have certain similarities with respect to their functions and the repertoire of molecules they express in the naïve and inflamed brain. However, their roles in different CNS pathologies differ greatly.

1.1.9 How do microglia detect changes in their microenvironment?

In the CNS, microglia are resident innate immune cells and are generally the first responders to inflammation or injury. As such, mechanisms must be in place that allow them to detect when the CNS has been damaged or invaded by pathogens. Research has shown that several cell types throughout the body and within the CNS are capable of detecting a number of evolutionarily conserved motifs on pathogens as well as molecules produced by CNS intrinsic cells following tissue damage. These molecules are collectively referred to as pathogen associated molecular patterns (PAMPs) and danger associated molecular patterns (DAMPs) respectively. Moreover, by virtue of several receptor-ligand interactions, microglia are incredibly sensitive to minute changes in the wellbeing of specific cell types.

Toll-Like Receptors (TLRs) are a class of pattern recognition receptors expressed by immune and non-immune cells that can detect PAMPs (Bsibsi et al. 2002, Crack et al. 2007). Though the Toll pathway was initially defined as a family of genes involved in the dorsal-ventral patterning of the Drosophila embryo, in mammals, TLRs have been shown to be involved in pathogen detection and in the initiation of immune responses (Kimbrell et al. 2001, Crack et al. 2007, Valanne et al. 2011). Both extracellular and intracellular TLRs have been found and they are characterized by a cytosolic motif termed the Toll/Il-1 Receptor domain (TIR) (Bowie et al. 2000, Crack et al. 2007). Though studies have shown that astrocytes (TLRs 2, 4, 5 and 9) and neurons (TLRs 2, 6 and 8) express some TLRs, it has also been shown that microglia express the mRNA for

all currently known murine TLR's (TLR 1-9) (Bowman et al. 2003, Kurt-Jones et al. 2004, Mishra et al. 2006, Kielian et al. 2002, Rasley et al. 2002, Dalke et al. 2002, Olson et al. 2004). This allows microglia to defend the brain from a wide range of pathogens. Specifically, studies have shown that microglia can detect bacterial lipopeptides, double stranded RNA, lipoplysaccharides from gram negative bacteria, bacterial flagellin, single stranded RNA and unmethylated CpG dinucleotides (Kielian et al. 2002, Clark et al. 2007). There is some debate as to whether astrocytes express TLR2 in vivo, as labs have had difficulty reproducing the data using in situ hybridization (Clark et al. 2007). These differences may be due to species differences used in experiments or even the route of PAMP administration (Clark et al.2007).

Though microglial activation status can change following the detection of specific stimuli, recent research also reveals that the lack of certain stimuli can also lead to microglial activation. Specifically, microglia express several receptors for ligands that are expressed by other cells in the CNS microenvironment. When these cells become damaged, they are no longer capable of expressing the microglial-specific ligand. This, in turn, causes the microglia to become activated. Through these interactions it is said that other cells of the CNS, namely neurons, are able to regulate microglial activation.

An important ligand-receptor pair that allows neurons to regulate microglial activation in the CNS is that between CD200 and CD200 receptor (CD200R). CD200 is a membrane glycoprotein of the immunoglobulin superfamily that is expressed by endothelial cells and neurons in the brain (Wright et al. 2000, Masocha 2009). CD200R is

expressed by myeloid cells in the brain including microglia and macrophages (Masoka 2009). Because of this unique expression pattern, many have pondered how the CD200-CD200R interaction could regulate both cell types. Studies have shown that in the healthy CNS when CD200 and CD200R are both present and able to interact, microglia display a ramified morphology, characteristic of their "homeostatic" activation status. Key studies performed by Hoek and colleagues clearly demonstrate that microglia in the spinal cord of unmanipulated CD200 knockout (KO) mice exhibit several features characteristic of activated microglia including shorter glial processes and elevated CD11b and CD45 expression. This clearly indicates that the CD200-CD200R interaction between neurons and microglia is required to prevent microglial activation in the non-inflamed brain. Moreover, to determine whether CD200-CD200R interactions also regulate the degree of microglial activation in specific disease processes, Hoek and colleagues examined microglial activation and the timeline of disease onset in CD200 KO mice with EAE. Interestingly, Hoek et al. found that in CD200 KO mice with myelin oligodendrocyte glycoprotein (MOG) induced EAE, the onset of EAE occurred 3 days before the onset in wild type C57BL/6 mice.

Because EAE requires activation of autoreactive T cells, some have speculated as to whether these studies demonstrate a faulty T cell compartment, mainly the hyperactivation of T cells. Hoek et al. have performed various in vitro and in vivo experiments and have not observed T cell dysregulation in CD200R knockout mice, strengthening the hypothesis that the problem lies within the myeloid compartment. Together, these results clearly demonstrate that CD200-CD200R interactions are required

in the brain to maintain homeostatic functions of microglia and prevent unwanted microglial activation.

CX3CL1-CX3CR1 interactions have also been shown to regulate microglial activation in the brain and spinal cord. The chemokine CX3CL1 (fractalkine) is expressed in various organs in the body including the brain, heart, kidney, lung and small intestine (Mizuno et al. 2003). In the healthy brain, the chemokine CX3CL1 is expressed by neurons while the receptor is expressed by myeloid cells including microglia and macrophages (Zujovic et al. 2000, Biber et al. 2007). However, in vitro studies have also concluded that TNFα and IFNγ stimulation can cause human astrocytes to express CX3CL1 (Yoshida et al. 2001). Though CX3CL1 exists as a membrane form, it can be cleaved to produce a soluble form as well. Parallel to these findings, studies have shown that the soluble form of fractalkine can be used as a chemoattractant for several leukocytes while the membrane bound form can facilitate adhesion of these cells (Yoshida et al. 2001, Bazan et al. 1997). Several essential functions of this ligand-receptor pair have been established. However, the role that this chemokine and receptor pair may play in neuronal-microglial communication has not been explored until recently.

Various in vitro studies have demonstrated that the CX3CL1-CX3CR1 interaction is neuroprotective. Using microglial cultures prepared from newborn rats, Zujovic et al. demonstrated that CX3CL1 lowered LPS induced TNFα release in a dose dependent manner. Interestingly, Zujovic and colleagues also demonstrated that when a neutralizing anti-CX3CL1 antibody was added to neuronal cultures prior to the addition of microglia,

neuronal death was detected (following the addition of microglia). This would illustrate that CX3CL1 expression by neurons is required to prevent neurotoxic microglial activation in the non-inflamed brain. However, these results have not been replicated in vivo. What both of these results reveal is that the CX3CL1-CX3CR1 interaction does allow neurons and microglia to communicate and that this "communication" leads to a neuroprotective microglial response. Though these in vitro studies were incredibly useful and informative, many qualities of in vitro studies, particularly with microglial cells, do not model what actually occurs in vivo.

To determine the roles of the CX3CL1-CX3CR1 interaction in the intact brain, Cardona et al. induced systemic inflammation in $CX3CR1^{+/GFP}$ mice (mice with functional CX3CR1 and green microglia) and $CX3CR1^{GFP/GFP}$ mice (mice that lack functional CX3CR1 but have green microglia) and examined microglial morphology and the degree of neuronal death in the hippocampus of the brain. Interestingly, Cardona and colleagues found that in LPS injected $CX3CR1^{+/GFP}$ mice, microglia maintained a ramified morphology, indicative of a "homeostatic" state. On the contrary, microglia from LPS injected $CX3CR1^{GFP/GFP}$ mice had shorter and thicker processes and a larger cell body, indicative of activated microglia (Cardona et al. 2006). These studies also nicely demonstrated that systemic inflammation in $CX3CR1^{GFP/GFP}$ mice caused a significant number of hippocampal neurons to become apoptotic, whereas no apoptotic neurons were detected in the $CX3CR1^{+/GFP}$ mice (Cardona et al 2006). These studies clearly show that the threshold for microglial activation is perhaps lowered in mice

lacking functional CX3CR1, and that the consequences of microglial hyperactivation to systemic inflammation in the absence of CX3CR1 is neuronal damage and death.

These studies clearly demonstrate that CX3CL1 is a means by which neurons can communicate with microglia. Moreover, these findings illustrate that the absence of neuronal CX3CL1 permits neurotoxic microglial activation in the diseased and inflamed brain.

A constitutively expressed molecule who's expression increases following microglial activation has also been shown to serve as a molecule through which B cells and neurons can regulate microglia (Tan et al. 2000, Mott et al. 2004). As discussed in a previous section, microglia constitutively express CD45, a protein tyrosine phosphatase (Tan et al. 2000). Though CD22, the ligand for CD45 was known to be expressed by B cells, Tan and colleagues discovered that neurons expressed and secreted CD22 as well. To investigate whether neurons regulate microglial activation state through CD45, primary murine microglial cultures were treated with CD40 ligand (CD40L) and a crosslinking antibody for CD45 (Tan et al. 2000). These studies demonstrated that activation of CD45 led to a significant decrease in the amount of TNFα produced by CD40 ligation. Tan and colleagues further illustrated the fact that CD45 expression by microglia is required to regulate the levels of TNFα produced following CD40 ligation by exposing primary microglial cultures from CD45 deficient mice to CD40L. As one may predict, the microglia from this experiment produced significantly higher levels of TNFα than CD45 expressing primary cultures.

These studies nicely illustrate that a molecule commonly used to identify microglial activation and distinguish them from CNS infiltrating macrophages in brain cell suspensions is also used by neurons to limit the degree of microglial activation. Moreover, it appears that microglial activation state in the healthy and inflamed brain is tightly regulated by a variety of ligands that are expressed by neurons. The redundancy in receptor-ligand interactions between these two cell types that specifically control microglial activation status strongly suggests that dysregulated microglial activation is not tolerated in the CNS. From another viewpoint, these interactions also allow microglia to monitor neuronal health.

1.2.0 What is systemic inflammation and how does it affect the CNS?

Viral and bacterial infections that occur outside of the CNS can cause immune responses within the brain and spinal cord. When viruses, bacteria or parasites invade the body, PAMPs, or in the case of tissue injury, DAMPS, are detected by cells of the innate immune system. Innate immune cells in the periphery including macrophages and neutrophils then produce inflammatory cytokines such as IL-1β, IL-6 and TNFα that can circulate in the blood (Perry 2004). It is well established from a variety of studies that systemic inflammation can lead to activation of microglia and astrocytes and changes in neurons. However, the methods by which these cells become activated are still being studied. In order for peripheral immune signals to affect cells within the brain, they must either (1) enter circumventricular (CVO) organs and diffuse directly from the blood into the brain parenchyma (though this is debatable) (2) activate endothelial cells forming the

blood vessels in the brain that can in turn activate perivascular microglia (3) be directly transported across the blood brain barrier (BBB) or (4) activate the vagus nerve which can then communicate with neuronal populations within the brainstem (Perry 2004, Dantzer 2006, Quan 2008).

Specific regions within the brain called CVOs are unique within the brain in that they lack a complete blood brain barrier (BBB). The blood vessels of most regions in the brain provide an extra level of protection for the CNS from a variety of molecules within the blood because the endothelial cells form tight junctions between one another. CVOs have fenestrated capillaries, and thus many believe that cytokines can freely diffuse from the blood into the brain parenchyma and interact with immune cells such as the perivascular microglia in these regions (Perry 2004). However, other studies have established that following systemic inflammation induced by LPS, perivascular phagocytic cells upregulate IL-1β in CVOs and this expression gradually shifts from the CVOs to adjacent brain nuclei (Konsman et al. 1999). Furthermore, Konsman and colleagues nicely demonstrate that the IL-1β is bioactive since iNOS mRNA can be detected at the interface between the CVOs and the adjacent neural nuclei. This study nicely illustrates that systemic inflammation can lead to activation of cells and the production of inflammatory cytokines in CVOs that can then propagate the signal into the brain parenchyma.

Several studies have also demonstrated that brain cells become activated following systemic inflammation due to endothelial cell production of inflammatory

molecules. Specifically, it is hypothesized that peripheral cytokines produced in response to systemic inflammation lead to the production of the rate limiting enzyme for prostanoid biosynthesis, cyclooxygenase-2 (COX-2), and membrane associated prostaglandin synthase-1 (mPGES-1) by endothelial cells in the brain (Engblom et al. 2003, Ching et al. 2007, Quan 2008). Depending on the dose and agent used to induce systemic inflammation, others have reported that perivascular microglia also induce COX-2 expression (Schiltz et al. 2002). These in turn lead to the production of prostaglandin E2 in the brain which has been shown to activate neurons (Quan 2008, Andreasson 2010). Several studies have demonstrated that systemic inflammation leads to production of circulating IL-1 and that interaction of IL-1 with its receptor on brain endothelial cells and some neurons can lead to the production of COX-2 (Schiltz et al. 2002, Ching et al. 2007). Ching and colleagues demonstrated that knockdown of endothelial cell IL-1R1 prevented COX-2 induction, hypothalamic neuronal activation and fever induced by intravenous (IV) and intracerebroventricular (ICV) injected IL-1. Curiously, endothelial specific knockdown of IL-1R1 suppressed intraperitoneal (IP) IL-1 induced fever but not neuronal activation in the hypothalamus (Ching et al. 2007). These results suggest that the IL-1 injected into the peritoneal cavity led to the activation of CNS neurons using another mechanism besides endothelial cell activation.

Several studies have illustrated that some cytokines are actively transported across the BBB. Although several cytokines are produced in the brain, many have also observed that cytokines that circulate in the blood can have effects on the CNS. For example, when cancer patients receive IV administration of TNFα for its anti-tumor properties, they

often experience several side effects that involve the CNS including headache, memory loss and acute blindness which are dose dependent and resolve following therapy (Steinmetz et al., 1988; Triozzi et al., 1990). These studies strongly suggest that the cytokines somehow cross the BBB to act directly on the brain. Following these studies, it was shown that TNFα and other cytokines, such as IL-1α were able to exert direct effects on the CNS because they are transported across the BBB. Studies by Gutierrez et al. demonstrate that following IV injection, radiolabeled murine TNFα (mTNFα) was found in its intact form in brain tissue. By administering an IV injection of unlabelled mTNFα along with the radiolabeled form, Gutierrez and colleagues demonstrated that TNFα was transported into the CNS via a saturable mechanism. Further studies from this same group found that the TNFα receptors on endothelial cells were required to transport the cytokine across the BBB (Pan et al. 2002). Specifically, in the absence of p55 and p75, the two TNFα receptors, influx of TNFα into the brain was completely abolished following IV administration (Pan et al. 2002). This suggests that specific cytokines bind directly to a receptor and are then transported, via this receptor, into the brain. TNFα is not the only cytokine that has been shown to cross the BBB. In similar studies, it has also been shown that cytokines such as IL-1β cross the BBB and that the type II IL-1 receptor plays a role in its transport (Banks et al. 1994, Skinner et al. 2009).

So far, mainly BBB dependent mechanisms of transferring peripheral immune signals to the brain have been discussed. However, studies have also nicely depicted that BBB independent mechanisms exist that transmit peripheral immune signals to the brain. Specifically, many have suggested that peripheral immune signals can be transmitted to

the brain via sensory nerves. In 1994, Bluthe and colleagues demonstrated that vagotomy could block LPS induced changes in social investigative behavior. Moreover, several studies have also demonstrated that the intact vagal nerve is essential for a number of physiological changes that occur following systemic immune challenge including hyperalgesia, fever, and increased levels of plasma corticosteroids (Quan 2008, Maier et al. 2006). Though many of these studies present convincing data, it seems that the route through which the inflammatory inducing agent is administered, the inflammatory inducing agent itself and the dose given can lead to debatable and contrasting results.

It is clear that peripheral immune molecules can either be transmitted themselves or transmit the ensuing inflammatory response to the CNS. The routes through which this occurs have been widely studied and discussed here. What is not yet well understood is (1) what the physiological criteria must be in order to determine which route is used in what instance or (2) whether all routes are used following all peripheral immune challenges (though given the data this seems unlikely).

1.2.1 How is systemic inflammation correlated with the development and/or pathogenesis of neurodevelopmental disorders?

Systemic inflammation, such as colds and flu viruses are commonly experienced by adults and children alike. In the adult, in addition to well known sickness behaviors such as fever, loss of appetite, and reduced activity, peripheral inflammation causes activation of CNS resident microglia and the acute influx of blood derived macrophages into the brain and spinal cord (Sedgwick et al. 1991, Perry et al. 1992, Perry 2004).

Though newborns, infants and toddlers also experience bouts of systemic inflammation, their brains and spinal cords are relatively immature. This adds an extra level of complexity in the comprehension of how peripheral inflammation experienced during periods of brain development can affect the ensuing development of the CNS. Several epidemiological studies have strongly suggested that systemic inflammation experienced during the perinatal period may play a role in the development and/or pathogenesis of several neurodevelopmental disorders including cerebral palsy, autism and schizophrenia. These studies loosely speculate that systemic inflammation experienced during the perinatal period causes glial activation and the production of inflammatory molecules that can affect developing oligodendrocytes and white matter and lead to the loss of neurons (Chew et al. 2006, Vargas et al. 2005). Though a great deal of research has been devoted to understanding neurodevelopmental disorders, the roles that microglia may have in these disorders is still relatively unknown.

Cerebral palsy (CP) is a neurodevelopmental disorder beginning in early childhood and persisting throughout the life of the individual (Bax et al. 2005). This group of disorders affects movement and posture and is believed to be the result of a disturbance in the developing fetal or infant brain (Bax et al. 2005, Makki et al. 2007). Clinical and epidemiological studies strongly suggest that the "disturbance" that leads to the development of periventricular leukomalacia (PVL), which is believed to precede many cases of CP, and CP itself, is systemic inflammation experienced during the perinatal period (Bax et al. 2005, Wang et al. 2006, Makki et al. 2007).

Systemic inflammation occurring in the pregnant mother, fetus or neonate is correlated with the increased incidence of PVL and CP (Damann et al. 2008). Haynes and colleagues clearly observed microglial and astrocyte activation and apoptotic oligodendrocytes in the periventricular white matter of brain tissue from deceased infants with PVL. Moreover, they also nicely illustrated that the microglia, astrocytes and possibly infiltrating macrophages expressed oxidative stress markers and markers of protein nitration (Haynes et al. 2003). Experimental studies have shown that when mice or rats receive ICV, IV, intrauterine, or maternal exposure to LPS, gliosis and elevated levels of TNFα, IL-1β and IL-6 in the blood or in brain tissue are commonly observed (Wang et al. 2006). These findings clearly demonstrate that microglia and other glial cells have intricate roles in the pathogenesis and perhaps the development of PVL and CP. However, their exact functions in this disorder and whether they exhibit phenotypes that promote and/or prevent the progression of the disorder is not well understood.

Studies have also demonstrated that systemic inflammation experienced during the perinatal period may contribute, in part, to the development of autism. Autism is a complex neurodevelopmental disorder characterized by impairments in social interaction, deficits in verbal and non-verbal communication and repetitive and stereotyped patterns of behavior (Vargas et al. 2005, Ashwood et al. 2009). Though the phenotypic heterogeneity of autism leads many to believe that a variety of factors contribute to the development of this disorder, including environmental and genetic, there is a growing consensus that the immune system is involved in its development and/or pathogenesis as well (Ashwood et al. 2009). Many have demonstrated that autistic patients have aberrant

immunological responses in the peripheral compartment and abnormal cytoarchitectural organization of the cerebral cortex and cerebellum (Gupta et al. 1998, Kemper et al. 1998, Wegiel et al. 2010). However, Vargas and colleagues published some of the first studies clearly illustrating glial activation and abnormal cytokine profiles in brain tissue from deceased autistic patients.

In postmortem autistic brains, Vargas and colleagues found microglial and astrocyte activation in cortical regions, white matter and in the cerebellum in areas with widespread purkinje and granule cell loss. They also found elevated expression of monocyte chemoattractant protein-1 (MCP-1) and IL-6 in postmortem brain tissue and cerebrospinal fluid (CSF) from living autistic patients and demonstrated that activated astrocytes were the main source of both of these inflammatory molecules in the brain (Vargas et al. 2005). Moreover, they also observed elevated TGF-β expression in postmortem brain tissue but not in CSF (Vargas et al. 2005). Interestingly, Vargas and colleagues found microglial nodules, or collections of activated microglia, in the postmortem autistic brains. This illustrates that there is persistent or chronic activation of microglia, as is seen in chronic neurodegenerative disorders such as Alzheimer's disease and Parkinson's disease, and is also observed in the brains of patients with neurodevelopmental disorders such as autism.

Though these studies elegantly depicted that glial cells are involved in the pathogenesis of autism, this study has also left many questions unanswered. During development, does a specific environmental or genetic insult occur that causes microglia

and astrocytes to become activated which then leads to abnormalities in the formation of the brain? Do abnormalities in brain development occur that then lead to microglial and astrocyte activation? Many researchers are undoubtedly attempting to answer these questions. However, with these results, we now know that glial cells are somehow involved in autism progression and can only speculate on their specific roles.

Schizophrenia is a chronic, severe and disabling psychotic disorder believed to result, in part, from inflammation experienced during the prenatal or perinatal period (Hickie et al. 2009, Meyer et al. 2010). The etiology of schizophrenia is currently unknown. However, many speculate that like autism, genetic, environmental and immunological factors contribute to its development (Hickie et al. 2009). In the schizophrenic brain, researchers have found elevated levels of the neurotransmitter dopamine, and abnormalities in the prefrontal cortex, striatum and limbic system (Breier et al. 1992, Seeman et al. 2000). In the periphery, studies show that schizophrenic patients have abnormal cytokine levels in the blood (O'Donnell et al. 1996, Potvin et al. 2008). These studies suggest that schizophrenic patients have abnormal levels of inflammatory molecules present in the blood and defects in specific regions of the brain. What is currently not well understood is whether immune cells of the brain are affected by the circulating inflammatory molecules and also how brain immune cells are involved in the pathogenesis of this disease.

Epidemiological studies strongly suggest that infections experienced during childhood can lead to the development of schizophrenia in the adult (Rantakallio et al.

1997). Using various imaging techniques, researchers have also demonstrated that activated glial cells, including microglia and astrocytes, could be detected in the brains of patients with schizophrenic psychosis, specifically in the hippocampus (Banati et al. 2009, Doorduin et al. 2009). Though these results strongly suggest that microglia may have a role in the pathogenesis of this disease, fundamental experiments have not yet been performed to illustrate when these cells become activated in the disorder, what inflammatory molecules they produce and whether they promote pathology or become activated to protect the brain from further pathology.

CHAPTER 2

MATERIALS AND METHODS

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

2.2.1 Antibodies used: Antibodies used for flow cytometry and immunohistochemistry are as follows: from BiogenIdec-murine anti-TLT2 (0.1ug/ml), from Abcam-rabbit anti-CX3CR1 (1mg/ml), from BD Pharmingen-rat anti mouse CD11b-FITC (0.5mg/ml), rat anti-mouse CD45-APC (0.2mg/ml), rat anti-mouse CD40-Biotin (0.5mg/ml), rat anti-mouse B7.2-Biotin (0.5mg/ml), rat anti-mouse MHC Class II-Biotin (0.5mg/ml), from R&D systems-rat anti-mouse TREM1-PE (0.2mg/ml), rat anti-mouse TREM2-PE (0.2mg/ml), rat anti-mouse TREM3-Biotin (0.5mg/ml), from BioLegend-rat anti-mouse Mannose Receptor (CD206)-Biotin (0.5mg.ml), from eBioscience-rat anti-mouse B7.H1 (0.5mg/ml), rat anti-mouse CD200R-PE (0.2mg/ml), from Vector Labs-murine anti-tomato lectin-Biotinylated (1mg/ml), from Chemicon-murine anti-NeuN-Biotinylated (1mg/ml), from Wako- rabbit Iba1 (0.5mg/ml).

2.2.2 Mouse Model of Systemic Inflammation: Wild type (WT) C57BL/6 or B10.D2 mice or TREM2 knockout (KO) mice at postnatal day 3 (P3), postnatal day 7 (P7), postnatal day 14 (P14), postnatal day 21 (p21) or 3 months were administered an intraperitoneal injection of lipopolysaccharide (LPS-Sigma) (5mg/kg) in a total volume of 100ul using a 25 gauge needle.

2.2.3 Quantitative RT-PCR (qRT-PCR): Expression of CCL2, CCL5, iNOS, Arginase 1 and TNFα was assessed by qPCR using cDNA from whole brains isolated from unmanipulated wild type (WT) and TREM2 knockout (KO) mice and in mice that received an intraperitoneal (IP) injection of lipopolysaccharide (LPS). For separate experiments, the expression of TREM1, TREM2, TREM3, TREM4, TREM4 splice variant and TREM5 was assessed using cDNA isolated from CD45lo and CD45hi populations in brains isolated from ICV injected WT mice. Total RNAs were purified from mice whole brains using Trizol reagent (Invitrogen, Carlsbad, CA). For reverse transcriptase (RT)-polymerase chain reaction (PCR), first strand cDNA was synthesized (Amersham Biosciences, NJ) and amplified by PCR by using specific primers as described in the table below.

Real-time PCR was performed using the CFX96 Real Time PCR Detection System (Bio-Rad) and primer sequences described below. The relative number of CCL2, CCL5, iNOS, Arginase 1, TNFα or TREM transcripts per hypoxanthine phosphoribosyl transferase (HPRT) transcripts was determined by using calibration standards for both HPRT and CCL2, CCL5, iNOS, Arginase 1, TNFα and TREMs. Briefly, PCR products served as standards for calibration of quantitative PCR. These standards were diluted to obtain a standard curve of 50pg, 5pg, 0.5pg, 0.05pg, 0.005pg and 0.0005pg for qPCR analysis. To minimize experimental variations from one sample to another, the copy number of CCL2, CCL5, iNOS, Arginase 1, TNFα and TREMs per sample was normalized to the expression of the housekeeping gene, HPRT. It was verified that the

copy number of HPRT transcripts was of the same order of magnitude in all samples being compared.

2.2.4 Primers used for qRT-PCR

Gene	Forward Primer 5'-3'	Reverse Primer 5'-3'	References
iNOS	GGCAGCCTGTGAGACCTTTG	GCATTGGAAGTGAAGCGTTTC	He et al. 2007
Arginase 1	CAGAAGAATGGAAGAGTCAG	CAGATATGCAGGGAGTCACC	Herbert et al. 2010
CCL2	TTGACCCGTAAATCTGAAGCTAAT	TCACAGTCCGAGTCACACTAGTTCAC	Ehrchen et al. 2010
CCL5	ATGAAGATCTCTGCAGCTGCCCTC	CTAGCTCATCTCCAAATAGTTGATG	Gamo et al. 2003
TNF alpha	CTGTGAAGGGAATGGGTGTT	GGTCACTGTCCCAGCATCTT	NM_013693
TREM1	TGCTGTATTGCCTTCGCTGT	TACAACAACAACAACAACAACAACA	Olson et al. 2003
TREM2	ACAGCACCTCCAGGATCAAG	CCACAGCCCAGAGGATGC	ENSMUSG00000022992
TREM3	TCTACCTCTCTCCTGACAATGTTATC	GGATGTCTTGGAAGGCTGTAAC	NM_021407.2
TREM4D	GCTTCAACTTCTTCACTGTCACC	GTGTTTTGTGGTAATAAGGGTAGGA	ENSMUST00000063481
TREM4SV	GCTTCAACTTCTTCACTGTCACC	TACAAAGCCCCTTTTGACACC	ENSMUST00000063481
TREM5	GCATGTTGTTGCCTGGAAGAC	GCTCTCCATCTCCCTGCCTA	NM- 177824

2.2.5 In situ hybridization analysis: In situ hybridization was performed on free-floating (25um) cryosections as previously described (Thrash et al. 2008, Melchior et al. 2010). Briefly, coronal sections were hybridized at 55^{o}C for 16 hours 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 um β-mercaptoethanol and 0.1% N-lauryl sarcosine. NeuN positive cells were visualized using biotinylated antibodies directed against NeuN and standard strep-avidin, horseradish peroxidase methodology. Sections were mounted onto Fisherbrand superfrost/plus slides and dehydrated with ethanol and chloroform. Slides were exposed for 3 days to Kodak-X-AR film and dipped in Ilford K-5 emulsion. After 3 weeks, slides were developed with Kodak D19 developer, fixed and counterstained with Mayer's hematoxylin.

2.2.6 Microglia and macrophage isolation from murine brain: Microglia and macrophages were isolated as previously described from the CNS of mice following halothane-mediated euthanasia (Carson et al. 1998). In brief, following euthanasia, the brains of mice were rapidly removed and mechanically dissociated. The cell suspension was separated on a discontinuous 1.03/1.088 Percoll density gradient and microglia/macrophages were collected from the interface as well as from the 1.03 Percoll fraction. Microglia and CNS infiltrating immune cells were identified by flow cytometry

using APC-conjugated antibodies against pan-CD45 and Fluorescein Isothiocyanate (FITC)-conjugated antibodies against CD11b (labeling microglia and macrophages). A BD FACS Calibur was used for flow cytometric quantification of immunoreactivity. Flow cytometric data were subsequently analyzed with FlowJo software (Tree Star Inc, Palo Alto, CA).

CHAPTER 3

Differential expression of TREMs by microglia and macrophages: Implications for cell type specific functions in the CNS

3.1 ABSTRACT

The Triggering Receptors Expressed on Myeloid cells (TREMs) are a family of orphan receptors that have been implicated in regulating innate and adaptive immune responses. Several studies have demonstrated that many of the TREMs are expressed by multiple myeloid cell types throughout the body. However, recent studies have identified that TLT2, a member of the TREM family, is expressed by T cells and B cells. We have previously shown that in the healthy brain, TREM2 is constitutively expressed by subsets of microglia. Here we tested whether other TREMs were expressed in the brain and determined the conditions regulating their expression. Using dual in situ hybridization and immunohistochemistry, we could not detect TREM1 or TREM3 expression in the brain. However, in contrast to what was found for TREM2, TREM4 is expressed by subsets of neurons in the healthy brain. Moreover, TREM4 expression does not change with LPS injection. The TREM receptors lack an intracellular signaling motif. As such, many signal intracellularly via DAP12, an adaptor molecule. In the brain, we observed that only subsets of microglia express this molecule. Interestingly, we found that in the brain, TLT2 is expressed by microglia, and though its expression is not developmentally regulated, its expression increases following systemic inflammation. Using RT-PCR we confirmed observations that TREM2 is more highly expressed on CD45lo microglia than

on CD45hi infiltrating macrophages. However, TREM3 expression is significantly higher on CNS infiltrating macrophages than on CNS resident microglia. As many of the TREMs are expressed by immune cells, studies show that their expression tends to change with inflammation. Using flow cytometry, we observed that TREM1 expression is found on CNS infiltrating macrophages following systemic inflammation. Moreover, a small population of macrophages express TREM3. These results demonstrate that TREMs are differentially expressed by cells within the brain and along with their roles in innate and adaptive immune responses, TREMs may also play roles in healthy brain and cognitive development as well as in CNS diseases.

3.2 INTRODUCTION

Cells of the innate immune system perform vital functions ranging from the recognition of pathogens to the generation of immune responses. Recently, several studies have shown that a family of immunomodulatory receptors called the Triggering Receptors Expressed on Myeloid cells (TREMs) play critical roles in both innate and adaptive immune responses. As such, studies have shown that several innate immune cells arising from the myeloid lineage, including monocytes, macrophages, dendritic cells, neutrophils, microglia, osteoclasts and platelets express at least one of the TREM family members. The TREMs belong to a family of structurally related proteins that include the TREM Like Transcripts (TLTs) (Ford et al. 2009). Interestingly, a single study has reported that TLT2 is expressed by B and T lymphocytes, cells arising from lymphoid lineage. These findings indicate that the TREMs may play important roles in both innate and adaptive immunity.

The TREM gene cluster is located on human chromosome 6p21 and on mouse chromosome 17C (Klesney-Tait et al. 2006, Ford et al. 2009). In humans, the TREM cluster encodes TREM1, TREM2, TLT1 and TLT2. In mice, the cluster encodes TREM1, TREM2, TREM3, TREM4, TREM5, TLT1, TLT2 and TLT4 (Klesney-Tait et al. 2006, Watari et al. 2008, Ford et al. 2009). In humans, TREM3 is a pseudogene and thus its protein is not expressed (Allcock et al. 2003). However, studies have also reported that mouse TREM5 is also a pseudogene (Chung et al. 2002). TREM4 is also known in the literature as PDC-TREM because it was found to be expressed by plasmacytoid dendritic

cells (PDC) by Watari and colleagues. Moreover, Watari et al. also found that PDC-TREM expression augments the production of type I interferons by PDC's. TREM1 and TREM2 in both species and TREM3 and TLT4 in mice all have an ectodomain, also believed to be the ligand binding domain, a transmembrane region and a short cytoplasmic tail that lacks signaling motifs. The transmembrane domains have a positively charged lysine residue that allows the TREMs to form a complex with and signal intracellularly through the DNAX Activating Protein of 12kDa (DAP12), the only known molecule that mediates TREM intracellular signaling (Bouchon et al. 2000). DAP12 is a transmembrane adaptor molecule that signals via immuno-receptor tyrosine based activation motifs (ITAM) (Wilson et al. 2000). Studies have revealed that this transmembrane adaptor molecule also signals for a variety of receptors in natural killer (NK) cells, granulocytes, monocytes/macrophages and dendritic cells (DCs) (Tomasello et al. 1998, Lanier et al. 2000, Wilson et al. 2000). Interestingly, only TREM1 and 2 in mouse and humans and TREM3, TLT1 and TLT4 in mouse signal through DAP12 (Klesney-Tait et al. 2006). Reports have illustrated that TLT1 contains an immuno-receptor tyrosine based inhibitory motif (ITIM) which can recruit phosphatases that can then decrease the activation of molecules involved in cell signaling (Vivier et al. 1997, Klesney-Tait et al. 2006).

The TREMs seem to regulate a range of immune responses by performing a variety of functions. For example, TREM1 has been found on neutrophils and CD14hi monocytes (Bouchon et al. 2000). CD14 is a co-receptor that works with TLR4 and MD2 in the detection of LPS (Leon et al 2008). Bouchon et al. demonstrated that in vitro

stimulation of CD14hi monocytes and neutrophils with LPS, heat inactivated gram positive bacteria or gram negative bacteria strongly induced TREM1 expression (Bouchon et al. 2000). These findings suggest that TREM1 plays a role in acute inflammatory responses. Bouchon and colleagues then performed a variety of informative studies on human tissue specimens and cells isolated from humans and mice and found that TREM1 amplifies immune responses generated by bacterial or fungal infections (Bouchon et al. 2001). Interestingly, Bouchon and colleagues illustrated that compared to neutrophils isolated from patients with aseptic systemic inflammatory response syndrome or unmanipulated WT mice, TREM1 was strongly induced on peritoneal neutrophils isolated from patients with septic shock caused by bacterial peritonitis and WT mice that received an IP injection of LPS. Moreover, administration of a TREM1 fusion protein prior to LPS injection blocked endotoxic shock and inflammatory responses in mice (Bouchon et al. 2001). These studies clearly illustrate that TREM1 serves to promote pro-inflammatory immune responses.

Contrary to many of the TREM1 findings, studies have shown that TREM2 serves to limit pro-inflammatory immune responses. TREM2 is expressed by dendritic cells, microglia/macrophages and osteoclasts. Studies have shown that TREM2 regulates osteoclastogenesis as peripheral blood mononuclear cells (PBMC) isolated from TREM2 or DAP12 deficient patients present with a failure to differentiate into osteoclasts with macrophage-colony stimulating factor (M-CSF) and receptor activator of nuclear factor κB ligand (RANKL) stimulation (Paloneva et al. 2003). Moreover, ex vivo generated

osteoclasts from DAP12 deficient patients displayed lower bone resorption rates (Paloneva et al 2003).

TREM2 has also been shown to inhibit the production of pro-inflammatory cytokines by macrophages and has been shown to promote the alternative activation of macrophages (Turnbull et al. 2006). Specifically, Turnbull and colleagues stimulated bone marrow derived macrophages (BMDM) isolated from WT and TREM2KO mice and demonstrated that the macrophages isolated from TREM2KO mice produced more TNFα and IL-6 in response to LPS, zymosan and CpG stimulation. Moreover, stimulation of peritoneal macrophages isolated from WT mice (without thioglycolate injection) with IL-4, a stimulus which leads to the production of alternatively activated cells, led to the induction of TREM2 (Turnbull et al. 2006). Overexpression of TREM2 by microglia has been shown to promote phagocytosis of apoptotic cells and cell debris, reduce the severity of EAE, a mouse model of multiple sclerosis, and limit the production of pro-inflammatory cytokines produced in microglial co-cultures with apoptotic neurons (Takahashi et al. 2005, Takahashi et al. 2007, Melchior et al. 2010).

Though the TREMs have been shown to primarily regulate immune responses, recent reports illustrate that TREM1 and TREM2 expression may play roles in cognitive development and in psychiatric disorders, respectively. Humans lacking functional TREM2 signaling develop a rare genetic disorder called Nasu-Hakola disease. This disease is characterized by the development of bone cysts, presenile cognitive dementia and death in the 40's or 50's (Madry et al. 2007). Because the only cell type in the brain

that expresses TREM2 are the microglia, these findings clearly illustrate that microglia are somehow able to regulate critical components of cognition (Thrash et al. 2009). A recent report has also demonstrated that patients with schizophrenia and bipolar disorder have elevated TREM1 expression on circulating monocytes (Weigelt et al. 2011). Curiously, they did not observe elevated expression of TREM1's signaling partner, DAP12, on patient monocytes (Weigelt et al. 2011). These findings definitely demonstrate that TREM1 expression is increased on the monocytes. However, as DAP12 expression does not concurrently increase, it is currently unknown whether the receptors are activated and subsequently, what this TREM1 induction means.

The TREM family of receptors have been shown to play critical roles in both innate and adaptive immune responses. A variety of studies have also illustrated that some TREMs are important in either the development or maintenance of cognitive functions and play roles in specific psychiatric disorders. There is a substantial amount of information that has yet to be revealed regarding the functions of these receptors, as few have been studied as much as TREM1 and TREM2. In this chapter, we will explore the expression patterns of the TREMs in the brain and will speculate as to why their expression is necessary in the brain.

3.3 RESULTS

3.3.1 The TREM family of receptors are expressed by different cell types in the brain

A variety of cell types found throughout the body have been shown to express TREM receptors. Studies have demonstrated that TREM1 is expressed by monocytes and neutrophils; TREM2 is expressed by microglia/macrophages, dendritic cells and osteoclasts; TREM3 has been found on two macrophage cell lines as well as a T cell line; TLT1 is expressed exclusively by megakaryocytes and platelets; and reports have indicated that TLT2 is the only TREM family member that is expressed by lymphocytes (Bouchon et al. 2001, Thrash et al. 2009, Chung et al. 2002, Washington et al. 2004, King et al. 2006). Though many have identified TREM expression in cells that reside mostly within the periphery, clear TREM2 expression has been found within the brain (Thrash et al. 2009, Schmid et al. 2009). Therefore, we have performed a variety of experiments to determine whether other TREMs are also expressed by cells within the brain. To determine whether TREM1, TREM2, TREM3, TREM4 or the major TREM signaling partner, DAP12 was expressed in the brain, we performed in situ hybridization (ISH) and detected their expression by using antisense ^{35}S or ^{33}P labeled riboprobes. We also performed immunohistochemistry (IHC) to visualize the cell types expressing the receptors by co-staining 3 month old murine brain sections with tomato lectin to stain for microglia, macrophages or blood vessels or with antibodies against NeuN to label neurons.

Using ISH, we did not detect TREM1or TREM3 expression in the unmanipulated adult murine brain (data not shown). In a previously published paper, our lab has shown that in the brain TREM2 is expressed by subsets of parenchymal microglia, as shown by the co-localization of lectin positive microglia/macrophages with the TREM2 riboprobes (black grains) (Fig. 3.1A-B) (Schmid et al. 2002, Schmid et al. 2009). Interestingly, autoradiograms of ISH illustrated that TREM4 was expressed in the hippocampus (Fig. 3.1C), cerebellum (Fig. 3.1D) and olfactory bulb (not shown) of the unmanipulated murine brain. TREM4 expression in the brain did not change following ICV injection of LPS (Fig. 3.1E-F). Surprisingly, we also observed that TREM4 was expressed by subsets of neurons throughout the brain via ISH and IHC (Figs. 3.1G-H). Because several TREMs are known to signal intracellularly using the adaptor molecule DAP12, we also analyzed the brain for DAP12 expression. Using dual in situ hybridization and immunohistochemistry, we could only detect DAP12 mRNA expression on subsets of microglia in the 3 month old murine brain (Figs. 3.1I-J).

3.3.2 The relative expression of TREMs 1, 2 and 3 differ on CD45lo and CD45hi populations

Microglia are the resident immune cells of the brain. Inflammation experienced within the brain compartment or in the periphery causes macrophages from the periphery to infiltrate the brain (Sedgwick et al. 1991, Ford et al. 1995, D'Mello et al. 2009, Puntambekar et al. 2011). In tissue sections, CNS resident microglia cannot be distinguished from CNS infiltrating macrophages, as they both express many of the same

molecules (Carson et al. 1998). However, when single cell suspensions are made from the inflamed brain, microglia can be distinguished from infiltrating macrophages because they express different levels of CD45, a protein tyrosine phosphate (Sedgwick et al 1991, Ford et al. 1995). Specifically, microglia express low levels of CD45 and infiltrating macrophages express high levels (Sedgwick et al. 1991, Ford et al. 1995). The literature shows that TREM family members are expressed on CNS resident microglia and on macrophages in the periphery. In order to determine what the relative amounts of TREM expression were on microglia and peripheral macrophages, we performed qRT-PCR using cDNA templates prepared from CD45lo microglia and CD45hi infiltrating macrophages isolated 24 hours post ICV injection of murine brains and sorted using flow cytometry.

Interestingly, at the RNA levels, our data show that TREM1 expression is higher on CD45lo microglia than CD45hi macrophages (Fig. 3.2). Also, we observed that TREM2 expression is significantly higher on CD45lo microglia than infiltrating macrophages (Fig. 3.2). This confirms findings reported by Schmid and colleagues. Lastly, we observed that TREM3 expression was significantly higher on CD45hi macrophages than on microglia (Fig. 3.2). At the RNA level we could not detect the membrane bound or splice variant forms of TREM4 nor could we detect TREM5 expression on microglia or macrophages (Fig. 3.2).

3.3.3. TREM protein expression is differentially induced on microglia following systemic inflammation.

With inflammation, the route through which immune cells in the brain become activated can affect the collection of receptors that they express. An intraperitoneal injection of LPS is detected in the periphery causing an increase in the amount of circulating pro-inflammatory cytokines. These cytokines can then activate cells within the brain by activating endothelial cells of the BBB, activating vagal nerve sensory afferents, being actively transported across the BBB or by entering and propagating immune signals through circumventricular organs (Perry 2004, Dantzer 2006, Quan 2008). This is often referred to as secondary activation of cells within the brain. We then tested whether the route by which immune cells were activated in the brain would affect the degree to which TREM expression changed.

Flow cytometric analysis of brain cell suspensions 24 hours post injection (IP) illustrated that TREM1 was not expressed by microglia in the naïve brain (Fig. 3.3A). However, low to negligible levels of TREM1 were induced on microglia isolated from IP LPS injected mice (Fig. 3.3B). Contrary to these findings, expression of TREM2 was detected on microglia in the unmanipulated brain (Fig. 3.3C). Following IP injection of LPS, microglia increase their expression of TREM2 (Fig. 3.3D). TREM3 expression could not be detected on microglia in the unmanipulated brain (Fig. 3.3E). However, following IP LPS injection, low to negligible levels of TREM3 were induced on microglia (Fig. 3.3F).

3.4 DISCUSSION

Many studies have demonstrated that TREMs are expressed by a variety of immune cells and are regulated by different types of inflammation. Moreover, findings illustrate that the TREMs play important roles in both innate and adaptive immune responses. Recently, critical studies have demonstrated that certain TREMs may also have important roles in cognitive development (TREM2) and perhaps even the pathogenesis of severe psychiatric disorders including schizophrenia and bipolar disorder (TREM1). Specifically, humans lacking functional TREM2 expression develop a rare genetic disorder characterized by presenile dementia, bone cysts and death in the 40's or 50's (Madry et al. 2007). This clearly indicates that lack of expression of a microglial protein can have primary psychological manifestations. Interestingly, studies have also shown that the expression of TREM1 is significantly elevated on monocytes isolated from patients with schizophrenia and bipolar disorder (Weigelt et al. 2011). Though expression of the TREM signaling partner does not change on monocytes from these patients, these findings would suggest that TREM1 somehow contributes to the chronic inflammatory response found in these patients or that TREM1 has a role in the pathogenesis of these disorders.

Until now, research has shown that TREM2 is the only TREM expressed in the brain, specifically by subsets of microglia (Fig. 3.1A) (Schmid et al. 2002, Thrash et al. 2008, Schmid et al. 2009). The results from the studies presented in this chapter, however, illustrate that other TREMs are expressed in the brain as well. Specifically, our

in situ hybridization studies illustrated that TREM4, also known as PDC-TREM in the literature, is expressed by subsets of neurons in the brain (Fig. 3.1G-H). This finding is surprising for a variety of reasons. First, most studies have found TREM or TLT expression on myeloid cells or on cells arising from lymphoid lineage. This is the first description of TREM expression on neurons in the brain. Moreover, the only known adaptor molecule that allows TREMs to signal inside the cell is DAP12. Our studies indicate that in the brain, DAP12 is expressed by subsets of microglia, but was not detected on any other cell types within the brain (Figs. 3.1E-F) (Thrash et al 2009). If TREM4 is indeed expressed by neurons, and lacks the intracellular signaling motif, what does it use to signal inside the cell once the receptor has been bound by the ligand?

Our results also indicate that in the brain, the relative expression of TREMs differ on CD45lo microglia and CD45hi infiltrating macrophages. Specifically, our RT-PCR data indicates that TREM1 expression, despite its high protein expression on CNS infiltrating macrophages, is higher on CNS resident microglia than on CNS infiltrating macrophages. Interestingly, at the protein level we find that TREM1 is highly expressed by CNS infiltrating macrophages and very low to negligible levels of TREM1 are found to be expressed on the microglial surface following systemic inflammation. Puntambekar et al. also demonstrates that TREM1 is expressed by CNS infiltrating macrophages 24 hours post ICV injection of LPS. We also found that TREM2 transcript expression is higher on microglia than on CNS infiltrating macrophages. This has previously been shown by TOGA analysis (Thrash et al. 2008) and in flow cytometric studies not shown. Though studies have reported that TREM3 is expressed by macrophage cell lines, we

observed that TREM3 RNA is significantly higher on CD45hi macrophages than on microglia (Fig. 3.2). However, in our flow cytometric studies, we only detected a very small population of CNS infiltrating macrophages expressing TREM3 protein following ICV injection of LPS (data not shown).

A variety of experiments have identified an obvious lack of correlation between mRNA and protein levels. However, there are a variety of reasons to explain why we observe differences in the mRNA and protein levels of TREM1 and TREM3. It is known that various post-transcriptional modifications occur within cells. However because we do not yet have a firm understanding of all of these mechanisms, we cannot yet accurately compute the amount of protein that will be generated from a known amount of mRNA (Greenbaum et al. 2003). Many cells also have several activation states that can regulate the amount of protein that is made. Once protein has been synthesized, there are also variations in the rates of protein degradation, leading to different protein half-lives (Greenbaum et al. 2003). Although several methods have been devised to determine mRNA and protein concentrations, we have not yet created perfect experimental setups that eliminate human error and noise that can be picked up (Greenbaum et al. 2003). Both of these will significantly affect the accuracy of our readings.

Our findings contribute to a body of research illustrating that CNS intrinsic immunity differs from CNS extrinsic immunity. Many studies demonstrate that the CNS is an immune privileged site. This is mainly due to findings that foreign tissue grafts survive for longer periods when placed inside the CNS parenchyma than when placed

under the skin (Medawar et al. 1948, Carson et al. 2006). A variety of studies also illustrate that CNS resident microglia serve to promote this immune privileged state. Studies by Magnus and colleagues illustrate that when microglia are exposed to IFNγ or supernatants from pro-inflammatory activated T-helper 1 cells, microglia upregulate B7-H1, a co-inhibitory molecule responsible for inhibiting T cell activation. Even though many studies have demonstrated that microglia can promote neuroprotective immune responses, studies also strongly suggest that microglia serve to promote CNS homeostasis. Moreover, a variety of labs have also shown that for some neuroinflammatory states, both CNS intrinsic and extrinsic immune responses are required for the resolution of the damage. Specifically, our lab and others have shown using the facial axotomy model that CNS infiltrating macrophages are required for activation of CD4+ T cells (Byram et al. 2004). However, CD4+ T cell mediated neuroprotection of the motoneurons in the facial nucleus required antigen presentation from microglia (Byram et al. 2004).

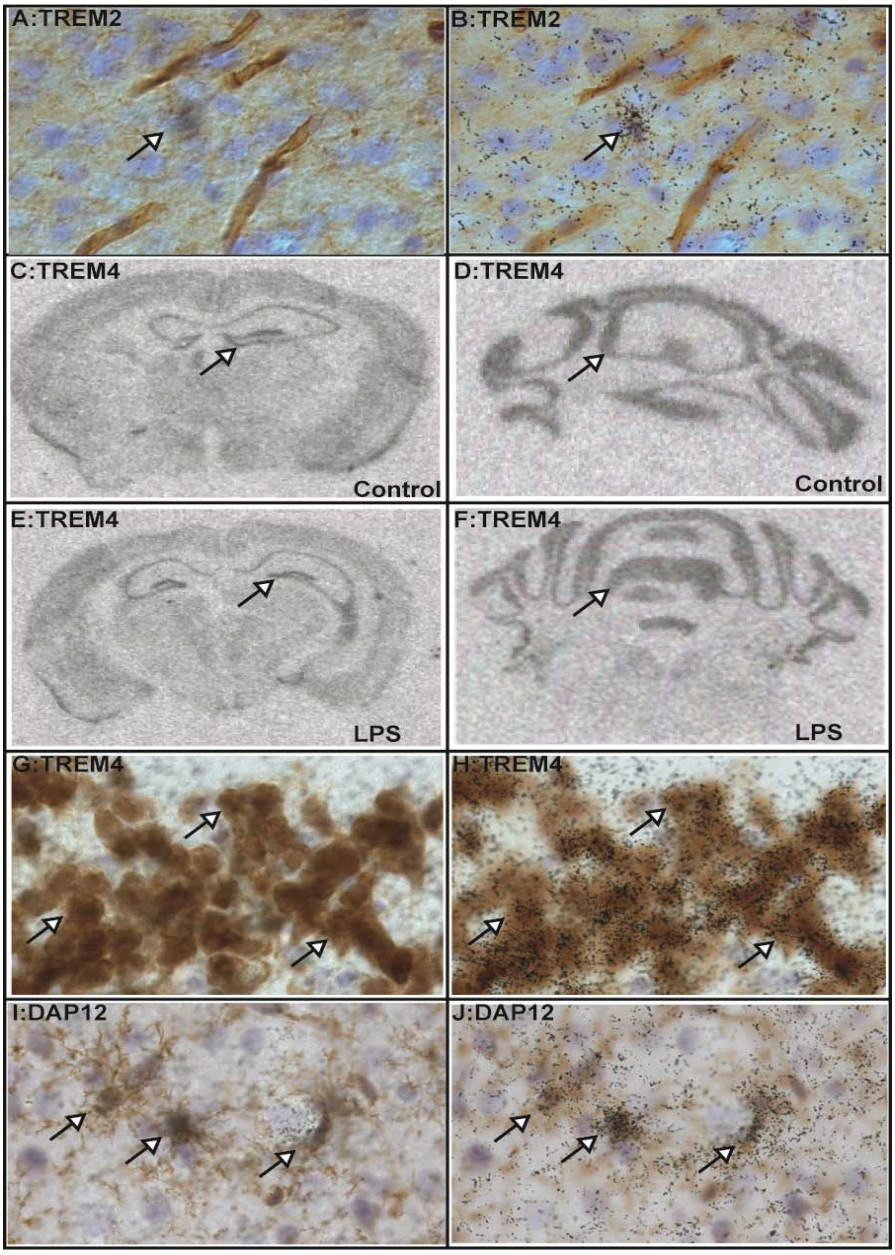
Our findings of cell type specific expression of TREM receptors in the brain may reflect on specific functions important for the CNS intrinsic and extrinsic compartments. Specifically, we find that the anti-inflammatory receptor, TREM2 is expressed by CNS resident microglia and is upregulated by many inflammatory stimuli in the developing, young adult and aged brain (data not shown). We also find that TREM1, a receptor associated with amplifying pro-inflammatory immune responses, is expressed by the CNS extrinsic compartment, the CNS infiltrating macrophages. The cell type specific TREM expression seems to polarize the CNS intrinsic and CNS extrinsic immune

compartments in regards to potential functions. However, because we find both cell types in the brain expressing TREMs following inflammation, this strongly suggests that their polarized functions either balance each other out and/or that both are required for the resolution of the inflammatory response and its effects on the CNS.

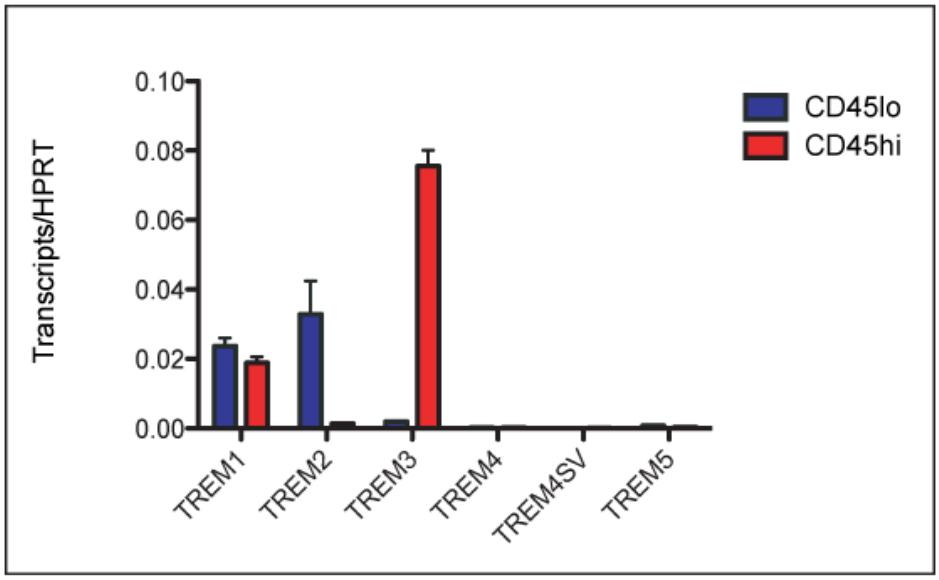
These studies contribute to the overall understanding and importance of TREM expression on different cell types throughout the body. In the brain it seems so far that TREM2 and TREM4 expression have essential roles in the healthy, unmanipulated state. So far the best understood TREM in the brain is TREM2. However, understanding the roles of TREM1, TREM2, TREM3 and TREM4 will undoubtedly prove to be helpful in our knowledge of TREM biology, the roles that TREMs have in brain development, homeostasis and disease and the roles of these receptors in their respective CNS intrinsic and CNS extrinsic compartments.

3.5 FIGURES AND LEGENDS

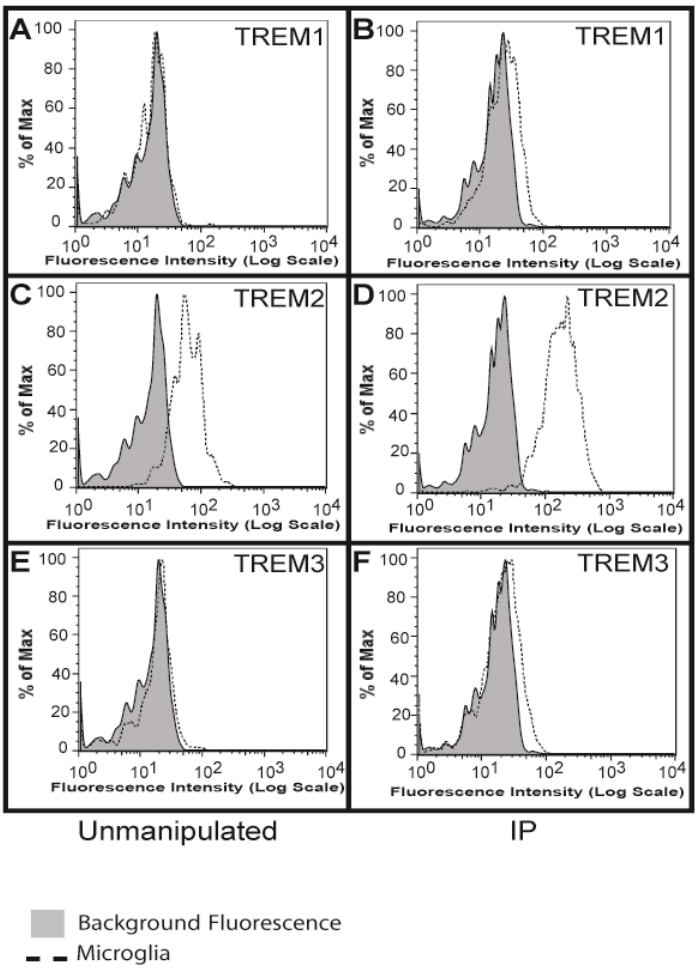
3.1 Cell type specific expression of TREMs and DAP12 in the brain. In situ hybridization analysis depicting 3 month old unmanipulated murine brain sections hybridized with ^{35}S labeled TREM2 **(A-B)** or ^{33}P labeled TREM4 **(C-H)** or DAP12 **(I-J)** riboprobes. TREM4 was also detected by autoradiogram analysis of 3 month old control **(C-D)** or LPS injected **(E-F)** murine brain sections hybridized with 33P labeled TREM4 riboprobes. Expression is indicated by white arrows pointing upwards. Probe is visualized by black grains in film emulsion. Blood vessels, microglia and macrophages are visualized in brown by tomato lectin **(A-B, I-J)**. Neurons are visualized in brown by NeuN **(G-H)**.Nuclei are visualized in blue by hematoxylin.


A:TREM2
B:TREM2
C:TREM4
Control
D:TREM4
Control
E:TREM4
LPS
F:TREM4
LPS
G:TREM4
H:TREM4
I:DAP12
J:DAP12

3.2 TREMs are differentially expressed on CD45lo and CD45hi populations. qRT-PCR and standard curve (copy number) analysis of TREM1, TREM2, TREM3, TREM4, TREM4SV and TREM5 mRNA induction using CD45lo (blue bar) and CD45hi (red bar) cDNA templates isolated 24 hours post ICV injection from whole brains of 3 month old mice.



3.3 TREM protein expression is differentially induced on microglia following systemic inflammation. Flow cytometric detection of microglial expression of TREM1 **(A-B)**, TREM2 **(C-D)** and TREM3 **(E-F)** from unmanipulated **(A, C, E)** and IP LPS injected **(B, D, F)** 3 month old mice assayed 24 hours post injection. Background fluorescence indicated by shaded histogram while microglia are depicted by dashed line.



CHAPTER 4

Systemic immune challenge causes tolerated and age-specific CNS immune responses in the developing and mature brain.

4.1 ABSTRACT

Neuroinflammation experienced during critical periods of CNS development has been implicated in the development and/or pathogenesis of neurodevelopmental disorders including cerebral palsy, schizophrenia and autism (Chew et al., 2006; Doorduin et al., 2008; Pardo et al., 2005). It has been hypothesized that during early childhood the brain is susceptible to systemic inflammation because this is a period of active brain maturation and synaptic rewiring. Focusing on microglia, the resident macrophages of the brain, we examined microglial phenotype and responses as a function of normal development. Here, using flow cytometry and dual in situ hybridization/immunohistochemistry we show that in the early postnatal brain microglia are in a pre-activated state that is non-polarized. We also show that microglial phenotypes display increasing diversity as a function of healthy aging. Because microglia are highly plastic cells and can change their phenotypes in response to environmental cues, we also examined microglial phenotypes and responses to systemic inflammation, and the degree of macrophage influx, as a function of normal development. Here we demonstrate that systemic inflammation induced by an intraperitoneal injection of LPS leads to widespread activation of microglia and a small transient influx of peripheral macrophages. Unlike the pro-inflammatory microglial activation observed in response to direct (intracranial injection of LPS)

activation of microglia, systemic inflammation led to the induction of TREM2, PD-L1, and CX3CR1 which could indicate anti-inflammatory microglial activation. Moreover, macrophage infiltration into the brain in response to systemic inflammation decreases as the brain develops. This data contributes to the growing literature demonstrating that the brain may have age-specific susceptibilities to insults associated with the development of neurodevelopmental disorders and potential therapies.

4.2 INTRODUCTION

Microglia are commonly referred to as the tissue macrophages of the central nervous system (CNS). As such, they not only perform common macrophage functions including the phagocytosis of apoptotic cells and cell debris, but they also serve as the first line of innate immune defense in the brain and spinal cord (Carson et al. 1998). A variety of research studies have revealed the dynamic roles that microglia have in the developing, mature and inflamed brain. Striking studies using two-photon microscopy have demonstrated that in the healthy brain, microglia are not quiescent, but instead are highly active surveyors of their environment (Nimmerjahn et al. 2005). Moreover, recent research has demonstrated that microglial activation is not as simple as was once thought. As mentioned, in the healthy brain microglia are quite active performing homeostatic functions (Nimmerjahn et al. 2005). When microglia detect a pathogen or CNS injury, they can adopt several different activation states including classical or alternative activation states (Mosser et al. 2008). Though substantial progress has been made in the understanding of microglia, several questions regarding the exact phenotypes and functions of these cells in different periods of brain development remain unanswered.

Brain development is a period of continuous and major changes. As the brain forms, a variety of cells appear, including neurons and glia, and these cells must migrate to the appropriate location and begin performing their duties in this dynamic environment (Rice et al. 2000). Microglia are no exception. It is documented mostly by immunohistochemical methods that microglial morphology and activation state change as

the brain develops (Hristova et al. 2010). Also, as numerous neurons are born as the brain develops, it is estimated that up to 50% undergo carefully controlled apoptosis after forming connections with their target cells (Raff et al. 1993, Schlegelmilch et al. 2011). Microglia are believed to be heavily involved in the phagocytosis of apoptotic neurons in the developing brain (Parnaik et al. 2000, Ferrer et al. 1990). This has been illustrated by studies showing that apoptotic cells in the cerebral cortex were engulfed by phagocytes in the brain during the first postnatal week of life (Ferrer et al. 1990).

Studies on microglial activation states have demonstrated how dynamic and plastic these cells truly are. Though microglia have long been considered as important immune effector cells in the brain, many studies have associated microglial activation with various types of neuropathology (Mosser et al. 2008). Recent findings suggest, however, that depending on the stimulus, microglia can adopt phenotypes associated with pathogen defense (classical activation) and/or anti-inflammatory, wound-healing responses (alternative activation) (Stein et al. 1992, Nair et al. 2003, Mosser et al. 2008). Depending on the specific type of microglial activation, these cells can either inadvertently promote tissue damage in the CNS by producing excess reactive oxygen species and pro-inflammatory cytokines when defending the host from pathogens, or microglia can promote anti-inflammatory, tissue repair functions by producing proline, a precursor to collagen (Mosser et al. 2008).

Inflammation experienced during the fetal or perinatal period has been implicated in playing a causative role in the development of several developmental brain disorders

(Chew et al. 2006). Several epidemiological, pathological and serological studies have revealed activated microglia in the postmortem brains of patients with autism, schizophrenia and cerebral palsy (CP) (Doorduin et al. 2003, Vargas et al 2005, Chew et al. 2006). From these studies, many have hypothesized that microglia play a role in the development and/or pathogenesis of these disorders. However, a complete understanding of microglial functions in the developing and in the developing but inflamed brain is lacking, ultimately hindering our comprehension of microglial roles in these disorders.

To understand the changing roles of microglia in the postnatal developing brain and their responses to systemic inflammation during this developmental period, we have examined the phenotypes of microglia and CNS infiltrating macrophages acutely isolated from the unmanipulated or inflamed (IP LPS) brain at different postnatal ages. We find that microglial phenotypes change dramatically as the healthy brain matures during the early postnatal period. However, age specific CNS immune responses to systemic inflammation are quite mixed with microglia expressing many pro-inflammatory markers yet upregulating anti-inflammatory markers, possibly to counteract the influx of pro-inflammatory macrophages.

4.3 RESULTS

4.3.1 Microglial phenotype changes as the brain develops postnatally.

Several studies have demonstrated that microglial morphology and activation state changes with healthy brain development (Hristova et al. 2010). Using immunohistochemical studies, Hristova and colleagues illustrated that microglia in the subcortical white matter areas displayed an activated morphology at birth that was gradually lost by the second postnatal week. Moreover, they also demonstrated that these microglia expressed activation markers including integrin subunits alpha 5 and 6 and the co-stimulatory molecule B7.2 (Hristova et al. 2010). Furthermore, the expression of these activation markers also subsided by the second postnatal week (Hristova et al. 2010). Though Hristova and colleagues clearly established that microglia during the early postnatal period have an activated phenotype, including morphology and the collection of activation markers, they did not illustrate whether microglia preferentially adopted a specific type of activation during this period or whether their activation was unbiased.

Using flow cytometry to analyze microglia acutely isolated from the CNS of mice ranging from early postnatal ages to 3 months, when the murine brain is relatively mature, we analyzed microglial expression of markers associated with classical or alternative activation. We chose to examine 4 developmental time points corresponding to the periods following birth (P2/P3), early dendritic spine and oligodendrocyte development (P7), early myelination (P14) and peak myelination (P21). Though microglia and CNS infiltrating macrophages cannot be distinguished in tissue sections,

they can be distinguished in single cell brain suspensions by their differential expression of CD45, also called leukocyte common antigen, that is expressed by all cells arising from the hematopoietic lineage (Sedgwick et al. 1991, Ford et al. 1995).

In single cell brain suspensions, antibodies against the integrin CD11b can be used to label myeloid cells while the CD45 low population indicates microglia (Fig. 4.1A). The overexpression of the anti-inflammatory molecule, TREM2, has been shown to increase microglial phagocytosis of apoptotic cells and cell debris, decrease microglial motility and decrease the severity of EAE, a mouse model of multiple sclerosis (Takahashi et al. 2005, Takahashi et al. 2007, Melchior et al. 2010). Though our lab has previously shown that microglial expression of TREM2 decreases as the brain ages, we found here that microglial expression of TREM2 is relatively high at postnatal day 2 (P2) and that it gradually declines as the brain develops (Fig. 4.1B). Another anti-inflammatory molecule that we found was abundant on microglia at P2 and decreased as the brain matured was the macrophage mannose receptor (MMR) (Fig. 4.1C). This molecule is not only known to mediate microglial/macrophage binding and ingestion of microorganisms with surface mannose residues, but it is consistently upregulated on alternatively activated myeloid cells (Stein et al. 1992). Interestingly, at P21 we observe two populations of MMR expressing microglia (Fig. 4.1C). One population does not express MMR beyond background while the other population expresses levels equivalent to those seen at P2. This observation may indicate that MMR expression cannot gradually decrease on microglia because its expression by some microglia is required for particular (currently unknown) developmental functions at P21.

Not all microglial activation markers followed the same patterns as TREM2 and MMR. Programmed Death-Ligand 1 (PD-L1) is a co-inhibitory molecule that is not only responsible for preventing T cell activation and cytokine production but is also upregulated on alternatively activated cells (Magnus et al. 2005). Interestingly, we observed that microglia had low expression of PD-L1 at P2 and this level remained constant as the brain matured (Fig. 4.1D). Fractalkine receptor (CX3CR1), a molecule whose expression is known to prevent neurotoxic microglial activation, is highly expressed by microglia during the early postnatal period and this level of expression is maintained as the brain develops (Fig. 4.1E). CD200R, a receptor known to prevent pro-inflammatory microglial activation, is expressed at low levels by microglia at P2 (Fig. 4.1F). However, like CX3CR1 and PD-L1, CD200R expression remains constant as the brain matures (Fig. 4.1F). Specifically, this data demonstrates that microglia express anti-inflammatory activation markers during the early postnatal period. However, the expression of some, but not all anti-inflammatory activation markers changes as the brain develops.

As microglia are the resident immune cells of the brain, they express molecules such as major histocompatibility complex class II (MHC Class II) that allow them to present antigen to T cells. We analyzed this molecule because it is quickly upregulated by microglia following several pathological conditions in the CNS. Moreover, it is a reliable marker of microglial activation (Hayes et al. 1987). Our studies illustrate that at P2, microglia express high, yet heterogeneous levels of MHC Class II that decrease as the brain develops (Fig. 4.1G). However, similar to the peak observed at P21 for MMR, at

this age, we also observe two populations of MHC Class II expressing microglia (Fig. 4.1G). We also analyzed the expression of molecules associated with classical activation of microglia associated with pathogen defense responses. CD40 and B7.2 are both co-stimulatory molecules that promote T cell activation and pro-inflammatory cytokine production (Kuchroo et al. 1995, Tan et al. 2000). However, CD40 has also been shown to promote microglial dependent induction of cholinergic neural differentiation in vitro (Jonakait et al. 2000). Interestingly, both CD40 and B7.2 are highly expressed by microglia during the early postnatal period, the expression is heterogeneous, and the expression gradually declines as the brain matures (Figs. 4.1H-I). Curiously, we observe two peaks for both CD40 (Fig. 4.1H) and B7.2 (Fig. 4.1I) at P21. This may indicate that the expression of these two molecules, often associated with classical activation of microglia, are also required by some microglia for developmental processes occurring at P21. The last receptor observed with this set of data was TLT2, a member of the TREM family of receptors. To date, studies have reported that TLT2 is expressed by neutrophils, macrophages in the lung and peritoneum and lymphocytes (King et al. 2006, Hashiguchi et al. 2008). Interestingly, this is the first report of TREM family expression on cells arising from the lymphoid lineage. Our flow cytometric studies illustrated that TLT2 protein is highly expressed by microglia during the early postnatal period (P2) and that its level of expression remains constant as the brain matures (Fig. 4.1J).

4.3.2 Markers of pro-inflammatory microglial activation are not upregulated following systemic inflammation.

To determine whether microglia in the early postnatal period respond to systemic inflammation by becoming classically activated, we administered IP injections of LPS to induce systemic inflammation in P3, P7, P14, P21 or 3 month old mice. These ages were chosen so that we could clarify whether microglial responses to systemic inflammation differed during critical periods of postnatal synaptogenesis, synaptic pruning and oligodendrocyte maturation. P3 was chosen because this is the earliest age that we could inject pups without causing maternal cannibalism. By 3 months, the murine brain is relatively mature. Twenty-four hours post injection we acutely isolated microglia from the CNS and performed flow cytometry to analyze microglial activation state.

Many in vitro studies have demonstrated that microglia express CD40 and B7.2 and increase the expression of these molecules following LPS stimulation (Kuchroo et al. 1995, Tan et al. 2000). It is unclear, however, whether systemic inflammation changes microglial expression of these two molecules during the early postnatal period. We found that both CD40 and B7.2 could be detected on microglia from unmanipulated mouse brains at P4, P8, P15 and P22 (Figs. 4.2A-F-dotted line). Systemic inflammation did not induce CD40 or B7.2 expression on microglia at P4, P8 or P15 (Figs. 4.2A-H-thick dark line) but did cause a slight induction of both (the first population) at P22 (Figs 4.2G-H thick dark line). We also observed that both P15 and P22 were periods where two populations of microglia could be detected in single brain suspensions from

unmanipulated and LPS injected mice (Figs. 4.2E-H). Again, this may indicate that specific populations of microglia expressing CD40 or B7.2 are required for specific but unknown developmental processes at these ages. Interestingly, in the mature murine brain (3 months) we could not detect CD40 (Fig. 4.2I-dotted line) or B7.2 (Figure 4.2J-dotted line) beyond background fluorescence. Moreover, systemic inflammation did not increase the expression of either at this age (Fig. 4.2I-J thick dark line).

4.3.3 Some markers of anti-inflammatory microglial activation are upregulated following systemic inflammation.

To determine whether microglia adopt alternative activation states following systemic inflammation during the early postnatal period, we administered IP injections of LPS to induce systemic inflammation in P3, P7, P14, P21 or 3 month old mice. Twenty-four hours post injection we acutely isolated microglia from the CNS and performed flow cytometry to analyze microglial activation state. Interestingly, we observed that at each age microglia from unmanipulated murine brains expressed high levels of TREM2, low PD-L1, high levels of CX3CR1 and high levels of the TREM family member, TLT2 (Fig. 4.3A-T-dotted line). Moreover, following systemic inflammation microglia increase their expression of TREM2, PD-L1, CX3CR1 and TLT2 at each age (Fig. 4.3A-T-thick, dark line). This clearly demonstrates that systemic inflammation causes microglia to increase their expression of some alternative activation markers. We have also analyzed mannose receptor expression on microglia following systemic inflammation and did not detect any increases in its expression.

4.3.4 Pro-inflammatory macrophages are actively recruited into the brain following systemic inflammation in a developmentally regulated manner.

Peripheral inflammation and inflammation within the brain cause the recruitment of blood derived cells into the brain (D'Mello et al. 2009). As previously mentioned, microglia can be distinguished from CNS infiltrating macrophages in single cell brain suspensions based on flow cytometric analysis of CD45 levels (Sedgwick et al. 1991, Ford et al. 1995). Microglia are CD11b+CD45lo and infiltrating macrophages are CD11b+CD45hi (Fig. 4.4A). To determine whether systemic inflammation experienced during different periods of postnatal brain maturation leads to differences in the number of macrophages that infiltrate the brain, we analyzed the number of CD45hi macrophages in the brain following systemic inflammation. Systemic inflammation led to the influx of macrophages into the brain at each age (Fig. 4.4B). However, the number of macrophages that infiltrated the brain differed with mouse age (Fig. 4.4B). Interestingly, the influx of macrophages into the brain at P8 was 6 fold higher than the number that infiltrated the 3 month old brain (Fig. 4.4B).

Chemokines are a family of small proteins that are involved in cellular migration and intercellular communication (Adler et al. 2006). Chemokines are widely known for recruiting immune cells to sites of infection and/or inflammation (D'Mello et al. 2009). To determine whether macrophages were actively recruited to the brain and also whether the age-specific recruitment of macrophages was due to age-specific expression of chemokines in the brain, we performed quantitative real time polymerase chain reaction

(qRT-PCR) to examine CCL2 (MCP1) and CCL5 (RANTES) mRNA expression using complementary DNA (cDNA) templates prepared from whole brains of unmanipulated or IP LPS injected mice of different ages. We specifically focused on these two chemokines because CCL2 is one of the strongest signals for monocyte/macrophage recruitment and CCL5 as well (Ambrosini et al. 2004). However, CCL5 also recruits a variety of other leukocytes (Ambrosini et al. 2004). Systemic inflammation caused more than 14 fold induction of CCL2 at P8 and greater than 13 fold induction of CCL2 at P15 (Fig. 4.4C). Though systemic inflammation also lead to more than 4 fold induction of CCL2 at 3 months, the induction observed at P8 and P15 were both statistically greater than the induction observed at 3 months (Fig. 4.4C). Interestingly, no statistically significant increases in CCL5 mRNA were observed at any age (Fig. 4.4D). This indicates that the macrophages were actively recruited to the CNS in part by CCL2 following systemic inflammation. This demonstrates that CCL2 plays a role in the age-specific macrophage recruitment into the CNS.

To determine the phenotype of CNS infiltrating macrophages we performed flow cytometric analysis looking at several known activation markers. CNS infiltrating macrophages expressed known pro-inflammatory markers, including the co-stimulatory molecules CD40 and B7.2 (data not shown). Microglia expressed low levels of Triggering Receptor Expressed on Myeloid cells-1 (TREM1), a pro-inflammatory receptor known to amplify TLR mediated immune responses (Bouchon et al. 2001) (Fig. 4.4E-black line). However, CNS infiltrating macrophages consistently expressed higher

levels of TREM1 than microglia (Fig. 4.4F-dashed line). This suggests that CNS infiltrating macrophages at each age promote pro-inflammatory responses.

Arginase-1 and iNOS are two well known enzymes used to identify alternative and classical activation states respectively (Mosser et al. 2008). Arginase-1 and iNOS compete for the substrate L-arginine (Pesce et al. 2009). Arginase-1 hydrolysis of arginine leads to the production of polyamines, proline and ultimately collagen, which can be used in wound healing and tissue repair (Pesce et al. 2009). The enzyme iNOS leads to the production of nitric oxide (NO) which is associated with pro-inflammatory microglia/macrophage responses (Mosser et al. 2008). Because we had determined that microglia expressed pro-inflammatory markers but induced anti-inflammatory markers following systemic inflammation, and that the CNS infiltrating macrophages were primarily pro-inflammatory, we decided to examine the neuroinflammatory environment. To examine the neuroinflammatory environment we performed qRT-PCR to look at Arginase-1 and iNOS mRNA using whole brain cDNA prepared from unmanipulated and IP LPS injected animals at different ages. We observed statistically significant induction of iNOS at P8 (Fig. 4.4G). However, the iNOS induction observed at P8 and P15 were statistically greater than the expression detected at 3 months (Fig. 4.4G). For Arginase-1 we observed significant induction at both P8 and P15 (Fig. 4.4H). Again, the inductions observed at both ages were significantly greater than the expression observed at 3 months (Fig. 4.4H).

4.3.5 TREM2 binding activity is found on neurons, microglia and CNS infiltrating macrophages

For many of the molecules examined thus far, the cell types that express their binding partners are known. Though several potential binding partners for TREM2 have been identified, the receptor remains largely an orphan (Klesney-Tait et al. 2006, Stefano et al. 2009). To determine what cell types in the brain express the TREM2 ligand, we used a fusion protein consisting of the extracellular binding region of the TREM2 receptor fused to the FC region of human IgG (Fig. 4.5A). Using immunohistochemistry, we observed that in the murine brain, the putative TREM2 ligand (red) is expressed by neurons (Iba1-green) (Fig. 4.5B). This is consistent with what other studies have reported (Hsieh et al. 2009). Using immunohistochemistry we did not detect TREM2 ligand expression by any other cell types in the brain.

We then used the TREM2 fusion protein to determine whether we could detect TREM2 ligand expression by microglia or CNS infiltrating macrophages by a more sensitive method, flow cytometry. Specifically, we analyzed microglia and CNS infiltrating macrophages acutely isolated from unmanipulated and IP LPS injected mice of different ages. We found that in the early postnatal period at P8, CNS resident microglia express the putative TREM2 ligand (Fig. 4.5C-thick dashed line) and this expression increases with systemic inflammation (Fig. 4.5C-thick black line). Moreover, at P8, CNS infiltrating macrophages also express TREM2 binding activity (Fig 4.5D- thin dashed line). In the mature murine brain (3 months) microglia express the TREM2 ligand

(Fig 4.5E-thick dashed line), but its expression does not increase with systemic inflammation (Fig. 4.5E-thick black line). At 3 months, CNS infiltrating macrophages also express the putative TREM2 ligand (Fig. 4.5F-thin dashed line). This demonstrates that TREM2 may allow microglia to communicate with neurons. However, as both microglia, in the postnatal developing brain, and CNS infiltrating macrophages express the TREM2 ligand, this may allow microglia to self activate the anti-inflammatory receptor and also allow the infiltrating, pro-inflammatory macrophages to activate the anti-inflammatory TREM2 on microglia.

4.3.6 TREM2 expression directly or indirectly regulates macrophage influx into the brain.

Several studies have reported that systemic inflammation induced by the administration of IP LPS leads to prolonged microglial activation (Masocha et al. 2009). To determine whether IP LPS leads to prolonged microglial activation in our model system and to examine the type of prolonged microglial activation state, we used flow cytometry to analyze the activation status of microglia acutely isolated from the CNS of unmanipulated and LPS injected mice of the aforementioned ages one week post injection. Though we observed TREM2 expression on microglia from the unmanipulated mouse at P22 (Fig. 4.6A-thick dashed line), only at this age did we observe sustained TREM2 expression 1 week post injection (Fig. 4.6A-thick black line). Interestingly, we also observed TLT2 expression on microglia from the unmanipulated mouse at P22 (Fig. 4.6B-dashed line) and we observed sustained TLT2 expression 1 week post injection

(Fig. 4.6B-thick black line). Twenty four hours post P14 injection, microglia isolated from WT mice exhibit elevated CD45 levels (Fig. 4.6C first peak-thin dotted line) and macrophage influx (Fig. 4.6C second peak-thin dashed line) is detected as compared to microglia isolated from unmanipulated WT mice (Fig 4.6C-thin black line). We also detected elevated CD45 levels on WT microglia 1 week post P14 injection (Fig. 4.6D-thin dashed line) as compared to microglia isolated from WT age matched mice (Fig. 4.6D-thin black line). This finding illustrates that when systemic inflammation is induced at P14, microglia remain activated up to 1 week post injection.

As TREM2 expression remained elevated on microglia one week post P14 injection, we decided to use mice lacking functional TREM2 receptor to determine whether this molecule was involved in regulating inflammation induced sustained microglial activation. Interestingly, in brains isolated from unmanipulated TREM2 knockout (KO) mice, macrophage influx was observed using flow cytometric analysis (Fig. 4.6E-thin black line, second peak). This is not observed in the unmanipulated wild type mouse. One day post P14 injection, cell suspensions acutely isolated from TREM2KO mouse brain exhibited microglial activation and macrophage recruitment comparable to wild type mice. Specifically, one day post P14 injection, microglia from IP LPS injected TREM2KO mice displayed elevated CD45, indicated by the shift in the large peak, and macrophage recruitment, indicated by the second peak (Fig. 4.6E-dashed line). Contrary to what is observed in WT mice, one week post P14 injection, microglial CD45 returned to baseline (Fig. 4.6F-dashed line, first peak) but macrophage influx was still observed in the TREM2KO brain (Fig. 4.6F-dashed line, second peak). These

findings strongly suggest that TREM2 plays a role in preventing macrophage influx into the unmanipulated brain and dampening macrophage influx following systemic inflammation.

Interestingly, we observed that like the WT mouse, one week post P14 and 3 month IP LPS injection, TLT2 remains elevated on microglia isolated from TREM2KO mice (Fig. 4.6G-H thick black line) as compared to microglia isolated from age matched unmanipulated TREM2KO mice (Fig. 4.6G-H thick dashed line).

4.3.7 TREM2KO mice exhibit chronic neuroinflammation in the unmanipulated brain.

After observing the altered macrophage recruitment in unmanipulated and IP LPS injected mice lacking functional TREM2, we decided to determine whether the CNS neuroenvironment displayed altered expression of inflammatory molecules. Specifically, we performed qRT-PCR looking at TNFα, iNOS, Arginase-1, CCL2 and CCL5 using whole brain cDNA from unmanipulated and IP LPS injected P15 TREM2KO mice. Using age matched cDNA generated from WT whole brain RNA, we observed that TREM2KO mice displayed 5 fold greater TNFα, 2 fold greater iNOS and 4 fold greater Arginase 1 in the unmanipulated brain compared to unmanipulated WT brains (Figs 4.7A-C). TREM2KO mice also displayed 2 fold greater CCL2 and 16 fold greater CCL5 in the unmanipulated brain as well as 6 fold greater induction of CCL5 in the TREM2KO IP LPS injected mouse brain when compared to the WT brain (Figs. 4.7 D-E). Because the unmanipulated TREM2KO mouse brain exhibits higher levels of many

of the inflammatory markers than WT mice, this indicates that these mice experience chronic neuroinflammation in their "healthy state." Interestingly, these mice do not exhibit any sickness behaviors that have been observed.

4.4 DISCUSSION

As the resident immunocompetent cells of the brain and spinal cord, studies have shown that microglia have essential roles in maintaining CNS homeostasis in the mature brain. Studies have also illustrated that microglia are very dynamic cells during brain development and gradually change their phenotypes and activation states (Hristova et al. 2010). Specifically, Hristova et al. demonstrated that at birth microglia in the subcortical white matter areas display an activated morphology and gradually adopt a stellate, resting morphology by the second postnatal week. Moreover, these cells also expressed many markers of activation including the co-stimulatory molecule B7.2, and integrin subunits alpha 5 and 6 (Hristova et al. 2010). Though these studies clearly indicate that microglia change as the brain matures postnatally, it was unclear whether these cells were merely activated, or whether they adopted a specific activation state. Our studies also confirm that microglia display a pre-activated state during the early postnatal period and that this activated state is lost by 3 months. Moreover, because microglia during the early postnatal period expressed molecules associated with both classical and alternative activation, this illustrates that this pre-activated state is non-polarized.

Interestingly, we also observed that microglia express high levels of the anti-inflammatory molecule, TREM2 during the early postnatal period. Takahashi and colleagues previously reported that TREM2 promotes the phagocytosis of apoptotic cells and cellular debris. This could indicate that high TREM2 levels during this period promote the phagocytosis of apoptotic cells during this important period of synaptic

pruning. We also observed that the expression of CX3CR1, PD-L1, CD200R and TLT2 remain relatively constant throughout postnatal development. These findings illustrate that CX3CR1, PD-L1, CD200R and TLT2 are not regulated by development. This may indicate that the constant expression of these four molecules is required for proper microglial physiology in both the developing and mature brain.

Several studies have indicated that LPS leads to microglial activation and microglial production of reactive oxygen species and pro-inflammatory molecules (Kuchroo et al. 1995, Tan et al. 2000). However, many of these studies have also been performed in vitro, where microglia are not in contact with cells normally found in their neuroenvironment. Subsequently, these results may not accurately reflect in vivo biology. A variety of studies have also hypothesized that systemic inflammation, and microglial activation is correlated with the increased incidence of many neurodevelopmental disorders (Doorduin et al. 2003, Vargas et al 2005, Chew et al. 2006). Vargas and colleagues clearly depicted that in the postmortem brains of autistic patients, microglial activation could be found in cortical areas and in the cerebellum. However, using immunohistochemistry, they also found that these cells expressed inflammatory molecules such as CCL2 and TGF-β (Vargas et al. 2005). Though these studies and others have unmistakably established that microglia are often activated in the brains of patients with neurodevelopmental disorders, they have failed to establish the roles of these activated cells.

In order to understand microglial roles in the postnatal developing brain following systemic inflammation, we performed experiments on microglia and CNS infiltrating macrophages acutely isolated from the murine CNS to examine how systemic inflammation affected microglial and macrophage phenotypes in the brain during the early postnatal period. Interestingly, we observed that microglia expressed pro-inflammatory molecules such as CD40 and B7.2 during the early postnatal period, and that systemic inflammation did not increase the expression of these two. Moreover, microglia also expressed several anti-inflammatory molecules including TREM2, PD-L1 and CX3CR1. Contrary to the pro-inflammatory molecules, systemic inflammation caused the increased microglial expression of all three of these molecules. This would strongly suggest that microglia are prepared to communicate with cells of the adaptive immune system during this early postnatal period. However, systemic inflammation also strongly promotes neuroprotective microglial activation. Specifically, the increased expression of TREM2 could indicate an increased ability to phagocytose cells, and also promotes wound healing and tissue repair functions. The increased PD-L1 may indicate that microglia become equipped with the ability to inhibit T cell proliferation and pro-inflammatory cytokine production if T cells were to enter the brain. Lastly, the increased CX3CR1 may occur to ensure that microglia do not adopt neurotoxic activation states during critical periods of postnatal brain development.

We also found that TLT2 expression increases on microglia 24 hours post IP LPS injection at P3, P7, P14, P21 and at 3 months. This clearly demonstrates that TLT2 expression on microglia is regulated by inflammation, as many of the TREMs and TLTs

are. Ongoing studies in our lab have demonstrated, by use of a TLT2 fusion protein, that the putative TLT2 ligands (s) are expressed by neurons in the brain. Given this information, the TLT2 receptor may serve as a newly identified route of communication between CNS neurons and microglia. However, we have also found that CNS neurons, microglia and CNS infiltrating macrophages express putative TREM2 ligands. This would indicate that TREM2 also allows microglia to communicate with neurons, infiltrating macrophages and self activate the TREM2 receptor. Moreover, the ability of microglia to communicate with these cell types differs with postnatal brain development and with inflammation. As TLT2 is not developmentally regulated, this demonstrates that the constant communication between the two cell types via this receptor is important during the postnatal period and in the mature brain. However, as TLT2 expression is regulated by inflammation, we can only wonder as to why the increased expression of TLT2 is necessary, and what is being communicated.

Systemic inflammation has also been shown to cause the recruitment of peripheral macrophages into the brain. Though we did observe macrophage recruitment into the brain at all ages, unexpectedly, the degree of macrophage recruitment was age-specific even though the mice received the same dose of LPS. Specifically, we established that at P8, the macrophage recruitment was 6 fold greater than that observed in the mature murine brain. This would suggest that the manner in which macrophages entered the brain differed with age. Though the timing of BBB closure has been debated for some time, the commonly accepted hypothesis is that the barrier is formed during embryogenesis and its development is complete at birth (Risau et al. 1990, Daneman et

al. 2010). This would indicate that other mechanisms are responsible for the observed age-specific recruitment of macrophages. Our results depict that the chemokine CCL2 is induced in the brain following systemic inflammation in an age-specific manner. However, it does not fully explain the macrophage infiltration results because CCL2 mRNA is relatively high in the inflamed brain at P15 as well, suggesting that we should also see high macrophage influx then.

Several studies have used TREM2KO mice to identify the importance of TREM2 expression. Moreover, people who lack functional TREM2 signaling develop Nasu-Hakola disease characterized by bone cysts, early onset cognitive dementia and death by their 40's or 50's (Thrash et al. 2009). However, the role of TREM2 in directly or indirectly regulating macrophage influx into the brain has not been reported. Turnbull and colleagues previously demonstrated that TREM2 expression inhibited cytokine production by macrophages exposed to TLR agonists. However, the macrophages used in their studies were isolated from either the peritoneum or bone marrow and then matured and stimulated in culture (Turnbull et al. 2006). In our studies, we detected high TNFα, iNOS, Arginase 1, CCL2 and CCL5 in the healthy, unmanipulated TREM2KO mouse brain. We have not yet explored what cell types are producing these inflammatory molecules in abundance.

Overall, we have used an ex vivo approach to understand how microglia would respond to systemic inflammation at different postnatal periods of brain development. We also examined the phenotypes of microglia and CNS infiltrating macrophages to

understand what their possible roles would be if systemic inflammation were experienced at these ages. Though we have not performed functional studies, the observed phenotypes following systemic inflammation would suggest that microglia at all ages would promote tissue repair and wound healing functions while the infiltrating macrophages would perform the pro-inflammatory, pathogen defense functions. Moreover, because microglia also express the pro-inflammatory molecules B7.2 and CD40 during the early postnatal period, these cells are also prepared to perform certain pathogen defense functions.

4.5 FIGURES AND LEGENDS

Figure 4.1 Microglia display a pre-activated but non-polarized phenotype during the early postnatal period that is lost as the brain matures. Flow cytometric detection of microglia isolated from the naive murine brain at postnatal day 7 (P7) **(A)**. Flow cytometric analysis of age-specific microglial expression of TREM2 **(B)**, Macrophage Mannose Receptor **(C)**, PD-L1 **(D)**, CX3CR1 **(E)**, CD200R **(F)**, MHC Class II **(G)**, CD40 **(H)**, B7.2 **(I)** and TLT2 **(J)** on microglia acutely isolated from the naïve murine brain at postnatal day 2 (P2) or postnatal day 3 (P3), postnatal day 7 (P7), postnatal day 14 (P14), postnatal day 21 (P21) and 3 months. Background is depicted as the grey shaded histogram.

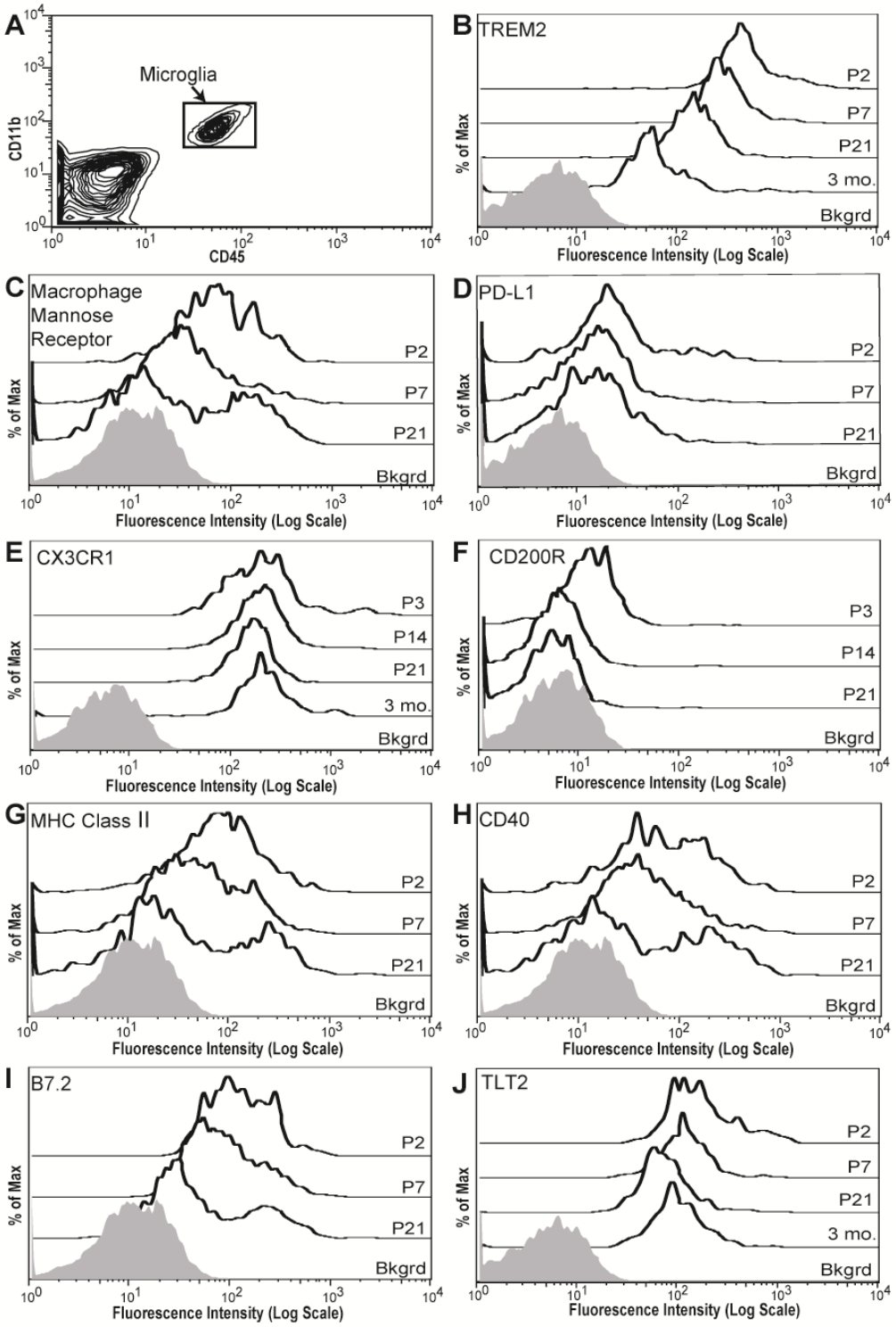

A
Microglia
CD11b
CD45
B
TREM2
P2
P7
P21
3 mo.
Bkgrd
% of Max
Fluorescence Intensity (Log Scale)
C
Macrophage
Mannose
Receptor
D
PD-L1
E
CX3CR1
P3
P14
F
CD200R
G
MHC Class II
H
CD40
I
B7.2
J
TLT2

Figure 4.2. Microglia express pro-inflammatory molecules during the early postnatal period but do not increase their expression of CD40 or B7.2 following systemic inflammation. Flow cytometric detection of microglial expression of CD40 from unmanipulated (dashed line) and IP LPS injected mouse (thick black line) at P4 **(A)**, P8 **(C)**, P15 **(E)**, P22 **(G)** and 3 months **(I)**. Flow cytometric detection of microglial expression of B7.2 from unmanipulated (dashed line) and IP LPS injected mouse (thick black line) at P4 **(B)**, P8 **(D)**, P15 **(F)**, P22 **(H)** and 3 months **(J)**. Background fluorescence is represented by the thin black lined (unmanipulated) and shaded (IP LPS) histograms.

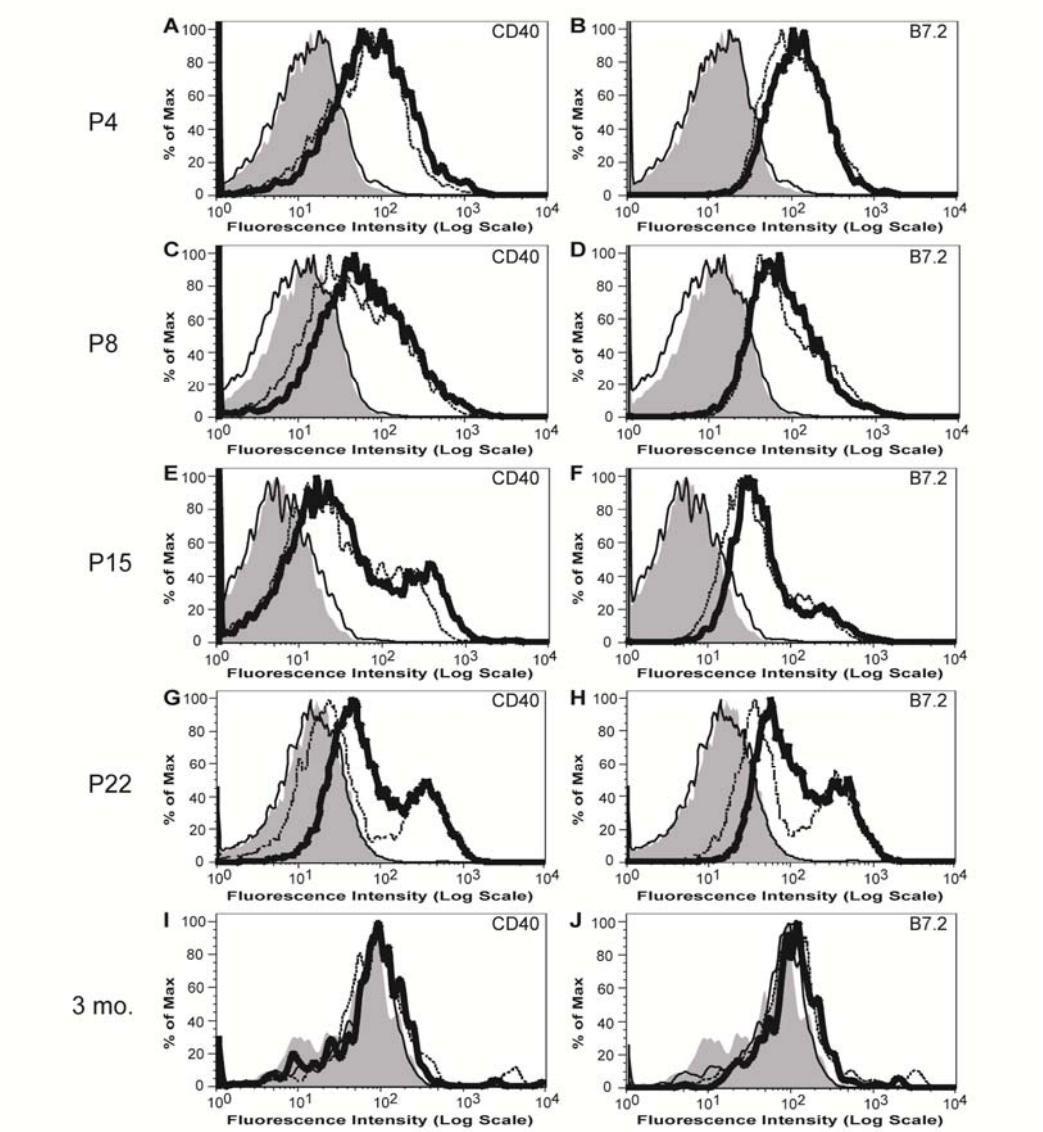

P4
P8
P15
P22
3 mo.
A
B
C
D
E
F
G
H
I
J
CD40
B7.2
% of Max
Fluorescence Intensity (Log Scale)

Figure 4.3 Systemic inflammation leads to acute microglial induction of the anti-inflammatory molecules TREM2, PD-L1 and CX3CR1 and TLT2, a TREM family member. Flow cytometric detection of microglial expression of TREM2 from unmanipulated (dashed line) and IP LPS injected mouse (thick black line) at P4 **(A)**, P8 **(E)**, P15 **(I)**, P22 **(M)** and 3 months **(Q)**. Flow cytometric detection of microglial expression of PD-L1 from unmanipulated (dashed line) and IP LPS injected mouse (thick black line) at P4 **(B)**, P8 **(F)**, P15 **(J)**, P22 **(N)** and 3 months **(R)**. Flow cytometric detection of microglial expression of CX3CR1 from unmanipulated (dashed line) and IP LPS injected mouse (thick black line) at P4 **(C)**, P8 **(G)**, P15 **(K)**, P22 **(O)** and 3 months **(S)**. Flow cytometric detection of microglial expression of TLT2 from unmanipulated (dashed line) and IP LPS injected mouse (thick black line) at P4 **(D)**, P8 **(H)**, P15 **(L)**, P22 **(P)** and 3 months **(T)**. Background fluorescence is represented by thin black lined (unmanipulated) shaded (IP LPS) histograms.

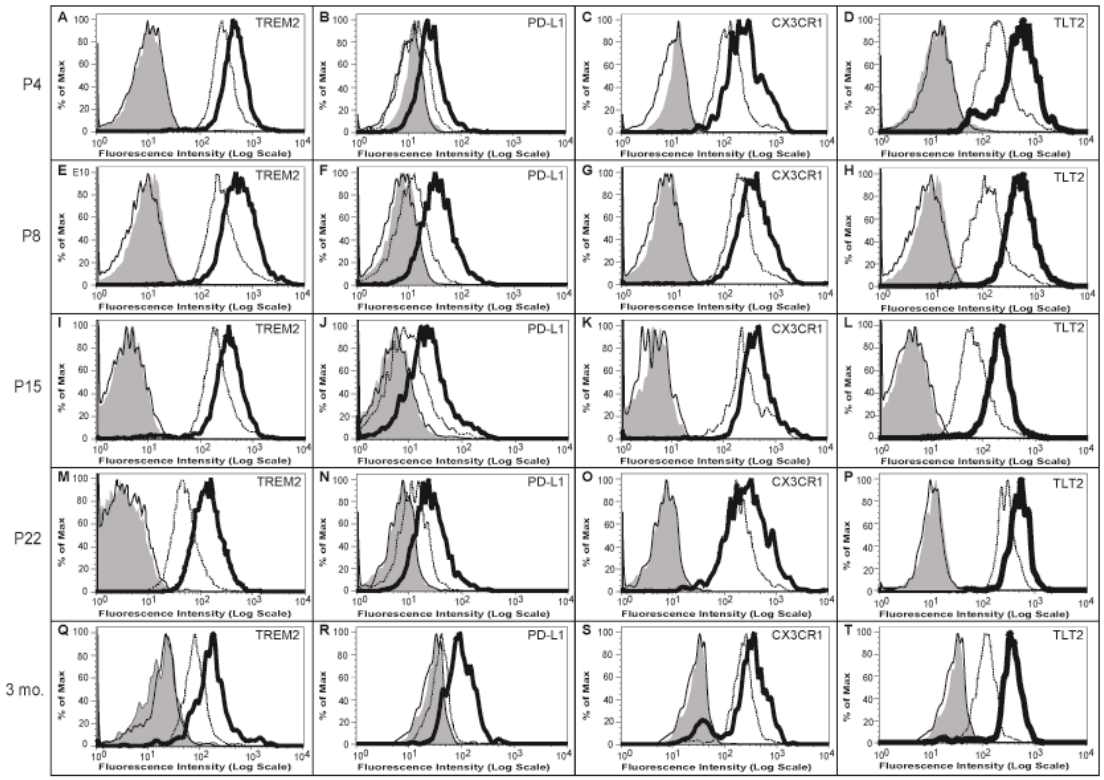

P4
P8
P15
P22
3 mo.
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
Q
R
S
T
TREM2
PD-L1
CX3CR1
TLT2
% of Max
Fluorescence Intensity (Log Scale)

Figure 4.4 Systemic inflammation causes the active recruitment of pro-inflammatory macrophages into the brain in a developmentally regulated fashion. Flow cytometric detection of microglia and CNS infiltrating macrophages isolated 24 hours post IP LPS injection from the brain **(A)**. Flow cytometric quantification of age specific macrophage influx into the CNS 24 hours post IP LPS injection **(B)**. qRT-PCR and standard curve (copy number) analysis of CCL2 **(C)** and CCL5 **(D)** mRNA induction using cDNA templates prepared from whole brains of P4, P8, P15 and 3 month old unmanipulated (light grey bar) and IP LPS injected (dark grey bar) mice. Flow cytometric detection of TREM1 expression by CNS resident microglia **(E, thick black lined histogram)**, and a greater induction by infiltrating macrophages **(F, long dashed histogram)**. Background fluorescence is represented by the shaded (IP LPS) histogram. qRT-PCR and standard curve (copy number) analysis of iNOS **(G)** and Arginase 1 **(H)** mRNA induction using cDNA templates prepared from whole brains of P4, P8, P15 and 3 month old unmanipulated (light grey bar) and IP LPS injected (dark grey bar) mice.

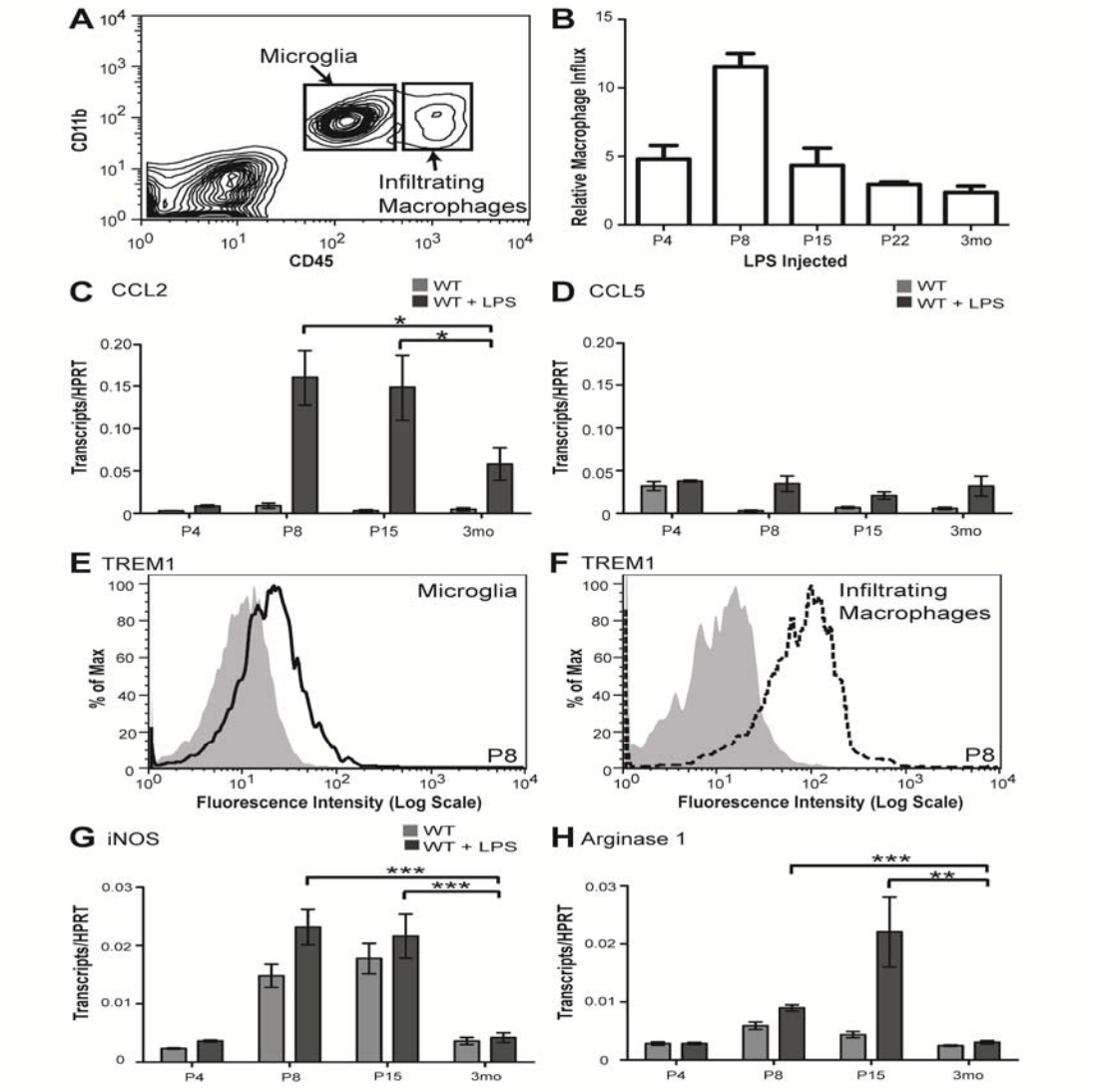

A
Microglia
Infiltrating Macrophages
CD11b
CD45
B
Relative Macrophage Influx
P4
P8
P15
P22
3mo
LPS Injected
C CCL2
WT
WT + LPS
Transcripts/HPRT
D CCL5
E TREM1
Microglia
P8
% of Max
Fluorescence Intensity (Log Scale)
F TREM1
Infiltrating Macrophages
G iNOS
H Arginase 1

Figure 4.5 TREM2 binding activity is detected on CNS neurons, microglia and CNS infiltrating macrophages. Illustration of TREM2 fusion protein used to identify TREM2 ligands **(A)**. Immunohistochemistry of 3 month old murine brain depicting Iba1 (green) expression of TREM2 ligand (red) **(B)**. Neurons expressing TREM2 ligands are depicted by white arrows. Flow cytometric detection of TREM2 ligands on microglia from unmanipulated (dark dashed line) and IP LPS injected mice (thick black line) at P8 **(C)** and 3 months **(E)**. Flow cytometric detection of TREM2 ligands on CNS infiltrating macrophages (light dashed line) at P8 **(D)** and at 3 months **(F)**. Background fluorescence represented by thin black lined (unmanipulated) and shaded (IP LPS) histograms.

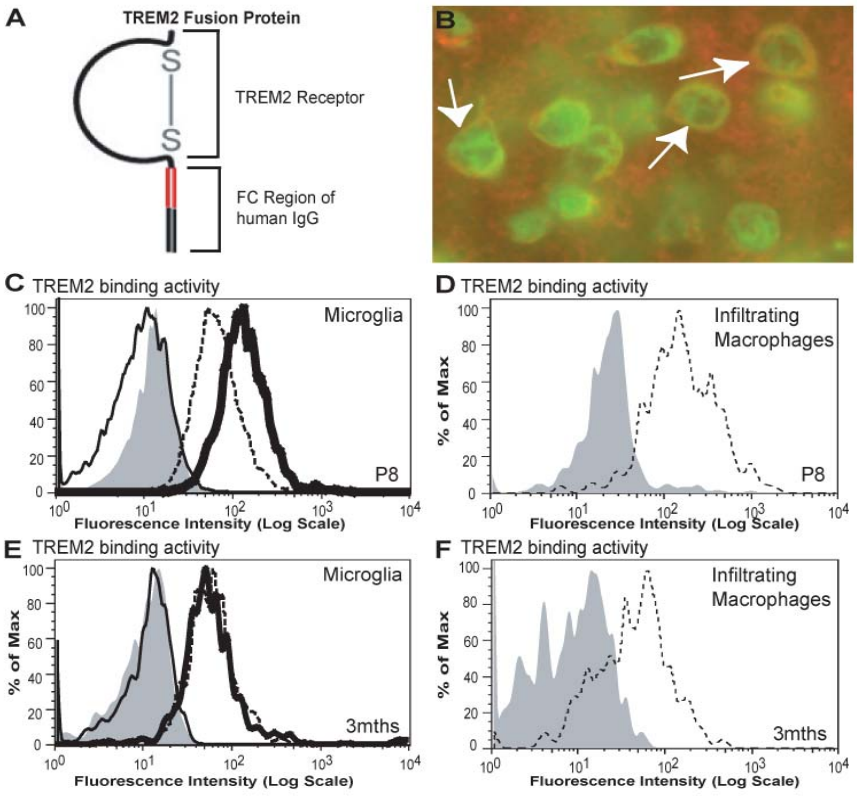


Figure 4.6 The absence of TREM2 directly or indirectly regulates macrophage influx into the brain. Flow cytometric analysis of microglial expression of TREM2 **(A)** and TLT2 **(B)** from unmanipulated (thick dashed line) WT mice and one week post P14 IP LPS injection (thick black line) of WT mice. Background fluorescence represented by thin black lined (unmanipulated) and shaded (IP LPS) histograms. Flow cytometric analysis of CD45 levels from CD11b+ population of single cell brain suspensions isolated from unmanipulated (thin black line) and IP LPS injected (thin dashed line) WT mice 24 hours post P14 injection **(C)** or one week post P14 injection **(D).** Flow cytometric analysis of CD45 levels from CD11b+ population of single cell brain suspensions isolated from unmanipulated (thin black line) and IP LPS injected (thin dotted line) TREM2KO mice 1 day post P14 injection **(E)** or 1 week post injection **(F)**. Flow cytometric analysis of microglial expression of TLT2 from unmanipulated (thick dashed line) and IP LPS injected (thick black line) TREM2KO mice one week post P14 IP LPS injection **(G)** or one week post 3 month old mouse injection **(H)**. Background fluorescence represented by thin black lined (unmanipulated) and shaded (IP LPS) histograms.

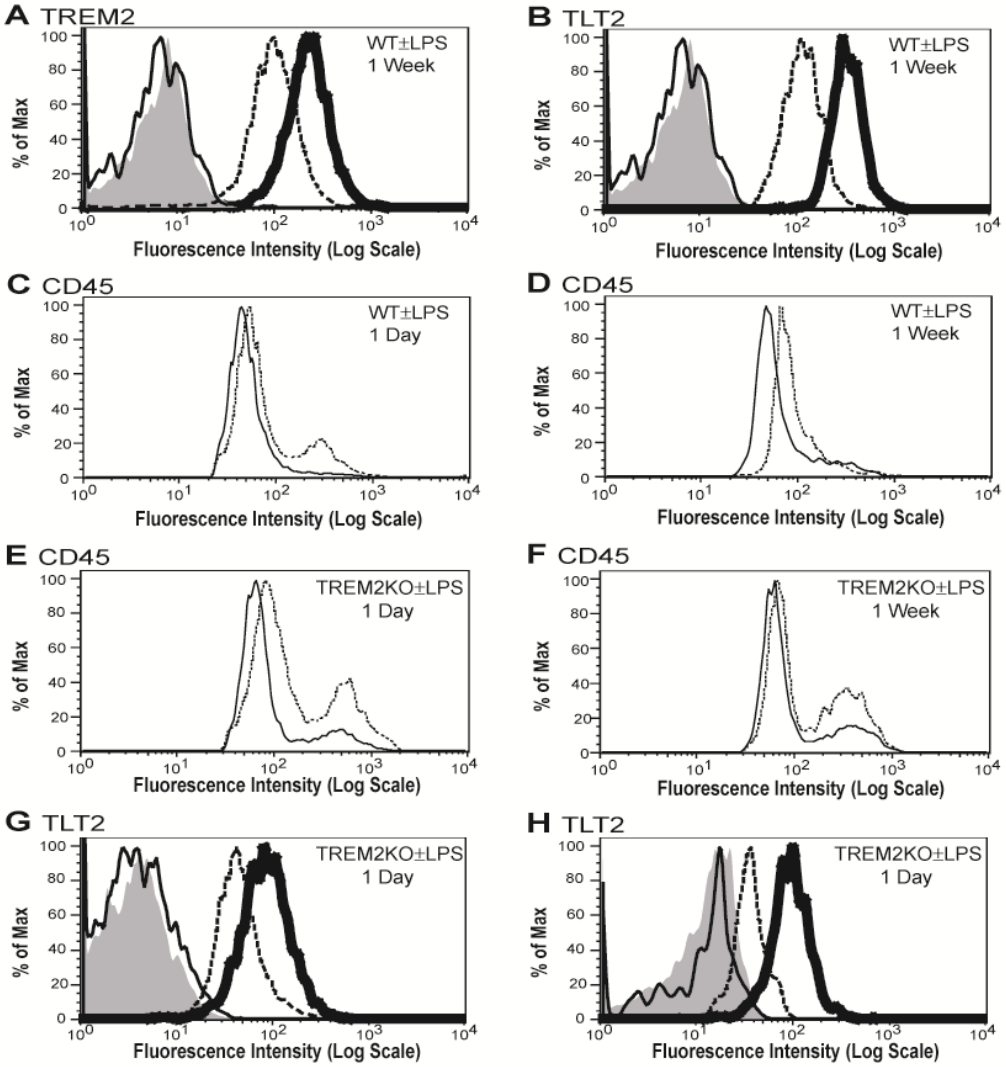
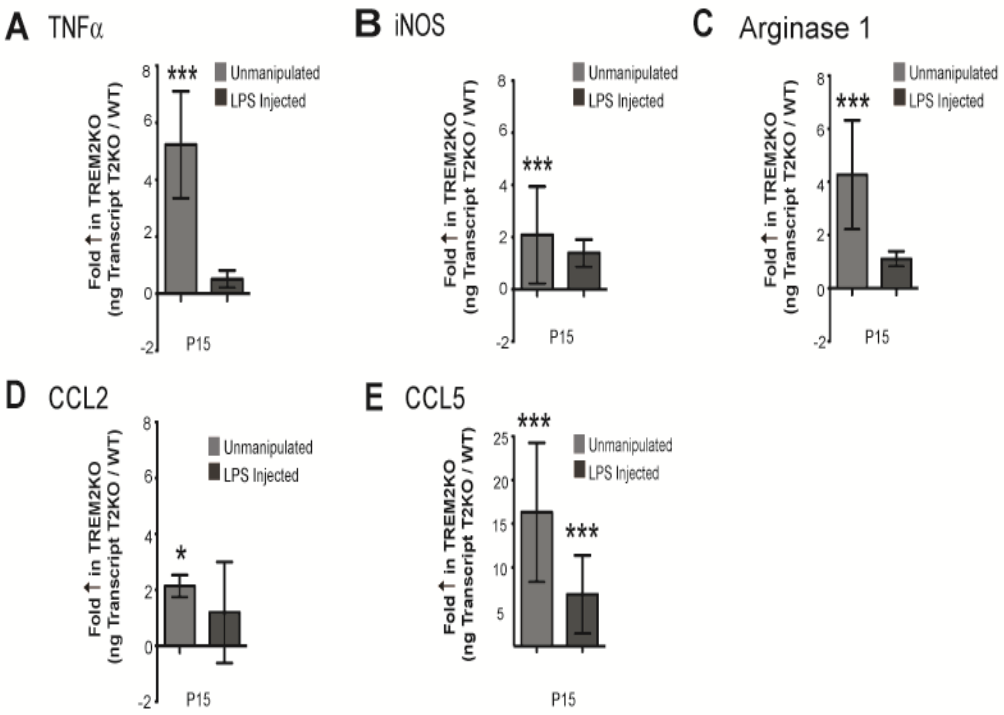

A TREM2
WT±LPS
1 Week
B TLT2
WT±LPS
1 Week
C CD45
WT±LPS
1 Day
D CD45
WT±LPS
1 Week
E CD45
TREM2KO±LPS
1 Day
F CD45
TREM2KO±LPS
1 Week
G TLT2
TREM2KO±LPS
1 Day
H TLT2
TREM2KO±LPS
1 Day
% of Max
Fluorescence Intensity (Log Scale)

Figure 4.7 TREM2KO mice exhibit chronic inflammation in the unmanipulated brain. qRT-PCR and standard curve (copy number) analysis of TNFα **(A)**, iNOS **(B),** Arginase 1 **(C)**, CCL2 **(D)** and CCL5 **(E)** fold mRNA induction (nanogram transcripts TREM2KO/WT) using cDNA templates prepared from whole brains of P15 unmanipulated (grey bar) and IP LPS injected (dark grey bar) mice.



CHAPTER 5

Healthy aging causes decreased microglial induction of anti-inflammatory molecules following LPS induced CNS inflammation

5.1 ABSTRACT

The activation of microglia in the healthy aged brain has been correlated to the development and/or pathogenesis of several neurodegenerative diseases. With healthy aging, the expression of pro-inflammatory molecules such as IL-1, IL-6, MHC Class II, B7.2 and IFNγ increase in the brain in the absence of pathology. Here we examine whether molecules associated with anti-inflammatory microglial activation can be found in the aged (15 month) brain and whether their expression changes following LPS induced CNS inflammation. Flow cytometric analysis of ex vivo microglia acutely isolated from the CNS of unmanipulated and ICV LPS injected mice illustrates that basal levels of the anti-inflammatory molecules TREM2, TREM2 binding activity and CX3CR1 can be found in the 3 month old brain while only TREM2 and CX3CR1 can be found in 15 month old aged brains. Moreover, LPS induced CNS inflammation causes increased microglial expression of TREM2, putative TREM2 ligands and CX3CR1 in the young adult brain, but only TREM2 in the aged brain, although CX3CR1 is also expressed by microglia in the aged brain. These data suggest that microglia in the aged brain do express anti-inflammatory molecules and that the expression of some, but not all, increase following LPS induced CNS inflammation.

5.2 INTRODUCTION

Dysregulated microglial activation is observed in the aging brain that primes these cells for future inflammatory immune responses. Under normal circumstances, homeostatic microglia in the healthy brain respond to inflammation or tissue injury by adopting a morphology characterized by swelling of the cell body, thickening of the processes, and expression of molecules associated with pathogen defense and/or tissue repair functions. Interestingly, research has shown that the brains of aged humans or rodents contain reactive microglia even in the absence of pathology. Moreover, following peripheral immune activation, microglia in aged brains exhibit exaggerated immune responses compared to younger individuals (Godbout et al. 2005). Generally a specific stimulus is required to activate microglia. However, neither the specific stimulus that leads to persistent microglial activation in the aged brain, nor the type of microglial activation that occurs following inflammation in the aged brain are well understood. The answers to these questions will undoubtedly clarify our understanding of microglial roles in the development of neurodegenerative diseases.

A variety of studies have shown that with normal brain aging, microglia acquire an activated phenotype (Frank et al. 2006, Dilger et al. 2008, Perry 2010). To support this, several immunohistochemical studies have demonstrated that microglial expression of MHC Class II increases with healthy aging (Perry et al. 1993, Ogura et al. 1994, Carson lab unpublished data). Interestingly, studies by Frank and colleagues have illustrated that healthy aging promotes the upregulation of not only MHC Class II, but

also B7.2 and IFNγ mRNA in the hippocampal region of the brain. Likewise, studies by Godbout et al. illustrate that the mRNA of the pro-inflammatory cytokines IL-1β and IL-6 also increases in the aged murine brain. Because Frank and colleagues also found a decrease in IL-10 mRNA expression, an anti-inflammatory cytokine, these findings strongly suggest that healthy aging promotes the production of pro-inflammatory immune responses in the brain (Godbout et al. 2005, Frank et al. 2006). Interestingly, Godbout and colleagues later found using ex vivo approaches that microglia in the aged brain upregulate the anti-inflammatory cytokine, IL-10. This demonstrates that microglial production of anti-inflammatory factors in the aged brain is not well understood at this point.

The neuronal expression of several specific molecules in the brain (CD200, CX3CL1, TREM2 ligands) has been shown to prevent neurotoxic microglial activation. Interestingly, studies performed by Frank and colleagues have also demonstrated that with healthy brain aging, the expression of CD200 mRNA in the brain decreases. Papers have shown that mice lacking functional CD200 have activated microglia and also exhibit a more rapid onset of EAE (Hoek et al. 2000). This demonstrates that neuronal CD200 is required to prevent the pro-inflammatory activation of microglia. These findings suggest that the age related decrease in CD200 mRNA may, in part, explain why microglia become activated with healthy aging.

In contrast, others have suggested that the observed increase in microglial activation markers may not indicate microglial activation, but instead mircroglial aging

and/or senescence (Streit et al. 2006). However, studies have shown that peripheral inflammation causes a more exaggerated immune response in the brains of aged individuals when compared to young adults. Specifically, Godbout and colleagues illustrated that systemic inflammation led to significantly higher levels of IL-1β and IL-6 mRNA and protein in the brains of aged mice than in the brains of young adult mice. Though these findings do not identify the cell types producing the two cytokines, the data strongly suggests that peripheral inflammation in the aged individual promotes an exaggerated neuro-immune response (Godbout et al. 2005). Moreover, the data also suggests that the cells producing the higher levels of IL-1β and IL-6 are primed. Though the elevated level of the discussed pro-inflammatory molecules may serve as markers of aged or senescent microglia, they also indicate that these cells are primed in the aged brain and thus could possibly contribute to the development and/or pathogenesis of several neurodegenerative diseases.

Several markers associated with the pro-inflammatory activation of microglia have been examined within the healthy and inflamed aged brain. However, data demonstrating whether microglia express anti-inflammatory markers in the healthy or inflamed aged brain is lacking. Here we will use an ex vivo approach to determine whether the expression of molecules associated with anti-inflammatory microglial activation, namely TREM2, TREM2 binding activity (used to detect putative TREM2 ligands) and CX3CR1 (1) are differentially expressed in the young adult (3 month) versus the aged (15 month) murine brain and (2) whether there is an age specific difference in their expression following systemic inflammation.

5.3 RESULTS

5.3.1 Microglia increase expression of anti-inflammatory markers following systemic inflammation at 3 months, but not at 15 months.

Several studies have demonstrated that microglia become activated with normal aging. Moreover, studies have demonstrated that microglia become primed with aging so that when systemic inflammation is experienced, higher levels of pro-inflammatory cytokines are produced within the aged brain than in the young adult brain (Godbout et al. 2005). To determine whether systemic inflammation in the young adult and aged murine brain causes differential expression of anti-inflammatory markers expressed by microglia, we isolated microglia from 3 month old or 15 month old ICV LPS injected mice 24 hours post injection and analyzed microglial expression of anti-inflammatory molecules TREM2, TREM2 binding activity and CX3CR1. We observed that microglia in the 3 month old unmanipulated brain express TREM2 and that inflammation causes an increase in its expression (Fig. 5.1A). At 15 months, however, we could detect low levels of TREM2 expression on microglia that increased slightly with inflammation (Fig. 5.1B). Interestingly, we also detected TREM2 binding activity (identifying putative TREM2 ligands) on microglia from the 3 month old murine brain (Fig. 5.2A). However, at 3 months inflammation only caused a percentage of the microglia to increase TREM2 binding activity (Fig. 5.2A). The majority of microglia were not positive for TREM2 binding activity in the 15 month old brain (Fig. 5.2B). Systemic inflammation in the 15 month old murine brain did not alter TREM2 binding activity on microglia. Though

CX3CR1 could be detected on microglia at 3 months, only a very small population of microglia induced their expression of CX3CR1 with inflammation (Fig. 5.3A). Interestingly, we could still detect CX3CR1 expression on microglia at 15 months but systemic inflammation did not change its expression (Fig. 5.3B).

5.4 DISCUSSION

Previously, studies have clearly illustrated that microglia become activated in the aged brain even in the absence of pathology. Moreover, studies have also shown that microglia exhibit exaggerated immune responses following inflammation in aged individuals, indicating that they are primed. Consequently, many have sought to examine why microglia become activated in the aged brain, what type of immune responses they promote in the aged brain following inflammation and whether this apparent physiological or pathological activation of microglia could somehow facilitate the development of neurodegenerative diseases. Though research has not yet revealed what causes microglial activation in the aged brain, many have demonstrated that the CNS microenvironment becomes increasingly pro-inflammatory with healthy aging. Several studies have illustrated that higher levels of MHC Class II, IL-1β and IFNγ are found in aged brains than in young adult brains (Godbout et al. 2005, Frank et al. 2006).

Many of the studies demonstrating the pro-inflammatory microenvironment of the aged brain have used immunohistochemical techniques or qRT-PCR. Though the research has definitely revealed important details regarding the microenvironment of the aged brain, many of these studies have not conclusively identified the cell types responsible for creating the pro-inflammatory CNS environment. Recently, data generated in our lab and others (unpublished, Henry et al. 2009) have demonstrated that CNS resident microglia become increasingly activated with healthy aging. Using flow cytometric studies of acutely isolated CNS resident microglia from unmanipulated 3, 8

and 15 month old mouse brains, our lab has found that microglia increase their expression of MHC Class II, a molecule important for antigen presentation, and the co-stimulatory molecules, B7.2 and CD40 (unpublished data) with healthy aging. These findings demonstrate that in the absence of any pathology, microglial expression of molecules associated with activation and pro-inflammatory responses increases with healthy aging. Moreover, our lab has also found that healthy aging alone led to the increased influx of macrophages into the brain (Carson lab unpublished data). This indicates that the aged CNS microenvironment is characterized by a constant level of chronic inflammation.

Recent findings also demonstrate that systemic inflammation causes exaggerated microglial inflammatory responses in the aged brain, indicating that these cells become primed with healthy aging (Carson lab unpublished data, Henry et al. 2009). Specifically, an ICV injection of LPS led to higher expression of MHC Class II, CD40 and B7.2 by microglia isolated from the whole brains of 8 month old and 15 month old mice than 3 month old mice (Carson lab unpublished data). Henry and colleagues observed that exaggerated microglial responses to systemic inflammation, indicated by the induction of IL-1β mRNA and protein by microglia isolated from IP LPS injected mice, was greater in aged mice than in young adult mice. Interestingly, recent studies have also demonstrated that microglia primed in the aged CNS do not only produce high levels of pro-inflammatory molecules. Data from both our lab (unpublished) and from Henry et al. illustrate that microglia in the aged brain also respond to inflammation by inducing higher levels of TREM2 and IL-10, both known to play roles in anti-inflammatory

immune responses. However, our lab has found that a polarized anti-inflammatory response is not generated either as mannose receptor, a well known marker of alternatively activated microglia/macrophages, is not induced in the aged brain following primary CNS inflammation.

In general, evidence regarding the presence of anti-inflammatory immune molecules in the aged brain is lacking. Frank and colleagues illustrated that IL-10 mRNA decreases in the hippocampal regions of the brain with aging. However, using ex vivo approaches, Godbout and colleagues clearly demonstrated that IL-10 protein increases in microglia isolated from the cortex. Though microglia are found throughout the brain, they are capable of forming different populations in different regions. Perhaps these findings suggest that microglia in the aged cortex express IL-10 while hippocampal microglia decrease their expression of IL-10.

Due to the unclear IL-10 findings in the aged brain, we sought to determine whether microglia (1) express anti-inflammatory molecules in the aged brain by examining the expression profile of several known molecules and (2) increase their expression of anti-inflammatory molecules following inflammation. Our findings here illustrate that we can detect TREM2 and CX3CR1 expression on microglia from the young adult and aged murine brains. Though TREM2 binding activity (labeling putative TREM2 ligands) can be found on microglia at 3 months, it cannot be detected in the aged brain. This may indicate that the tissue repair and anti-inflammatory functions of TREM2 are more apparent in the young (data not shown) and young adult brain, but not in the

aged brain. However, we have also detected TREM2 binding activity on neurons (data not shown). We have not yet determined whether TREM2 binding activity decreases by 15 months. However, using immunohistochemical methods, we have found that TREM2 binding activity on neurons decreases from the early postnatal period to 3 months. These findings illustrate that with healthy aging, microglia also express some markers associated with anti-inflammatory immune responses.

Previous data from our lab demonstrates that TREM2 expression by CNS resident microglia increases following LPS induced CNS inflammation in an age-dependant manner (Carson lab unpublished data). Specifically, we found that there is a greater induction of TREM2 expression on microglia following LPS induced primary CNS inflammation in the 3 month old brain than in the 15 month old brain (Carson lab unpublished data). Here we have confirmed these results. Interestingly, we have also found that a small population of CNS resident microglia increase their expression of putative TREM2 ligands in the 3 month old brain but not in the aged brain. This may indicate that at 3 months, microglia are able to self activate the anti-inflammatory TREM2 receptor following inflammation, but are not capable of doing so in the aged brain. Lastly, though we observed CX3CR1 expression by microglia at 15 months, we could not detect an increase in its expression following inflammation.

Overall, this data demonstrates that microglia in the young adult brain are more capable of mounting anti-inflammatory immune responses than in the aged brain. However, we cannot dismiss the fact that TREM2 expression does increase following

LPS induced CNS inflammation at 15 months and that CX3CR1 is still present on microglia at this age. This demonstrates that mechanisms to prevent pro-inflammatory activation of microglia are present in the 15 month old brain. Current findings would indicate that the exaggerated immune responses observed in the aged brain are not polarized. However, future research will need to be performed to better understand whether the presence and induction of anti-inflammatory molecules by microglia in the aged CNS is functionally significant in a microenvironment where microglia are primed and numerous pro-inflammatory molecules are also found.

5.5 FIGURES AND LEGENDS

Figure 5.1 Inflammation causes increased expression of TREM2 in the young adult but not in the aged brain. Flow cytometric detection of microglial expression of TREM2 **(A)** in unmanipulated (dashed line) and ICV LPS injected (thick black line) 3 month old mice. Flow cytometric detection of microglial expression of TREM2 **(B)** in unmanipulated (dashed line) and ICV LPS injected (thick black line) 15 month old mice. Background fluorescence is represented by thin black lined (unmanipulated) and shaded (IP LPS) histograms.

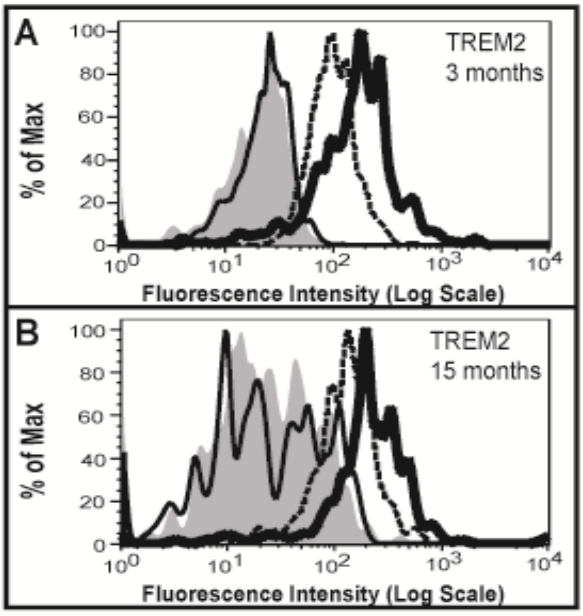


Figure 5.2 Inflammation causes increased TREM2 binding activity in young adult but not in the aged brain. Flow cytometric detection of microglial expression of TREM2 binding activity **(A)** in unmanipulated (dashed line) and ICV LPS injected (thick black line) 3 month old mice. Flow cytometric detection of microglial expression of TREM2 binding activity **(B)** in unmanipulated (dashed line) and ICV LPS injected (thick black line) 15 month old mice. Background fluorescence is represented by thin black lined (unmanipulated) and shaded (IP LPS) histograms.

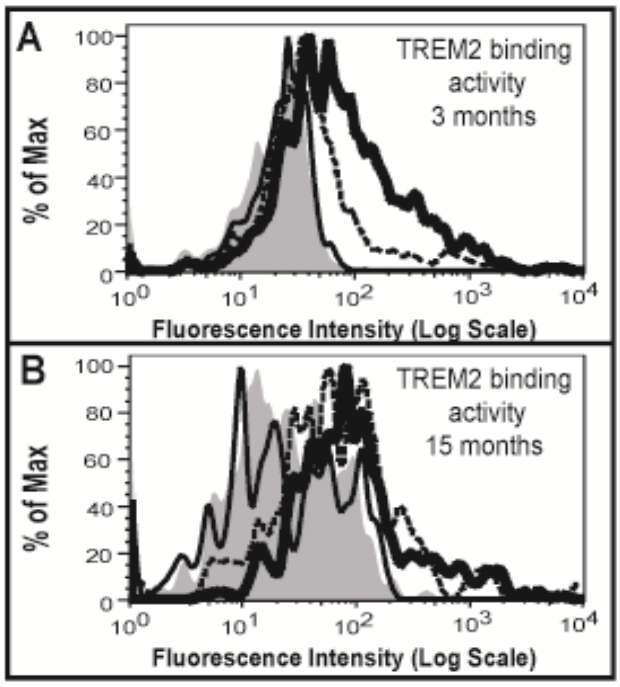
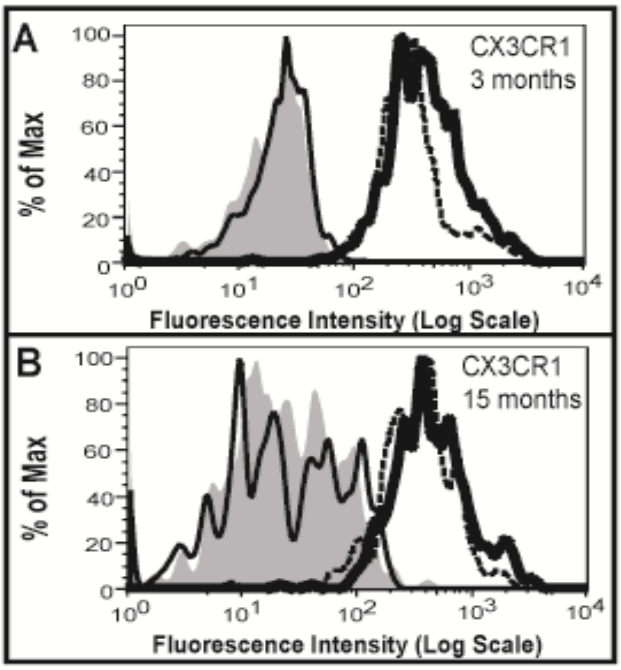


Figure 5.3 Inflammation causes increased expression of CX3CR1 in young adult but not in the aged brain. Flow cytometric detection of microglial expression of CX3CR1 **(A)** in unmanipulated (dashed line) and ICV LPS injected (thick black line) 3 month old mice. Flow cytometric detection of microglial expression of CX3CR1 **(B)** in unmanipulated (dashed line) and ICV LPS injected (thick black line) 15 month old mice. Background fluorescence is represented by thin black lined (unmanipulated) and shaded (IP LPS) histograms.



CHAPTER 6

DISCUSSION

Microglia are the resident immune cells of the brain. As such, their activation is associated with a range of conditions in the healthy and inflamed CNS. In the healthy brain, microglia maintain homeostasis by continuously sampling their environment (Nimmerjahn et al. 2005). When CNS infection and/or inflammation occurs, microglia respond appropriately by changing their activation state. Recent studies illustrate that microglial activation is not an "all or nothing" event. Instead, like peripheral macrophages, microglia exhibit a range of activation states associated with pathogen defense, wound healing and tissue repair functions (Colton et al. 2006, Mosser et al. 2008).

Though microglial activation has been observed in the developing brain, the type of activation and the functions associated with the particular activation state (s) have not been closely examined. Studies by Hristova and others have clearly demonstrated that microglia in subcortical white matter areas of the brain convert from an activated phenotype during the early postnatal period to a resting or homeostatic phenotype observed during the second postnatal week. Though they also identified that several markers of microglial activation changed with healthy postnatal brain development, whether microglia adopt a specific type of activation state during this period is unknown.

Systemic inflammation experienced during the perinatal period and subsequent microglial activation have recently been associated with the development and/or

pathogenesis of several neurodevelopmental disorders (Chew et al., 2006; Doorduin et al., 2008; Pardo et al., 2005). Moreover, epidemiological, immunohistochemical and imaging techniques have shown that microglia are activated in the brains of patients of various ages with autism, schizophrenia and cerebral palsy (Vargas et al. 2005, Banati et al. 2009, Doorduin et al. 2009, Haynes et al. 2003). These findings strengthen the hypothesis that microglial activation in the brains of these patients is associated with the pathogenesis of these disorders. However, because microglial functions in the postnatal developing brain are not well defined, the potential roles that microglia may have in these and other neurodevelopmental disorders are unknown.

Interestingly, studies have also shown that microglia become activated in the brain with healthy aging (Frank et al. 2006, Dilger et al. 2008, Perry 2010). This activation is also associated with increased expression of pro-inflammatory molecules such as B7.2, IFNγ, IL-1β and IL-6 (Godbout et al. 2005, Frank et al. 2006). There is currently some debate as to whether anti-inflammatory molecules are upregulated in the aged brain as hippocampal IL-10 mRNA expression has been shown to decrease in the aged brain while cortical IL-10 protein expressed by microglia has been shown to increase (Frank et al. 2006, Godbout et al. 2009). Currently, it is unknown what causes microglia to become activated in the healthy aged brain. It is also unknown whether microglia become activated with age or whether the increased expression of inflammatory molecules serves as an indicator of aged or senescent microglia. However, because systemic inflammation causes greater induction of pro-inflammatory molecules

in the brains of aged mice than in the brains of young adult mice, this demonstrates that microglia do become primed in the aged brain.

In this dissertation, we have examined:

1. The expression of TREM family members in the healthy and inflamed brain.
2. Microglial phenotype during critical periods of postnatal brain development and age-specific CNS immune responses to systemic inflammation during these periods.
3. Microglial expression of anti-inflammatory molecules in the aged brain and microglial responses to LPS induced CNS inflammation in the aged brain.

Interestingly, the TREM receptors seem to be expressed by a variety of cell types within the brain and their roles in innate and adaptive immune responses continue to be identified. TREM1 seems to amplify TLR mediated pro-inflammatory responses throughout the body (Bouchon et al. 2001). However, recent findings suggest that TREM1 may also be involved in the pathogenesis of schizophrenia and bipolar disorder (Weigelt et al. 2011). Though TREM2 expression has consistently been found on CNS resident microglia, we find that TREM3 RNA is highly expressed by CNS infiltrating macrophages. Though studies have shown that TREM3 signals through DAP12, which has an ITAM, we do not yet understand the importance of TREM3 expression. We found that TREM4 is expressed in the brain by subsets of neurons in the olfactory bulb, hippocampus and cerebellum. This is interesting because TREMs, as stated in the name,

are usually expressed by myeloid derived cells throughout the body. This finding, along with TLT2 expression by lymphocytes, demonstrates that TREMs are also expressed by cells other than those derived from the myeloid lineage (King et al. 2006). As we have found that DAP12 is expressed by subsets of microglia in the brain, we still do not understand how TREM4 signals intracellularly, and what its functions are. The TREM family of receptors seem to play a host of roles in innate and adaptive immune responses, brain development and even behaviors that are currently not well understood.

Confirming the results observed by Hristova and colleagues, using an ex vivo approach we also observed that microglia have an activated phenotype during the early postnatal period. However, we found that (1) the activation state is non-polarized and (2) the activation state is lost as the brain matures (3 months). We used several markers of anti-inflammatory (Mannose Receptor, TREM2, PD-L1, CX3CR1) or pro-inflammatory (CD40, B7.2) microglial activation and observed that microglia express intermediate to high levels of both during the early postnatal period. As the brain matures, the expression of several anti-inflammatory and pro-inflammatory markers decrease. This may indicate that the high expression of these receptors and ligands are required by microglia for specific functions needed during postnatal brain development and that they are not required as much in the mature brain. These findings could also indicate that during the early postnatal period microglia express a host of molecules, which may not necessarily serve specific functions, but may indicate a very immature phenotype of microglia during this period.

Interestingly, the expression of CD200, CX3CR1, PD-L1 and TLT2 remains the same throughout postnatal brain maturation. This may indicate that these are required to constantly promote and/or prevent a specific type of microglial activation state and that dysregulated expression of these may affect the homeostatic capabilities of these cells. For example, studies have shown that microglial expression of CD200R and CX3CR1 is required to prevent neurotoxic microglial activation in the brain (Hoek et al. 2000, Cardona et al. 2006).

Our studies have also illustrated that systemic inflammation causes age-specific immune responses. Specifically, we find that microglia express markers of pro-inflammatory activation during the early postnatal period but not in the mature brain. Moreover, following systemic inflammation, microglia increase the expression of anti-inflammatory molecules (TREM2, PD-L1 and CX3CR1). This demonstrates that microglia are prepared to mount pro-inflammatory immune responses during early postnatal periods. However, systemic inflammation increases the ability of microglia to perform wound healing and tissue repair functions. Moreover, increased PD-L1 expression may serve to prevent T cell activation if T cells were recruited while increased TREM2 may also promote microglial phagocytosis and prevent the production of pro-inflammatory cytokines. Furthermore, the increased microglial expression of neuroprotective molecules may serve to protect the brain from the pro-inflammatory macrophages that infiltrate the CNS.

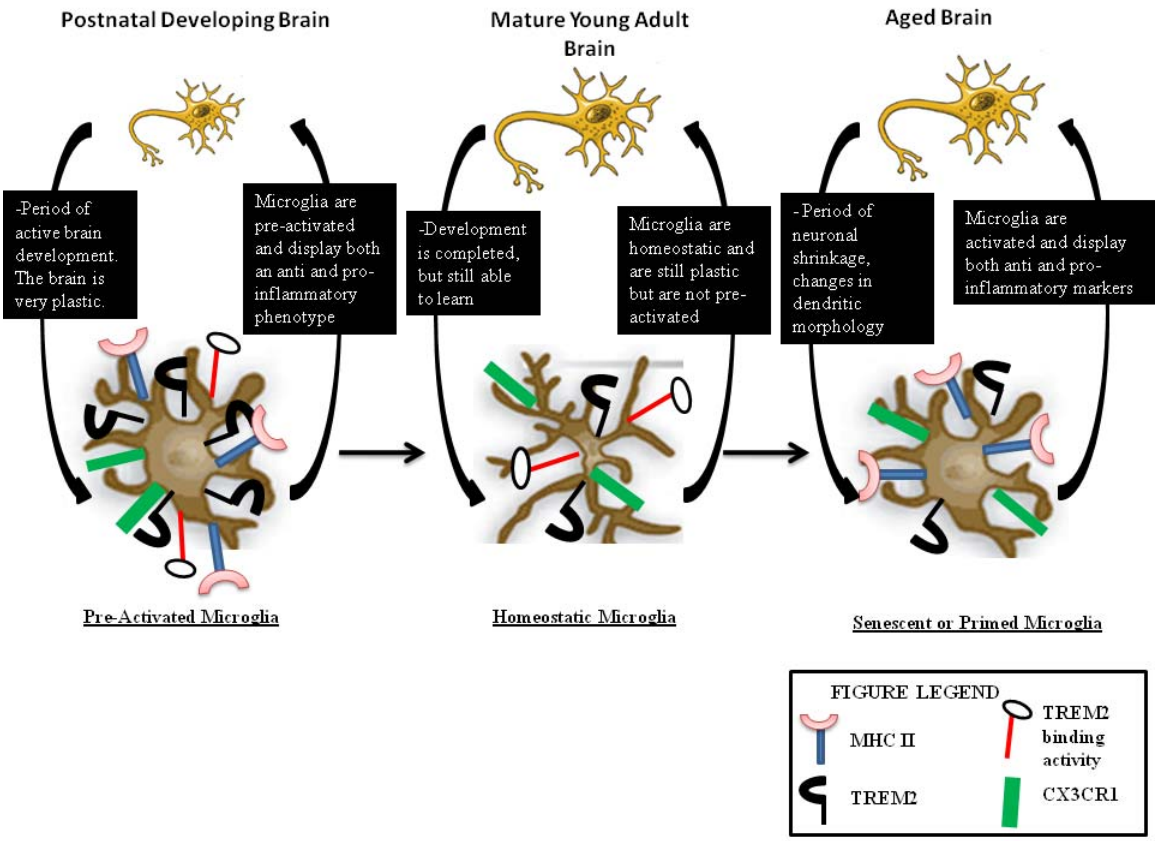
Overall, these studies suggest that if systemic inflammation equivalent to the cold or flu is directly experienced by the fetus or neonate during the perinatal period, microglia promote protective immune responses. This may occur to balance out the effects of the pro-inflammatory macrophages. Interestingly, neuronal damage is not observed (data not shown, Ethell lab). We have not yet tested microglial functions following systemic inflammation and we have not examined microglial phenotypes in rodent models of neurodevelopmental disorders. We also have not determined whether the same changes in microglial phenotype would occur in response to viral infections.

Several studies have shown that in the aged brain, higher levels of various pro-inflammatory factors can be detected. Data concerning the expression of anti-inflammatory molecules in the aged brain is unclear. Interestingly, we have found that microglia in the aged brain do express TREM2, putative TREM2 ligands and CX3CR1 which promote neuroprotective microglial functions (unpublished data, Carson lab). Moreover, our lab has shown that with LPS induced CNS inflammation, microglia increase expression of TREM2. This clearly demonstrates that microglia definitely express molecules associated with anti-inflammatory activation in the aged brain. However, more studies need to be performed to understand the significance of the expression of these anti-inflammatory molecules in relation to the various pro-inflammatory cytokines that have been found in the aged brain.

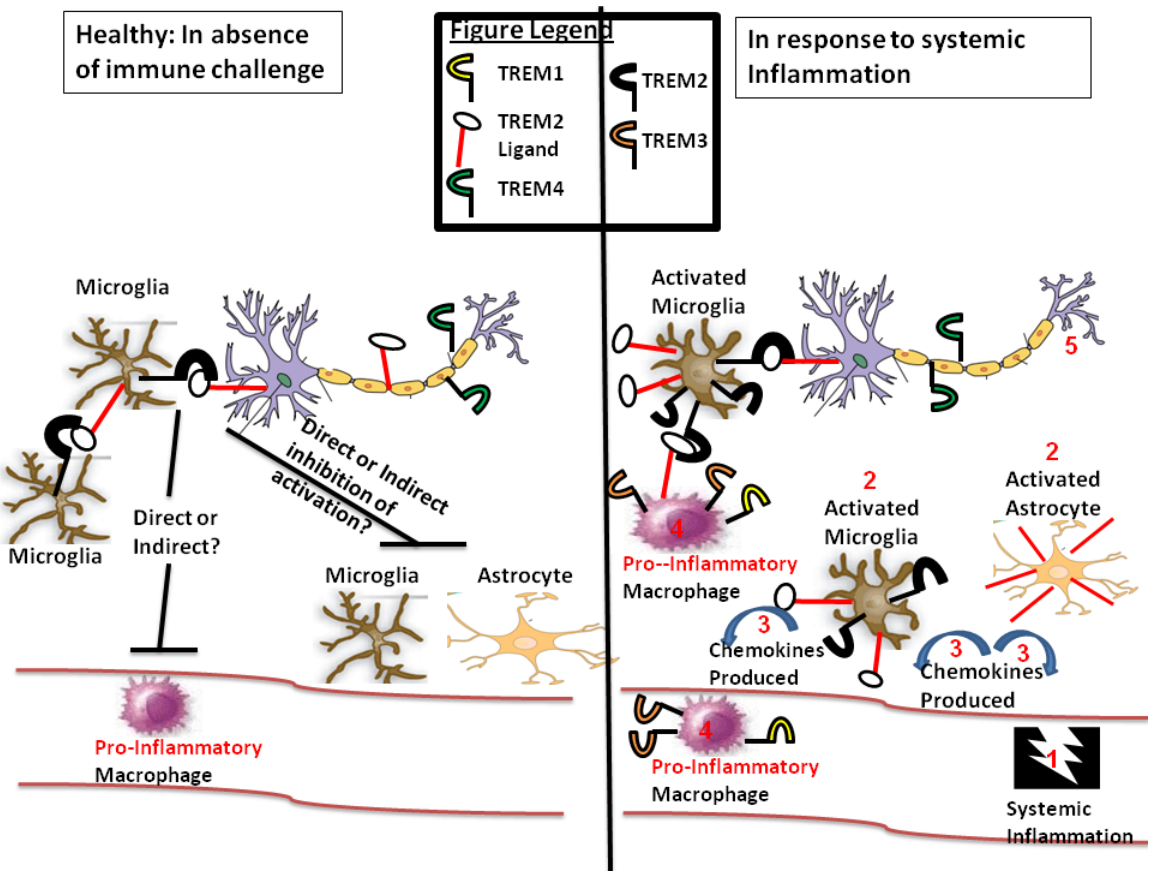
Understanding of microglial phenotype and microglial responses in the healthy developing brain, healthy aged brain and following inflammation is not only critical to

our comprehension of microglial physiology, but will also allow us to understand the roles of these cells in various types of neuropathology. We have clearly shown here that CNS immune responses in the healthy and inflamed brain are age related. Therefore when studying microglial phenotype, one must consider the "age" of the microglia and the processes occurring in the CNS at that time. Continued research in these areas will not only help scientists to understand microglial roles in neurodevelopmental disorders, but will also serve as important stepping stones for the development of therapeutics or the identification of preventative mechanisms for neurodevelopmental disorders.

Developmental & Aging Model



Systemic Inflammation & TREM2 Model



REFERENCES

1. Agarwal, S., Piesco, N.P., Johns, L.P., Ricelli, A.E. (1995) Differential expression of IL-1β, TNFα, IL-6 and IL-8 in human monocytes in response to lipopolysaccharides from different microbes. Journal of Dental Research 74 (4): 1057-1065.

2. Akiyama, H., McGeer, P.L. (1990) Brain microglia constitutively express beta-2 integrins. Journal of Neuroimmunology. 30 (1): 81-93.

3. Allcock, R.J., Barrow, A.D., Forbes, S., Beck, S., Trowsdale, J. (2003) The human TREM gene cluster at 6p21.1 encodes both activating and inhibitoroy silge IgV domain receptors and includes NKp44. European Journal of Immunology. 33 (2): 567-577.

4. Ambrosini, E., Aloisi, F. (2004) Chemokines and glial cells: A complex network in the central nervous system. Neurochemical Research. 29 (5): 1017-1038.

5. Andjelkovic, A.V., Nikolic, B., Pachter, J.S., Zecevic, N. (1998) Macrophages.microglial cells in human central nervous system during development: an immunohistochemical study. Brain Research. 814 (1-2):13-25.

6. Andreasson, K. (2009) Emerging roles of PGE2 receptors in models of neurological disease. Prostaglandins & Other Lipid Mediators. 91 (3-4): 104-112.

7. Banati, R., Hickie, I.B. (2009) Therapeutic signposts:using biomarkers to guide better treatment of schizophrenia and other psychotic disorders. The Medical Journal of Australia. 190(4):S26-S32.

8. Banks, W.A., Kastin, A.J., Ehrensing, C.A. (1994) Blood borne interleukin-1α is transported cross the endothelial blood-spinal cord barrier of mice. Journal of Physiology. 479: 257-264.

9. Bax, M., Goldstein, M., Rosenbaum, P., Paneth, N. (2005) Proposed definition and classification of cerebral palsy. Developmental Medicine and Child Neurology. 47: 571-576.

10. Bazan, J.F., Bacon, K.B., Hardiman, G., Wang, W., Soo, K., Rossi, D., Greaves, D.R., Zlotnik, A., Schall, T.J. (1997) A new class of membrane-bound chemokine with a CX#C motif. Nature. 385: 640-644.

11. Biber, K., Neumann, H., Inoue, K., Boddeke, W.G.M. (2007) Neuronal 'On' and 'Off' signals control microglia. doi:10.1016/j.tins.2007.08.007.

12. Bluthé, R.M., Walter, V., Parnet, P., Lavé, S., Lestage, J., Verrier, D., Poole, S., Stenning, B.E., Kelley, K.W., Dantzer, R. (1994) Lipopolysaccharide induces sickness behaviour in rats by a vagal mediated mechanism. Comptes rendus de l'Academie de sciences. 317(6):499-503.

13. Bouchon, A., Hernandez-Munain, C., Cella, M., Colonna, M. (2001) A DAP12-Mediated pathway regulates expression of Cc Chemokine receptor 7 and maturation of human dendritic cells. Journal of Experimental Medicine. 194 (8): 1111-1122.

14. Bouchon, A., Dietrich, J., Colonna, M. (2000) Cutting edge: inflammatory responses can be triggered by TREM-1, a novel receptor expressed on neutrophils and monocytes. Journal of Immunology. 164 (10): 4491-4495.

15. Bouchon, A., Facchetti, F., Weigand, M.A., Colonna, M. (2001) TREM-1 amplifies inflammation and is a crucial mediator of septic shock. Nature. 410 (6832): 1103-1107.

16. Bowie, A., O'Neil, L.J. (2000) The interleukin-1 receptor/Toll-like receptor superfamily: signal generators for pro-inflammatory interleukins and microbial products. Journal of Leukocyte Biology. 67: 508-514.

17. Bowman, C.C., Rasley, A., Tranguch, S.L., Marriott, I. (2003) Cultured astrocytes express Toll like receptors for bacterial products. Glia. 43: 281-291.

18. Byram, S.C., Carson, M.J., DeBoy, C.A., Serpe, C.J., Sanders, V.M., Jones, K.J. (2004) CD4-Positive T cell-mediated neuroprotection requires dual compartment antigen presentation. The Journal of Neuroscience. 24 (18): 4333-4339.

19. Cardona, A.E., Pioro, E.P., Sasse, M.E., Kostenko, V., Cardona, S.M., Dijkstra, I.M., Huang, D., Kidd, G., Dombrowski, S., Dutta, R., Lee, J., Cook, D.N., Jung, S., Lira, S.A., Littman, D.R., Ransohoff, R.M. (2006) Control of microglial neurotoxicity by the fractalkine receptor. Nature Neuroscience. 9 (7) 917-924.

20. Carson, M.J., Reilly, C.R., Sutcliffe, J.G., Lo, D. (1998) Mature microglia resemble immature antigen presenting cells. Glia. 22: 72-85.

21. Carson, M.J., Doose, J.M., Melchior, B., Schmid, C.D., Ploix, C.C. (2006) CNS immune privilege: hiding in plain sight. Immunological Reviews. 213: 48-65.

22. Carson, M.J., Thrash, J.C., Walter, B. (2006) The cellular response in neuroinflammation: The role of leukocytes, microglia and astrocytes in neuronal death and survival. Clinical Neuroscience Research. 6 (5): 237-245.

23. Carson, M.J., Crane, J., Xie, A.X., Silva, G.A. (2008) Modeling CNS microglia: the quest to identify predictive models. Drug discovery today: Disease Models. 5(1): 19-25.

24. Chew L, Takanohashi A, Bell M. (2006) Microglia and inflammation: Impact on developmental brain injuries. Mental retardation and developmental disabilities research reviews. 12: 105-112.

25. Ching, S., Zhang, H., Belevych, N., He, L., Lai, W., Pu, X., Jaeger, L.B., Chen, Q., Quan, N. (2007) Endothelial-specific knockdown of Interleukin-1(IL-1) type 1 receptor differentially alters CNS responses to IL-1 depending on its route of administration. The Journal of Neuroscience. 27(39): 10476-10486.

26. Chung, D.H., Seaman, W.E., Daws, M.R. (2002) Characterization of TREM-3, an activating receptor on mouse macrophages: definition of a family of single Ig domain receptors on mouse chromosome 17. European Journal of Immunology. 32: 59-66.

27. Conde, J.R., Streit, W.J. (2006) Microglia in the aging brain. Journal of Neuropathology and Experimental Neurology. 65 (3): 199-203.

28. Crack, P.J., Bray, P.J. (2007) Toll-like receptors in the brain and their potential roles in neuropathology. Immunology and Cell Biology. 85: 476-480.

29. Dalpke, A.H., Schafer, M.F., Frey, M. Zimmerman, S., Tebbe, J., Weihe, E., Heeg, K. (2002) Immunostimulatory CpG DNA activates murine microglia. Journal of Immunology. 168: 4854-4863.

30. Dantzer, R. (2001) Cytokine-induced sickness behavior: mechanisms and implications. Annals of the New York Academy of Sciences. 933:222-234.

31. D'Mello, C., Le, T., Swain, M.G. (2009) Cerebral microglia recruit monocytes into the brain in response to tumor necrosis factor α signaling during peripheral organ inflammation. The Journal of Neuroscience. 29 (7): 2089-2102.

32. Demeur, C.E., Yang, L.P., Byun, D.G., Ishihara, H., Vezzio, N., Delespesse, G. (1995) Human naïve CD4 T cells produce interleukin-4 priming and acquire a Th2 phenotype upon repetitive stimulations in neutral conditions. The European Journal of Immunology. 25 (9): 2722-2725.

33. Doorduin, J., de Vries, E.F.J., Willemsen, A.T.M., de Groot, J.C., Dierckx, R.A., Klein, H.C. (2009) Neuroinflammation in schizophrenia related psychosis: A PET study. The Journal of Nuclear Medicine. 50(11):1801-1807.

34. Ehrchen, J.M., Roebrock, K., Foell, D., Nipe, N., von Stebut, E., Weiss, J.M., Munck, N., Viemann, D., Varga, G., Muller-Tidow, C., Schuberth, H., Roth, J., Sunderkotter, C. Keratinocytes determine Th1 immunity during early experimental leishmaniasis. PLoS (2010) 10.1371/journal.ppat.1000871.

35. Engblom, D., Saha, S., Engstrom, L., Westman, M., Audoly, L.P., Jakobsson, P.J., Blomqvist, A. (2003) Microsomal prostaglandin E synthase-1 is the central switch during immune-mediated pyresis. Nature Neuroscience. 6 (11): 1137-1138.

36. Ferrer, L., Bernet, E., Soriano, E. del Rio, T., Fonseca, M. (1990) Naturally occurring cell death in the cerebral cortex of the rat and removal of dead cells by transitory phagocytes. Neuroscience. 39(2):451-458.

37. Ford, A.L., Goodsall, A.L., Hickey, W.F., Sedgwick, J.D. (1995) Normal adult ramified microglia separated from other central nervous system macrophages by flow cytometric sorting. The Journal of Immunology. 154: 4309-4321.

38. Ford, J.W., McVicar, D.W. (2009) TREM and TREM-like receptors in inflammation and disease. Current Opinion in Immunology. 21: 38-46.

39. Frank, M.G., Barrientos, R.M., Biedenkapp, J.C., Ridy, J.W., Watkins, L.R., Maier, S.F. (2006) mRNA upregulation of MHC II and pivotal pro-inflammatory genes in normal brain aging. Neurobiology of aging. 27 (5): 717-722.

40. Gamo, K., Kiryu-Seo, S., Konishi, H., Aoki, S., Matsushima, K., Wada, K., Kiyama, H. (2008) G-Protein coupled receptor screen reveals a role for chemokine receptor CCR5 in suppressing microglial neurotoxicity. The Journal of Neuroscience 28: 11980-11988.

41. Germain, R.N., Miller, M.J., Dustin, M.L., Nussenzweig, M.C. (2006) Dynamic imaging of the immune system: progress, pitfalls and promise. Nature Reviews Immunology. 6:497-507.

42. Ginhoux, F., Greter, M., Leboeuf, M., Nandi, S., See, P., Gokhan, S., Mehler, M.F., Conway, S.J., Ng, L.G., Stanley, E.R., Samokhvalov, I.M., Merad, M. (2010) Fate mapping analysis reveals that adult microglia derive from primitive macrophages. Science. 330: 841-845.

43. Godbout, J.P., Chen, J., Abraham, J., Richwine, A.F., Berg, B.M., Kelley, K.W., Johnson, R.W. (2005) Exaggerated neuroinflammation and sickness behavior in aged mice after activation of the peripheral innate immune system. The FASEB Journal. 19 (10): 1329-1331.

44. Greenbaum, D., Colangelo, C., Williams, K., Gerstein, M. (2003) Comparing protein abundance and mRNA expression levels on a genomic scale. Genome Biology. 4:117-117.8

45. Greter, M., Heppner, F.L., Lemos, M.P., Odermatt, B.M., Goebles, N., Laufer, T., Noelle, R.J., Becher, B. (2005) Dendritic cells permit immune invasion of the CNS in an animal model of multiple sclerosis. Nature Medicine. 11: 328-334.

46. Guayao, W.U., Sidney, M.M. (1998) Arginine metabolism: nitric oxide and beyond. The Biochemical Journal. 3336: 1-17.

47. Gupta, S., Aggarwal, S., Rashanravan, B., Lee, T. (1998) Th1-and Th2-like cytokines in CD4+ and CD8+ T cells in autism. Journal of Neuroimmunology. 85(1): 106-109.

48. Gutierrez, E.G., Banks, W.A., Kastin, A.J. (1993) Murine tumor necrosis factor alpha is transported from blood to brain in the mouse. Journal of Neuroimmunology. 47: 169-176.

49. Haynes, R.L., Folkerth, R.D., Keefe, R.J>, Sung, I., Swzeda, L.I. (2003) Nitrosative and oxidative injury to premylinating oligodendrocytes in periventricular leukomalacia. Journal of Neuropathological Experiments and Neurology. 62 (5): 441-450.

50. He, T., Xing, Y., Zhao, X., Ai, M. (2007) Interaction between iNOS and COX-2 in hypoxia induced retinal neovascularization in mice. Archives if medical research. 38: 807-815.

51. Henry, C.J., Huang, Y., Wynne, A.M., Godbout, J.P. (2009) Peripheral lipopolysaccharide (LPS) challenge promotes microglial hyperactivity in aged mice that is sssociated with exaggerated induction of both pro-inflammatory IL-1β and anti-inflammatory IL-10 cytokines. Brain, Behavior and Immunity. 23 (3): 309-317.

52. Herbert, D.R., Orekov, T., Roloson, A., Ilies, M., Perkins, C., O'Brien, W., Cederbaum, S., Christianson, D.W., Zimmermann, N., Rothenberg, M.E., Finkelman, F.D. (2010) Arginase I suppresses Il-12/Il-23p40-driven intestinal inflammation during acute schistosomiasis. The Journal of Immunology. 184; 6438-6446.

53. Hickie, I.B., Banati, R., Stewart, C.H., Lloyd, A.R. (2009) Are common childhood or adolescent infections risk factors for schizophrenia and other psychotic disorders? The Medical Journal of Australia. 190:S17-S21.

54. Hoek, R.M., Ruuls, S.R., Murphy, C.A., Wright, G.J., Goddard, R., Zurawski, S.M., Blom, B., Homola, M.E., Streit, W.J., Brown, M.H., Barclay, A.N., Sedgwick, J.D. (2000) Down-regulation of the macrophage lineage through interaction with OX2 (CD200). Science. 290: 1768-1771.

55. Howard M, Paul WE. (1983) Regulation of B-cell growth and differentiation by soluble factors. Annual Reviews of Immunol. 1:307–33.

56. Hristova, M., Cuthill, D., Zbarsky, V., Acosta-Saltos, A., Wallace, A., Blight, K., Buckley, S.M.K., Peebles, D., Heuer, H., Waddington, S.N., Raivich, G. (2010) Activation and deactivation of periventricular white matter phagocytes during postnatal mouse development. Glia. 58: 11-28.

57. Hsieh, C.L., Koike, M., Spusta, S., Niemi, E., Yenari, M., Nakamura, M.C., Seaman, W.E. (2009) A role for TREM2 ligands in the phagocytosis of apoptotic neuronal cells by microglia. Journal of Neurochemistry. 109 (4): 1144-1156.

58. Hutchins, K.D., Dickson, D.W., Rashbaum, W.K., Lyman, W.D. (1990) Localization of morphologically distinct microglial populations in the developing human fetal brain: implications for ontogeny. Developmental Brain Research. 55: 95-102.

59. Jonakait, G.M., Wen, Y., Wan, Y., Ni, L. (2000) Macrophage cell conditioned medium promotes cholinergic differentiation of undifferentiated progenitors and synergizes with nerve growth factor action in the developing basal forebrain. Experimental Neurology. 161(1):285-296.

60. Kielian, T., Mayes, P. Kielian, M. (2002) Characterization of microglial responses to *Staphylococcus aureus*: effects on cytokine, costimulatory molecule, and Toll like receptor expression. Journal of Neuroimmunology. 130:86-99.

61. Kimbrell, D.A., Beutler, B. (2001) The evolution and genetics of innate immunity. Nature Reviews. Genetics. 2(4) 256-267.

62. King, R.G., Herrin, B.R., Justement, L.B. (2006) Trem-like transcript is expressed on cells of the myeloid/granuloid and B lymphoid lineage and is up-regulated in response to inflammation. The Journal of Immunology. 176 (10): 6012-6021.

63. Klesney-Tait, J., Trunbull, I.R., Colonna, M. (2006) The TREM family and signal integration. Nature Immunology. 7 (12): 1266-1273.

64. Konsman, J.P., Kelley, K., Dantzer, R. (1999) Temporal and spatial relationships between lipopolysaccharide-induced expression of fos, interleukin-1β and inducible nitric oxide synthase in rat brain. Neuroscience. 89 (2): 535-548.

65. Kreider, T., Anthony, R.M., Urban, J.F., Gause W.C. (2007) Alternatively activated macrophages in helminth infections. Current Opinion in Immunology. 19: 448-453.

66. Kurt-Jones, E.A., Chan, M., Zhou, S., Wang, J., Reed, G., Bronson, R., Arnold, M.M., Knipe, D.M., Finberg, R.W. (2004) Herpes simplex virus 1 interaction with Toll-like receptor 2 contributes to lethal encephalitis. Proceedings of the National Academy of Sciences. 101 (5): 1315-1320.

67. Lanier, L.L., Bakker, A.B.H. (2000) The ITAM-bearing transmembrane adaptor DAP12 in lymphoid and myeloid cell function. Immunology Today. 21 (12): 611-614.

68. Leon, C.G., Tory, R., Jia, J., Sivak, O., Wasan, K.M. (2008) Discovery and development of Toll-Like Receptor 4 (TLR4) antagonists: a new paradigm for treating sepsis and other diseases. Pharmaceutical Research. 25 (8) DOI: 10.1007/s11095-0089571.

69. Lichanska, A.M., Browne, C.M., Henkel, G.W., Murphy, K.M., Ostrowski, M.C., McKercher, S.R., Maki, R.A., Hume, D.A. (1999) Differentiation of the mononuclear phagocyte system during mouse embryogenesis: the role of transcription factor PU.1. Blood. 94 (1): 127-138.

70. Madry, H., Prudlo, J., Grgic, A., Freyschmidt, J. (2007) Nasu-Hakola Disease (PLOSL): Report of five cases and review of the literature. Clinical Orthopaedics & Related Research. 454: 262-269.

71. Maier, S.F., Goehler, L.E., Fleshner, M., Watkins, L.R. (2006) The role of the vagus nerve in cytokine to brain communication. Annals of the New York Academy of Sciences. 840: 289-300.

72. Magnus, T., Schreiner, B., Korn, T., Jack, C., Guo, H., Antel, J., Ifergan, I., Chen, L., Bischof, F., Bar-Or, A., Wiendl, H. (2005) Microglial expression of the B7 family member B7 homolog 1 confers strong immune inhibition: Impications for immune responses and autoimmunity in the CNS. The Journal of Neuroscience. 25 (10): 2537-2546.

73. Martinez, F.O., Helming, L., Gordon, S. (2009) Alternative activation of macrophages:An immunologic functional perspective. Annual Review of Immunology. 27: 451-483.

74. Masocha, W. (2009) Systemic lipopolysaccharide (LPS)-induced microglial activation results in different temporal reduction of CD200 and CD200 receptor gene expression in the brain. Journal of Neuroimmunology. 214: 78-82.

75. McKenzie AN, Culpepper JA, de Waal Malefyt R, Briere F, Punnonen J, et al. (1993) Interleukin 13, a T-cell-derived cytokine that regulates human monocyte and B-cell function. Proc. Natl. Acad. Sci. USA90:3735–39.

76. McMahon, E.J., Bailey, S.L., Castenada, C.V., Waldner, H., Miller, S.D. (2005) Epitope spreading initiates in the CNS in two mouse models of multiple sclerosis. Nature Medicine. 11: 335-339.

77. Medawar, P.B. (1948) Immunity to homologous graftes skin. III. The fate of skin homografts transplanted to the brain, to subcutaneous tissue, and to anterior chamber of the eye. British Journal of Experimental Pathology. 29: 58-69.

78. Melchior, B., Garcia, A.E., Hsiung, B.K., Lo, K.M., Doose, J.M., Thrash, J.C., Stalder, A.K., Staufenbiel, M., Neumann, H., Carson, M.J. (2010) Dual induction of TREM2 and tolerance related transcript, Tmem176b, in amyloid transgenic mice: implications for vaccine-based therapies for Alzheimer's disease. ASN Neuro. 2 (3): e00037.

79. Meyer, U., Feldon, J., Dammann, O. (2011) Schizophrenia and autism: both shared and disorder-specific pathogenesis via perinatal inflammation? Pediatric Research. 69: 26R-33R.

80. Mishra, B.B., Mishra, P.K., Teale, J.M. (2006) Expression and distribution of Toll-like receptors in the brain during murine neurocysticercosis. Journal of Neuroimmunology. 181:46-56.

81. Mizuno, T., Kawanokuchi, J., Numata, K., Suzumura, A. (2003) Production and neuroprotective functions of fractalkine in the central nervous system. Brain Research. 979: 65-70.

82. Moqbel, R., Ying, S., Barkans, J., Newman, T.M., Kimmitt, P., Wakelin, M., Taborda-Barata, L., Meng, Q., Corrigan, C.J., Durham, S.R., Kay, A.B. (1995) Identification of messenger RNA for IL-4 in human eosinophils with granule localization and release of the translated product. 155 (10) 4939-4947.

83. Mora, A.L., Torres-Gonzalez, E., Rojas, M., Corredor, C., Ritzenthaler, J. (2006) Activation of alveolar macrophages via the alternative pathway in herpesvirus induced lung fibrosis. The American Journal of Respiratory Cell and Molecular Biology. 35: 466-473.

84. Mosser, D.M., Edwards, J.P. (2008) Exploring the full spectrum of macrophage activation. Nature Reviews Immuology. 8: 958-969.

85. Mott, R.T., Ait-Ghezala, G., Town, T., Mori, T., Vendrame, M., Zeng, J., Ehrhart, J., Mullan, M., Tan, J. (2004) Neuronal expression of CD22: Novel mechanism for inhibiting microglial proinflammatory cytokine production. Glia. 46:369-379.

86. Nair, M.G., Cochrane, D.W., Allen, J.E. (2003) Macrophages in chronic type 2 inflammation have a novel phenotype characterized by the abundant expression of Ym1 and Fizz1 that can be partly replicated in vitro. Immunology Letters. 85 (2): 173-180.

87. O'Donnell, M.C., Catts, S.V., Watd, P.B., Liebert, B., Lloyd, A., Wakefield, D., McConaghy, N. (1996) Increased production of interleukin-2(IL-2) but not soluble interleukin-2 receptors (sIL-2R) in unmedicated patients with schizophrenia and schizophreniform disorder. Psychiatry Research. 65: 171-178.

88. Olson, J.K., Zamvil, S.S., Miller, S.D. (2003) Efficient technique for immortalization of murine microglial cells relevant for studies in murine models of multiple sclerosis. Journal of Neuroscience Methods 128(1-2): 33-43.

89. Olson, J.K., Miller, S.D. (2004) Microglia initiate central nervous system innate and adaptive immune responses through multiple TLRs. The Journal of Immunology. 173: 3916-3924.

90. Owhashi, M., Arita, H., Niwa, A. (1998) Production of eosinophil chemotctic factor by CD8+ T cells in *Toxocara canis*-infected mice. 84: 136-138.

91. Owhashi, M., Arita, H., Hayai, N. (2000) Identification of a novel eosinophil chemotactic cytokine (ECF-L) as a chitinase family protein. The Journal of Biological Chemistry. 275: 1279-1286.

92. Paloneva, J., Mandelin, J., Kialainen, A., Bohling, T., Prudio, J., Hakola, P., Haltia, M., Konttinen, Y.T., Peltonen, L. (2003) DAP12/TREM2 deficiency results in impaired osteoclast differentiation and osteoporotic features. The Journal of Experimental Medicine. 1988 (4): 669-675.

93. Pan, W., Kastin, A.J. (2002) TNFα transport across the Blood-Brain Barrier is abolished in receptor knockout mice. Experimental Neurology. 174: 193-200.

94. Parnaik, R., Raff, M.C., Scholes, J. (2000) Differences between the clearance of apoptotic cells by professional and non-professional phagocytes. Current Biology. 10(14):857-860.

95. Perry, V.H., Andersson, P.B. (1992) The inflammatory response in the CNS. Neuropathology and Applied Neurobiology. 18: 454-459.

96. Perry, V.H. (2004) The influence of systemic inflammation on inflammation in the brain: implications for chronic neurodegenerative disease. Brain, behavior and immunity. 18: 407-413.

97. Pesce, J.T., Ramalingam, T.R., Wilson, M.S., Mentik-Kane, M.M., Thompson, R.W., Cheever, A.W., Urban, J.F., Wynn, T.A. (2009) Retnla (Relmα/Fizz1) suppresses helminth-induced Th2 type immunity. PLoS Pathogens. 5(4) e1000393. doi:10.1371/journal.ppat.1000393.

98. Potvin, S., Stip, E., Sephery, A.A., Gendron, A., Bah, R., Kouassi, E. (2007) Inflammatory cytokine alterations in schizophrenia:A systematic quantitative review. Biological Psychiatry. 63(8):801-808.

99. Puntambekar, S.S., Davis, D.S., Hawell, L., Crane, J., Byus, C.V., Carson, M.J. (2011) LPS-induced CCL2 expression and macrophage influx into the murine central nervous system is polyamine dependent. Brain, Behavior and Immunity. 25 (4): 629-639.

100. Quan, N. (2008) Immune to brain signaling: How important are the Blood-Brain Barrier independent pathways. Molecular Neurobiology. 37(2-3): 142-152.

101. Raff, M.C., Barres, B.A., Burne, J.F., Coles, H.S., Ishizaki, Y., Jacobson, M.D. (1993) Programmed cell death and the control of cell survival: lessons from the nervous system. Science. 262 (5134): 695-700.

102. Rantakallio, P., Jones, P., Moring, J., Von Wend, L. (1997) Association between central nervous system infections during childhood and adult onset schizophrenia and other psychoses: A 28-year follow up. International Journal of Epidemiology. 26:837-843.

103. Rasley, A., Anguita, J., Marriott, I. (2002) *Borrelia burgdorferi* induces inflammatory mediator production by murine microglia. Journal of Neuroimmunology. 130:22-31.

104. Saadani-Makki, F., Kannan, S., Lu, X., Janisse, J., Dawe, E., Edwin, S., Romero, R., Chugani, D. (2008) Intrauterine administration of endotoxin leads to motor deficits in a rabbit model: a link between prenatal infection and cerebral palsy. American Journal of Obstetrics and Gynecology. 199(6):651 e1-7.

105. Sanders, V.M., Jones, K.J. (2006) Role of immunity in recovery from a peripheral nerve injury. Journal of Neuroimmune Pharmacology. 10.1007/s11481-005-9004-0.

106. Schiltz, J.C., Sawchenko, P.E. (2002) Distinct brain vascular cell types manifest inducible cyclooxygenase expression as a function of the strength and nature of immune insults. The Journal of Neuroscience. 22(13): 5606-5618.

107. Schlegelmilch, T, Henke, K., Peri, F. (2011) Microglia in the developing brain: from immunity to behavior. Current Opinion in Neurobiology. 21 (1):5-10.

108. Schmid, C.D., Sautkulis, L.N., Danielson, P.E., Cooper, J., Hasel, K.W., Hilbush, B.S., Sutcliffe, J.G., Carson, M.J. (2002) Heterogeneous expression of the triggering receptor expressed on myeloid cells-2 on adult murine microglia. Journal of Neurohemistry. 83 (6): 1309-1320.

109. Schmid, C.D., Melchior, B., Masek, K., Puntambekar, S.S., Danielson, P.E., Lo, D.D., Sutcliffe, J.G., Carson, M.J. (2009) Differential gene expression in LPS/IFNgamma activated microglia and macrophages: in vitro versus in vivo. Journal of Neurochemistry. 109 Suppl 1: 117-125.

110. Seder, R.A., Paul, W.E., Dvorak, A.M., Sharkis, S.J., Kagey-Sobotka, A., Niv, Y., Finkelman, F.D., Barbieri, S.A., Galli, S.J., Plaut, M. (1991) Mouse splenic and bone marrow cell populations that express high-affinity Fc_{ε} receptors and produce interleukin 4 are highly enriched in basophils. 88:2835-2839.

111. Seder, R.A., Boulay, J.L., Finkelman, F., Barbier, S., Ben-Sazzon, S.Z. (1992) CD8+ T cells can be primed in vitro to produce IL-4. 148: 1652-1656.

112. Sedgwick, J.D., Schwender, S., Imrich, H., Dorries, R., Butcher, G.W., Meulen, V.T. (1991) Isolation and direct characterization of resident microglial cells from the normal and inflamed central nervous system. Proceedings of the National Academies of Sciences. 88 (16) 7438-7442.

113. Skinner, R.A., Gibson, R.M., Rothwell, N.J., Pinteaux, E., Penny, J.I. (2009) Transport of interleukin-1 across cerebromicrovascular endothelial cells. British Journal of Pharmacology. 156: 1115-1123.

114. Soulet, D., Rivest, S. (2008) Bone marrow derived microglia: myth or reality? Current Opinion in Pharmacology. 8: 508-518.

115. Stein, M., Keshav, S., Harris, N, Gordon, S. (1992) Interleukin 4 potently enhances murine macrophage mannose receptor activity: A marker of alternative immunologic macrophage activation. The Journal of Experimental Medicine. 176 (1): 287-292.

116. Stefano, L., Racchetti, G., Bianco, F., Passini, N., Gupta, R.S., Bordignon, P.P., Meldolesi, J. (2009) The surface –exposed chaperone, Hsp60, is an agonist of the microglial TREM2 receptor. Journal of Neurochemistry. 110 (1): 284-294.

117. Steinmetz, T., Schaadt, M., Gahl, R., Schenk, V., Diehl, V., Pfreundschuh, M., (1988) Phase I study of 24-hour continuous intravenous infusion of recombinant human tumor necrosis factor. Journal of Biological Response Modifiers. 7(5): 417-423.

118. Steppan, C.M., Brown, E.J., Wright, C.M., Bhat, S., Banerjee, R.R., Dai, C.Y., Enders, G.H., Silberg, D.G., Wen, X., Wu, G.D., Lazar, M.A. (2001) A family of tissue-specific resistin-like molecules. Proceedings of the National Academies of Sciences. 98: 502-506.

119. Tan, J., Town, T., Mullan, M. (2000) CD45 inhibits CD40L-induced microglial activation via negative regulation of the Src/p44/42 MAPK pathway. The Journal of Biological Chemistry. 275 (47):37224-37231.

120. Takahashi, K., Rochford, C.D.P., Neumann, H. (2005) Clearance of apoptotic neurons without inflammation by microglial triggering receptor expressed on myeloid cells-2. The Journal of Experimental Research. 201 (4): 647-657.

121. Takahashi, K., Prinz, M., Stagi, M., Chechneva, O., Neumann, H. (2007) TREM2-Transduced myeloid precursors mediate nervous tissue debris clearance and facilitate recovery in an animal model of multiple sclerosis. PLoS. 4(4): e124.

122. Tan, J., Town, T., Mullan, M. (2000) CD45 injibits CD40L-induced microglial activation via negative regulation of the Src/p44/42 MAPK pathway. Journal of Biological Chemistry. 275: 37224-37231.

123. Thomas, M.L. The leukocyte common antigen family. (1989) Annual Reviews of Immunology. 7: 339-369.

124. Thrash, J,C., Torbett, B.E., Carson, M.J. (2009) Developmental regulation of TREM2 and DAP12 expression in the murine CNS: implications for Nasu-Hakola disease. Neurochemical Research. 34 (1): 38-45.

125. Tomasello, E., Olcese, L., Vely, F., Geourgeon, C., Blery, M., Moqrich, A., Gautheret, D., Djabali, M., Mattei, M.G., Viver, E. (1998) Gene structure, expression pattern, and biological activity of mouse killer cell activating receptor-associated protein (KARAP)/DAP-12. The Journal of Biological Chemistry. 273: 34115-34119.

126. Triozzi, P.L., Kinney, P., Rinehart, J.J. (1990) Central nervous system toxicity of biological response modifiers. Annals of the New York Academy of Sciences. 594:347-354.

127. Turnbull, I.R., Gilfillan, S., Cella, M., Aoshi, T., Miller, M., Piccio, L., Hernandez, M., Colonna, M. (2006) TREM-2 Attenuates macrophage activation. The Journal of Immunology. 177 (6): 3520-3524.

128. Valanne, S., Wang, J.H., Ramet, M. (2011) The drosophila toll signaling pathway. Journal of Immunology. 186: 649-656.

129. Vargas, D.L., Nascimbene, C., Krishnan, C., Zimmerman, A.W., Pardo, C. (2005) Neuroglial activation and neuroinflammation in the brain of patients with autism. Annals of Neurology. 57(1): 67-81.

130. Colton, C.A., Mott, R.T., Sharpe, H., Xu, Q., Van Nostrand, W.E., Vitek, M.P. (2006) Expression profiles for macrophage alternative activation genes in AD and in mouse models of AD. Journal of Neuroinflammation. 27: 3-27.

131. Vivier, E., Daeron, M. (1997) Immunoreceptor tyrosine-based inhibition motifs. Immunology Today. 18 (6): 286-291.

132. Wang, X., Rousset, C,I, Hagberg, H., Mallard, C. (2006) Lipopolysaccharide-induced inflammation and perinatal brain injury. Seminars in Fetal ans Neonatal Medicine. 11: 343-353.

133. Washington, A.V., Schubert, R.L., Quigley, L., Disipio, T., Feltz, R., Cho, E.H., McVicar, D.W. (2004) Hemostasis, Thrombosis and Vascular Biology. 104 (4): 1042-1047.

134. Watari, H., Sekine, E., Inuoe, S., Nakagawa, R., Kaisho, T., Taniguchi, M. (2008) PDC-TREM, a plasmacytoid dendritic cell specific receptor, is responsible for augmentd production of type I interferon. Proceedings of the National Academies of Sciences of the United States of America. 105 (8): 2993-2998.

135. Weigelt, K., Carvalho, L.A., Drexhage, R.C., Wijkhuijis,A., Wit, H.D., van Beveren, N.J., Birkenhager, T.K., Bergink, V., Drexhage, H.A. (2011) TREM-1 and DAP12 expression in monocytes of patients with severe psychiatric disorders. EGR3, ATF3 and PU.1 as important transcription factors. Brain, Behavior and Immunity.

136. Wegiel, J., Kuchna, I., Nowicki, K., Imaki, H., Wegiel, J., Marchi, E., Yong Ma, S., Chauhan, A., Chauhan, V., Bobrowicz, T.W., de Leon, M., Saint Louis, L.A., Cohen, I.L., London, E., Brown, W.T., Wisniewski, T. (2010) The neuropathology of autism: defects of neurogenesis and neuronal migration and dysplastic changes. Acta Neuropathologica. 119)6): 755-770.

137. Wilson, M.J., Lindquist, J.A., Trowsdale, J. (2000) Immunologic Research. 22 (1): 21-42.

138. Wright, G.J., Puklavec, M.J., Willis, A.C., Hoek, R.M., Sedgwick, J.D., Brown, M.H., Barclay, A.N. (2000) Lymphoid/Neuronal cell surface OX2 glycoprotein recognizes a novel receptor on macrophages implicated in the control of their function. Immunity. 13: 233-242.

139. Yoshida, H., Imaizumi, T., Fujimoto, K., Matsuo, N., Kimura, K., Cui, X., Matsumiya, T., Tanji, K., Shibata, T., Tamo, W. (2001) Synergistic stimulation, by tumor necorsis factor-alpha and interferon-gamma, of fractalkine expression in human astrocytes. Neuroscience Letters. 303: 132-136.

140. Yoshimoto, T., Bendelac, A., Watson, C., Hu-Li, J., Paul, W.E. (1995) Role of NK1.1+ T cells in a TH2 response and in immunoglobulin E production. Science. 270: 1845-1847.

141. Zujovic, V., Benavides, J., Vige, X., Carter, C., Taupin, V. (2000) Fractalkine modulates TNFα secretion and neurotoxicity induced by microglial activation. Glia. 29: 305-315.