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TREM2 and Inflammatory Regulation of Microglial Functions Over the Lifespan and Across Species

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by

Abdullah Muhammad Madany

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Dissertation Committee:
- Dr. Monica J. Carson, Chairperson
- Dr. Iryna Ethell
- Dr. Meera Nair
- Dr. Byron Ford

The Dissertation of Abdullah Muhammad Madany is approved:

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Committee Chairperson

University of California, Riverside

ABSTRACT OF THE DISSERTATION

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The proper development and function of the mammalian central nervous system (CNS) depend critically on the activity of immune cells referred to as microglia. The microglia are the resident macrophages of the CNS and act as the primary immune regulator in the CNS during normal development, adult tissue homeostasis, and in injury and disease. In this thesis, we discuss the exciting developments in our understanding of microglial biology from their in vivo developmental origin, to their participation in CNS homeostasis and pathophysiological states, such as systemic inflammation induced by Lipopolysaccharide (LPS), and neurodegeneration due to aging. We will also look at microglial *ex vivo* response to *S. aureus* and Synaptosomes. And finally, the in vitro development and maturation of human microglial-like cells (iMGL) differentiated from hematopoietic progenitor cells (iHPCs). We see that microglia are a versatile cell and possess the capacity to morphologically and functionally adapt to their ever-changing environment. Even in a resting state, the processes

of microglia are highly dynamic and perpetually scan the CNS. Studies of microglia development, homeostasis, and function give us insights into how to modulate them during the course of CNS diseases. Importantly, we now have the technologies to study human microglia function in a pure *in vitro* setting, which should vastly enhance our understanding of these cells and how various mutations contribute to altered functions.

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LIST OF ABBREVIATIONS

ALT: *Alternaria*

APC: Allophycocyanin

BAL: Bronchio-Alveolar Lavage

BBB: Blood Brain Barrier

BMDM: Bone Marrow Derived Macrophages

CD: cluster of differentiation

CD45: leukocyte common antigen

CD47: integrin associated protein

CD93: Cluster of Differentiation 93

CNS: Central Nervous System

CPC: Condensation Particle Counter

DAMP: Danger Associated Molecular Pattern

DAP12: DNAX activating protein of 12 kDa

ELISA: Enzyme-Linked Immunosorbent Assay

FITC: Fluorescein

GSL: Griffonia Simplificolia Lectin

H&E: Hematoxylin and Eosin

HPRT: Hypoxanthine Guanine Phosphoribosyl Transferase

IHC: Immunohistochemistry

iMGL: human microglial-like cells

IP: Intraperitoneal

IPLPS: Intraperitoneal Lipopolysaccharide

ITAM: Immuno-receptor Tyrosine based Activation Motifs

ITIM: Immuno-receptor Tyrosine based Inhibitory Motifs

KO: Knock Out

LAP: Laser Aerosol Particle Sizer

LPS: Lipopolysaccharide

M-CSF: Macrophage-Colony Stimulating Factor

MFI: Mean Florescence Intensity

MG: Microglia

MP: Macrophages

PAMP: Pathogen Associated Molecular Pattern

PBMC: Peripheral Blood Mononuclear Cell

PE: Phycoerythrin

PM: Particulate Matter

qPCR: quantitative Polymerase Chain Reaction

RELMα: Resistin-Like Molecule alpha

S. aureus: *Staphylococcus aureus*

SiglecH: Sialic acid binding Ig-like lectin H

SMPS: Scanning Mobility Particle Size

TAM: Tyro3, Axl and Mer

TGFβ: Transforming Growth Factor beta

TLR: Toll Like Receptor

TLR2: Toll Like Receptor 2

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

TMB: Tetramethylbenzidine

TNFa: Tumor Necrosis Factor alpha

TREM1: Triggering Receptor Expressed on Myeloid cells 1

TREM2: Triggering Receptor Expressed on Myeloid cells 2

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

1.1 Microglia as CNS immune cells

Microglia are the resident immune cells of the central nervous system (CNS). The CNS has previously been defined as being immune privileged. However, recent studies have shown that the CNS is actually interactive with the peripheral immune system and itself being immune proficient (Ransohoff, Kivisakk, & Kidd, 2003). Despite this change in viewpoint, as the only myeloid cell population resident in the CNS, the focus of the bulk of research into microglia function has still unfortunately been that of an immune modulator. Microglial cells are not "resting" but however, constantly survey the CNS and their processes to interact with axonal myelin and dendritic synapses (Nimmerjahn, Kirchhoff, & Helmchen, 2005; Paolicelli et al., 2011; Schafer et al., 2012; Wake, Moorhouse, Jinno, Kohsaka, & Nabekura, 2009). Microglia can sense the slightest change in their environment, which results in mounting a response to restore homeostasis. More recent studies of microglial cells have started to show they have many potentially non-immunological functions in development, maintenance of homeostasis, and tissue repair following injury or disease of the CNS. These studies are beginning to redefine many aspects of microglial physiology giving insight into immune regulation of disease. We now know that microglia originate from the yolk sac,

that migrate and colonize in the neuroepithelium during early embryogenesis (Ginhoux et al., 2010).

Microglia continue development neonatally, which happens when committed myeloid precursors differentiate into microglia. This developmental process is under strict program control at different specific stages by a variety of signaling molecules such as transcription factors, growth factors, cytokines, and chemokines. Key factors involved in microglial differentiation and maturation includes Runx1, PU.1, M-CSFR, and TREM2.

Runx1 binds to the enhancer elements of the PU.1 gene and results in modulation of its expression during hematopoiesis (Huang et al., 2008) and also regulates additional genes necessary for hematopoietic development.

PU.1; protein is constitutively expressed in both naive and activated rodent microglia as well as human microglia (Smith et al., 2013; Walton et al., 2000) and is essential for microglia development. The PU.1 deficient mice lack mature macrophages and neutrophils (McKercher et al., 1996), and as a result are immunocompromised and eventually die in days after birth (Anderson et al., 1998). It was also shown that the loss of PU.1 impedes the maturation of yolk-sac-derived myeloid progenitors, which is evidenced when these cells lack the

myeloid differentiation markers CD11b, CD64, and macrophage colony-stimulating factor receptor (M-CSFR) (M. C. Olson et al., 1995).
M-CSFR is a tyrosine kinase transmembrane receptor expressed throughout on mononuclear myeloid cells. The receptor consists of an extracellular ligand-binding portion and a cytoplasmic portion that are responsible for signal transduction to Jak-STAT, and Erk1/2 following ligand with dimerization with the receptor. A recent study has shown that M-CSF can induce PU.1 expression in Hematopoietic stem cells (HSCs) both *in vitro* as well as *in vivo* (Mossadegh-Keller et al., 2013). M-CSFR is critical for the regulation of mononuclear differentiation, development, chemotaxis, and survival. However, it is not necessarily a growth factor receptor for all hematopoietic cells, since M-CSFR (ligand-receptor) mutant mice result in a loss of tissue macrophages only, but not in langerhans cells or microglia (Felix et al., 1990). There has been a growing list of ligand-receptor interactions that regulate the mature microglia phenotype. Despite this, we found that the addition of M-CSF is crucial for the differentiation of iHPCs into human microglial-like cells (iMGL)(Abud et al., 2017). One such receptor is triggering receptor expressed on myeloid cells 2 (TREM2).

TREM2 is expressed exclusively on microglia in the CNS. For years TREM2's actual ligands have been unknown. However, thanks to recent *in vitro* as well as *in vivo*, we now know one such ligand to be Apolipoprotein E (APOE) (Atagi et al., 2015; Bailey, DeVaux, & Farzan, 2015). Triggering receptor expressed on

myeloid cells 2 (TREM2) binds negatively charged lipid ligands, probably through the positively charged arginine residues (R^+) that are present in its extracellular domain. Upon ligand binding, the TREM2-associated adaptor DAP12 is tyrosine phosphorylated by the protein kinase SRC and recruits the tyrosine protein kinase SYK. SYK phosphorylates the adaptors LAT (linker for activation of T-cells family member 1) and/or LAT2, which in turn recruit various signaling mediators and adaptors, including phospholipase Cγ (PLCγ) (which degrades phosphatidylinositol-3,4,5-trisphosphate (PIP3) into inositol trisphosphate (IP3) and diacylglycerol (DAG)), lymphocyte cytosolic protein 2 (LCP2; also known as SLP76), proto-oncogene vav (VAV1), growth factor receptor-bound protein 2 (GRB2) and members of the son of sevenless homologue (SOS) family. Ultimately, these pathways lead to Ca^{2+} mobilization, activation of protein kinase Cθ (PKCθ), activation of the RAS–ERK pathway and actin remodeling. SYK also activates the phosphoinositide 3-kinase (PI3K)–AKT pathway as well as the E3 ubiquitin-protein ligase CBL, which negatively regulates the TREM2 pathway. TREM2 also associates with DAP10, which recruits and activates PI3K. TREM2 can be cleaved from the cell surface by a disintegrin and metalloproteinase domain-containing protein 10 (ADAM10) and γ-secretase, thereby releasing soluble TREM2 (sTREM2) (Marco Colonna & Yaming Wang, 2016). In brief TREM2 may contribute to immune regulation by its interaction with its adaptor protein DAP-12 that can result in downstream ITAM-signal following interacting with neurons (Takahashi, Rochford, & Neumann, 2005). In the mouse DAP12

expression is restricted to microglia cells and is thought to play a major role in the CNS's innate immune functions. Mutations in TREM-2 have been associated with increased risk of Alzheimer's disease (AD), showing the importance of proper immune regulation mediated signals, for the maintenance of CNS homeostasis (Jonsson et al., 2013). Humans that are actually lacking a functional TREM2 develop a rare genetic disorder called Nasu-Hakola disease. The development of bone cysts and early onset cognitive dementia are hallmarks of this disease (Cella et al., 2003; Thrash, Torbett, & Carson, 2009). Osteoclasts have hematopoietic cells precursor, and an alteration in the TREM2/DAP12 signaling pathway could lead to bone cysts by an alteration in programmed osteoclast apoptosis or by an excessive paradoxical osteoclastic hyperfunction (M.M. Bianchin, H.M. Capella, D.L. Chaves, *et al. 2*004)

1.2 Microglia function in CNS development

Microglia play a crucial role in neuronal synaptic homeostasis. These functions includes, synaptic pruning as well as synapse maturation. The microglial participation in the process of neuronal development depends on synaptogenic signals as well as neurotrophic factors. Some of these signaling pathways are actually initiated by microglia. The microglial DAP12 signal is essential for adequate expression of tyrosine kinase receptor B (TRK-B) that detects neurotrophic factor therefore, when there is DAP12 deficiency, the results is diminished synaptic function (Roumier et al., 2004). The classical complement

cascade is another signaling pathway, which acts as potential microglial mediator of synaptic development. The complement system actually appears to facilitate microglial synaptic pruning. During neuronal development, the complement protein C1q is expressed localized to synaptic compartments in the postnatal CNS. It mediates synaptic pruning by its interaction and activation of C3 resulting in phagocytosis and synaptic elimination. The C3 receptor is expressed on microglia and in mice deficient in either C1q or C3, there is a defect in synaptic elimination, which results excessive synaptic connections retention (Stevens et al., 2007). A follow up study showed that it was really the complement component C3 and its receptor that mediated engulfment of presynaptic terminals of the developing neurons. The inhibition of neural activity with tetrodotoxin actually enhanced synaptic engulfment by microglia rather than decrease it (Schafer et al., 2012).

Microglia having another broader role in neuronal synaptic homeostasis, this is synapse maturation of synaptic and potentially remodeling of synapses, which in recent studies, it occurs in developing hippocampus and cortex. The fractalkine receptor (CX3CR1), expressed specifically by microglia in the developing CNS, has been demonstrated to be necessary for hippocampal synapse development. In the developing hippocampus, CX3CR1 deficiency results in an increased dendritic spine densities and less mature synapses, despite there being no deficits in phagocytosis of postsynaptic elements (Paolicelli et al., 2011). The

selective elimination of defective synapses is critical for the establishment of mature functional neuronal circuits during CNS development since excessive neuronal processes are able to hinder mature neuronal circuit formation. Therefore, this function is essential for normal CNS development and is mediated in fractalkine signaling (Paolicelli et al., 2011). More recent studies have shown that microglia modulate synaptic activity by their regulation of synapse densities, glutamatergic receptors, and dendritic spine numbers (K. Ji, Akgul, Wollmuth, & Tsirka, 2013). Microglia also actively participate in remodeling synaptic architecture of the visual cortex due to the synaptic plasticity (Tremblay, Lowery, & Majewska, 2010). Collectively, these studies suggest that microglia, without a doubt; participate in neuronal synaptic development and maturation.

1.3 Microglia function in healthy adulthood

We see that microglia are indispensable in normal CNS development. However, there still remain additional non-immunological roles following the completion of the CNS maturation. Recent studies revealed that microglia interact with neurons during development, and that these interactions carry over into the adult CNS. Disruption of these interactions can have a severe negative impact on neuronal function and CNS homeostasis. These vital homeostatic microglia functions help in the establishment and maintenance of the overall health of the adult CNS. In the maintenance of a healthy CNS, neuronal survival is crucial. Various neurotrophic factors that are used to promote differentiation during development

also act as specific neuronal survival mediators during adulthood. Therefore, it's important for microglia to contribute by release of trophic factors that promote their survival. Studies have shown neuronal supportive component for microglia function, which is apparent in the inhibition CX3CR1, or depletion of IGF1 on microglia, which results in the promotion of cell death of layer V cortical neurons. In addition, IGF-1 is needed to induce neural progenitor cells to differentiate into oligodendrocytes, which are needed for the homeostasis of the CNS(J. Hsieh et al., 2004). Microglia also secrete additional trophic factors other such as basic fibroblast growth factor, platelet-derived growth factor, hepatocyte growth factor, epidermal growth factor, nerve growth factor, in addition to the brain-derived neurotrophic factors which play roles in the maintenance of neuronal homeostasis and CNS function (Yamagata et al., 1995). Therefore microglia are seen to be essential facilitators of neuronal health and survival in the CNS.

Microglia are ubiquitously expressed and distributed in the adult CNS. However, this distribution is not at all uniform. In the CNS, microglia density varies from regions with high density such as the substantia nigra, to regions with low density such as the molecular layer of the cerebellum (Lawson, Perry, & Gordon, 1992).These microglia occupy their own local regions, and recent *in vivo* imaging studies reveal that they continually probe the neurons and other glia cells within their local region of coverage (Nimmerjahn et al., 2005). Differences in microglia density and morphology across the CNS resulted in studies into possible

functional differences in the adult naive state. By looking at surface expression of immune molecules only subtle differences were found in rodents (de Haas, Boddeke, & Biber, 2008); despite this, treating microglia as a heterogeneous population is gaining popularity. Recent studies suggest that there is heterogeneity in the microglia response to local inflammatory challenges. However, whether these differences in response to inflammation are due to heterogeneous microglia or their environment is not clear (Scheffel et al., 2012). Recent studies have found that IRF8 participates in the maintenance of microglia homeostasis by regulating apoptosis since they saw that IRF8 deficient cells were resistant to stimuli that induce programmed cell death (Gabriele et al., 1999). Additional studies into the role of IRF8 in the maintenance of microglial homeostasis have revealed that IRF-8 deficiency results in increased microglial abundance in the adult CNS with increased signs of activation. This suggest that IRF8 plays an important role in maintaining microglial homeostasis in the adult CNS, and additionally may potentially control their activation state (Minten, Terry, Deffrasnes, King, & Campbell, 2012).

1.4 Microglia function in injury and disease

To this point we have been discussing microglia function during normal development and adult homeostasis. However, this is not always the case and things can go awry at any point. Due to their local regional specification and their plastic nature, microglia are the most critical component of the innate immune

system in the CNS. While microglia continuously scan their microenvironment, they maintain immune vigilance and quickly respond to foreign materials that enter the parenchyma. Microglia express receptors that are able to respond to a variety of pathogens such as fungal, bacterial, and viral. Part of the innate system utilized by microglia, results in swift control of invading pathogens and preparation for the arrival of adaptive immune cells such as T cells and B cells.

Since microglia are ubiquitously distributed throughout the CNS, they can easily mount a response against almost every form of infection. These microglia are equipped intracellular pattern-recognition receptors that can recognize PAMPs, such as TLR2, TLR4, and TLR9, that detect bacterial lipopeptides, bacterial lipopolysaccharides, and bacterial DNA, respectively (J. K. Olson & Miller, 2004). Foreign bacterial material results in microglia activation, and the responses to these TLRs immune cells can induce CNS damage (Hoffmann et al., 2007). Bacteria such as *Staphylococcus aureus* (*S. aureus*) can cause brain abscesses and bacterial meningitis.

When they are activated, microglia secrete many proinflammatory cytokines such as IL-1β, IL-6, and TNFα and chemokines well as reactive nitrogen and oxygen species (RNS and ROS) following a potential bacterial infection. In response to bacteria microglia can even proliferate (Lutsik, Stekhnovich, & Lutsik, 1991). Additionally, microglia have other cellular defense mechanisms against bacteria

that help in the pathogen detection, promotion of inflammation, and facilitates phagocytosis, bacterial destruction and neuroprotection. As we have there is a close relationship between microglia and their neuronal environment more specifically the neurons themselves. Neuronal protein accumulation in disregulation of microglia has become the hallmark of many neurological diseases. It turns out that the microglia when they become reactive can eventually contribute to a chronic neuroinflammatory state, despite their initial role being one of neuroprotection.

Alzheimer's disease is a neurodegenerative disorder whose prevalence and extreme severity have made it one of the most studied neurological diseases. Yet there has been no successful established treatment proposed. For this reason, there has been an intense research drive to understand and determine a potential treatment option.

Alzheimer’s disease is characterized by the excessive accumulation of beta-amyloid in plaques, derived from amyloid precursor protein (APP) in the CNS as well as tau protein tangles inside neurons. This extreme pathology and drastic neuronal involvement results in progressive cognitive decline in Alzheimer’s patients. It has been shown that microglia express several genes that have been suggested to have roles in the resolution of Alzheimer’s disease neuropathology. CD33 can actually interfere with the ability of microglia to phagocytose beta-

amyloid(Griciuc et al., 2013). On the other hand, genes such as TREM2 have been seen to be essential for phagocytosis of beta-amyloid plaque. This clearance of deposit can only go so far, it will reach a point when the microglia become overwhelmed and have little contribution to the progression of the disease but may somehow at this point regulate neurotoxicity. In conclusion, these finding suggests that the phagocytic system in the CNS is crucial for the resolution of material accumulation be it domestic or foreign. Regardless the precise role of microglia in the course CNS injuries and diseases, there remain the need further study microglial function. The fact is that how microglia transition from proinflammatory cytokine secretion to phagocytosis to recruiting other myeloid cells, becomes necessary for the understanding of neurological injury or disease.

1.5 Macrophage assist in microglial function

Myeloid cells are professional immune cells that can invade tissues in response to injury. Different immune cells invade tissue at different rates. For example, neutrophils migrate quickly to the sight of injury and are short lived as compared to macrophages. Upon activation, microglia secrete chemoattractants that promote the recruitment of myeloid cells such as neurotrophils and monocytes into the CNS (Spanaus et al., 1997). Despite this phenomenon, other populations of non-CNS resident immune cells have received much less attention with regard to their developmental origin and turnover contrast to the microglia.

Macrophages need signal from CCR2 to extravagate into the CNS. The CNS infiltrating macrophages have distinct morphological and phenotypical differences when compared to the microglia. However, most conventional modes of measurement can't make a distinction between these two populations, illustrating the profound effect of the local microenvironment. Microglia and macrophages express most of the same molecules due to their common hematopoietic origin. A major limitation in the *in vivo* microglia studies conducted is dissecting microglia-specific contributions from those of macrophages. The way we distinguish between microglia and macrophages from the CNS is due to their slight difference in the amount of CD45 they express. *Ex vivo* analysis of microglia shows they are considered to be $CD45^{low}$ while macrophages $CD45^{high}$ (Carson, Reilly, Sutcliffe, & Lo, 1998), and *in vivo* it has been postulated that TMEM119 is a markers to distinguish microglia from macrophages (Bennett et al., 2016). One of the main features till date in microglia combating invasion or the accumulation of material in the CNS is through phagocytosis. And it has been a consensus in the literature, that macrophages are more phagocytic than microglia. The TREM2 on microglia help with its ability to phagocytose. Also, it has been suggested that TREM2 plays a role in preventing macrophage infiltration into the healthy CNS and dampening macrophage infiltration in response to systemic inflammation. So then, the interplay between resident immune cells and the peripheral immune cells is crucial to understanding the role of microglia in the CNS.

1.6 Microglia in the aging CNS

Age has been considered the single most important risk factor for sporadic neurological diseases. And with human lifespan increasing, these diseases are increasingly in the prevalent (Ferri et al., 2005). With increased age, there is also the increased potential for mechanical injuries. As a result, the role of inflammation in disease progression and physical injury is of much interest as is microglial contribution to this end. The microglia neuronal interactions are affected by age on the part of the neuron with degeneration, as well as the microglia with a loss of features. A large amount of immature neurons die during brain development as a result of defective differentiation, migration, or the failure to form proper neuronal circuits that requires elimination. This neuronal cell death can be induced by microglia, which in turn, clean up the resultant cellular debris (Wakselman et al., 2008). This cleanup is carried out through phagocytosis. It is important to note that this occurs without initiating an inflammatory response. This noninflammatory phagocytosis is mediated through microglial TREM2 signal (Takahashi et al., 2005). The TREM2 signal, as we know, results in the phosphorylation of the adaptor protein DAP12; the complex then induces the phagocytosis. Another molecule is MFG-E8 that is also expressed by microglia, and induces phagocytosis of apoptotic neurons through activation of the CD47 signaling pathway (Li et al., 2012). We see that microglia are essential for phagocytosis and clearance of apoptotic and dead cells from the CNS during developing in the healthy adult CNS, and thus maintaining homeostasis.

However, with age there is a decline in the function of the adaptive immune system In the CNS. There is increased oxidative stress resulting in a chronic proinflammatory state (Cevenini, Monti, & Franceschi, 2013). So there is a resulting perpetual double-edged sword with the onset of neurodegeneration and the persistent state of microglial activation.

Aging changes the microglial ramified morphology, density, and distribution within the CNS. Interestingly, as the CNS ages, there is altered microglial morphological features and the quality and abundance of regulatory factors also decrease as the numbers and distribution of microglia progressively lessens, indicating that aging irreparably alters microglial function. In aged microglia, there is a loss of the ramified morphology resulting in less processes, smaller dendritic arbors and less branched as compared to younger microglia (Damani et al., 2011).There is also a loss in the total number and density of the actual microglia in various CNS areas such as the visual and auditory cortices (Tremblay et al., 2010). These might be in part to the low rate of basal microglia proliferation (Lawson et al., 1992). Aging microglia can't properly regulate neuronal activity and synapses. We know from recent studies is that microglia constitutively regulate synaptic structure and neuronal activity. The microglial processes are seen to make continuous contact with neuronal synaptic structures and influence synaptic stability (Schafer et al., 2012; Wake et al., 2009). Microglia are also able to modulate neuronal activity by directly contacting neuronal somata with their

processes (Li et al., 2012). Together these show two ways, in which microglia stay in contact with the neurons in their environment, which happens in an activity-dependent manner. In the aged microglia, there is a decline in motility of processes and migration rates in the CNS (Damani et al., 2011). As a result, microglia can't make their normal timely connections with the neurons, which results in a decrease of neuronal synaptic activity. Aging microglia may also alter their neurotrophic functions. The priming of microglia in the aging CNS is of interesting, as it is one of the potential components by which systemic inflammation in elderly individuals lead to cognitive decline. We know that microglia change in their structural and activation state with age, which can be potentially associated with a decline of neuroprotective function of the CNS (Streit & Xue, 2014). This can account for the increased susceptibility to various neurodegenerative diseases. Aging results in the change in cell surface molecules to those associated with a primed microglia phenotype and enhanced cytokine synthesis (Wynne, Henry, & Godbout, 2009). The primed phenotype of microglia can be associated with both the loss of molecules involved in maintaining the naive phenotype such as the inhibitory molecules such as the inflammatory IL-10 and the activation of the proinflammatory IL-1β (Henry, Huang, Wynne, & Godbout, 2009), due to the change in their environment.

With this, we see that aging changes in baseline of microglial activation. In the adult CNS, the microglia are maintained under a perpetual steady state conditions through a balance between mitosis and apoptosis (Wirenfeldt et al., 2007). However, aging seems to interfere with this equilibrium. In addition to changes in structure, microglia demonstrate aging changes in their immune profile. Microglia in the aged CNS shows increased baseline activation and resemble activated microglia, with increased expression of CD11b and MHCII (Perry, Matyszak, & Fearn, 1993; Rogers, Luber-Narod, Styren, & Civin, 1988). More recent studies showed that this proinflammatory profile extended into the increased expression of CD11b, CD68, F4/80, FcR and CD11c in the aged CNS (Hart, Wyttenbach, Perry, & Teeling, 2012). There is also increase in the expression levels of inflammatory cytokines such as IL1beta, IL6, and TNFα in these aged microglia (Sheng, Griffin, Royston, & Mrak, 1998). We know that the microglial regulatory molecule TREM2 expression significantly decreases with age. These studies shed light for the need to study microglia in their relative context and design experiment accordingly.

CHAPTER 2

Material and Methods

2.1 Animals

Wild type (C57BL/6) mice were purchased from Jackson laboratories and housed in our vivarium. The TREM2 KO mice were previously described (Turnbull et al., 2006) and graciously donated by Marco Colonna. The CCR2 KO mice were previously described (Boring et al., 1997). All the crosses were done in house. All mice were bred and housed at the University of California Riverside animal facility with a 12/12-h light/dark cycle (lights on at 06.00 h) under specific pathogen free conditions. Both male and female mice were used in all experiments. Preference was not given to any one sex, and an even distribution of the sexes where done to the best of the ability of experimental design. The Institutional Animal Care and Use Committee at UCR approved all of the animal studies.

2.2 Systemic Inflammation

Wild type C57BL/6, TREM2 KO, CCR KO or CCR2/TREM2 dKO mice were administered an intraperitoneal injection of 5mg/kg of lipopolysaccharide (LPS)(Sigma-Aldrich, Saint Louis, MO, USA). The response to the IPLPS mimics a response to systemic infection. Animals were euthanized either 24hr or 1 week post injections.

2.3 Microglia and macrophage isolation

The microglia and macrophages were isolated immediately after euthanization. In brief, the extracted brains were mechanically separated and made into a single cell suspension. The single cell brain suspension was separated by a discontinuous 1.03/1.088 g/ml Percoll (Sigma-Aldrich, Saint Louis, MO, USA) density gradient (Carson et al., 1998; Puntambekar et al., 2011). The cell interface of the two layers as well as the top layer contained the microglia from the brains of naive mice, or microglia and macrophages from the brains of the IPLPS treated mice.

2.4 RNA isolation

Animals were anesthetized and transcardially perfused with Saline. The brains were harvested, and the right and left hemispheres where mechanically separated immediately. The RNA was isolated from either a single hemisphere or both. In brief, as previously described (Puntambekar et al., 2011), the brain tissue was homogenized and total RNA was extracted using Trizol (Invitrogen, Carlsbad, CA, USA), followed by a phase separation with chloroform (Fisher Scientific, Fair lawn, NJ, USA) and finally RNA was precipitated with Isopropanol (Fisher Scientific, Fair lawn, NJ, USA).

Collected cultured human cells were stored in Ambion RNAlater, a RNA stabilizing reagent prior to their isolation. The RNA was then isolated using the

RNeasy Mini Kit (Qiagen, Valencia, CA, USA) following the manufacturer's protocol.

2.5 qPCR

The extracted RNA was converted into cDNA using the first strand cDNA Kit (GE healthcare, Pittsburgh, PA, USA) following the manufacturer's protocol using a CFX96 Real Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA). The relative number of gene transcripts was determined using the calibration standards method for each of the tested genes. The regular PCR products performed with the same gene primers were used to generate the standards for qPCR. The resulting standards were diluted to obtain a standard curve of 50pg, 5pg, 0.5pg, 0.05pg, 0.005pg and 0.0005 pg for the qPCR analysis. Care was taken to make sure that the copy number of HPRT transcripts was in the same order of magnitude in all samples tested. This is because each gene tested was normalized to the expression of HPRT.

The following primers were used:

HPRT: F:CCCTCTGGTAGATTGTCGCTTA, R:AGATGCTGTTACTGATAGGAAATCGA

TREM1: F:TGCTGTATTGCCTTCGCTGT, R:TACAACAACAACAACAACAACAACA

TREM2: F:ACAGCACCTCCAGGAATCAAG, R:CCACAGCCCAGAGGATGC

TREM3: F:GATGTGGGGCTGTACCAGTG, R:GCTGTAACACCTTAGGCCCAG

TREM4: F:GCTTCAACTTCTTCACTGTCACC R:GTGTTTTGTGGTAATAAGGGTAGGA

TREM5: F:GGATGTTGTTGCCTGGAAGAC R:GCTCTCCATCTCCCTGCCTA

Iba1: F:TGCCAGCCTAAGACAACCAG, R:GTTTGGACGGCAGATCCTCA

TLT2: F:GTAACACGGCTGGGCATTT, R:TGTAACGGTGCCACTGGTATC

CCL2: F:TTGACCCGTAAATCTGAAG, R:TCACAGTCCGAGTCACACT

CCL5:F:ATGAAGATCTCTGCAGCTGCCCTC, R:CTAGCTCATCTCCAAATAGTTGATG

Arg1: F:CAGAAGAATGGAAGAGTCAG, R:CAGATATGCAGGGAGTCACC

iNOS: F:GGCAGCCTGTGAGACCTTTG, R:GCATTGGAAGTGAAGCGTTTC

TNFα: F:CTGTGAAGGGAATGGGTGTT, R:GGTCACTGTCCCAGCATCTT

TGFβ: F:ATCGACATGGAGCTGGTGAA, R:CATAGATGGCGTTGTTGCG

IL-1β: F:GGAAGGCAGTGTCACTCATTGT, R:GGTCCTCATCCTGGAAGCTCC

IL-1 RN: F:CAGTTCCACCCTGGGAAGGT, R:AGCCATGGGTGAGCTAAACAGGACA

P2Y12: F:AGGCTTTGGGAACTTATGC, R:GGGTGGTATTGGCTGAGGTG

2.6 FACS and Flow Cytometer Analysis

Acutely isolated mouse microglia cells were analyzed by the FACSCalibur (BD Biosciences, Drive Franklin Lakes, NJ, USA) flow cytometer. Mouse cells were centrifuged and resuspended in ice-cold sterile filtered FACS buffer (1X HBSS, 5% FBS, and 0.05mM EDTA). The mouse cell populations were determined by using fluorescently conjugated antibodies APC-conjugated anti CD45, PE-conjugated anti FcR (eBiosciences Inc, San Diego, CA, USA). The engulfment particles were labeled with FITC- pHrodo conjugated dye (Invirogen, Carlsbad, CA, USA). The microglia receptor expressions were labeled with anti CD47, anti CD93, anti TLR2 and anti SiglecH (eBiosciences Inc, San Diego, CA, USA) and anti Tyro3, anti Axl and anti Mer (R&D systems, Minneapolis, MN, USA).

Human cells cultures were analyzed by the FACSCalibur or FACSARIA (BD Biosciences, Drive Franklin Lakes, NJ, USA) flow cytometer. Human cells were centrifuged and resuspended in ice-cold sterile, filtered and degassed FACS buffer (1X DPBS, 2% BSA, and 0.05mM EDTA). The iMGL were then incubated with human Fc block (BD Bioscience, Drive Franklin Lakes, NJ, USA) for 15 min on ice. For detection of human hematopoietic surface markers, cells were stained with anti CD11b-FITC, anti CD45-APC/Cy7 (Tonbo Biosciences, San Diego, CA, USA), anti CD11c-APC (BD Biocsience, Drive Franklin Lakes, NJ, USA), and anti CD192 (CCR)-brilliant violet (Biolegend, San Diego, CA, USA) and directly ran on the FACSARIA. The FACSCalibur was used for analysis of the human phagocytosis experiment. The cell population was determined by using fluorescently conjugated anti CD11b-PE and anti CD45-APC/Cy7 (Tonbo Biosciences, San Diego, CA, USA). The flow cytometric data was analyzed using FlowJo software V.10.1 (Tree Star, Inc, Ashland, OR, USA).

2.7 Immunohistochemistry

All animals were anesthetized and transcardially perfused with Saline, followed by 4% paraformaldehyde (PFA)(Sigma, Saint Louis, MO, USA). Brains were extracted and post-fixed in 4% PFA overnight at 4°C. The brains were then removed from the 4% PFA and placed in a 30% sucrose in 4% PFA solution until they sunk at 4°C (this step was for cryoprotection). The frozen brains were then cut into free-floating coronal sections of 25µm using the Leica CM1850 cryostat

(Leica Biosystems Inc., Buffalo Grove, IL, USA). Sections were stored in cytoskeleton buffer with sucrose (CBS) at -80°C before IHC. Floating brain sections were blocked for 1 hr at room temperature (RT) in PBS containing 10% normal goat serum (NGS)(Vector Laboratories Inc, Burlingame, CA, USA) and 0.2% Triton X-100 (Sigma, Saint Louis, MO, USA) and then incubated overnight at 4°C in primary antibody vGlut1 (1:200) (Invitrogen life technologies, Eugene, OR, USA) and GAD65 (1:200) (BD Biosciences Pharmingen, San Diego, CA, USA). The sections were washed, and incubated for 1 hr at RT with Alexa Fluor conjugated secondary antibody (Invitrogen life technologies, Eugene, OR, USA) diluted to 1:2000 then washed again. The sections were then mounted on slides (Superfrost Plus–VWR) and cover slipped with Prolong gold with DAPI (Invitrogen life technologies, Eugene, OR, USA).

2.8 Imaging

The imaging of the coronal brain tissue sections were acquired using a BD CARVII Confocal Imager (BD Biosystems, Franklin Lakes, NJ, USA), which utilized the Metamorph Imaging Software (Ver. 7.7.0.0, Sunnyvale, CA, USA) on a Zeiss inverted microscope. Images of the hippocampus were taken in the CA1 region closest to the pyramidal layer. Images were captured in multidimensions at 63x with a 30-step z-stacks 0.59 µm/pixel thickness that encompassed the neuronal soma and processes. Human cells were imaged in bright field. Images of cell cultures were captured on an Evos XL Cell Imaging microscope.

2.9 Volocity image analysis

Confocal images, 15 regions images per hippocampus captured in z-stacks were analyzed using Volocity Imaging Software (Perkin Elmer, Waltham, MA, USA). Neuronal synaptic puncta was calculated based on their fluorescence. The puncta were visualized using the XYZ views in Volocity imaging software and calculated using the “find points” function in the measurement section. With this, the total number of puncta was calculated. Finally, the number of puncta was divided by the total area of the image to get the number of puncta per 100μm^2.

2.10 Synaptosome isolation

Synaptosomes were isolated from whole adult wild type brains as previously described in (Zhang et al., 2008). Following isolation, we confirmed synaptosome protein by western blotting**.** The cells were lysed with RIPA lysis buffer with protease inhibitors. An equal amount of protein was loaded for each lane as determined by the Pierce BCA Protein Assay Kit (Life Technologies Corporation, Grand island, NY, USA). Nitrocellulose membranes were blocked in TBS with 0.1% Tween and 5% non-fat milk. The following antibodies were used anti-Synaptophysin 1:1000 (Millipore, Temecula, CA, USA) anti-HDAC-1 1:200 (Santa Cruz, Dallas, TX, USA). Primary antibodies were incubated overnight at 4°C and IR-secondary antibodies for 1 hour at room temperature. Proteins were

detected using the Odyssey imager (LI-COR Biotechnology, Lincoln, NE, USA). The synaptosomes where then labeled with the pHrodo dye following manufactures protocol (Invirogen, Carlsbad, CA, USA). The synaptosome particles were resuspended at a protein concentration of 0.2ug/*µl* in phagocytosis buffer.

2.11 Phagocytosis

1-1.5 x10^5 acutely isolated mouse microglia cells were assayed in 1ml of DMEM, supplemented with 5% FBS or 1-2 x10^5 iMGL cells were assayed in 1ml of (base media) for 60 minutes at 37°C under slight agitation. 100µl of pHrodo *S. aureus* (Invirogen, Carlsbad, CA, USA) or isolated Synaptosomes added 60, 30 or 15 minutes before the completion of the assay in a reverse time course so that all cells were assayed for the same amount of time. pHrodo dye fluoresces once the phagolysosomal internalized particles. To stop the phagocytosis assay we added excess ice-cold media and immediately centrifuged. Cells were then resuspended and stained for flow cytometry. The mean fluorescence intensity (MFI) of cell phagocytosing particles was determined. A quantitative estimation of phagocytosis was obtained by subtracting the various time point by the 0 time point.

2.12 Differentiation of iHPCs to iMGL

iHPCs were handled and stored in accordance with CDI's guideline. The iMGL were cultured as previously described (Abud et al., 2017). At the start of day 0 the iCell Hematopoietic Progenitor Cells (iHPCs) (Cellular Dynamic International, Madison, WI, USA) were thawed following the manufacturer's protocol and then washed using iMGL base differentiation medium: phenol-free DMEM/F12 (1:1), insulin (0.02 mg/ml), holotransferrin (0.011 mg/ml), ITS-G, 2%v/v, B27 (2% v/v), N2 0.5%, v/v, monothioglycerol 200 mM, Glutamax 1X, NEAA 1X, and additional insulin (5 mg/ml; Sigma) and filtered through a 0.22 mm filter) and centrifuged at 300 x g for 6 min at room temperature (RT). Following centrifugation steps, the supernatant was aspirated and discarded then the iHPCs were gently suspended in complete differentiation medium which is the base medium supplemented with M-CSF 25 ng/ml, IL-34 100 ng/ml (PeproTech, Rocky Hill, NJ, USA), and TGFb-1 50 ng/ml (Militenyi, Bergisch Gladbach, Germany) added fresh each time. Cell density was adjusted to seed at 1-2 $x10^5$ cells in 2 mL of complete medium per well in growth factor-reduced Matrigel coated 6-well plates. iMGL were supplemented with 1 mL per well of fresh RT complete differentiation medium on every other day. On day 12 differentiating iMGL were collected and centrifuged at 300x g for 6 min at RT, then a half the supernatant was replaced with fresh RT complete differentiation media. After 25 days of culturing, iMGL were transferred to a maturation media, which is complete, differentiation media supplemented with CD200 100 ng/ml (Novoprotein, Summit, NJ, USA) and CX3CL1 100 ng/ml

(PeproTech, Rocky Hill, NJ, USA) and cultured for three additional days to complete the iMGL maturation.

2.13 Mouse chamber

The chamber was made of clear acrylic sheets with external dimensions of 40 x 32 x 25 $inch^3$ (length x width x height) for approximately 540 L in volume. Inside the chamber, two perforated aluminum plates separated the inlet (from upper left) and the outlet (lower right) to enable uniform dispersion of injected aerosols. The large size of the chamber accommodates up to six mouse cages simultaneously for exposure tests. On the top of the chamber, an LED warm light string was attached to a timer switch to provide a 12:12 hour light cycle. The whole chamber was covered with blackout cloth to ensure zero light contamination from outside. A ¼ inch inlet from the upper left of the chamber was used for injection while four ½ inch exhaust ports located in the lower right chamber ensured that the in-chamber pressure remained the same as atmospheric. Another ¼ inch outlet located in the lower right of the chamber was used for instrument sampling and monitoring. The chamber was flushed with clean air with at least ten chamber volumes before and after each test to avoid contamination.

2.14 Aerosol generation

Lab compressed air passed through a clean air system consisting of silica gel (to absorb water moisture), activated carbon (to absorb hydrocarbon), hopcalite (to absorb CO) and purafil (to absorb NOx). Pressure at both the inlet and outlet of the clean air system were monitored and controlled by a pressure regulator. During the test, the inlet pressure remained controlled at 60 psi while the outlet pressured was 25 psi, resulting in an aerosol flow at 6 L/min (monitored by a mass flow controller) coming out of the nebulizer. The nebulizer converted *Alternaria* liquid filtrate solution into an aerosol spray (May et al., 1973) and the spray went through a diffusion dryer for physical absorption of water vapor. This flow can saturate the 540 L mouse chamber with *Alternaria* aerosol and maintain the saturation concentration through continuous injection.

2.15 Sample monitoring system

Four instruments were attached from the sample port of the chamber. Aerosol concentration and size distribution were monitored by a Scanning Mobility Particle Sizer (SMPS). The SMPS is widely used as a standard method for characterization of particles smaller than 1 μm in diameter. (Wang and Flagan, 1990., Mulholland et al., 2006). It provides size from 2 to 1000 nm with high resolution (107 channels). A Condensation Particle Counter (CPC) was used to measure airborne particle number concentration for the SMPS to provide overall particle size distribution. The Laser Aerosol Particle sizer (LAP) served as a

supplemental instrument for monitoring particle size ranging from 200 nm to 40 μm with high resolution of up to 128 channels. A DustTrak (TSI) provided information about the total $PM_{2.5}$ concentration. A modified ammonia (NH_3) analyzer provided measurements of the NH_3 level inside the chamber.

2.16 Operation of the chamber

Biological material introduction and dose preparation. Lab compressed air passed through a clean air system before entering an atomizer. A stainless-steel atomizer generated aerosols from a 0.26 g/mL solution of *Alternaria* filtrate in ultrapure water. The wet aerosol passed through a heated copper coil at 52°C to transform water moisture into vapor and the water vapor was absorbed by passing through a diffuser dryer filled with indicating silica gel, which was replaced daily. These dry aerosols produced were injected into the mouse chamber. The chamber held mice in conventional mouse cages with wire tops to hold food as well as enable free flow of air into the cages, and hydrogel containers in the bottom to provide water along with conventional wood shaving bedding. Injected aerosols were shown to saturate the chamber with *Alternaria* particulates by 2 hours of continuous injection. In this study, tests continued for 96 hours with continuous aerosol injection. During the injection period, instruments monitored the aerosol concentration and size distribution, as well as the overall Particulate Matter (PM) concentration. At the end of the exposure period, ammonia levels were monitored to ensure that the mice were under a

non-toxic environment. The chamber was flushed with clean air of at least ten chamber volumes before and after each test to avoid contamination.

2.17 Statistics

All data was graphed using Prism6 (GraphPad Software, San Diego, CA, USA) and analyzed using a one or two-way ANOVA with either a Tukey or Sidak's posttest. The statistics from the microglia and macrophage genotype changes with age and activation used the Tukey's posttest. The statistics from the phagocytosis assay compares the genotypes or microglial activation states at the end point of the assay and used the Tukey's posttest. The statistics from the acutely isolated cells compare the genotype with in a cell type with the Sidak's posttest or the other cell types to the activated microglia with the Tukey's posttest. All data are presented as the mean ± SEM. For all data, statistical significance was assigned one star designating $p < 0.05$, two stars designating $p < 0.01$, three stars designating $p < 0.001$ and four stars designating $p < 0.0001$.

CHAPTER 3

Differential regulation of neuronal development by blood derived and CNS resident immune cells

3.1 ABSTRACT

Microglia play an important role in the central nervous systems (CNS) development and maturation. It regulates neuronal development in an activation dependent manner. We determined that Triggering Receptor Expressed on Myeloid cells 2 (TREM2) mutations directly affect microglial and not neuronal development and function since neurons don't express TREM2. Here we demonstrate that systemic inflammation induced by Lipopolysaccharide (LPS) or mutations in microglial genes result in aberrant neuronal environment and defects in the blood-brain barrier (BBB) structure in as little as 24 hours and synaptogenesis which becomes evident after one week. These results indicate that in order to determine and better understand how mutations in microglia result in neuronal degenerative diseases, and aberrant response to physical injury, we first need to understand microglia functions in the context of the development of their neuronal environment.

3.2 INTRODUCTION

Microglia are universally expressed in all regions of the CNS and act as specialized type of tissue macrophage (Carson et al., 2007; Lawson et al., 1992) that supports neuronal function during development and maturation. The PU.1 transcription factor is a protein that encoded by SPI1 and activates gene expression during myeloid cell development (Pham et al., 2013). The expression of PU.1 target genes are not consistent in all cell types or specific to the CNS, despite the difference in cellular concentration of PU.1 and their regulation of neighboring transcription factors, its presence is needed for mouse postnatal survival. Importantly, PU.1-deficient mice result in a complete loss of microglia and mature macrophages, showing that PU.1 is key in regulating genes involved in differentiation and maturation of hematopoietic cells in the CNS(Beers et al., 2006; McKercher et al., 1996). However, as of now a complete and comprehensive profile of PU.1 target genes involved in microgliogenesis remains uncharacterized. So to clarify the biological role of PU.1 in regulation of microglial development, we attempted to characterize the hematopoietic target genes in the CNS and identify the presence of microglial specific cell profile in p1 pups. This was done to definitively indicate which cellular markers in the CNS are immune cells specifically expressed.

Unlike neurons, microglia are generated when embryonic yolk sac myeloid precursors travel to the brain and differentiate (Eglitis & Mezey, 1997). Triggering

Receptor Expressed on Myeloid cells-2 (TREM2) is enriched on microglia as compared to all other types of myeloid cells (Schmid et al., 2009) and in the CNS only expressed by microglia. Data has shown a new and crucial role for microglia in the contribution to neuronal proliferation, the control of differentiation, and formation of synaptic connections (Graeber, 2010; Hughes, 2012). So mutations in microglia alter their surveillance of their environment and their editing of synapses. We know a single point mutation in microglial TREM2 can result in an aberrant phenotype and that the microglial phenotype is also shaped by the CNS environment and developmental stage (Carson et al., 2007; Hong et al., 2016). However, the reverse is also true in that microglia shape neuronal development. So we wanted to see how mutations in TREM2 affect the neuronal environment development.

Microglia are an important component of the neurovascular unit (NVU)(Spindler & Hsu, 2012). The NVU comprises microglia as well as endothelial cells, neurons, basal lamina, pericytes, and astrocyte end feet that function as a physical blood-brain barrier (BBB). The BBB ensures homeostasis and adequate functioning of the brain by having non-fenestrated endothelial cells with tight junctions and express only specific membrane transporters that controls trafficking in and out of the CNS. Microglial activation as a result of brain injury or neurodegeneration and diseases impair the BBB (Dickstein et al., 2006; Zipser et al., 2007). We wanted to know how the CNS resident and blood derived immune

cells that are allowed to pass the BBB, contributes to neuronal development. We already know that in response to systemic inflammation, microglia both acquire new functions and decrease some homeostatic functions. In addition, during systemic inflammation an additional myeloid population derived from the blood, appears in the CNS. However, it is not clear how activated microglia and infiltrating macrophages alter functions in the context of neurodevelopment. So this is what we hoped to clarify.

Microglia were shown to contribute to the modification and the elimination of synaptic structures (Tremblay et al., 2010)and more specifically, to the synaptic pruning during postnatal development in mice (Paolicelli et al., 2011). The synaptic developmental component of neuronal development can be measured by the degree of pruning of presynaptic sites. Since we know microglia play a role in synaptic pruning, the elimination of synapses during brain maturation we can test how microglial mutations contribute to defective pruning. By the quantification of the formation of synaptic sites, we will get insight into microglial contribution to pruning.

Systemic inflammation, a normal childhood event, can be modeled by simple IPLPS challenge of mouse pups. Lipopolysaccharide (LPS) is a gram-negative bacterial endotoxin, which elicits a rapid innate immune response. Here we administer LPS intraperitoneal at p14 and analyzed its effects at p15 and at p21.

Intraperitoneal injection of LPS in mice results in microglial activation and macrophage infiltration into the brain that peaks between 24 hours post-injection. We hypothesized immune cells play key regulatory roles in neuronal development and maturation, and that immune cells regulate this neuronal synaptic maturation in an activation dependent manner. We tested the consequences of immune modulation in the presence and absence of IPLPS immune challenge given during a critical period in neuronal synaptogenesis.

3.3 RESULTS

3.3.1 TREM2 is microglia specific in the CNS.

When PU.1 is knocked out (KO), there is an absence of microglia in the CNS. We wanted to address the controversy of neurons also expressing TREM2. We decided to determine whether the CNS displayed altered expression of TREM family of molecules when PU.1 was knocked out. Specifically, we performed qPCR looking at TREM1, TREM2, TREM3, TREM4, TREM5, and iba1 with whole brain cDNA from p1 PU.1 KO mice comparing them to the congenic wild type whole brain cDNA. PU.1 KO mice expressing TREM1and its expression is greater than what is seen in wild type (Figure 3.1 A). TREM2 is completely depleted when PU.1 is knocked out. (Figure 3.1 B). TREM3 like TREM4 expression in PU.1 mice was extremely low and similar to increase in the expression in wild type mice (Figure 3.1 C-D). Though TREM5 was also similar between PU.1 KO and wild type, there expression was significantly detected (Figure 3.1 E). The PU.1 KO mice have significantly less Iba1 than wild type (Figure 3.1 F). However, it is interesting that they were still expressing it. Since the PU.1 mice showed loss of expression of the CNS microglial markers TREM2, this indicates that its expression is specific to myeloid cells in the CNS and not expressed by neurons. So when TREM2 is subsequently knocked out, the resulting phenotype will be microglial specific.

3.3.2 TREM2 KO brains are pre inflamed at postnatal day 15.

After showing that PU.1 was required for microglia formation we knocked out PU.1 there was an absence of an inflammatory state. We decided to determine whether the CNS neuroenvironment displayed altered expression of inflammatory molecules when the TREM2 was knocked out. Specifically, we performed qPCR looking at TLT2, TNFα, CCL2, CCL5, IL-1β, IL-1RN, Arg1, and iNOS with whole brain cDNA from naive and IPLPS treated p15 TREM2 KO mice comparing them to the congenic wild type whole brain cDNA. Our previous flow cytometric studies showed that TLT2 protein like TREM2 is highly expressed by microglia during the early postnatal period. Here we observed that TREM2 KO mice though expressing TLT2 were not able to induce its expression with activation (Figure 3.2 A). They displayed a multiple fold increase in TNFα at baseline, however we were not able to induce it much with activation (Figure 3.2 B). With CCL5 unlike CCL2 TREM2 KO mice also displayed a significant increase in the expression in the naive as well as the IPLPS treated mice as compared to the wild type brain (Figure 3.2 C-D). CCL2 binds to CCR 2/4 and is important for monocyte chemotaxis while CCL5 bind CCR1/3/5 and is important for leukocyte (T cell) recruitment. IL-1β is only induced in wild type not TREM2 KO mice and the IL-1RN remained low in both the naive and IPLPS treated compared to wild type (Figure 3.2 E-F). Naive TREM2 KO mice have greater Arginase 1 and iNOS compared to naive wild type brains (Figure 3.2 G-H). Since the naive TREM2 KO mice show higher levels of expression of many

inflammatory markers than wild type mice, this indicates that these mice are preinflamed. Interestingly, these mice do not exhibit as great of a response as to IPLPS treatment as do the wild type, potentially due to their persistent exposure chronic neuroinflammation in their naive state.

3.3.3 TREM2 KO and IPLPS disrupt the BBB at postnatal day 15.

Previous studies have shown that components of the NVU such as astrocytes, play a key role in maintenance of the BBB (Janzer & Raff, 1987). This suggests the interaction of the endothelial cells with the neural environment is the basis of maintaining the integrity of BBB with potential microglial interactions as well. Here we see that IPLPS treatment results in significant microglia activation and fibrinogen infiltration into the brain (Figure 3.3 B) indicating loss of BBB integrity as compared to the naive (Figure 3.3 A). Interestingly, when TREM2 is knocked out, we also see significant fibrinogen infiltration even in the naive state (Figure 3.3 C) that increases following IPLPS treatment (Figure 3.3 D). These results indicate proper microglial function is required for the maintenance of an intact BBB.

3.3.4 Immune challenge inhibits normal excitatory synaptic development.

IPLPS induced immune challenge at p14 results in a visual loss of the excitatory vGlut1 expression by p21 in the CA1 region of the hippocampus of wild type mice while inhibitory GAD65 stays relatively unchanged (Figure 3.4 A). Specifically, we

performed IHC analysis of looking at vGlut1 and GAD65 puncta expression in brain sections from naive and IPLPS treated p21 Wild type, TREM2 KO, CCR2 KO, and CCR2/TREM2 dKO mice. Our results indicate that IPLPS treatment and microglial TREM2 deficiency activates pruning of excitatory synapses (Figure 3.4 B). We also find that macrophages are required for immune challenged activation of excitatory synaptic pruning while, TREM2 deficiency is sufficient to activate immune challenged excitatory synaptic pruning, even in the absence of macrophages (Figure 3.4 C). Interestingly, neither IPLPS immune challenge nor TREM2 deficiency alters inhibitory synaptic pruning (Figure 3.4 D-E). Our findings give direct proof of CNS immune cells influencing neuronal development and maturation. Giving a further need to understand microglial functions, and to determine the effects they have on their environment in a naive state as well as the diseased state. We take this further my analyzing which cells are influencing the Synapse maturation by analyzing cocultures of neurons with microglia or macrophages. We test the viability of these cultures with neuronal MAP2 staining (Figure 3.4 F). We then can analyze their expression of vGlut1 and GAD65 on the neuronal dendrites (Figure 3.4 G). This data is then used to get an idea of the individual cell types contribution to synapse maturation.

3.4 DISCUSSION

These results suggest that PU.1 plays a pivotal role in the regulation of microglia specific genes regulation. As TREM2 is a microglial specific gene in the CNS is not expressed (Thrash et al., 2009). Recently, studies have shown TREM1 expression in non-myeloid endothelial cells (Chen, Laskin, Gordon, & Laskin, 2008). While TREM1 is known to be regulatory induced in the context of inflammation, it is unclear how epithelial cells regulate it (Bouchon, Hernandez-Munain, Cella, & Colonna, 2001). On the other hand, TREM3 and TREM4 are low expression genes, so it stands for reason that knocking out PU.1 had no effect on their expression. Studies have also reported that TREM5 is also a pseudo gene (Chung, Seaman, & Daws, 2002) and as such it is not unexpected that knocking out PU.1, had no effect on it. The classical IHC marker for CNS microglia is the intracellular ionized calcium binding adapter molecule 1 (Iba1). However, it should be noted that Iba1 is not myeloid-specific outside the brain (Imai, Ibata, Ito, Ohsawa, & Kohsaka, 1996) and it could be possibly labeling some other type of cell such as Lymphoid (Kelemen & Autieri, 2005).

The TREMs regulate a range of immune responses and perform various functions. These TREMs belong to a family of related proteins that includes the TREM Like Transcripts (TLTs)(Ford & McVicar, 2009). We have

previously shown that As TLT2 is not developmentally regulated. However, here we show TLT2 expression is regulated by inflammation and is TREM2 dependent. Loss of TREM2 results in an inflammatory profile that in some extent resembles IPLPS treatment.

Some recent studies have suggested that microglial activation may be related to BBB disruption. Here we have show that IPLPS treatment which microglia are activated result in the disruption of the BBB. This disruption result in the influx of vascular material, which result in persistent CNS inflammatory state. It has been suggested that produced reactive oxygen species (ROS) by activated microglia directly impair BBB(Sumi et al., 2010).

IPLPS immune challenge and TREM2 deficiency activates excitatory synaptic pruning in the stratum radiatum of the hippocampus. We showed that IPLPS immune challenge decreases vGlut1 puncta levels. But in CCR2 KO mice IPLPS does not result in decreased vGlut1 puncta levels indicating this mechanism is macrophage dependent. However, TREM2 deficiency suppresses the effects observed in CCR2 KO mice, and vGlut1 puncta levels decrease. Synaptic pruning in response to systemic inflammation involves macrophages. However, here we show that TREM2 forms its own independent mechanism of synaptic pruning. We also saw that C1q KO had

increased vGlut1 in the naive that decreased when TREM2 was knocked out (data not shown). Since neither IPLPS immune challenge or TREM2 deficiency alters GAD65 inhibitory synaptic pruning. We can speculate that this regulation is synapse specific.

3.5 FIGURES AND LEGEND

Figure 3.1. Microglia specific TREM2 lost in PU.1 KO. Bar graphs of HPRT normalized qPCR data showing loss of microglial-enriched genes in the PU.1KO mice. **(A)** TREM1, **(B)** TREM2, **(C)** TREM3, **(D)** TREM4 **(E)** TREM5, and **(F)** Iba1 measured in postnatal day 1 mice pups. Data is represented as mean ± SEM and analyzed using an unpaired t test with a two-tail P value. Statistical annotation represents greatest p value for TREM2 expression. Wild type (n = 6), and PU.1 KO (n = 3). *P < 0.05; **P < 0.01; ***P < 0.001 and ****P < 0.0001.

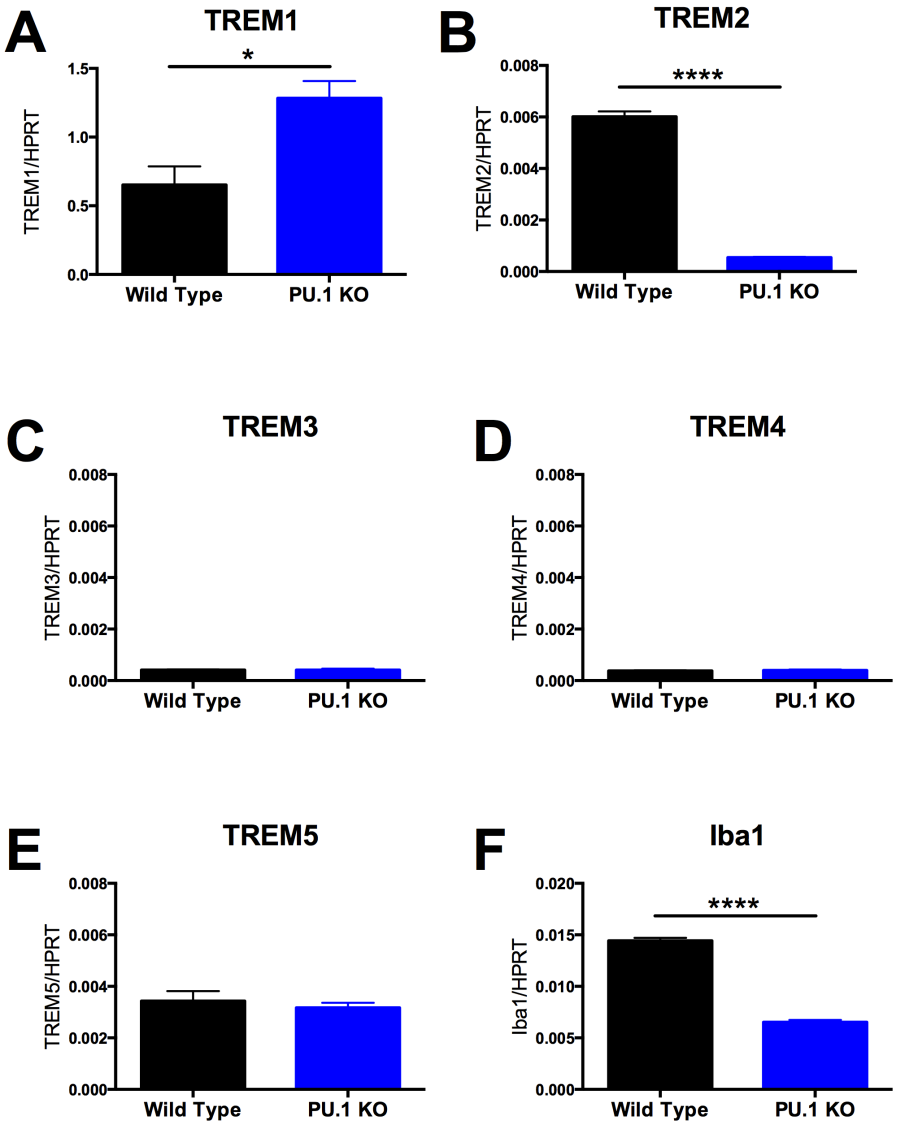

A
TREM1
*
TREM1/HPRT
1.5
1.0
0.5
0.0
Wild Type
PU.1 KO
B
TREM2
****
TREM2/HPRT
0.008
0.006
0.004
0.002
0.000
Wild Type
PU.1 KO
C
TREM3
TREM3/HPRT
0.008
0.006
0.004
0.002
0.000
Wild Type
PU.1 KO
D
TREM4
TREM4/HPRT
0.008
0.006
0.004
0.002
0.000
Wild Type
PU.1 KO
E
TREM5
TREM5/HPRT
0.008
0.006
0.004
0.002
0.000
Wild Type
PU.1 KO
F
Iba1
****
Iba1/HPRT
0.020
0.015
0.010
0.005
0.000
Wild Type
PU.1 KO

Figure 3.2. TREM2 KO brains don't up regulate typical inflammatory molecules at postnatal day 15. Bar graphs of HPRT normalized qPCR data TREM2 KO mice. **(A)** TLT2, **(B)** TNFα, **(C)** CCL2, **(D)** CCL5, **(E)** IL-1β, **(D)** IL-1RN, **(E)** Arg1, and **(F)** iNOS measured in naive or IPLPS treated postnatal day 15 mice pups. Data is represented as mean ± SEM and analyzed using one-way ANOVA followed by Tukey's corrected multiple comparison post hoc test. Wild Type naive (n = 8), IPLPS Wild Type (n = 8), TREM2 KO naive (n = 8), IPLPS TREM2 KO (n = 7) *P < 0.05; **P < 0.01; ***P < 0.001 and ****P < 0.0001.

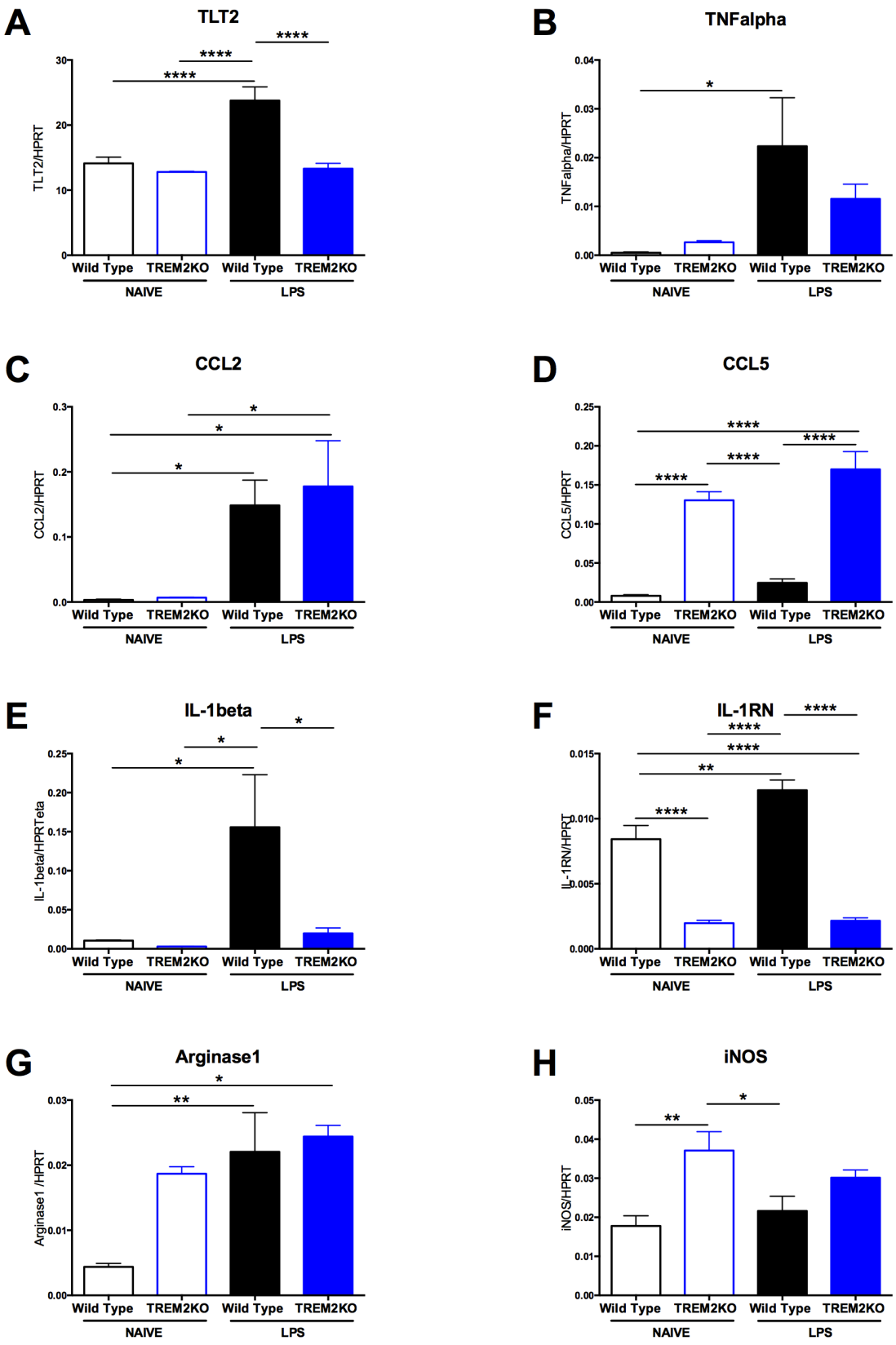

A
TLT2
TLT2/HPRT
B
TNFalpha
TNFalpha/HPRT
C
CCL2
CCL2/HPRT
D
CCL5
CCL5/HPRT
E
IL-1beta
IL-1beta/HPRTeta
F
IL-1RN
IL-1RN/HPRT
G
Arginase1
Arginase1 /HPRT
H
iNOS
iNOS/HPRT
Wild Type
TREM2KO
Wild Type
TREM2KO
NAIVE
LPS

Figure 3.3. IPLPS immune challenge and TREM2 KO disrupt the BBB. (A) Imaging of the wild type naive hippocampus and cortex showing the expression of Lectin and Fibrinogen. **(B)** Imaging of the wild type IPLPS hippocampus and cortex showing the expression of Lectin and Fibrinogen. **(C)** Imaging of the TREM2 KO naive hippocampus and cortex showing the expression of Lectin and Fibrinogen. **(D)** Imaging of the TREM2 KO IPLPS hippocampus abd cortex showing the expression of Lectin and Fibrinogen.

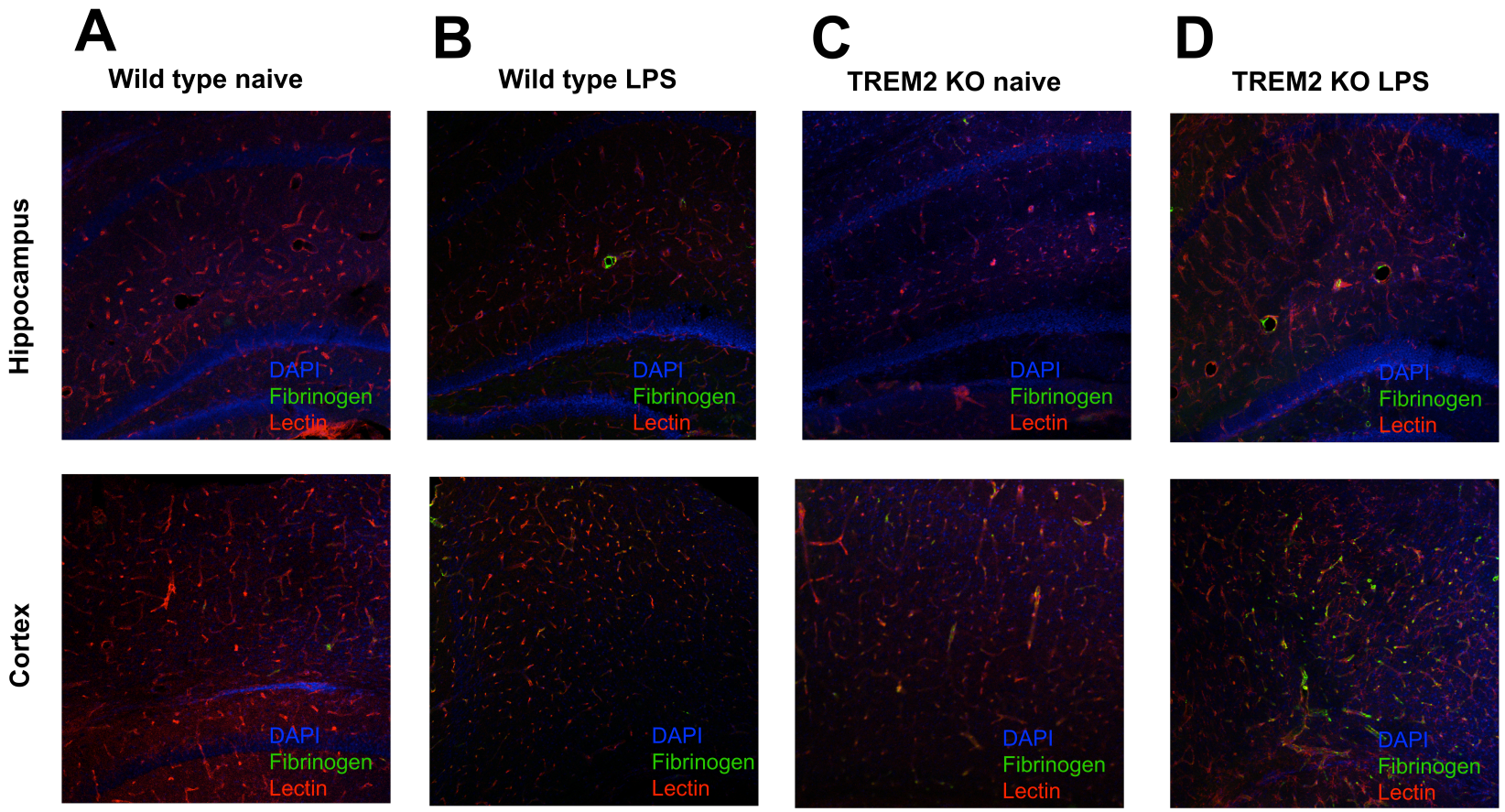
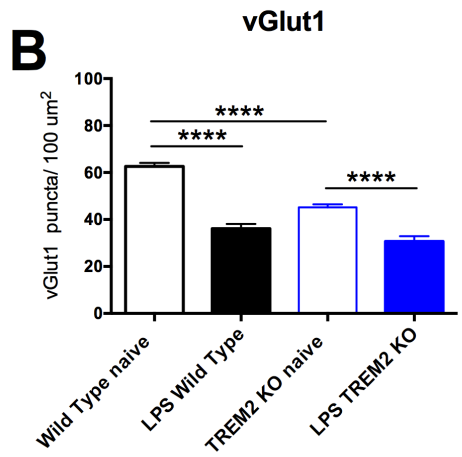

A
Wild type naive
B
Wild type LPS
C
TREM2 KO naive
D
TREM2 KO LPS
Hippocampus
Cortex
DAPI
Fibrinogen
Lectin

Figure 3.4. IPLPS Immune challenge and TREM2 KO inhibit normal excitatory synaptic development. (A) Confocal imaging of the CA1 region of the hippicampus showing the expression of vGlut1 and GAD65. **(B-C)** Quantitative analysis of vGlut1 and GAD65 puncta here, the bar graphs show the number of puncta per 100μm area for wild type and TREM2 KO from the naive and IPLPS treated animals. **(C-E)** Quantitative analysis of vGlut1 and GAD65 puncta here, the bar graphs show the number of puncta per 100μm^2 area for CCR2 KO, and CCR2/TREM2 dKO from the naive and IPLPS treated animals. **(F-G)** Images of neuronal culture for analysis of neurons, vGlut1 and GAD65 puncta per 25μm area for neuron only, p15 microglia naive/IPLPS, p15 macrophages, and adult microglia, cocultures. Data is represented as mean ± SEM and analyzed using one-way ANOVA followed by Tukey's corrected multiple comparison post hoc test. Wild Type naive (n = 8), IPLPS Wild Type (n = 8), TREM2 KO naive (n = 8), IPLPS TREM2 KO (n = 7), CCR2 KO naive (n = 5), IPLPS CCR2 KO (n = 6), CCR2/TREM2 KO naive (n = 6), IPLPS CCR2/TREM2 KO (n = 6). *P < 0.05; **P < 0.01; ***P < 0.001 and ****P < 0.0001.

A
Wild Type naive
Wild Type LPS
vGlut1
GAD65
vGlut1
GAD65
GAD65
vGlut1
GAD65
vGlut1


B
vGlut1
vGlut1 puncta/ 100 um2
100
80
60
40
20
0
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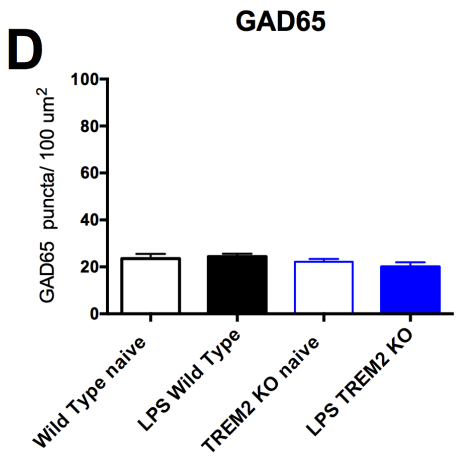
Wild Type naive
LPS Wild Type
TREM2 KO naive
LPS TREM2 KO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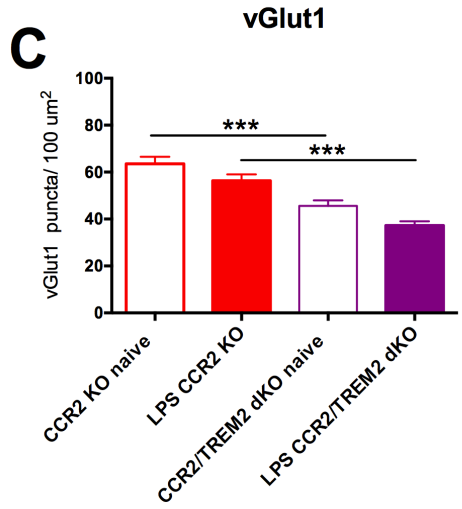
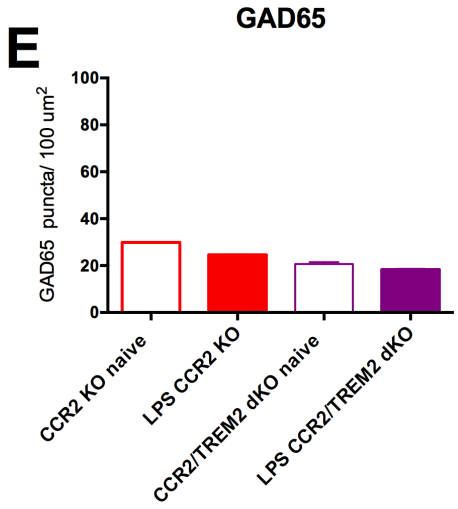
GAD65
GAD65 puncta/ 100 um2
100
80
60
40
20
0
Wild Type naive
LPS Wild Type
TREM2 KO naive
LPS TREM2 KO


C
vGlut1
vGlut1 puncta/ 100 um2
100
80
60
40
20
0
***
***
CCR2 KO naive
LPS CCR2 KO
CCR2/TREM2 dKO naive
LPS CCR2/TREM2 dKO


E
GAD65
GAD65 puncta/ 100 um2
100
80
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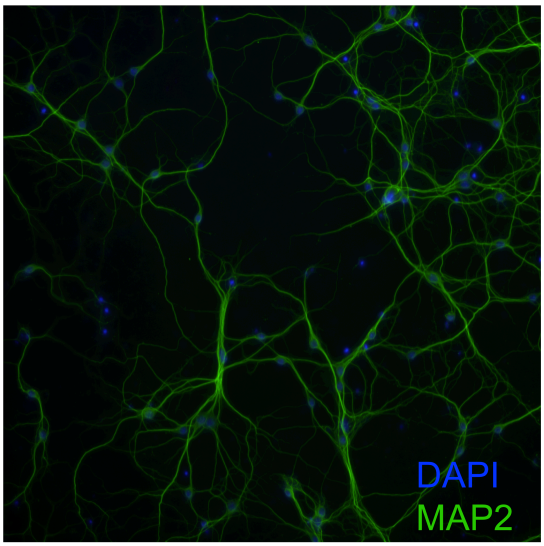
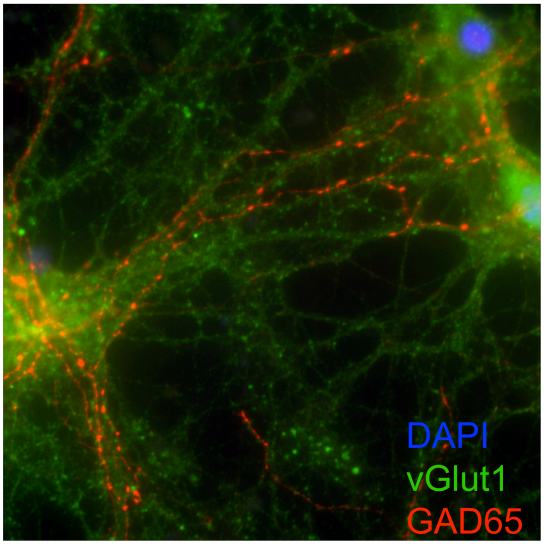
20
0
CCR2 KO naive
LPS CCR2 KO
CCR2/TREM2 dKO naive
LPS CCR2/TREM2 dKO


F
DAPI
MAP2


G
DAPI
vGlut1
GAD65

CHAPTER 4

TREM2 regulation of microglial phagocytosis is age-, activation- and target-dependent

4.1 ABSTRACT

Microglia are the resident macrophages of the central nervous system (CNS) and act as the primary phagocyte in the CNS during normal development, tissue homeostasis and disease. In the CNS, Triggering Receptor Expressed on Myeloid cells 2 (TREM2) is expressed only by microglia. We and others have previously demonstrated that TREM2 deficiency decreases phagocytosis using microglia cell lines and primary neonatal glial cultures. Because cultured microglia display significantly different phenotypes from microglia differentiated *in vivo,* we sought to confirm that TREM2 regulated phagocytosis in microglia acutely isolated from an intact mouse brain. While many reports of cultured microglia display low percentages of phagocytosing cells, we found that nearly all microglia assayed immediately after isolation from brain tissue displayed phagocytic activity. While phagocytic activity decreased with age, at the ages examined, activated microglia show greater phagocytosis of both targets than those isolated from untreated mice. At postnatal day 15 (p15), TREM2 deficiency decreased phagocytosis only in activated microglia. This effect was target dependent. TREM2 deficiency decreased the percentage of microglia phagocytosing *S. aureus* but not the amount phagocytosed per cell. By contrast,

TREM2 deficiency did not alter the percentage of microglia phagocytosing synaptosomes but did decrease the amount phagocytosed per cell. In the quantification of surface expression of receptors for pathogenic (TLR2) and for cellular targets (Tyro3, Axl, and Mer), we see that activation regulated expression of these molecules but TREM2 deficiency did not. Our data suggest that TREM2 deficiency has little effect on homeostatic phagocytosis but has large effects on inflammation-associated phagocytosis. Thus, we speculate that TREM2 mutations may increase the risk of Alzheimer's disease due to the cumulative effect of altered responses to lifelong environmental insults.

4.2 INTRODUCTION

Being the resident macrophages of the CNS, microglia act as the primary phagocyte during normal development, tissue homeostasis and disease. During the different stages of development and maturation, microglial core functions tend to shift with their ever-changing environment (Shemer, Erny, Jung, & Prinz, 2015; Zietlow, Dunnett, & Fawcett, 1999). Postnatally preference is placed on synaptic pruning, which is a key phagocytic function. In adulthood phagocytosis is important in homeostasis maintenance and repair. While with aging where there is neurodegeneration that results in exacerbated generation of reactive oxygen species (ROS) and inflammatory mediators the phagocytosis focus shifts more towards repair.

At all ages microglia function to maintain tissue homeostasis by phagocytosing to resolve injury and attenuate disease. Basically maintaining homeostasis depends on eliminating extracellular particles and apoptotic neural material (Linnartz, Wang, & Neumann, 2010). Many studies of microglial phagocytosis have cultured microglia from cell lines or primary cultures, but we now know that microglia in these culture environments and exposure to serum components and other nonneuronal factors that change microglia phenotype and genotype. Therefore we are no longer studying the same cells from the intact brain. However in lots of ways we know that *in vitro* conditions couldn't reliably reproduce many *in vivo* aspects. So studying *ex vivo* microglia phagocytosis can

in a lot of ways bridge some of the gaps we have in our understanding of what's occurring in the CNS.

During development microglia actively phagocytose synaptic material and play a key role in postnatal development (Paolicelli et al., 2011). Microglia phagocytose the immature synapses as well as inactive synapses in the adult CNS (Paolicelli et al., 2011; Tremblay, Lowery, & Majewska, 2010). Evidence has shown that early synaptic loss in neurological diseases such as Alzheimer's disease is due to microglial-mediated pathways (Hong et al., 2016). Though we know microglia phagocytose through out life, we don't know if or how microglia phagocytosis changes *in vivo* over the lifetime. So, what of our current knowledge from *in vitro* studies is recapitulated *in vivo* and what age does it correspond with? Studying *ex vivo* microglia phagocytosis from different ages will give us insight.

TREM2 plays a crucial role in modulating microglial immune responses through iTAM, and microglia phagocytosis (Bouchon, Hernandez-Munain, Cella, & Colonna, 2001; Otero et al., 2012; Takahashi, Rochford, & Neumann, 2005). Neuronal cells increase expression of TREM2 ligands during apoptosis, which then stimulates phagocytosis by microglial cells (Hsieh et al., 2009). TREM2 deficiency reduces phagocytosis and increases production of proinflammatory molecules (Takahashi et al., 2005; Xiang et al., 2016). While the reverse is also true where the over expression of TREM2 promotes phagocytosis and reduces

the proinflammatory response (Satoh et al., 2013). However microglia use different receptors to recognize and phagocytose different target so different tools are used to phagocytose synaptosomes as opposed to pathogenic targets. In response to bacteria-induced inflammation surface features of bacteria are recognized by microglial toll like receptors (TLRs), which regulate and speed up the maturation of phagosomes (Blander & Medzhitov, 2004; Russell & Yates, 2007). There is also an additional macrophage population that comes into play. We don't know to what extent specific receptors identified from *in vitro* studies are involved in phagocytosis *in vivo*? In this study we hope start to get a picture of this.

Aging microglia display specific morphological features, such as pancaking and blunter processes resulting in loss of their ramification. Brain aging is associated with neuroinflammation. While activation of microglia *in vivo* can be neuroprotective in juvenile mice it can also be neurotoxic in aging mice (Sawada, Sawada, & Nagatsu, 2008). Increased production of ROS and enhances oxidative stress result from oxidative damage of mitochondrial DNA in aged microglia are few among many microglia phonotypical changes that are associated with aging (Ojo, Rezaie, Gabbott, & Stewart, 2015; Streit & Xue, 2014). Though aging microglia express proinflammatory markers and functionally they are far less motile then juvenile microglia (Damani et al., 2011). A combination of these factors can influence microglial functional changes with

their environment. We know microglia phenotype changes with age but how does this influence phagocytosis capability. Microglia and macrophage phagocytosis is critical in injury and neurodegenerative diseases. So *ex vivo* studies will validate *in vitro* protocols and parameters specific to particular disease states and ages. This is important in modeling disease *in vitro* and *in vivo*.

Since microglial phagocytosis decreases when TREM2 is removed. We wanted an *ex vivo* setting to characterize and simultaneously quantify microglial and macrophage phagocytosis. Then to determine how IPLPS immune challenge, TREM2 deficiency, and age altered microglia and macrophage phagocytosis of homeostatic and pathogenic targets. Finally to determine what surface receptors if any contributed to the observed phagocytosis differences.

4.3 RESULTS

4.3.1 Microglia phenotype changes with age and activation.

During injury and disease, microglia acquire an activated phenotype, and peripheral macrophages infiltrate and assist in microglial function. Injury and disease are accompanied by inflammation of the CNS and for the purposes of our studies we simulate inflammation with an intraperitoneal injection of 5mg/kg of lipopolysaccharide (LPS). Microglia are defined as being FcR and CD45 positive and for the most parts are indistinguishable from the CNS infiltrating macrophages however, we can separate the two populations based on their CD45 expression levels. That is microglia are $CD45^{low}$ while infiltrating macrophages are $CD45^{high}$ (Figure 4.1 A). Activated microglia increase their surface expression of FcR and CD45 24h post IPLPS stimulation at all ages tested (Figure 4.1 B). However the rate of increase in surface expression of CD45 changes with age with the p15 animals shifting the most and the 2 years old barely shifts at all (Figure 4.1 C). To observe precise microglia and macrophage phenotype changes we used statistics from all experiments. With activation wild type and TREM2 KO microglia significantly increase their CD45 surface expression at p15 and 3 months but not 2 years. While FcR surface expression significantly increased at all ages tested (Figure 4.1 D-E). These data suggest that age affects the extent of IPLPS stimulation of microglia activations and the macrophage infiltration.

4.3.2 pH sensitive labeling allows for the detection of active phagocytosis.

To test the consequences of TREM2 deficiency of *ex vivo* microglia and macrophages on phagocytosis of particles we opted for a pH sensitive pHrodo fluorescence indicator method, where the particles fluoresce only upon entry into the phagolysosome (Figure 4.2 A). Since TREM2 has previously been shown to promote phagocytosis of pathogenic bacteria we used commercially available pHrodo labeled *S. aureus* particles. We also tested TREM2 effect on phagocytosis of synaptosomes. Therefore we isolated and enrichment synaptosomes from whole brains (Figure 4.2 B). We then labeled these synaptosomes with pHrodo dye and confirmed that the particles were pH sensitive (Figure 4.2 C-D). For the analysis of phagocytosis, microglia were separated from macrophages based on their CD45 expression (Figure 4.2 E). In these two populations we see active phagocytosis of pHrodo particles represented by an increase in green fluorescence, plotted across the 60-minute assay (Figure 4.2 F-G). With this accurate means of detecting phagocytosis we can clearly and confidently say the changes in MFI are a result of active phagocytosis.

4.3.3 TREM2 regulates microglial-phagocytosing numbers.

We wanted to determine the capacity of CNS immune cells to phagocytose the different targets and go a step further and see how age and activation state of these cells were influenced by this phenomena. We were especially intrigued to

see if and how TREM2 deficiency altered this in an *ex vivo* setting. We hypothesized that our *ex vivo* microglia would behave similarly to microglia from other studies, which showed TREM2 deficiency resulted in decreased phagocytosis as compared to wild type. However we saw that the naive *ex vivo* microglia where able to phagocytose the *S. aureus* with a continuous increased amount through out the 60 minute assay at all ages. When we took the assay out to 3 hours all the microglia phagocytosed (Data not shown), however we used the 1hour assay to minimize the time from harvest to analysis. The number the microglia phagocytosing significantly increased with their activation (Figure 4.3 A-C). In these assays TREM2 deficiency showed no apparent effect. We then showed that the number of microglia phagocytosing synaptosomes was much less than *S. aureus* (Figure 4.3 D-F). Here we see TREM2 deficiency affecting the number of microglia phagocytosing synaptosome with activated wild type microglia phagocytosing more than the TREM2 KO's at p15 and 3 months. For the most part macrophages from the animal phagocytose *S. aureus* at similar rates as the microglia (Figure 4.4 A-C). With the main difference being a decrease in the rate of TREM2 KO macrophage phagocytosis as compared to wild type at p15. This difference was also seen when we compared the rate of macrophage phagocytosis of the synaptosomes (Figure 4.4 D-F). Therefore TREM2 regulates the number of microglia phagocytosing synaptosomes at p15 and 3 months and the number of macrophages phagocytosing both *S. aureus* and synaptosome only at p15.

4.3.4 TREM2 regulates the amount microglia phagocytose.

To further examine whether TREM2 deficiency altered *ex vivo* phagocytosis we looked at the amount of *S. aureus* and synaptosomes each of the phagocytosing microglia phagocytosed. Similar to the rate of phagocytosis we showed that activation of microglia increase the amount of *S. aureus* they phagocytose at all ages (Figure 4.5 A-C). Interestingly the amount that activated TREM2 KO microglia are able to phagocytose is significantly less than wild type but only at p15. This difference or any other difference between wild type and TREM2 KO is not seen when looking at the amount of synaptosomes being phagocytosed (Figure 4.5 D-F). The macrophages from the same animal phagocytose more *S. aureus* than microglia (Figure 4.6 A-C). With a decrease in the amount that TREM2 KO macrophage phagocytosed as compared to wild type only at p15. This difference was not seen when we compared the amount of synaptosomes macrophages phagocytosed (Figure 4.6 D-F). Therefore TREM2 also regulates the amount of *S. aureus* microglia and macrophages from the activated animals phagocytose only at p15.

4.3.5 Microglial and macrophages phagocytosis changes with age.

Finally we determine the capacity of CNS immune cells to phagocytose the different targets age becomes a significant factor. The number of naive and activated microglia of phagocytosing the *S. aureus* and synaptosomes gradually decreases with age (Figure 4.7 A-B). As well as the amount the naive and

activated microglia are able to phagocytose (Figure 4.7 C-D). We see similar effects when analyzing the macrophage populations (Figure 4.7 E-H). This can be seen to correspond with the FcR and CD45 receptor expression.

4.3.6 Activation-induced up regulation of Tyro 3 and Axl in microglia is TREM2-dependent in postnatal day 15 but not in adult.

In order to get a better understanding and characterization of the different ages and activation states we analyzed various receptors expressed on acutely isolated microglia and macrophages. We looked at the TAM family of receptors. Tyro3, Axl and Mer. Flow cytometry plots were analyzed (Figure 4.8 A, F, K) and graphed. We see that basically all microglia and macrophages expressed Tyro 3 (Figure 4.8 B-C). Where Tyro3 receptors are upregulated with activation and there are no TREM2 differences (Figure 4.8 D-E). We see that basically all microglia and most macrophages expressed Axl (Figure 4.8 G-H). Where Axl receptors are upregulated with activation and the resulting macrophages expression is dependent on TREM2 (Figure 4.8 I-J). Finally we see that basically all microglia and most macrophages expressed Mer also (Figure 4.8 L-M). Where Mer receptors is upregulated with activation (Figure 4.8 N-O).

4.3.7 Activated microglial up regulation of TLR2 is TREM2 dependent at post natal day 15 but not in adult.

We analyzed the phagocytic molecules expression (Figure 4.9 A, F). With TLR2 and CD93 we see that the number of microglia expressing them decreases with age (Figure 4.9 B-C). Interestingly WT microglia upregulated TLR2 at p15 only while the TREM2 KO animals don't. (Figure 4.9 D-E). We see that the percent of CD93 microglia increases to macrophage levels with activation (Figure 4.9 G-H). While the amount these microglia express is still significantly less than the macrophages (Figure 4.9 I-J).

4.3.8 In TREM2 deficient mice, more microglia and macrophages express SiglecH at postnatal day 15 not in adult.

The phagocytic inhibitor CD47 and SiglecH were analyzed on these same microglia and macrophages (Figure 4.10 A, F). Very few microglia express CD47 as compared to macrophages (Figure 4.10 B-C). There are also higher amounts expressed on these macrophages (Figure 4.10 D-E). We see that more p15 TREM2 KO microglia express SiglecH then wild type but not at 3 months (Figure 4.10 G-H). The amount these microglia express is not affected by activation (Figure 4.10 I-J).

4.4 DISCUSSION

Understanding the mechanisms of microglial phagocytic function has been elusive. Since microglia phenotypes are ever changing with their environment (Shemer et al., 2015; Zietlow et al., 1999). We need to put the type of microglia cells we study in to context. Therefore we cannot properly elucidate amyloid plaque build up clearance by microglia using mice pups. By the same token we cannot use aged microglia to get a good understanding mechanisms involved in synaptic pruning during brain development.

In this study we looked at a variety of factors that contribute to microglia phagocytosis function such as age, activation state receptor deficiency and target. For the most part many groups have done numerous of studies on understanding how a few key phagocytic genes contribute to the phagocytosis of homeostatic and pathogenic targets (Blander & Medzhitov, 2004; Fu, Shen, Xu, Luo, & Tang, 2014). TREM2 mutations have been confirmed over and over again with compelling evidence to contribute significantly to countless microglia functions including phagocytosis (C. L. Hsieh et al., 2009; Kleinberger et al., 2014; Melchior et al., 2010; Savage et al., 2015; Sharif et al., 2014; Takahashi et al., 2005).So building on what is known about microglia phagocytosis we decided to look at not only different ages but also different activation states across the ages. Are finding correlate with a few previously published studies indicating that TREM2 deficiency results in decreased phagocytosis (C. L. Hsieh et al., 2009;

Kleinberger et al., 2014; Melchior et al., 2010; Savage et al., 2015; Sharif et al., 2014; Takahashi et al., 2005). However we find that using acutely isolated *ex-vivo* microglia this trend does not hold up across the board, which is fine in its own right. We only see difference in phagocytosis by TREM2 deficient microglia during early development. It's important however to note that juvenile microglia have been used for the majority of microglia cell lines and primary cultures.

Although many studies analyze microglia and macrophages together as a single population our previous studies have contributed to the understanding of both microglia and macrophages made contributions to clearance of debris and injured neurons (Schilling et al., 2005; Schmid et al., 2009). There is evidence that TREM2 is expressed by tissue macrophages (Bouchon et al., 2001; Colonna, 2003; Turnbull et al., 2006), which infiltrate into the brain during inflammation. We show that these macrophages regardless of the classified subtypes, classical (M1) or alternatively activated (M2) or a combination of the two for the most part behave similarly to microglia even in the absence of TREM2, except during early development. And since in a physiological inflammation context partials are typically phagocytosed by both macrophages and microglia (Lauber, Blumenthal, Waibel, & Wesselborg, 2004; Schilling et al., 2005) further giving evidence that for the most part TREM2 KO animals are phonotypical normal. Suggesting that they can somewhat compensate for the loss of TREM2. We know that TREM2 is selectively expressed by microglia *in*

vivo in the naive mouse brains. Though for the phagocytosis of synaptosomes and *S. aureus* TREM2 is important, we know that TREM2 is not the only receptor responsible for mediating phagocytosis.

The Tyro3, Axl and Mer are the TAM family of proteins and have been identified as a distinct subfamily of receptor-type protein tyrosine kinase (PTK) (Lai & Lemke, 1991). This family of proteins binds to two ligands growth-arrest-specific 6 (GAS6) and protein S (Stitt et al., 1995). When TAM receptor binds to its ligands it triggers its activation, which result in the recruitment, phosphorylation, and activation of subsequent downstream targets. TAM's bind their ligands which in turn bind to exposed phosphatidylserine on neurons (Grommes et al., 2008; Wu, Singh, Georgescu, & Birge, 2005) It has been shown that microglia express all three members of TAM receptors (R. Ji et al., 2013). And we see that microglia lacking functional TAM receptors produced increased amounts of proinflammatory cytokines when stimulated with IPLPS (R. Ji, Meng, Li, & Lu, 2015). Those indicating that though TAM receptors are associated with homeostatic regulation they some how regulates activation response in microglia. TAM knockout mice studies have shown their participation of the receptors in phagocytic regulation, mainly through Mer and Axl (Scott et al., 2001). We see in our studies that with IPLPS stimulation all TAM family of receptors gets induced only in the juvenile age but not with adults.

In most neurodegenerative diseases, high levels of immune factors stimulate microglia activation. This activation occurs through TLR receptors, and their coreceptors CD47 (Lehnardt, 2010; Strowig, Henao-Mejia, Elinav, & Flavell, 2012). There are inhibitors of phagocytosis such as CD47, which is expressed on cells, and block phagocytosis by binding to signal regulatory protein-α (SIRPα) (Gitik, Liraz-Zaltsman, Oldenborg, Reichert, & Rotshenker, 2011). We saw no apparent difference in CD47 expression between WT and TREM2 microglia in the naive or activated state suggesting that this type of inhibition signal was not contributing to differences in TREM2 mutant microglial function. Polysialylation on the cell surface proteins of neurons may act as a phagocytosis inhibitor signal; on the other hand desialylation may promote phagocytosis of neuronal components. It has been shown that SiglecH (in mice) can promote microglial phagocytosis (Kopatz et al., 2013). We see that SiglecH is upregulated in the TREM2 KO microglia. Leading us to believe it is acting as potentially compensation.

In conclusion our data shows that TREM2 deficiency has little effect on homeostatic phagocytosis but has large effects on injury or inflammation-associated phagocytosis. The differences in phagocytosis depend greatly on the age of the microglia. Since microglia are long lived there is inherent changes that occur that affect their overall function. These changes are important to note when selecting the appropriate microglia model to use. For the most part TREM2

deficient mice are similar to their wild type counterparts. Thus, we speculate that TREM2 mutations may increase the risk of Alzheimer's disease due to the cumulative effect of altered responses to lifelong environmental insults.

4.5 FIGURES AND LEGENDS

Figure 4.1. Characterizing microglia and macrophages phenotype changes with age and IPLPS activation. (A) Microglia can be identified as being FcR^{+} and $CD45^{+}$ and macrophages are distinguished based on their $CD45^{high}$ expression compared to microglia using flow cytometry **(B)** Flow cytometry plots of CD45 positive cells shows the shift in microglia FcR and CD45 as well as the infiltrating macrophage populations with IPLPS treatment at all ages. **(C)** Histogram plots show a decrease in microglia CD45 shift with increased age. Quantification of microglial and macrophages CD45 **(D)** and FcR **(E)** changes with age and activation. Data is represented as mean ± SEM from independent experiments. Samples are p15 naive/IPLPS n=30/24, 3 months naive/ IPLPS n=29/31, 2years naive/IPLPS n=6/6 respectfully. Statistical differences were calculated by one-way ANOVA with a Tukey's posttest. *P < 0.05; **P < 0.01; ***P < 0.001 and ****P < 0.0001.

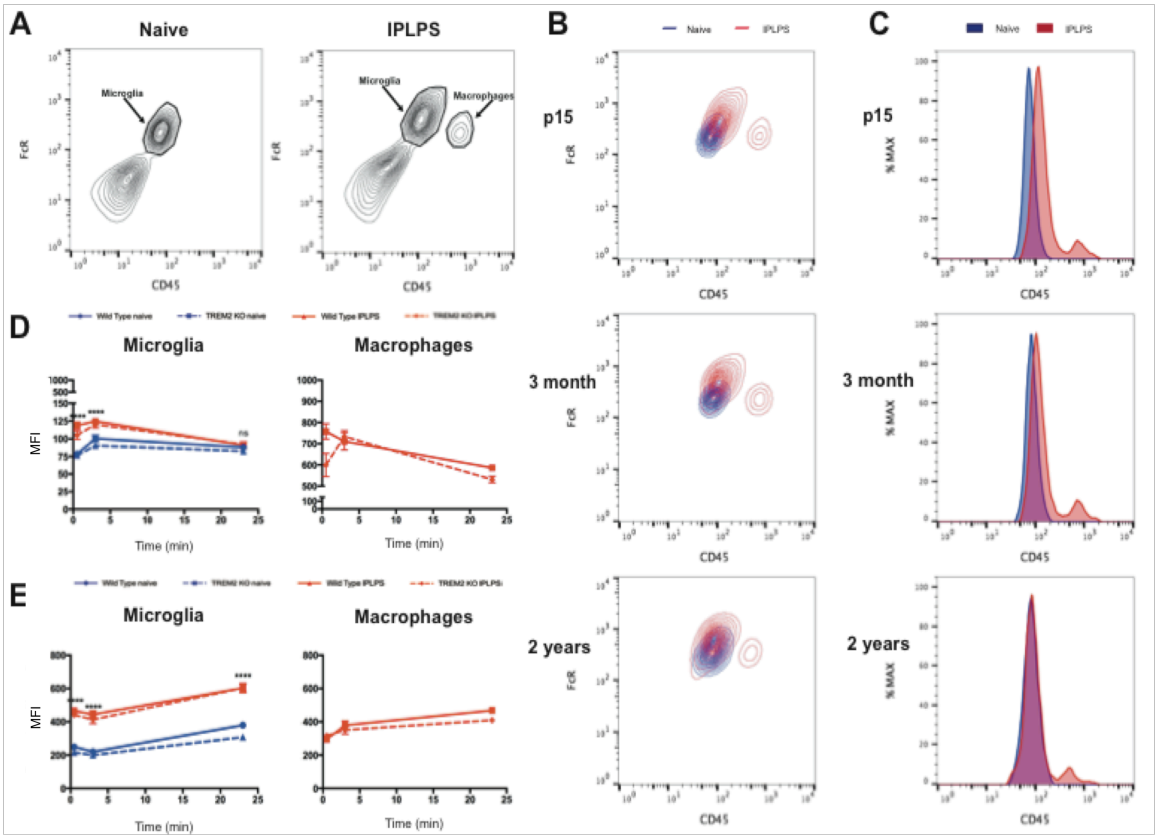

A
Naive
IPLPS
Microglia
Macrophages
FcR
CD45
B
Naive
IPLPS
p15
3 month
2 years
C
% MAX
D
Wild Type naive
TREM2 KO naive
Wild Type IPLPS
TREM2 KO IPLPS
Microglia
Macrophages
MFI
Time (min)
E

Figure 4.2. Characterization of microglial and macrophage *ex vivo* phagocytosis. (A) The pHrodo-labeled target only fluoresces once it enters the phagolysosome. **(B)** Western blot of Synaptophysin and HDAC1 (Histone Deacetylase 1) from wild type adult synaptosome purification. The pHrodo-labeled synaptosomes are fluorescent under acidic **(D)** but not neutral **(C)** conditions. The scale bar = 80μm. **(E)** Flow cytometric separation of microglia and macrophages into two distinct populations based on their CD45 expression. Analysis these separate populations pHrodo positive microglia **(F)** and macrophage **(G)** over the time course of the 60-minute assay is used to measure active phagocytosis.

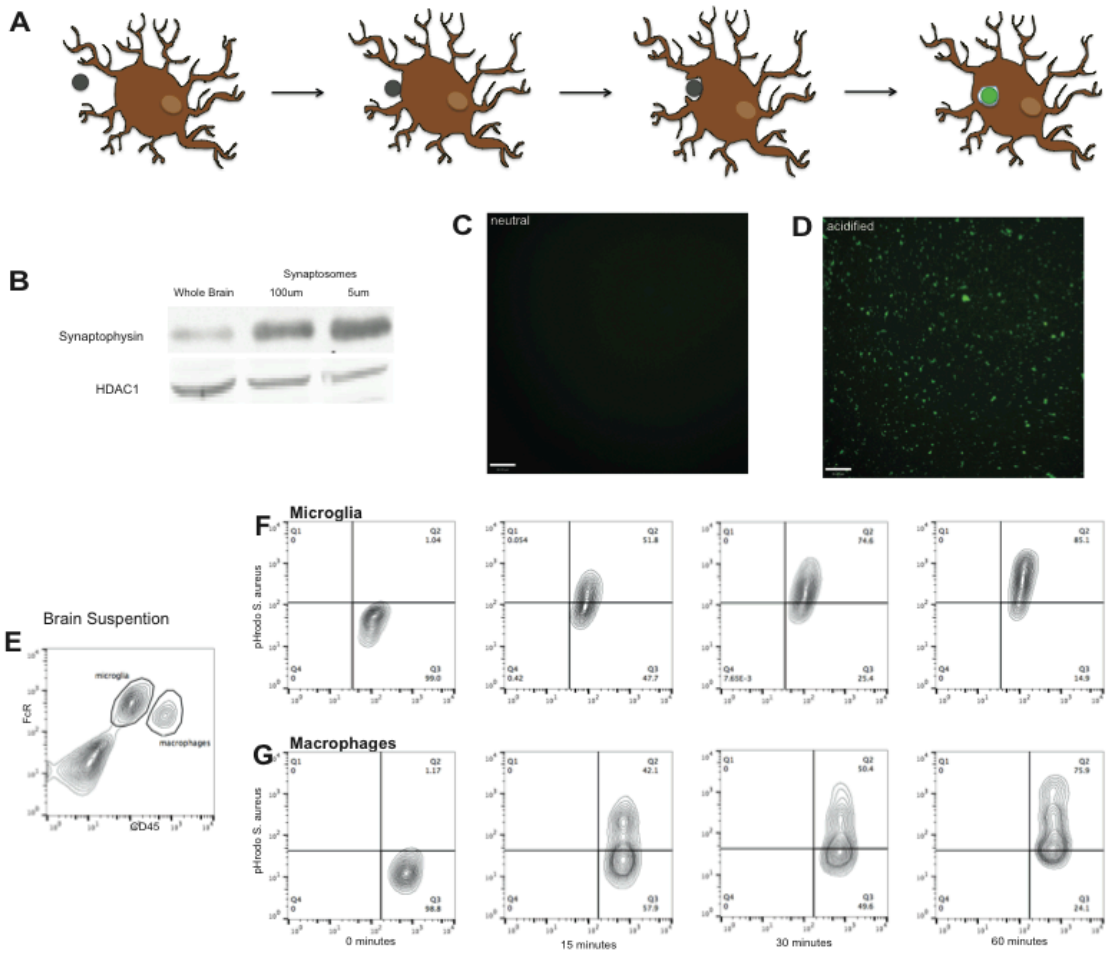

A
B
Synaptosomes
Whole Brain
100um
5um
Synaptophysin
HDAC1
C
neutral
D
acidified
E
Brain Suspention
microglia
macrophages
FcR
CD45
F
Microglia
pHrodo S. aureus
G
Macrophages
0 minutes
15 minutes
30 minutes
60 minutes

Figure 4.3. TREM2 regulates the number of microglia phagocytosing synaptosomes but not *S. aureus* in an age and activation dependent manner. Graphs of the flow cytometric data showing the percent of microglia phagocytosing pHrodo *S. aureus* **(A-C)** or pHrodo-labeled synaptosomes **(D-F)** over the 60 minutes assay, from p15 **(A** and **D)**, 3 months **(B** and **E)** and 2 years **(C** and **F)** old mice. Data is represented as mean ± SEM from independent experiments. Phagocytosis of *S. aureus* WT p15 IPLPS n=11, p15 and WT 3 months naive n=10, TREM2 KO p15 and 3 months IPLPS n=7, WT IPLPS and TREM2 KO naive 3 months n=6, 2 years naive n=4, 2 years IPLPS n=3. Phagocytosis of synaptosomes p15 IPLPS and 2 years naive n=4, p15 naive, 3 months and 2 years IPLPS n=3. Statistical differences were calculated by two-way ANOVA. *P < 0.05; **P < 0.01; ***P < 0.001 and ****P < 0.0001.

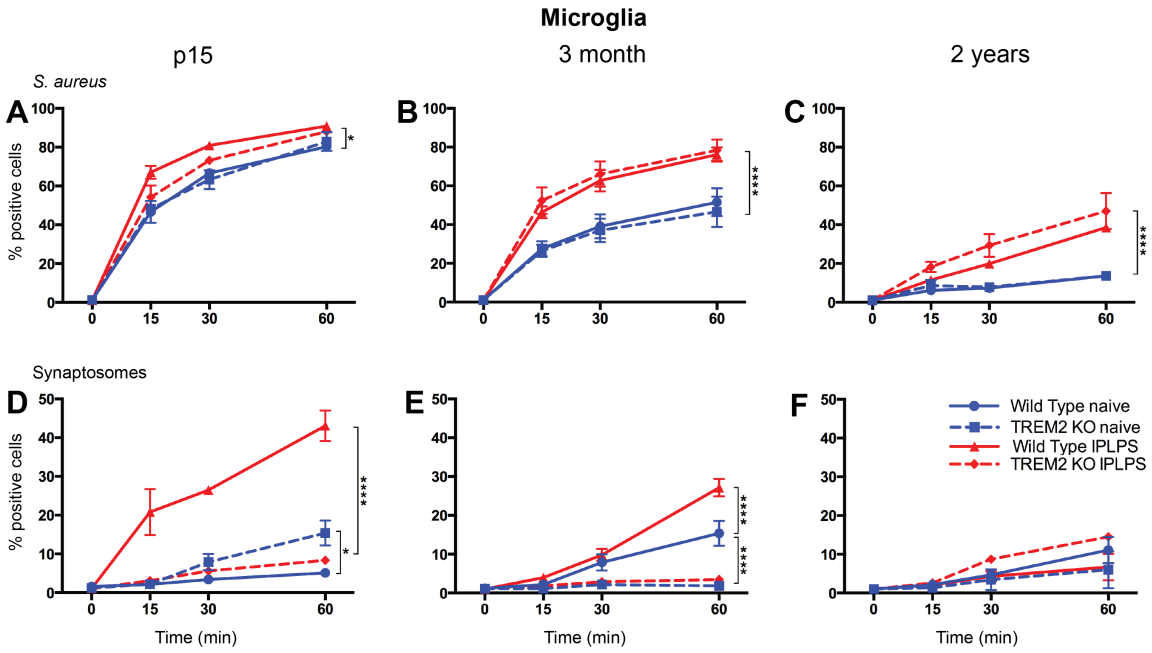

Microglia
p15
3 month
2 years
S. aureus
A
B
C
% positive cells
Synaptosomes
D
E
F
Time (min)
Wild Type naive
TREM2 KO naive
Wild Type IPLPS
TREM2 KO IPLPS

Figure 4.4. TREM2 regulates the number of macrophages phagocytosing in an age dependent manner. Graphs of the flow cytometric data showing the percent of macrophages phagocytosing pHrodo *S. aureus* **(A-C)** or pHrodo-labeled synaptosomes **(D-F)** over the 60 minutes assay, from p15 **(A** and **D)**, 3 months **(B** and **E)** and 2 years **(C** and **F)** old mice. Data is represented as mean ± SEM from independent experiments. Phagocytosis of *S. aureus* WT p15 IPLPS n=11, TREM2 KO p15 and 3 months IPLPS n=7, WT IPLPS 3 months n=6, 2 years IPLPS n=3. Phagocytosis of synaptosomes p15 IPLPS n=4, 3 months and 2 years IPLPS n=3. Statistical differences were calculated by two-way ANOVA. *P < 0.05; **P < 0.01; ***P < 0.001 and ****P < 0.0001.

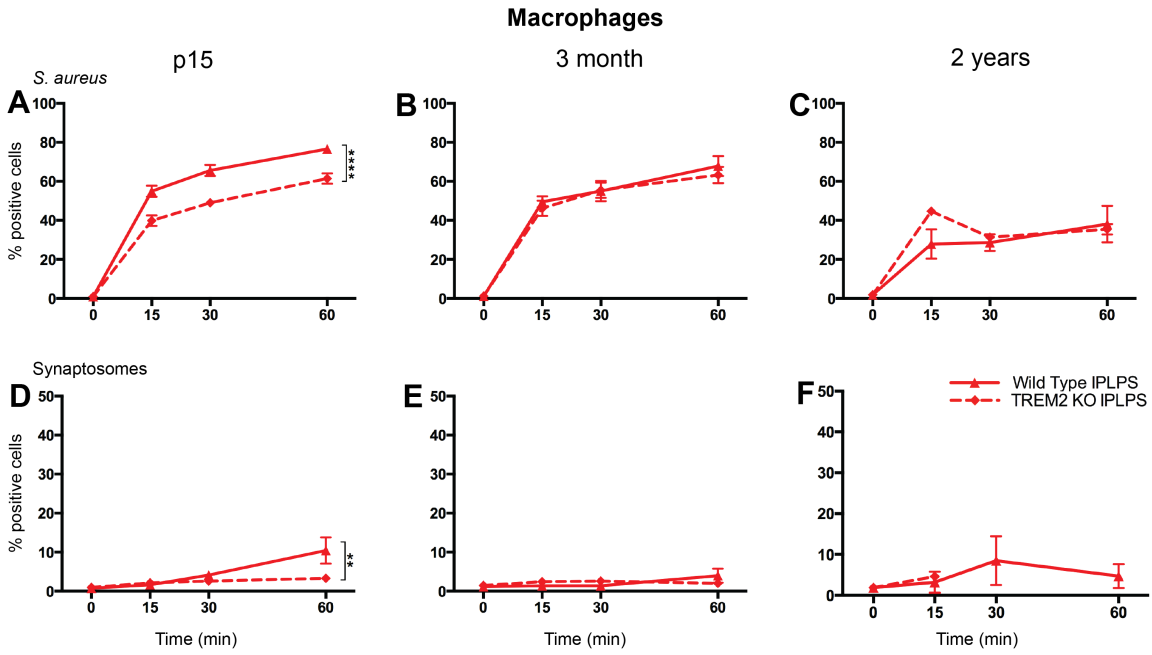

Macrophages
p15
3 month
2 years
S. aureus
A
B
C
% positive cells
****
Synaptosomes
D
E
F
**
Wild Type IPLPS
TREM2 KO IPLPS
Time (min)
Time (min)
Time (min)

Figure 4.5. TREM2 regulates the amount of *S. aureus* but not synaptosomes phagocytosed by microglia in an age and activation dependent manner. Graphs of the flow cytometric data showing the Mean Fluorescence Intensity (MFI) of microglia that phagocytosed pHrodo *S. aureus* **(A-C)** or pHrodo-labeled synaptosomes **(D-F)** over the 60 minutes assay, from p15 **(A** and **D)**, 3 months **(B** and **E)** and 2 years **(C** and **F)** old mice. Data is represented as mean ± SEM from independent experiments, Phagocytosis of *S. aureus* WT p15 IPLPS n=11, p15 and WT 3 months naive n=10, TREM2 KO p15 and 3 months IPLPS n=7, WT IPLPS and TREM2 KO naive 3 months n=6, 2 years naive n=4, 2 years IPLPS n=3. Phagocytosis of synaptosomes p15 IPLPS and 2 years naive n=4, p15 naive, 3 months and 2 years IPLPS n=3. Statistical differences were calculated by two-way ANOVA. *P < 0.05; **P < 0.01; ***P < 0.001 and ****P < 0.0001.

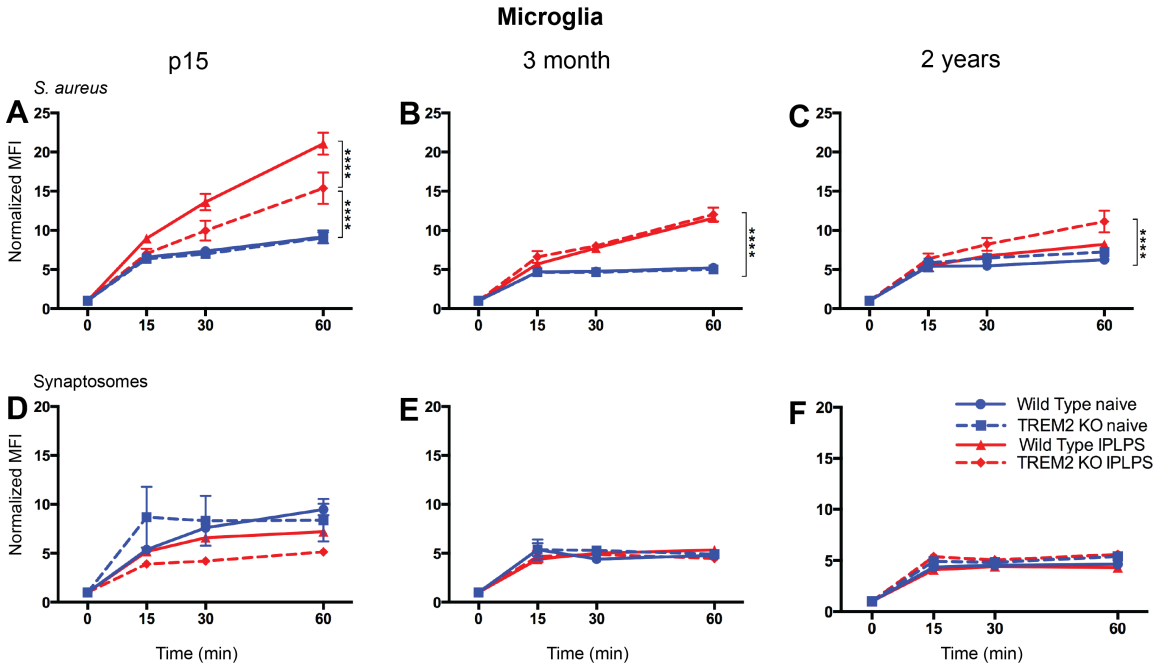

Microglia
p15
3 month
2 years
S. aureus
A
B
C
Normalized MFI
****
****
****
Synaptosomes
D
E
F
Normalized MFI
Time (min)
Time (min)
Time (min)
Wild Type naive
TREM2 KO naive
Wild Type IPLPS
TREM2 KO IPLPS

Figure 4.6. TREM2 regulates the amount of *S. aureus* but not synaptosomes phagocytosed by macrophages in an age and activation dependent manner. Graphs of the flow cytometric data showing the Mean Fluorescence Intensity (MFI) of macrophages that phagocytosed pHrodo *S. aureus* **(A-C)** or pHrodo-labeled synaptosomes **(D-F)** over the 60 minutes assay, from p15 **(A** and **D)**, 3 months **(B** and **E)** and 2 years **(C** and **F)** old mice. Data is represented as mean ± SEM from independent experiments. Phagocytosis of *S. aureus* WT p15 IPLPS n=11, TREM2 KO p15 and 3 months IPLPS n=7, WT IPLPS 3 months n=6, 2 years IPLPS n=3. Phagocytosis of synaptosomes p15 IPLP n=4, 3 months and 2 years IPLPS n=3. Statistical differences were calculated by two-way ANOVA. *P < 0.05; **P < 0.01; ***P < 0.001 and ****P < 0.0001.

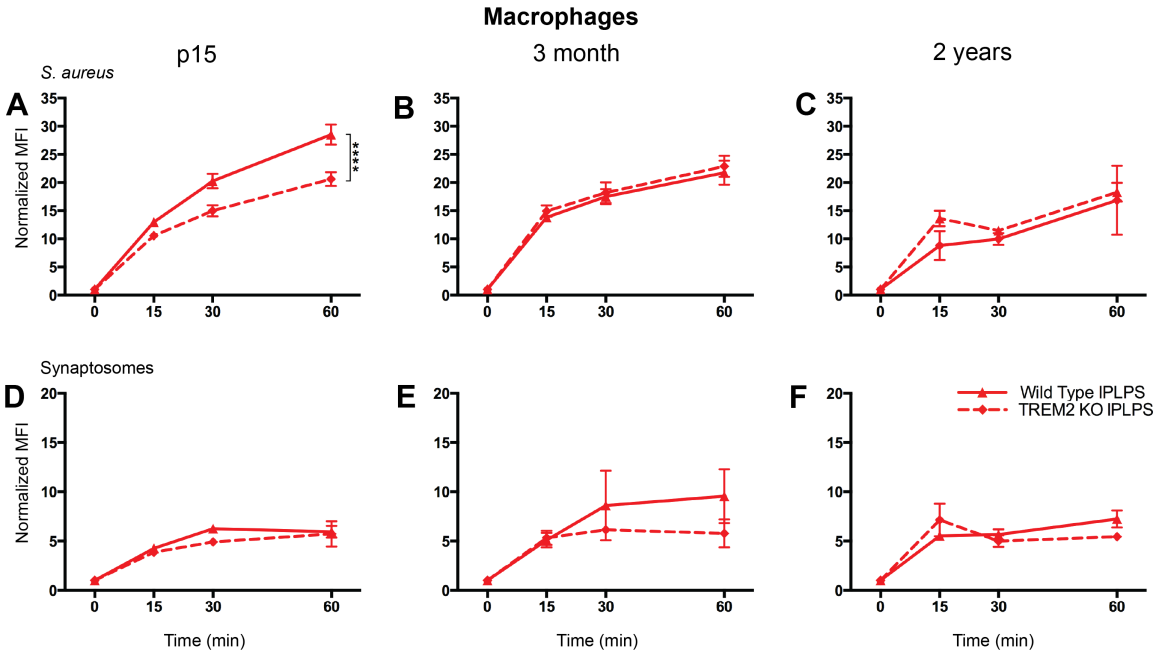

Macrophages
p15
3 month
2 years
S. aureus
A
B
C
D
E
F
Normalized MFI
Synaptosomes
Time (min)
Wild Type IPLPS
TREM2 KO IPLPS
****

Figure 4.7. The percent of phagocytosing microglia and the amount they phagocytose decreases with age. Graphs of the flow cytometric data showing the percent of microglia phagocytosing pHrodo *S. aureus* **(A)** or pHrodo labeled synaptosomes **(B)** after 60 minutes over lifespan. The percent of macrophages phagocytosing pHrodo *S. aureus* **(E)** or pHrodo labeled synaptosomes **(F)** after 60 minutes over lifespan. The MFI of microglia that phagocytosed pHrodo *S. aureus* **(B)** or pHrodo labeled synaptosomes **(D)** after 60 minutes over lifespan. The percent of macrophages that phagocytosed pHrodo *S. aureus* **(G)** or pHrodo labeled synaptosomes **(H)** after 60 minutes over lifespan. Data is represented as mean ± SEM from independent experiments, Phagocytosis of *S. aureus* WT p15 IPLPS n=11, p15 and WT 3 months naive n=10, TREM2 KO p15 and 3 months IPLPS n=7, WT IPLPS and TREM2 KO naive 3 months n=6, 2 years naive n=4, 2 years IPLPS n=3. Phagocytosis of synaptosomes p15 IPLPS and 2 years naive n=4, p15 naive, 3 months and 2 years IPLPS n=3. Statistical differences were calculated by two-way ANOVA. *P < 0.05; **P < 0.01; ***P < 0.001 and ****P < 0.0001

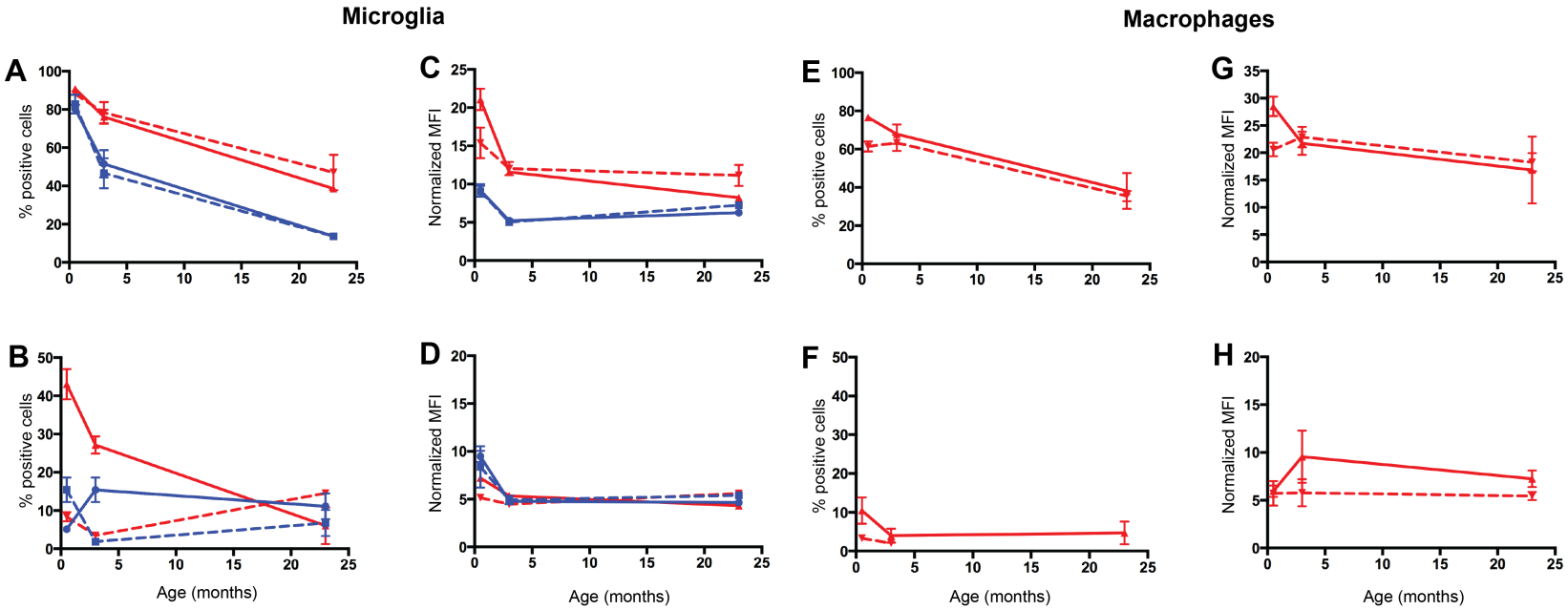

Microglia
Macrophages
A
B
C
D
E
F
G
H
% positive cells
Normalized MFI
Age (months)

Figure 4.8. Activation-induced up regulation of Tyro 3 and Axl in juvenile microglia is TREM2-dependent. Flow cytometric analysis of Tyro3 **(A)**, Axl **(F)** and Mer **(K)** on microglia in purple with background fluorescence in grey from untreated 3 months old adult mice. Each contour plot is representative of three independent experiments. The percent of positive cells are graphed in **(B-C, G-H, L-M)** while their MFI's are graphed in **(D-E, I-J, N-O)** with the WT and TREM2 KO microglia and macrophages being normalized to WT 3 months naive microglia. Graphical data is represented as mean ± SEM from independent experiments, n = 3 (p15) and n = 4 (3 months). Statistical differences were calculated by two-way ANOVA. *P < 0.05; **P < 0.01; ***P < 0.001 and ****P < 0.0001.

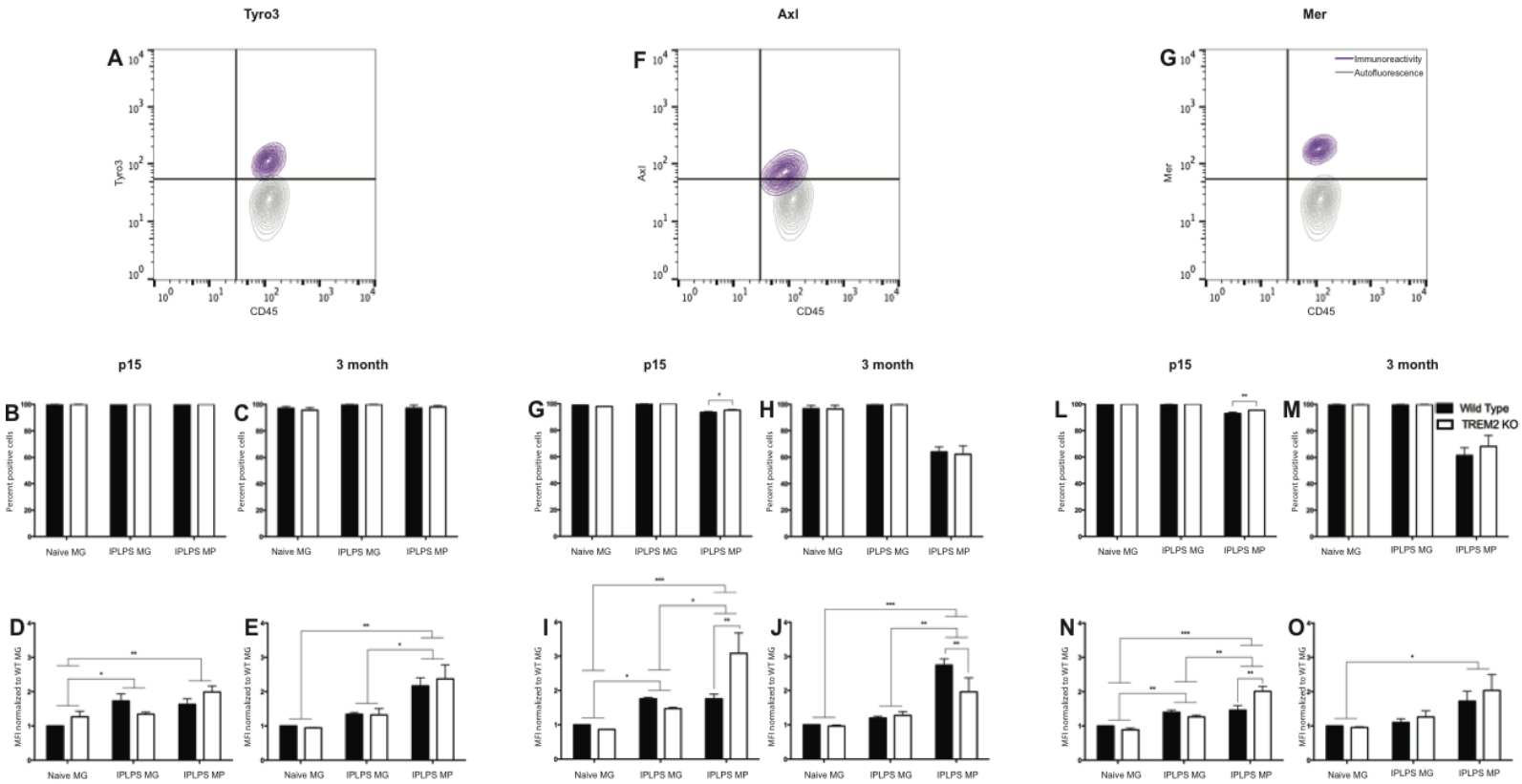

Tyro3
Axl
Mer
A
F
G
Tyro3
Axl
Mer
CD45
Immunoreactivity
Autofluorescence
p15
3 month
B
C
G
H
L
M
Percent positive cells
Wild Type
TREM2 KO
Naive MG
IPLPS MG
IPLPS MP
D
E
I
J
N
O
MFI normalized to WT MG

Figure 4.9. Microglial up regulation of TLR2 is TREM2 dependent in juvenile mice. Flow cytometric analysis of TLR2 **(A)** and CD93 **(F)** on microglia in purple with background fluorescence in grey from untreated 3 months old adult mice. Each contour plot is representative of three independent experiments. The percent of positive cells are graphed in **(B-C, G-H)** while their MFI's are graphed in **(D-E, I-J)** with the WT and TREM2 KO microglia and macrophages being normalized to WT 3 months naive microglia. Graphical data is represented as means ± SEM from independent experiments, n = 3 (p15) and n = 4 (3 months). Statistical differences were calculated by two-way ANOVA. *P < 0.05; **P < 0.01; ***P < 0.001 and ****P < 0.0001.

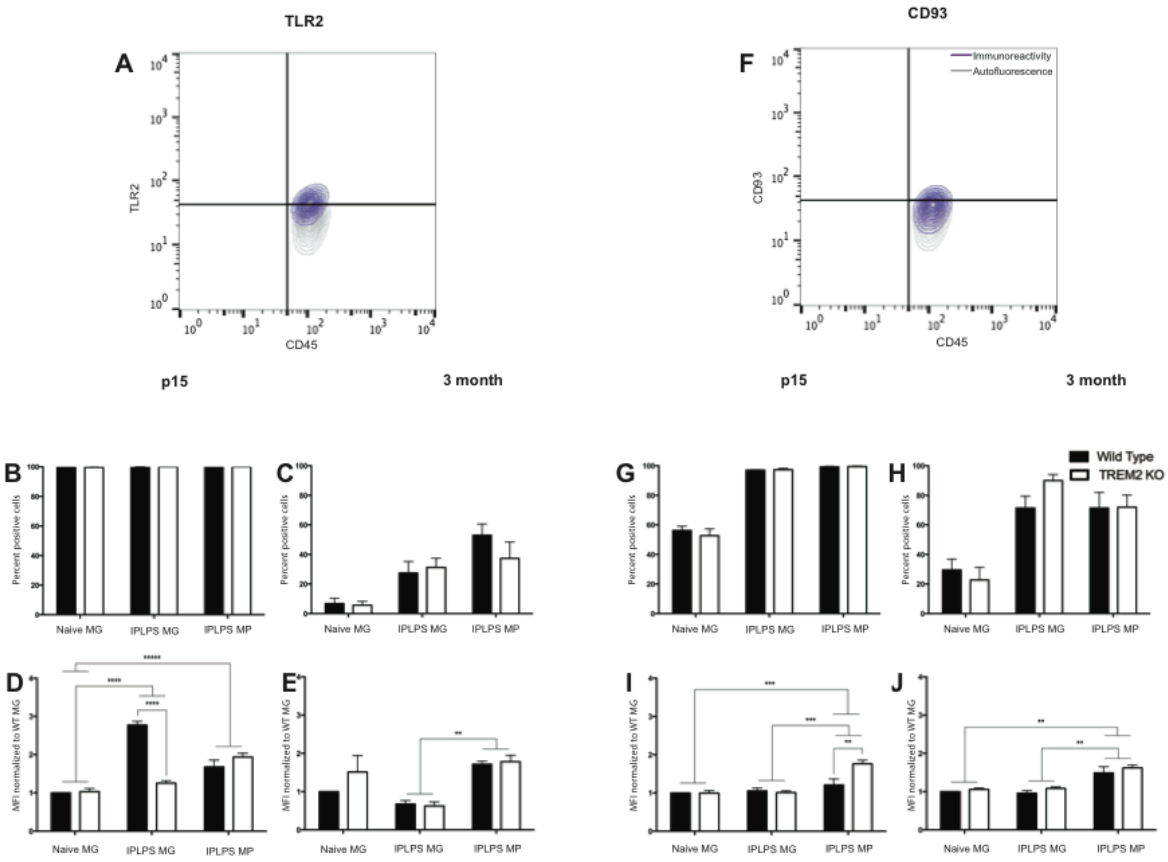
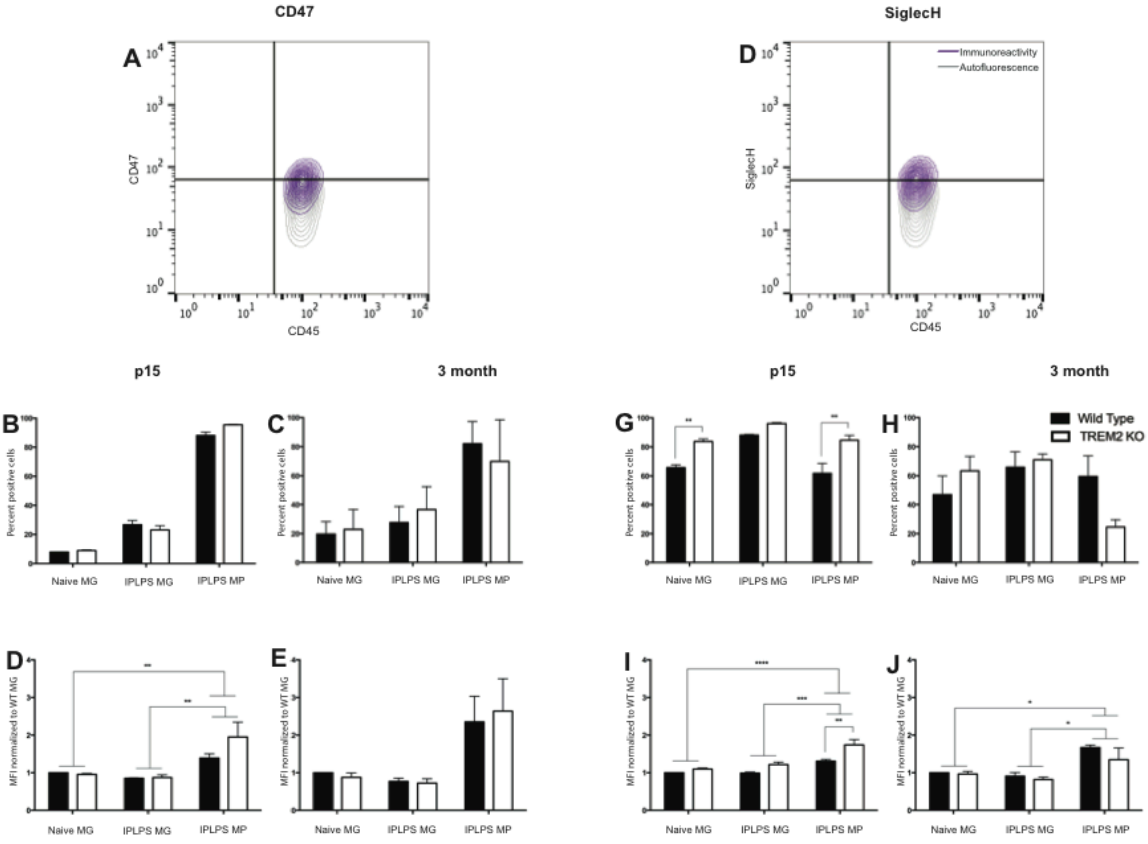

TLR2
A
TLR2
CD45
p15
3 month
CD93
F
Immunoreactivity
Autofluorescence
CD93
CD45
p15
3 month
B
C
G
H
Percent positive cells
Naive MG
IPLPS MG
IPLPS MP
Wild Type
TREM2 KO
D
E
I
J
MFI normalized to WT MG

Figure 4.10. In the absence of TREM2 more microglia and macrophages express SiglecH. Contour plot depict flow cytometric analysis of CD47 **(A)** and SiglecH **(F)** in microglia from untreated mice on microglia in purple with background fluorescence in grey from untreated 3 months old adult mice. Each Contour plot is representative of three experiments. The percent of positive cells are graphed in **(B-C, G-H)** while their MFI's are graphed in **(D-E, I-J)** with the WT and TREM2 KO microglia and macrophages being normalized to WT 3 months naive microglia. Graphical data is represented as mean ± SEM from independent experiments, n = 3 (p15) and n = 4 (3 months). Statistical differences were calculated by two-way ANOVA. *P < 0.05; **P < 0.01; ***P < 0.001 and ****P < 0.0001.


CD47
SiglecH
A
D
Immunoreactivity
Autofluorescence
CD47
SiglecH
CD45
CD45
p15
3 month
p15
3 month
B
C
G
H
Wild Type
TREM2 KO
Percent positive cells
Naive MG
IPLPS MG
IPLPS MP
D
E
I
J
MFI normalized to WT MG

CHAPTER 5

Characterizing iHPC-derived human microglia-like cells

5.1 ABSTRACT

Microglia are resident immune cells of the central nervous system (CNS) and as such, have important roles in its development tissue homeostasis and neurologic diseases or injury. The extreme difficulty in acquiring viable human microglia has limited their study and potential translational applications, which have shown effectiveness in animal models of CNS disorders. Here we show that human microglial-like cells (iMGL) can be differentiated from hematopoietic progenitor cells (iHPCs) and be used to study microglial function. The exposure to the defined factors M-CSF, IL-34 and TGFβ are sufficient without a need to culture with astrocytes to differentiate iHPCs to iMGL. These iMGL are functional and able to phagocytose *S. aureus* as well as synaptosomes. These finding give us the ability to generate a renewable source of human microglia, which can be used to study CNS disorders.

5.2 INTRODUCTION

Microglia are a specialized type of tissue macrophage that supports brain and spinal cord function and are found in all regions (Carson et al., 2007; Lawson et al., 1992). Triggering Receptor Expressed on Myeloid cells-2 (TREM2) is enriched on microglia as compared to all other types of myeloid cells (Schmid et al., 2009). Lack of TREM2 or mutations in TREM2 alter microglial surveillance of their environment, their phagocytosis of debris/toxins, and their editing of synapses as well as their response to injury and pathogens. A single point mutation in microglial TREM2 can result in an aberrant phenotype. The microglial phenotype is shaped by its CNS environment and is developmental stages, as it transitions from neurogenesis and synaptogenesis (Hong et al., 2016)to aging and neurodegeneration. The acquisition of this specific microglial phenotype is a result of multiple regulatory contributions provided by microglial interactions with other CNS cells (Carson et al., 2007). These CNS cells express and release molecules recognized by microglial receptors generating environmental cues that determines microglial phenotypical state. The hope is that we can somehow artificially provide cues that mimic this environment.

Fetal primary microglia and cell lines are in common use. However, based on numerous studies, we know that these cells diverge from the profile of what we see *in vivo* and express markers not typically expressed by microglia. So how

can we model human microglia physiology when cultured mouse microglia are non-predictive of its own *in vivo* biology (Schmid et al., 2009).

Recent genome wide association studies revealed that mutations in several genes expressed by microglia are sufficient to increase risk for many prevalent neurologic diseases including Alzheimer's disease. Highlighting a further need to expand on our understanding of human microglial function but not only in disease but also healthy individuals. One such gene identified was TREM2. Many groups including ours have been studying the effects of TREM2 on microglia physiology and whole body homeostasis using mouse models. However, it is difficult to use this model to test human genes, therefore the need for the generation of human microglia-like cells iMGL arised.

Many iPSC derived microglia protocols with a variety of culture conditions and media cocktails have been proposed in recent years. However, some of the shortcomings described (Muffat et al., 2016) include generation of intermediates that are neuronal and not of the myeloid lineage and the final cell only expressing a few of the microglial markers. We generated a protocol for the differentiation of human iPSC into iMGL with a Hematopoietic Progenitor Cells (iHPCs) intermediate, which is consistent with how microglia develop *in vivo*. Unlike neurons, microglia are generated when embryonic yolk sac myeloid precursors travel to the brain and spinal cord and differentiate (Eglitis & Mezey, 1997;

Kierdorf et al., 2013). An important advantage that all groups studying stem cell derived microglia, agree on is that health and diseased individuals can be used to generate patient-derived iPSCs, which can easily be expanded. This gives us unique opportunities to examine disease and the contribution of their genetic risk factors.

Thus far, mouse models of human neurological diseases, in which there are mutations in, or deletion of microglia functions, have been insightful in understanding their effect on brain and spinal cord disorders. However the transition of these finding into a clinical application have been lacking. This is primarily due to the lack of a relatively available source of human microglia. A solution to this, rather than trying to use human fetal tissue, which comes with its on set of caveats or postpartum adult CNS tissue, which has shown low viability in the tested protocol, is to use a relatively available renewable source such as iPSCs that can be differentiated into iMGL (Pandya et al., 2017). On the plus side, defining culture condition that adequately differentiate iPSCs to iMGL will give us further insight into culturing the finicky primary mouse cultures and cell lines to better mimic the human neuronal environment. We know that there have been challenges in generating functional microglia that are distinct from other CNS myeloid cells from iPSCs, are due the specific cues needed for their differentiation and maturation. Here we hope to address these concerns and put

precedence on our iMGL being function in common microglia functions such as phagocytosis.

5.3 RESULTS

5.3.1 iMGL differentiated from iHPCs.

For the purpose of these experiments, we used a commercial source of Hematopoietic Progenitor Cells (iHPCs), which is a pure population that express the glycoprotein CD34 on their surface and are derived from human induced pluripotent stem cells (iPSCs)(Cellular Dynamic International, Madison, WI, USA) and differentiated them into iMGL. This is the second step of a two-step protocol recently defined that goes from iPSCs→iHPCs→iMGL (Abud et al., 2017). These experiments were done in part to characterize these iMGL and define an efficient environment for their generation in collaboration with the Blurton-Jones lab. Using the Cellular Dynamic guide's protocol the iHPCs should be spherical after plating (Figure 5.1 A). However, The iHPCs were cultured in a serum free differentiation media containing; M-CSF-1, IL-34, and TGFb-1 and within a week (6DIV), we see processes beginning to extend from the cells (Figure 5.1 B) and clusters of proliferating cells. The protocol typically yielded 4-6 x10^6 million iMGL from the 1x 10^6 iHPCs vial. By week 2 (15DIV), we see the iMGL start to more resemble microglia (Figure 5.1 C). It is at this point where we can see the iMGL expressing the myeloid-associated transcription factor PU.1 and the microglial enriched gene TREM2 (Abud et al., 2017). We also see that the iMGL morphology is quit different from that of monocyte derived macrophages that are more amoeboid (Figure 5.1 D). We waited a month (28DIV) to test iMGL expression the microglial enriched genes profile as a result of altered culturing

conditions or to compare iMGL to monocytes and monocyte derived macrophages or to do the phagocytosis assay.

5.3.2 iMGL have a microglial genotype.

Before the protocol was defined, we tried different culture environments. The purpose was to determine what the necessary and sufficient condition to induce microglial differentiation. The thought is that if microglia are yolk sac derived and differentiated into mature microglia in a neuronal environment how best can we recreate this environment. We started with differentiating the iMGL in the presence of astrocytes; we then found they were not necessary. We also tested different cytokine cocktails and found that the addition of the TGFβ resulted in a robust induction of microglial genes CX3CR1: fractalkine receptor key in microglia migration. In the CNS microglia express CX3CR1 and P2Y12: a purinergic receptors key in neuronal signaling and primary mechanism by which microglia sense tissue damage (data not shown) as compared to cultures lacking TGFβ. In contrast the addition of TGFβ resulted in a reduced TREM2 as compared to cultures lacking TGFβ (Figure 5.2 A). However it is important to note that TREM2 is significantly induced over what we see in macrophages or other monocytes. In better understanding the phenomena by which iMGL gene profile changes, we wanted to look at identifying microglia specific markers in out cultures. Ben Barres group recently published transmembrane protein 119 (Tmem119) as a microglial specific gene in both human and mouse (Bennett et

al., 2016). When we tested TMEM119, we saw that our iMGL expressed it however; the TGFβ cultures didn't further induce its expression (Figure 5.2 B). Another microglial specific gene OLFML3 (Butovsky et al., 2014)was described as being a unique TGF-beta-dependent. We see that in the absence of TGFβ in our cultures OLFML3 is not expressed (Figure 5.2 C). These finding help us feel confident in the iMGL that we generated by our defined protocol needs the exogenous TGFβ.

5.3.3 iMGL have a microglial phenotype.

In order to get a better understanding of what is phenotypicaly going on with the iMGL, we analyzed various receptors expressed on Hematopoietic cells. We looked at the receptor expression of CD11b: Integrin alpha M and is a myeloid lineage that is frequently used to identify macrophages and microglia, CD45: leukocyte common antigen and is a protein expressed specifically on hematopoietic cells, CD11c: transmembrane protein expressed most highly by dendritic cell but can also be found on macrophages, and CCR2 specific mediator of monocyte chemotaxis and is involved in monocyte infiltration into the brain. Then the iMGL expression of these receptors was profiled in comparison to monocytes and monocyte derived macrophages. iMGL were striking similarity to monocytes but quite distinct from the monocyte derived macrophages (Figure 5.3 A-B). When analysing the intensity of Monocyte receptor we see that they strikingly distinct for background fluorescence (Figure 5.3 C, E, G, I) we want to

see how iMGL diverged from monocyte were the profile looked similar, using monocyte derived macrophages as a control. Quantification of the comparative analysis shows that iMGLs are $CD11b^{+}$ similar to monocytes but different from monocyte derived macrophages (Figure 5.3 D). iMGL are $CD45^{low}$ despite levels increasing with maturation as compared to monocyte derived macrophages but have higher CD45 expression them monocytes (Figure 5.3 F). iMGLs are $CD11c^{-}$ similar to monocytes but different from monocyte derived macrophages (Figure 3 H). And iMGLs have lower CCR2 than monocytes and monocyte derived macrophages (Figure 5.3 J). These results suggest that iMGL have a unique phenotype and are easily distinguished from macrophages and other myeloid cells.

5.3.4 iMGL are functional in phagocytosing.

We wanted to determine the capacity of iMGL to phagocytose *S. aureus* and synaptosomes, and then see how maturation with CD200 and CX3CL1, and activation with TNF for 24h influence this phenomenon. In our previous study of mouse *ex vivo* microglial model, we see that age and activation affected microglial phagocytosis (Madany et al. 2017 unpublished). Here we wanted to determine in the iMGL as defined by their $CD11b^{+}$ and $CD45^{low}$ expression (Figure 5.4 A) were functional as microglia in their capability to phagocytose the different targets. In this study we hypothesized that the iMGL were less professional than the mouse *ex vivo* microglia so we allowed them to

phagocytose for 90 minutes (Figure 5.4 B-E) as compared to the previous 60 minutes. The iMGL were able to phagocytose both targets. However, the number the microglia phagocytosing *S. aureus* or synaptosomes didn't significantly increased with 5ng/ml or 50ng/ml activation, or maturation of the iMGL (Figure 5.4 G, I). We also showed that similar to mouse *ex vivo* microglia the number iMGL-phagocytosing synaptosomes was much less than *S. aureus*. To further examine whether activation and maturation altered iMGL phagocytosis, we looked at the amount of *S. aureus* and synaptosomes each of the phagocytosing iMGL phagocytosed. Similar to the rate of phagocytosis, we showed that activation of iMGL didn't increase the amount of *S. aureus* or synaptosomes they phagocytose nor maturation (Figure 5.4 F, H). Therefore its important to determine the exact mechanism in which iMGL should be activated to resemble what has been shown in our previous study as in other studies. However, it is important to note that these iMGL are functional in addition to expressing a microglial profile that is unique from other hematopoietic cells.

5.4 DISCUSSION

In this study, we have validated a robust protocol for the derivation of microglia-like cells from human pluripotent stem cells (Abud et al., 2017). These cells were derived in a serum free conditions that better simulates the CNS environment. We demonstrated that iMGL are geneotypicaly similar to primary fetal and adult human microglia in the expression of TREM2, TMEM119 and OLFML3 as well as being phenotypicaly different from monocytes and macrophages. We also show that iMGL are phagocytic and able to phagocytose *S. aureus* and synaptosomes. So we can now compare our rodent-derived data with human data. Though other protocol for the differentiation of iMGL have need recently published, they don't use serum free conditions or they co-culture with astrocytes therefore, we think our method is unique. We demonstrated that not only M-CSF and IL-34 that have CSF-1R as their receptor but also TGFβ supported the differentiation into iMGL. Although these factors are sufficient for the differentiation, CD200 and CX3CR1 are required for iMGL maturation (Abud et al., 2017).

TREM2-deficient mouse microglial cell lines and primary microglia showed significantly reduced clearance of amyloid plaques(Xiang et al., 2016). It is important to note that *in vivo* the amyloid plaque deposition is similar in wild type and TREM2 KO mice. However the TREM2 KO mice showed reduced microglia accumulations around amyloid plaques (Wang et al., 2016). We can now test this in human cells by generating microglia from individuals with mutations in TREM2

and studying how the function of human TREM2 mutant microglia differs from microglia from individuals with normal TREM2. Also TREM2 is known to be involved in phagocytosis of apoptotic neurons (Colonna, Turnbull, & Klesney-Tait, 2007; Takahashi et al., 2005). However, TREM2's role in pathological setting where there is exacerbated CNS neuronal degeneration is less known and potentially controversial. Therefore with this model we can test some of these consequences.

A potential application of iMGL technology is to generate microglia from an individual's own genetic background and test for function and response to therapeutics or graft microglia in an unconditioned CNS since there will be no need for Immuno suppressants and allow them to travel to the affected area. This is a first step toward personalized medicine. Another potential applications of iMGL would be in drug discovery were they can model disease and do toxicity screening

5.5 FIGURES AND LEGENDS

Figure 5.1. Characterizing iMGL morphology during differentiation. (A) iCell Hematopoietic Progenitor Cells plating for 24 hours shows the expected morphology as described by the Cellular Dynamics International's user's guide. **(B)** Differentiating iMGL 6 DIV showing change in morphology from the hematopoietic progenitors, arrow indicates cluster that is proliferating, Scale bar, 100 μm^2. **(C)** Differentiating iMGL15 DIV showing further morphology changes from earlier iMGL, arrow indicates initiation of ramification, Scale bar, 100 µm **(D)** PBMC macrophages in culture show a distanced amoeboid morphology quit different from that of the iMGL.

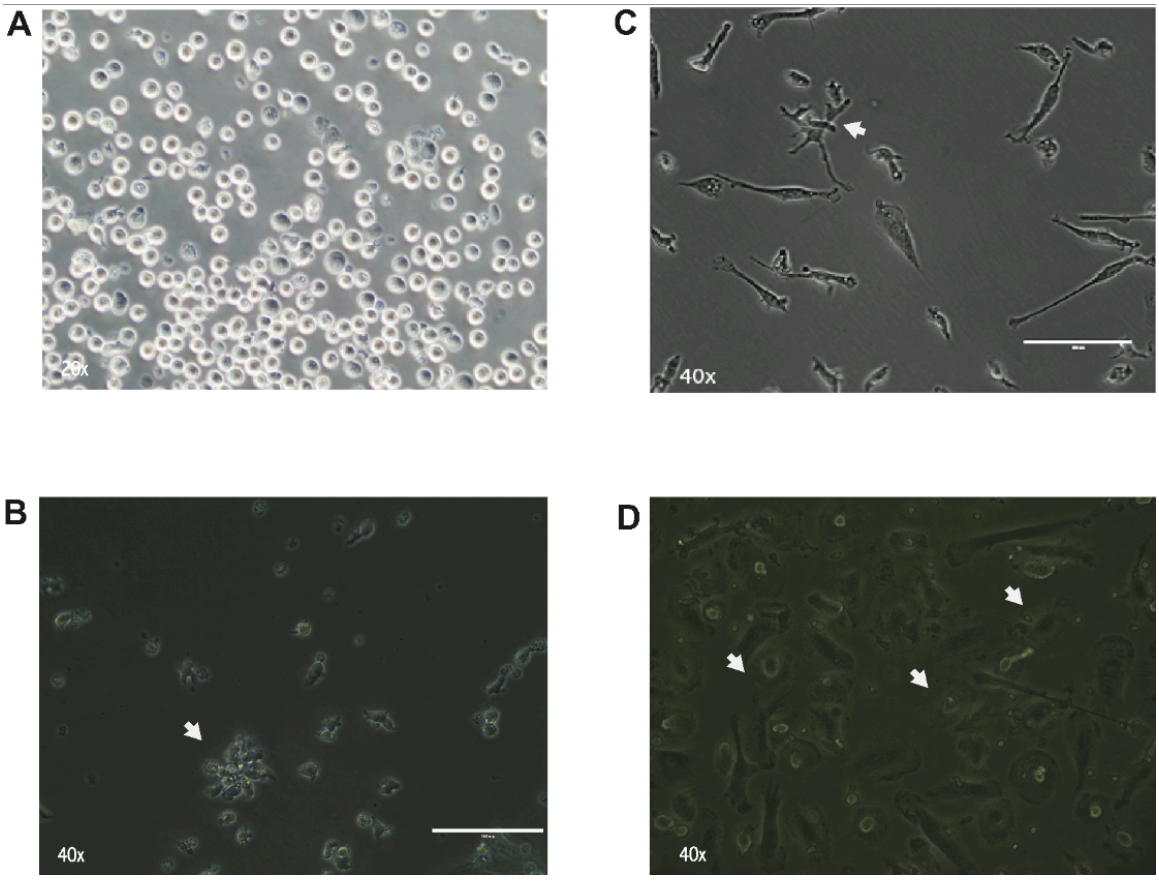

A
20x
C
40x
B
40x
D
40x

Figure 5.2. iMGL gene profile is highly dependent on the culture environment. Bar graphs of HPRT normalized qPCR data showing microglial enriched genes **(A)** TREM2, **(B)** TMEM119, and **(C)** OLFML3 measured in iMGL generated in different culture environments. Data is represented as mean ± SEM and analyzed using one-way ANOVA followed by Tukey's corrected multiple comparison post hoc test. Statistical annotation represents greatest p value for iMGL, cultured with or without the TGFβ. GM-CSF/M-CSF +Astrocytes (n = 3), GM-CSF/M-CSF/TGFβ + Astrocytes (n = 3), M-CSF/TGFβ + Astrocytes (n = 3), and GM-CSF without Astrocytes (n = 3). *P < 0.05; **P < 0.01; ***P < 0.001 and ****P < 0.0001.

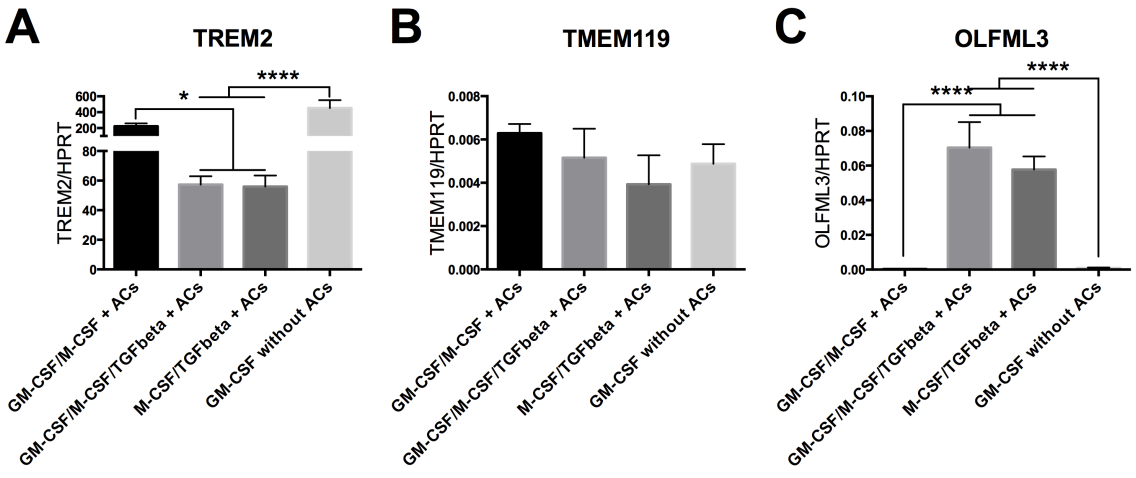


Figure 5.3. iMGL up regulation hematopoietic markers but not to the extent of macrophages. (A) Comparative analysis shows that iMGLs (green) are $CD45^{low}$, and $CD11b^{+}$ similar to Monocytes (orange) but different from $CD45^{high}$ MD-Macrophages (red 1DIV, blue 2DIV). **(B)** Comparative analysis shows that iMGLs (green) don't express CD11c unlike MD-Macrophages (red 1DIV, blue 2DIV) and that iMGLs (green) are $CCR2^{low}$, as compare to Monocytes (orange) and MD-Macrophages (red 1DIV, blue 2DIV). **(C, E, G, I)** Histogram of CD11b, CD45, CD11c, and CCR2 intensity of Monocytes in orange with background fluorescence in grey from the normalization of MFI. **(D, F, H, J)** Graphs of the flow cytometric MFI data showing that iMGLs (green) are different from the Monocytes (orange) in CD45 and CCR2 and MD-Macrophages (red 1DIV, blue 2DIV) in (CD11b, CD45, CD11c, and CCR2). Monocyte (n = 1), iMGL (n = 6), Macrophage 1 DIV (n = 1), and Macrophage 2 DIV (n = 1).

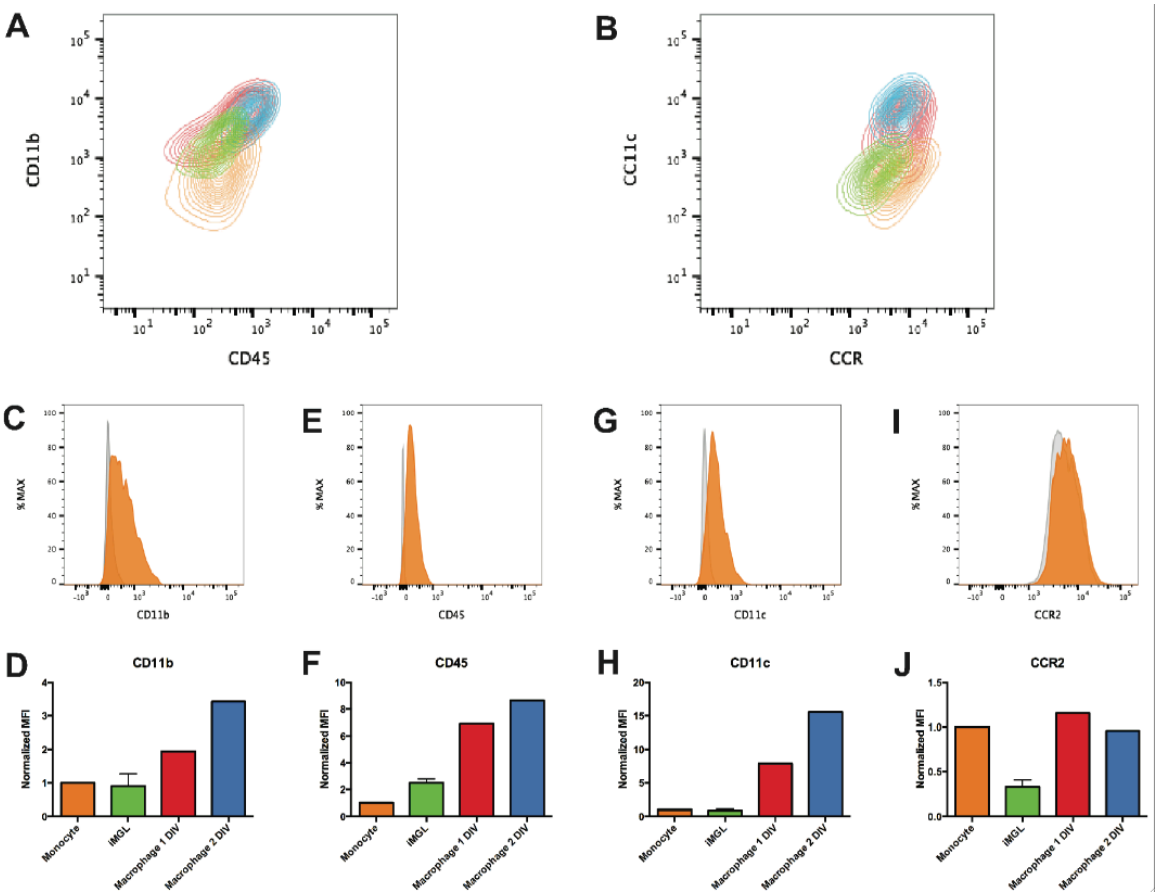
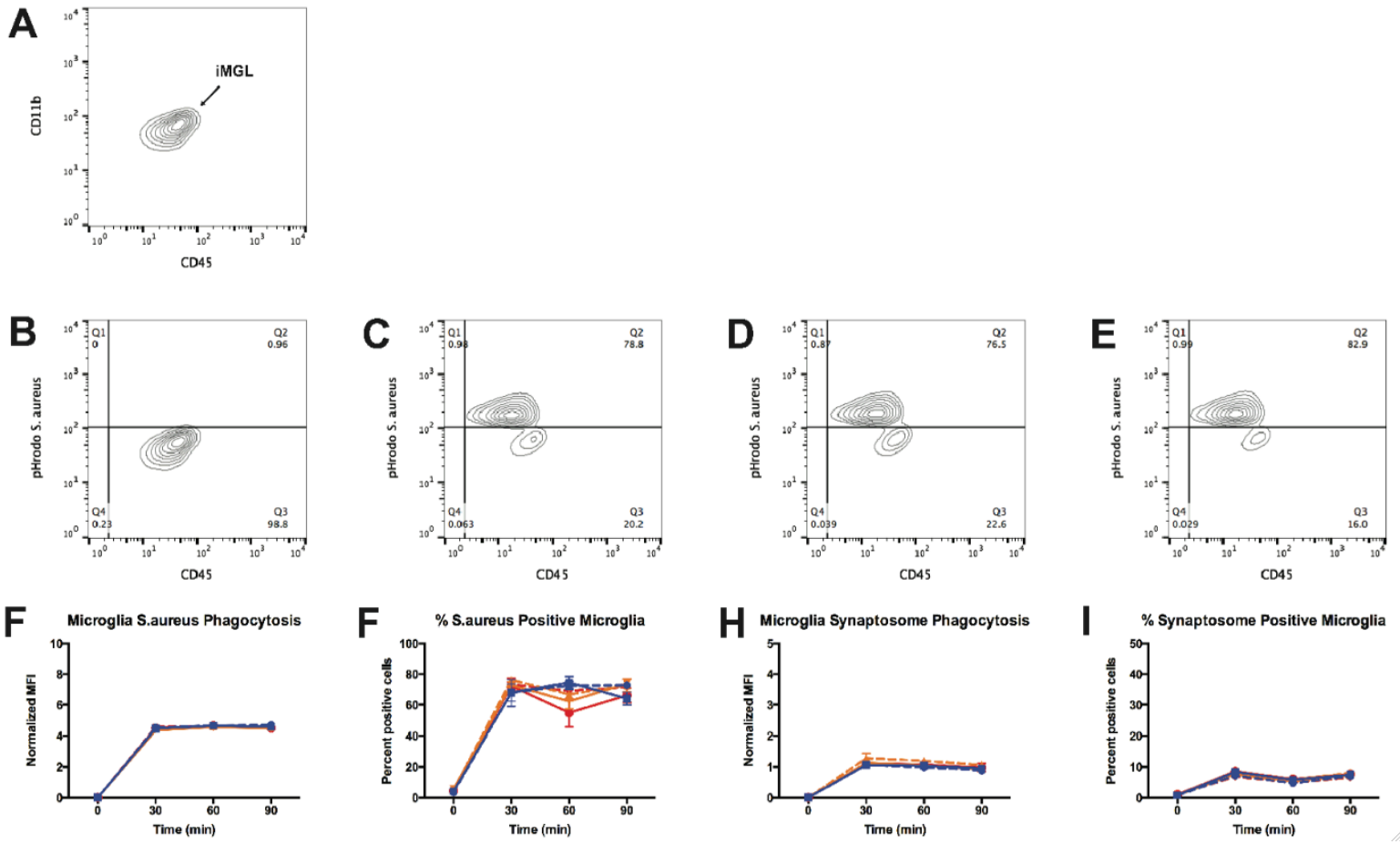

A
CD11b
CD45
B
CC11c
CCR
C
% MAX
CD11b
E
% MAX
CD45
G
% MAX
CD11c
I
% MAX
CCR2
D
CD11b
Normalized MFI
Monocyte
iMGL
Macrophage 1 DIV
Macrophage 2 DIV
F
CD45
Normalized MFI
Monocyte
iMGL
Macrophage 1 DIV
Macrophage 2 DIV
H
CD11c
Normalized MFI
Monocyte
iMGL
Macrophage 1 DIV
Macrophage 2 DIV
J
CCR2
Normalized MFI
Monocyte
iMGL
Macrophage 1 DIV
Macrophage 2 DIV

Figure 5.4. TNFalpha activation doesn't regulate the number iMGL phagocytosing or amount *S. aureus* nor synaptosomes phagocytosed. (A) Flow cytometric separation of microglia populations based on their CD11b and CD45 expression. **(B-E)** Flow cytometric analysis of these distinct populations for pHrodo positive microglia over the time course of the 90-minute assay is used to measure active phagocytosis. Graphs of the flow cytometric data showing the Mean Fluorescence Intensity (MFI) of microglia that phagocytosed pHrodo *S. aureus* **(F)** or pHrodo-labeled synaptosomes **(H)**. And data showing the percent of microglia phagocytosing pHrodo *S. aureus* **(G)** or pHrodo-labeled synaptosomes **(I)** over the 90 minutes assay. Data is represented as mean ± SEM and analyzed using one-way ANOVA followed by Tukey's corrected multiple comparison post hoc test of phagocytosis of *S. aureus* or pHrodo-labeled synaptosomes by human young microglia naive (n = 3), human mature microglia naive (n = 3), human young microglia 5ng/ml TNFα (n = 3), human mature microglia 5ng/ml TNFα (n = 3), human young microglia 50ng/ml TNFα (n = 3), and human mature microglia 50ng/ml TNFα (n = 3). *P < 0.05; **P < 0.01; ***P < 0.001 and ****P < 0.0001.


A
iMGL
CD11b
CD45
B
pHrodo S. aureus
Q1
0
Q2
0.96
Q4
0.23
Q3
98.8
CD45
C
Q1
0.98
Q2
78.8
Q4
0.063
Q3
20.2
D
Q1
0.87
Q2
76.5
Q4
0.039
Q3
22.6
E
Q1
0.99
Q2
82.9
Q4
0.029
Q3
16.0
F
Microglia S.aureus Phagocytosis
Normalized MFI
Time (min)
F
% S.aureus Positive Microglia
Percent positive cells
Time (min)
H
Microglia Synaptosome Phagocytosis
Normalized MFI
Time (min)
I
% Synaptosome Positive Microglia
Percent positive cells
Time (min)

CHAPTER 6

Aerosolized *Alternaria* fungus results in inflammation within brains of mice

6.1 ABSTRACT

The inhalation of pollutants be they natural or man-made, can cause inflammation in the pulmonary system. In this study we wanted to determine if the inhalation of a high concentrations of a fine aerosol particulates in the size range of $PM_{2.5}$ (particle size $\leq$ 2.5 μm) of a common fungus *Alternaria*, which we know to cause primary extensive inflammation in the lungs when inhaled, would result in any detectable inflammation of the central nervous system (CNS). We constructed an environmental chamber able to simultaneously control particle size distribution and mass concentration of the aerosolized suspensions of the 100nm *Alternaria* particulates. The 96-hour of continuous exposure to this aerosol induced lung pathology in C57BL/6J mice with interstitial infiltrates. Since we know that microglia, the resident immune cells of the CNS are sensitive to changes in their environment, and are able to rapidly respond to systemic inflammation, we were curious to determine how the *Alternaria* that has a robust pulmonary response would affect the CNS So we analyzed a panel of CNS immune specific markers and Microglial markers in both forebrain and brainstem. This was done in mice that were exposed aerosolized *Alternaria* in a chamber

constantly for a week. We found that *Alternaria* exposed animals showed inflammation in the CNS and microglia activation in brain region specific manner.

6.2 INTRODUCTION

We show that systemic inflammation from an intraperitoneal injection of lipopolysaccharide (LPS) can result an immunological response that has the potential effect; a shift in the immunological profile of the CNS. Inhalation is another way in which we introduce foreign molecules into our system that can result in an immunological response and subsequently, inflammation of the lungs and systemic activation. Inhaled toxicants whether man-made or natural, can cause inflammation in both the pulmonary system and CNS. For example, exposure to ozone has been reported to result in acute inhalation and significant increase in inflammatory mRNA levels in the brain. And just a short-term inhalation of ozone was seen to alter the activity of sympathetic nerves and catecholamine neurons (Soulage et al., 2004). The anatomical description of the CNS regions activated by O3 inhalation has been able to provide evidence that the exposure activated the CNS through the vagus nerves. (Gackiere, Saliba, Baude, Bosler, & Strube, 2011) It has also been shown that diesel exhaust particles caused microglial activation and upregulation of oxidative stress in a region specific manner and suggested region vulnerability since different areas were more activated (Levesque et al., 2011).

A lot of the literature has focused mainly on man-made toxicants that cause

robust phenomena. Immune compromised people also have exacerbated response. Air pollution exposures were more toxic to hypertensive rats in terms of vascular leakage increased, pulmonary injury as compared to controls. (U. P. Kodavanti et al., 2000)**.** It has also been shown that several oxidative stress parameters were compromised in older animals, and that chemical exposure was able to worsen these effects. These ROS were seen at both the mitochondrial and cytosolic levels (P. R. Kodavanti, Royland, Richards, Besas, & Macphail, 2011). Though chronic exposure to man-made air pollutants in suitable animal models may provide insight into the physiological consequences of these alterations, little has been done to see how natural occurring particles, if so, can lead to similar effects. Therefore, we purpose this study was to determine if inhalation of an aerosolized, naturally occurring irritant, can cause changes in the expression of neuroimmune biomarkers within the CNS. For this study we used the extract of a common fungus to Southern California, *Alternaria*. These particles are known to cause extensive inflammation in the lungs when inhaled and has also been shown to cause asthma following chronic exposure and exacerbated effects in compromised individual. We show something as common as *Alternaria* can affect the CNS immune profile.

6.3 RESULTS

6.3.1 Aerosolized *Alternaria* exposure changes CNS immune profile in a reign dependent manner.

We decided to determine whether the *Alternaria* exposure altered expression of inflammatory molecules following the week exposure. Specifically, we performed qPCR looking at P2Y12, TGFβ, Arginase1 (Arg1), and iNOS with brainstem or rest of the whole brain cDNA from aerosolized *Alternaria* exposed adult mice comparing them to their naive cDNA counterparts. Here we show significant increase in P2Y12 expression, a primary purinergic receptor used by microglia to detect cellular damage following *Alternaria* exposure in the brainstem but not the rest of the brain (Figure 6.1 A-B). There was a significant decrease in TGFβ expression in brainstem but not the rest of the brain did not reach significant following *Alternaria* exposure (Figure 6.1 C-D) indicating reduced expression of an anti-inflammatory biomarkers. TNFα expression, a tumor necrosis factor that in a pro-inflammatory biomarkers. (Figure 6.1 E-F). There were also a significant decrease in arginase1 expression in brainstem but the rest of the brain did not reach significant following *Alternaria* exposure (Figure 6.1 G-H) indicating reduced expression of a neuroprotective biomarkers. iNOS was reduced however, it did not reach significance (Figure 6.1 I-J). The ratio of Arg1 to iNOS is used to determine the polarization of immune cells and inevitable the type of

activation, we see there is no preference to one over another in any brain region (Figure 6.1 K-L). Chamber animals showed robust changes in mRNA expression within brainstem but little to no change in mRNA expression from the rest of the brain.

6.3.2 Aerosolized *Alternaria* specifically activates microglia in a region dependent manner.

We decided to determine how immune cell specific the *Alternaria* exposure was in the CNS. There were altered expressions of inflammatory molecules following the week exposure regulating or regulated by microglia or macrophages. Specifically, we performed flow cytometry of microglia and looked at CD11b, CD36, TLR2, TLR4, TREM2, and Tyro3 with brainstem or rest of the brain isolated microglia from aerosolized *Alternaria* exposed adult mice, comparing them to their congenic counterparts. Here we show significant robust increase in CD11b expression, following *Alternaria* exposure in both the brainstem as well as the rest of the brain (Figure 6.3 A-B). No significant change was detected in CD36 nor the TLR's 2 and 4, tested from brainstem, nor the rest of the brain following *Alternaria* exposure (Figure 6.3 C-H). There was also a significant decrease in TREM2 expression in brainstem but not in the rest of the brain, where it did not reach significance following *Alternaria* exposure (Figure 6.3 I-J) indicating microglial activation. Finally we saw no change in Tyro3 Figure 6.3 G-H) following the exposure to *Alternaria*. Chamber animals showed robust

changes in microglial profile in brainstem but little to no chances, but not as much from the rest of the brain. When we looked at the percent positive cells we saw no difference between the brainstem and the rest of the brain for all the molecules tested (Figure 6.3 M-X).

6.4 DISCUSSION

We see that the aerosolized *Alternaria* fungal particles are sufficient to cause a shift in the mRNA expression of neuroimmune biomarkers as measured by qPCR. However, this shift in the expression of neuroimmune biomarkers occurs in a region specific manner. Suggesting that the mode of activation needs to be further studied. This is such as the vagus nerve as has need previously described. Increased expression of the microglial-specific DAMP receptor P2Y12 expression along with the concurrent decrease in expression of protective and anti-inflammatory biomarkers (TGF-β and Arginase 1, respectively) is indicative of an altered CNS following *Alternaria* exposure. Flow cytometric analysis of microglial activation detected a significant decrease in the percentages of microglia expressing the PAMP receptor, TLR2. Taken together these data indicate that inhalation exposure to a natural fungal allergen is sufficient to induce significant inflammation-associated changes in the region of the CNS controlling respiration.

Further evidence of this perturbed CNS becomes obvious when we analyzed the immune cells. Interestingly, there was no macrophage infiltration following *Alternaria* exposure. However, the microglia were activated. There was an

increase in CD11b expression on microglia in all regions of the CNS tested. The brainstem showed alteration in TREM2 expression. This decrease in TREM2 upon activation, gives us a little insight into the type of microglial activation. We know from our previous studies that when LPS is given IC rather than IP, we see a decrease in TREM2 expression on microglia. So from this study we conclude that a short-term exposure to a fungal pathogen is sufficient to cause CNS alterations.

Here we generated and characterized a novel murine exposure chamber equipped to disperse a natural fungal allergen continuously and equipped to monitor sustained aerosolized dispersion of the natural allergen for 96-hours. The nebulized *Alternaria* aerosol particles used in our study were an average size of approximately 100 nm and are well within the size range of $PM_{2.5}$ which are well defined as the size of ambient air pollutants able to provoke potent inflammatory responses. Indeed, such fine aerosol particles are capable of penetrating deep into the alveolar spaces in the lung (Barnewall et al. 2015, Jayaraj et al. 2017). Therefore, continuous exposure to this particle suspension ensured wide distribution throughout the lung, and avoided the known artefactual ingestion of a large proportion of allergen solutions when applied intranasally. In addition, continuous exposure over several days avoided the transient effects of short term or sporadic exposure in a nebulizer or other similar device, in which

acute allergic hypersensitivity reactions may induce repetitive bursts of transient CNS responses that are not characteristic of chronic effects.

6.5 FIGURES AND LEGENDS

Figure 6.1. Chamber exposure to *Alternaria* changes microglial profile. Bar graphs of HPRT normalized qPCR data showing shift in CNS profile in the *Alternaria*-exposed mice. Brainstem **(A)** P2Y12, **(C)** TGFβ, **(E)** TNFα, **(G)** Arginase1, **(I)** iNOS, and **(K)** Arg1/iNOS and the rest of the brain **(B)** P2Y12, **(D)** TGFβ, **(F)** TNFα, **(H)** Arginase1, **(J)** iNOS, and **(L)** Arg1/iNOS measured after aerosolized *Alternaria* extract (Chamber) continuously exposure for 5 days and compared to naive animals. This was done to determine if the treatment would lead to changes in neuroimmune markers. Data is represented as mean ± SEM and analyzed using an unpaired t test with a two-tail P value. Statistical annotation represents greatest p value for microglial marker expression from the brainstem. Naive brainstem (n = 3), Chamber brainstem (n = 3), Naive rest of brain (n = 3), and Chamber rest of brain (n = 3). *P < 0.05; **P < 0.01; ***P < 0.001 and ****P < 0.0001.

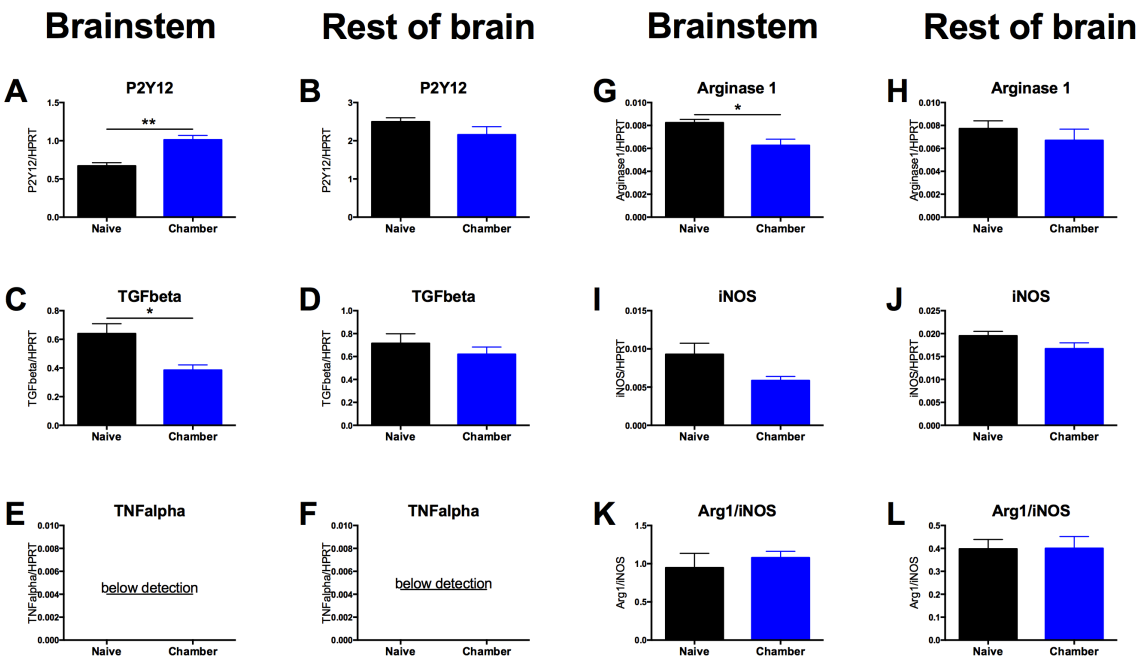
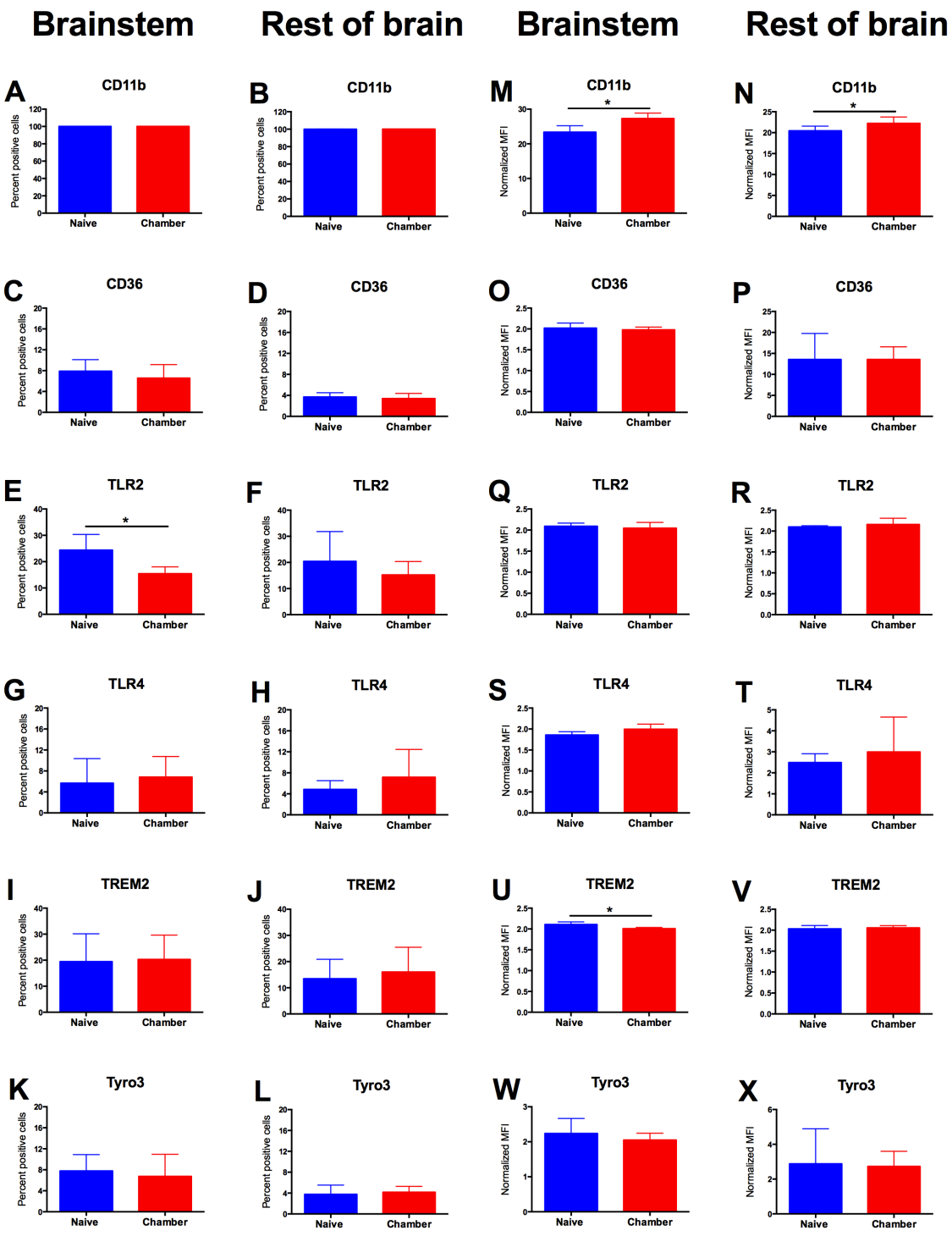

Brainstem
Rest of brain
Brainstem
Rest of brain
A
P2Y12
P2Y12/HPRT
**
Naive
Chamber
B
P2Y12
P2Y12/HPRT
Naive
Chamber
G
Arginase 1
Arginase 1/HPRT
*
Naive
Chamber
H
Arginase 1
Arginase 1/HPRT
Naive
Chamber
C
TGFbeta
TGFbeta/HPRT
*
Naive
Chamber
D
TGFbeta
TGFbeta/HPRT
Naive
Chamber
I
iNOS
iNOS/HPRT
Naive
Chamber
J
iNOS
iNOS/HPRT
Naive
Chamber
E
TNFalpha
TNFalpha/HPRT
below detection
Naive
Chamber
F
TNFalpha
TNFalpha/HPRT
below detection
Naive
Chamber
K
Arg1/iNOS
Arg1/iNOS
Naive
Chamber
L
Arg1/iNOS
Arg1/iNOS
Naive
Chamber

Figure 6.2. Change in microglial phenotype following chamber exposure to *Alternaria*. Bar graph depicting the mean flurecent intensity (MFI) of the brainstem **(A)** CD11b, **(C)** CD36, **(E)** TLR2, **(G)** TLR4, **(I)** TREM2, and **(K)** Tyro3 and the rest of the brain **(B)** CD11b, **(D)** CD36, **(F)** TLR2, **(H)** TLR4, **(J)** TREM2, and **(L)** Tyro3 measured after aerosolized *Alternaria* extract (Chamber) continuously exposure for 5 days and compared to naive animals.
Bar graph depicting the persencent positive cells of the brainstem **(M)** CD11b, **(O)** CD36, **(Q)** TLR2, **(S)** TLR4, **(U)** TREM2, and **(W)** Tyro3 and the rest of the brain **(N)** CD11b, **(P)** CD36, **(R)** TLR2, **(T)** TLR4, **(V)** TREM2, and **(X)** Tyro3 measured after aerosolized *Alternaria* extract (Chamber) continuously exposure for 5 days and compared to naive animals. This was done to determine if the treatment would lead to changes in neuroimmune markers. Data is represented as mean ± SEM and analyzed using an unpaired t test with a two-tail P value. Statistical annotation represents greatest p value for microglial marker expression from the brainstem. Naive brainstem (n = 4), Chamber brainstem (n = 4), Naive rest of brain (n = 6), and Chamber rest of brain (n = 6). *P < 0.05; **P < 0.01; ***P < 0.001 and ****P < 0.0001.


Brainstem
Rest of brain
Brainstem
Rest of brain
A
CD11b
Percent positive cells
Naive
Chamber
B
CD11b
Percent positive cells
Naive
Chamber
M
CD11b
*
Normalized MFI
Naive
Chamber
N
CD11b
*
Normalized MFI
Naive
Chamber
C
CD36
Percent positive cells
Naive
Chamber
D
CD36
Percent positive cells
Naive
Chamber
O
CD36
Normalized MFI
Naive
Chamber
P
CD36
Normalized MFI
Naive
Chamber
E
TLR2
*
Percent positive cells
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Chamber
F
TLR2
Percent positive cells
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Chamber
Q
TLR2
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Naive
Chamber
R
TLR2
Normalized MFI
Naive
Chamber
G
TLR4
Percent positive cells
Naive
Chamber
H
TLR4
Percent positive cells
Naive
Chamber
S
TLR4
Normalized MFI
Naive
Chamber
T
TLR4
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I
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J
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U
TREM2
*
Normalized MFI
Naive
Chamber
V
TREM2
Normalized MFI
Naive
Chamber
K
Tyro3
Percent positive cells
Naive
Chamber
L
Tyro3
Percent positive cells
Naive
Chamber
W
Tyro3
Normalized MFI
Naive
Chamber
X
Tyro3
Normalized MFI
Naive
Chamber

CHAPTER

Discussion

The fact that microglia play multiple critical roles in human neurological diseases, such as clearance of cellular debris and protein aggregates or foreign material and regulating toxicity is well established. Defects in microglia can in turn lead to their inability to perform some of these physiological functions and result in contribution to the exacerbation of disease pathology. For this reason, in a large variety of neuronal pathologies, microglia are thought to be promising targets for therapeutic intervention. The thought is that microglia replacement therapy can play a critical role in resolving many CNS diseases such as Alzheimer's disease. However, for this reason a better understanding of the mechanisms underlying their ability to resolve neuronal dysfunction is needed.

Recent studies focusing on the role of microglia in the healthy brain, and in the contexts of neurodegeneration, have found evidence that suggests that microglia play a roles in neuronal remodeling and this regulation is most crucial in the actual developing and maturation of synapses. The interaction between microglia and synapses is dependent upon molecular signaling as well as physical contact. We know microglia are motile and surveys their environments. They are also involved in modulating neurons throughout life while they are surveying. In the context of the developing CNS, there are phagocytic signaling pathways directly

regulating the neuronal microglia interactions. Microglia at a later stage are important for the synapse elimination by pruning and engulfment of synaptic element. Finally there are interactions that occur during apoptosis and neuron degeneration.

Although we know microglia to be long-lived in the CNS, it remains possible that aged microglia may actually be replaced or reinforced by BMDM cells capable of carrying out the functions of young microglia. Following infiltration other myeloid cells could potentially perform surveillance and scavenging functions similar to microglia in the CNS. These cells could exert therapeutic effects particularly in specific circumstances when selective loss and dysfunction of microglia or microglia senescence are observed in disease processes like Alzheimer's disease and in the aged brain. As we know macrophages to be more phagocytic than microglia, studies have shown BMDM to be more efficient than resident microglia in eliminating amyloid plaque. Studies in slice cultures have shown that when microglia are deprived in a region cultured cells that acquire a ramified morphology can replenish them.

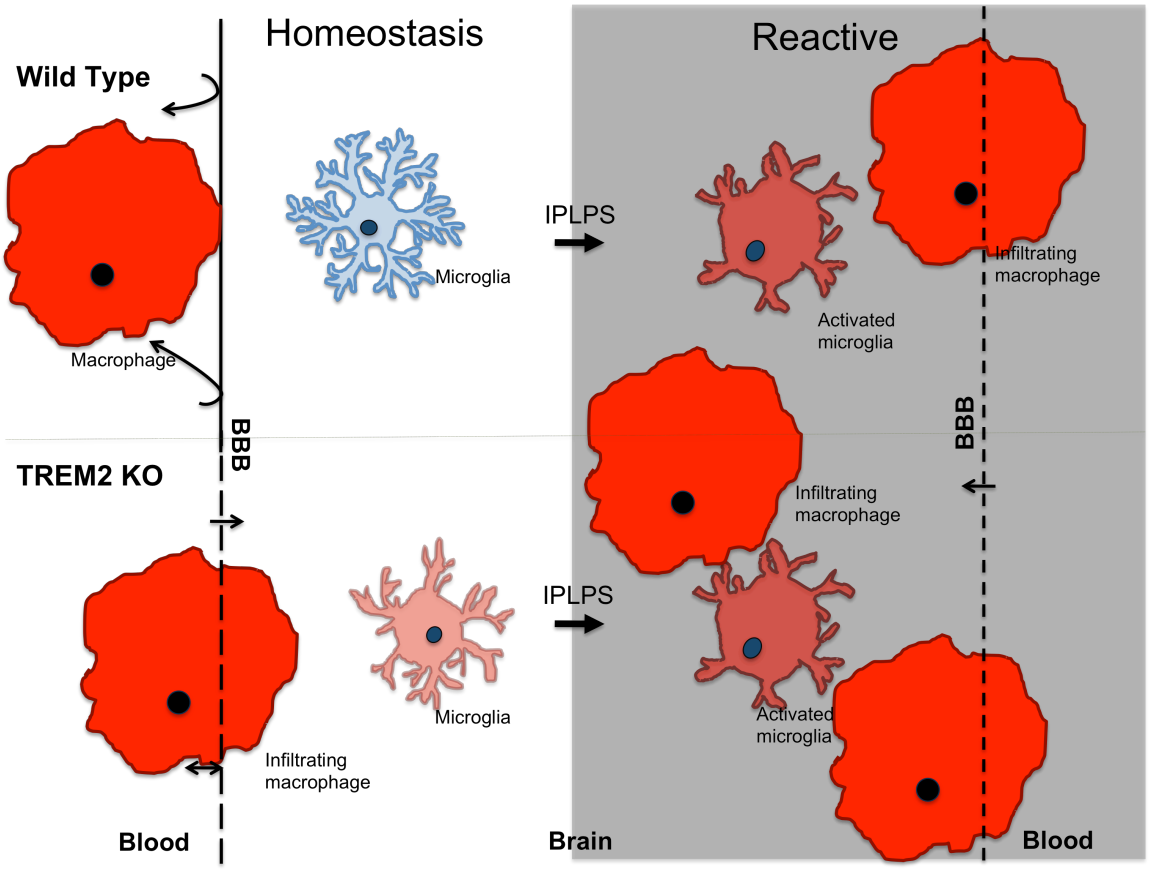
In response to IPLPS stimuli microglia become activated and there is also an infiltration of peripheral macrophages that aid in immune regulation. When TREM2 is depleted there is a pre existing activation state hat can be a result of decrease BBB integrity. The TREM2 KO mice have a decrease in vGlut1

synapses. The decrease in vGlut1 synapses following IPLPS immune challenge requires macrophages as well as C1q signals. When microglia phagocytose we see that TREM2 expression is critical especially in the activated state. Therefore TREM2 is crucial for CNS immune regulation (models).

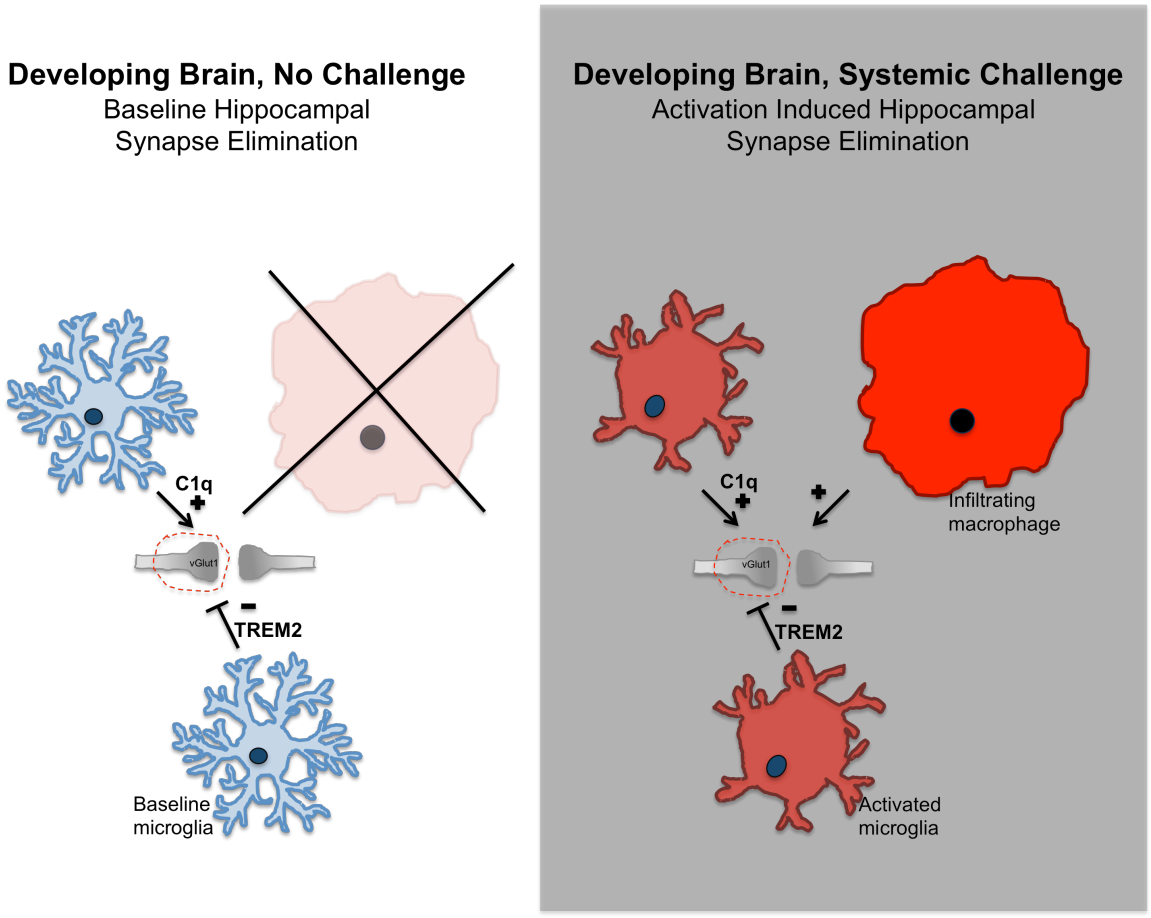
We how short-term inhalation of *Alternaria* can alter the activity and immune profile of the CNS it can also specifically activate microglia cells for the most mart this CNS activation was region specify with the blunt of the activation occurring in the brainstem. However Microglia activation through increased CD11b of microglia seems to occur globally. There were significant alteration of P2Y12, TGFβ, and Arginase1 in the mRNA levels. This regional specificity of the neuronal changes observed under our conditions of exposure is more representative of relevant physiological responses rather than a toxic influence of *Alternaria* on the CNS. Our study supports the notion that common fungal particles can exert neurological effects. So from this we can conclude that the use of this chamber can be used to test an array of aerosolized particles that are common in our environment and see which have direct effects on our CNS. Therefore chronic exposure to air pollutants in suitable animal models may provide additional insight into the physiological consequences of these alterations.

To get a better understanding of these microglial cells is the use of *in vivo, ex vivo,* in addition to *in vitro* strategies. As we have seen microglia are highly reactive cells that respond quickly to a plethora of neuronal manipulation. Despite this fact the *in vitro* assays such as isolated cell culture, acute slice, and slice cultures are important and necessary strategies for dissecting mechanism and function, however there is a need to confirmed *in vivo* the physiological relevance and molecular mechanisms observed. This is because these models only give us a snap shot of what is really going on. So the future of microglial *in vivo* studies include the use of technical advancements in the combinatorial approach of live imaging and molecular biology, which will indeed include new mouse lines. As for *in vitro* studies they may significantly advance our understanding of the function of human microglia as they interact with other CNS cells and the molecular mechanisms underlying these interactions. Since we have no *in vivo* human model this remains the closes translational approach. The knowledge of molecules and function of human microglia is important for advancing our understanding of basic biological mechanisms as well as for the promise of developing novel diagnostic and therapeutic strategies associated with CNS disease and injury.

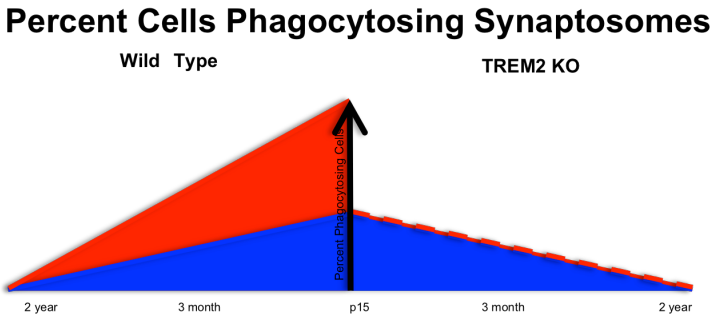
TREM2 deficiency results in decrease BBB integrity and reactivated microglia

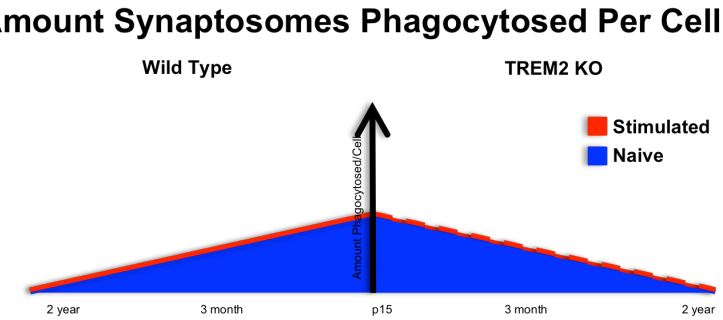


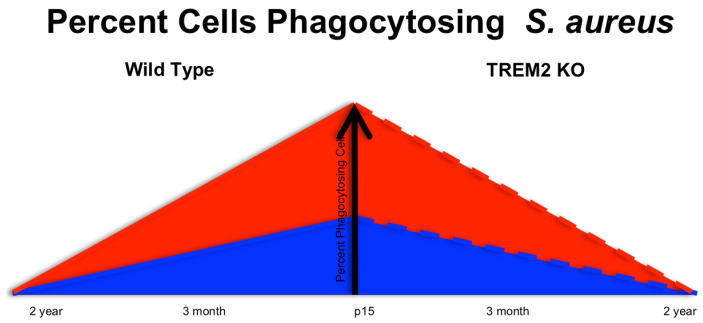
TREM2 is required for proper synapse elimination

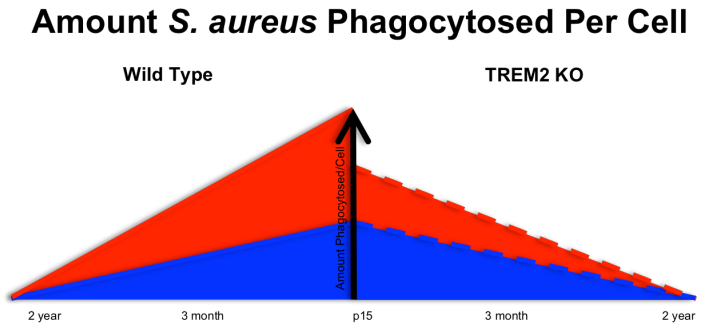


Effect of in vivo stimuli and TREM2 sufficiency on microglial phagocytosis

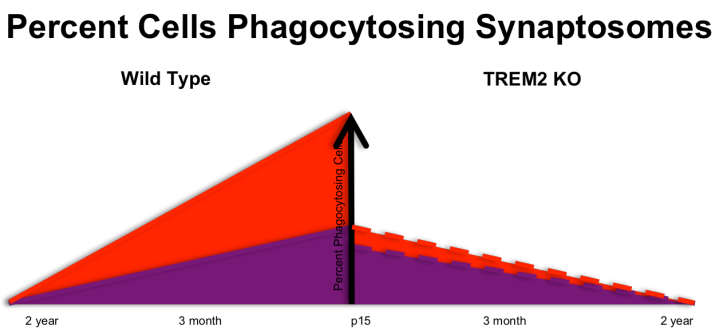


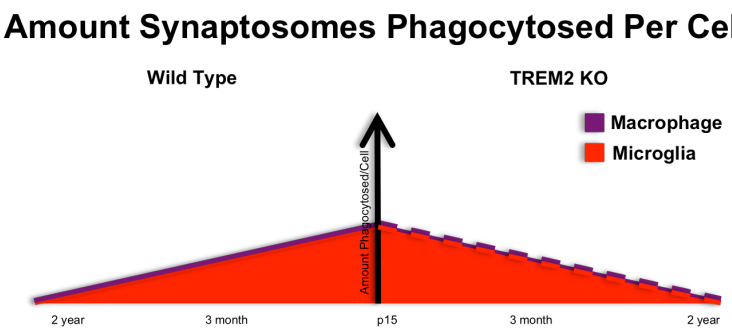


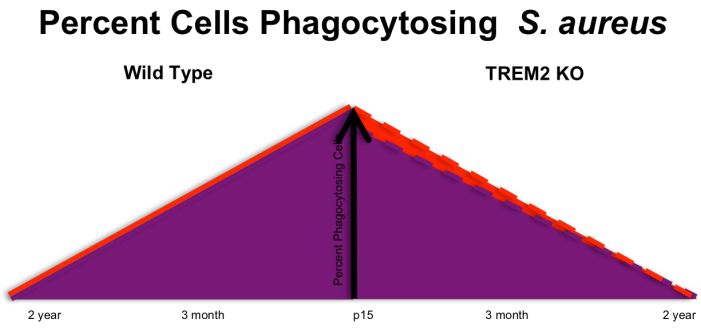


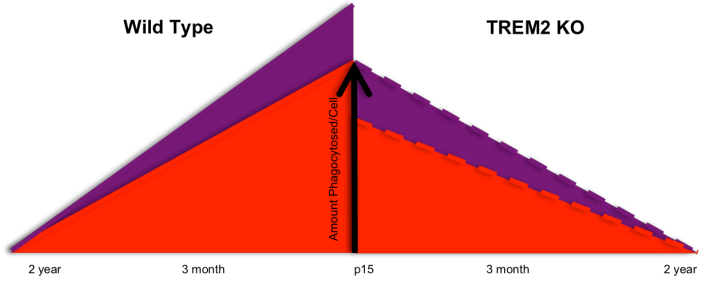


Effect of cell type and TREM2 sufficiency on microglial and macrophage phagocytosis









REFERENCES

Abud, E. M., Ramirez, R. N., Martinez, E. S., Healy, L. M., Nguyen, C. H. H., Newman, S. A., . . . Blurton-Jones, M. (2017). iPSC-Derived Human Microglia-like Cells to Study Neurological Diseases. *Neuron, 94*(2), 278-293 e279. doi: 10.1016/j.neuron.2017.03.042

Anderson, K. L., Smith, K. A., Conners, K., McKercher, S. R., Maki, R. A., & Torbett, B. E. (1998). Myeloid development is selectively disrupted in PU.1 null mice. *Blood, 91*(10), 3702-3710.

Atagi, Y., Liu, C. C., Painter, M. M., Chen, X. F., Verbeeck, C., Zheng, H., . . . Bu, G. (2015). Apolipoprotein E Is a Ligand for Triggering Receptor Expressed on Myeloid Cells 2 (TREM2). *J Biol Chem, 290*(43), 26043-26050. doi: 10.1074/jbc.M115.679043

Barnewall RE, Benson EM, Brown MA, Fisher DA, Lindsay AS, Simmons AA, Anderson MS (2015) Characterization of a large animal aerosol exposure system for aerosolizing four strains of Burkholderia pseudomallei. *Journal of Aerosol Science* 84: 21-38.

Bailey, C. C., DeVaux, L. B., & Farzan, M. (2015). The Triggering Receptor Expressed on Myeloid Cells 2 Binds Apolipoprotein E. *J Biol Chem, 290*(43), 26033-26042. doi: 10.1074/jbc.M115.677286

Beers, D. R., Henkel, J. S., Xiao, Q., Zhao, W., Wang, J., Yen, A. A., . . . Appel, S. H. (2006). Wild-type microglia extend survival in PU.1 knockout mice with familial amyotrophic lateral sclerosis. *Proc Natl Acad Sci U S A, 103*(43), 16021-16026. doi: 10.1073/pnas.0607423103

Bennett, M. L., Bennett, F. C., Liddelow, S. A., Ajami, B., Zamanian, J. L., Fernhoff, N. B., . . . Barres, B. A. (2016). New tools for studying microglia in the mouse and human CNS. *Proc Natl Acad Sci U S A, 113*(12), E1738-1746. doi: 10.1073/pnas.1525528113

Blander, J. M., & Medzhitov, R. (2004). Regulation of phagosome maturation by signals from toll-like receptors. *Science, 304*(5673), 1014-1018. doi: 10.1126/science.1096158

Boring, L., Gosling, J., Chensue, S. W., Kunkel, S. L., Farese, R. V., Jr., Broxmeyer, H. E., & Charo, I. F. (1997). Impaired monocyte migration and reduced type 1 (Th1) cytokine responses in C-C chemokine receptor 2 knockout mice. *J Clin Invest, 100*(10), 2552-2561. doi: 10.1172/JCI119798

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. *J Exp Med, 194*(8), 1111-1122.

Butovsky, O., Jedrychowski, M. P., Moore, C. S., Cialic, R., Lanser, A. J., Gabriely, G., . . . Weiner, H. L. (2014). Identification of a unique TGF-beta-dependent molecular and functional signature in microglia. *Nat Neurosci, 17*(1), 131-143. doi: 10.1038/nn.3599

Carson, M. J., Bilousova, T. V., Puntambekar, S. S., Melchior, B., Doose, J. M., & Ethell, I. M. (2007). A rose by any other name? The potential consequences of microglial heterogeneity during CNS health and disease. *Neurotherapeutics, 4*(4), 571-579. doi: 10.1016/j.nurt.2007.07.002

Carson, M. J., Reilly, C. R., Sutcliffe, J. G., & Lo, D. (1998). Mature microglia resemble immature antigen-presenting cells. *Glia, 22*(1), 72-85.

Cella, M., Buonsanti, C., Strader, C., Kondo, T., Salmaggi, A., & Colonna, M. (2003). Impaired differentiation of osteoclasts in TREM-2-deficient individuals. *J Exp Med, 198*(4), 645-651. doi: 10.1084/jem.20022220

Cevenini, E., Monti, D., & Franceschi, C. (2013). Inflamm-ageing. *Curr Opin Clin Nutr Metab Care, 16*(1), 14-20. doi: 10.1097/MCO.0b013e32835ada13

Chen, L. C., Laskin, J. D., Gordon, M. K., & Laskin, D. L. (2008). Regulation of TREM expression in hepatic macrophages and endothelial cells during acute endotoxemia. *Exp Mol Pathol, 84*(2), 145-155. doi: 10.1016/j.yexmp.2007.11.004

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. *Eur J Immunol, 32*(1), 59-66. doi: 10.1002/1521-4141(200201)32:1<59::AID-IMMU59>3.0.CO;2-U

Colonna, M. (2003). DAP12 signaling: from immune cells to bone modeling and brain myelination. *J Clin Invest, 111*(3), 313-314. doi: 10.1172/JCI17745

Colonna, M., Turnbull, I., & Klesney-Tait, J. (2007). The enigmatic function of TREM-2 in osteoclastogenesis. *Adv Exp Med Biol, 602*, 97-105.

Damani, M. R., Zhao, L., Fontainhas, A. M., Amaral, J., Fariss, R. N., & Wong, W. T. (2011). Age-related alterations in the dynamic behavior of microglia. *Aging Cell, 10*(2), 263-276. doi: 10.1111/j.1474-9726.2010.00660.x

de Haas, A. H., Boddeke, H. W., & Biber, K. (2008). Region-specific expression of immunoregulatory proteins on microglia in the healthy CNS. *Glia, 56*(8), 888-894. doi: 10.1002/glia.20663

Dickstein, D. L., Biron, K. E., Ujiie, M., Pfeifer, C. G., Jeffries, A. R., & Jefferies, W. A. (2006). Abeta peptide immunization restores blood-brain barrier integrity in Alzheimer disease. *FASEB J, 20*(3), 426-433. doi: 10.1096/fj.05-3956com

Eglitis, M. A., & Mezey, E. (1997). Hematopoietic cells differentiate into both microglia and macroglia in the brains of adult mice. *Proc Natl Acad Sci U S A, 94*(8), 4080-4085.

Felix, R., Cecchini, M. G., Hofstetter, W., Elford, P. R., Stutzer, A., & Fleisch, H. (1990). Impairment of macrophage colony-stimulating factor production and lack of resident bone marrow macrophages in the osteopetrotic op/op mouse. *J Bone Miner Res, 5*(7), 781-789. doi: 10.1002/jbmr.5650050716

Ferri, C. P., Prince, M., Brayne, C., Brodaty, H., Fratiglioni, L., Ganguli, M., . . . Alzheimer's Disease, International. (2005). Global prevalence of dementia: a Delphi consensus study. *Lancet, 366*(9503), 2112-2117. doi: 10.1016/S0140-6736(05)67889-0

Ford, J. W., & McVicar, D. W. (2009). TREM and TREM-like receptors in inflammation and disease. *Curr Opin Immunol, 21*(1), 38-46. doi: 10.1016/j.coi.2009.01.009

Fu, R., Shen, Q., Xu, P., Luo, J. J., & Tang, Y. (2014). Phagocytosis of microglia in the central nervous system diseases. *Mol Neurobiol, 49*(3), 1422-1434. doi: 10.1007/s12035-013-8620-6

Gabriele, L., Phung, J., Fukumoto, J., Segal, D., Wang, I. M., Giannakakou, P., . . . Morse, H. C., 3rd. (1999). Regulation of apoptosis in myeloid cells by interferon consensus sequence-binding protein. *J Exp Med, 190*(3), 411-421.

Gackiere, F., Saliba, L., Baude, A., Bosler, O., & Strube, C. (2011). Ozone inhalation activates stress-responsive regions of the CNS. *J Neurochem, 117*(6), 961-972. doi: 10.1111/j.1471-4159.2011.07267.x

Ginhoux, F., Greter, M., Leboeuf, M., Nandi, S., See, P., Gokhan, S., . . . Merad, M. (2010). Fate mapping analysis reveals that adult microglia derive from primitive macrophages. *Science, 330*(6005), 841-845. doi: 10.1126/science.1194637

Gitik, M., Liraz-Zaltsman, S., Oldenborg, P. A., Reichert, F., & Rotshenker, S. (2011). Myelin down-regulates myelin phagocytosis by microglia and macrophages through interactions between CD47 on myelin and SIRPalpha (signal regulatory protein-alpha) on phagocytes. *J Neuroinflammation, 8*, 24. doi: 10.1186/1742-2094-8-24

Graeber, M. B. (2010). Changing face of microglia. *Science, 330*(6005), 783-788. doi: 10.1126/science.1190929

Griciuc, A., Serrano-Pozo, A., Parrado, A. R., Lesinski, A. N., Asselin, C. N., Mullin, K., . . . Tanzi, R. E. (2013). Alzheimer's disease risk gene CD33 inhibits microglial uptake of amyloid beta. *Neuron, 78*(4), 631-643. doi: 10.1016/j.neuron.2013.04.014

Grommes, C., Lee, C. Y., Wilkinson, B. L., Jiang, Q., Koenigsknecht-Talboo, J. L., Varnum, B., & Landreth, G. E. (2008). Regulation of microglial phagocytosis and inflammatory gene expression by Gas6 acting on the Axl/Mer family of tyrosine kinases. *J Neuroimmune Pharmacol, 3*(2), 130-140. doi: 10.1007/s11481-007-9090-2

Hart, A. D., Wyttenbach, A., Perry, V. H., & Teeling, J. L. (2012). Age related changes in microglial phenotype vary between CNS regions: grey versus white matter differences. *Brain Behav Immun, 26*(5), 754-765. doi: 10.1016/j.bbi.2011.11.006

Henry, C. J., Huang, Y., Wynne, A. M., & Godbout, J. P. (2009). Peripheral lipopolysaccharide (LPS) challenge promotes microglial hyperactivity in aged mice that is associated with exaggerated induction of both pro-inflammatory IL-1beta and anti-inflammatory IL-10 cytokines. *Brain Behav Immun, 23*(3), 309-317. doi: 10.1016/j.bbi.2008.09.002

Hoffmann, O., Braun, J. S., Becker, D., Halle, A., Freyer, D., Dagand, E., . . . Weber, J. R. (2007). TLR2 mediates neuroinflammation and neuronal damage. *J Immunol, 178*(10), 6476-6481.

Hong, S., Beja-Glasser, V. F., Nfonoyim, B. M., Frouin, A., Li, S., Ramakrishnan, S., . . . Stevens, B. (2016). Complement and microglia mediate early synapse loss in Alzheimer mouse models. *Science, 352*(6286), 712-716. doi: 10.1126/science.aad8373

Hsieh, C. L., Koike, M., Spusta, S. C., Niemi, E. C., Yenari, M., Nakamura, M. C., & Seaman, W. E. (2009). A role for TREM2 ligands in the phagocytosis of apoptotic neuronal cells by microglia. *J Neurochem, 109*(4), 1144-1156. doi: 10.1111/j.1471-4159.2009.06042.x

Hsieh, J., Aimone, J. B., Kaspar, B. K., Kuwabara, T., Nakashima, K., & Gage, F. H. (2004). IGF-I instructs multipotent adult neural progenitor cells to become oligodendrocytes. *J Cell Biol, 164*(1), 111-122. doi: 10.1083/jcb.200308101

Huang, G., Zhang, P., Hirai, H., Elf, S., Yan, X., Chen, Z., . . . Tenen, D. G. (2008). PU.1 is a major downstream target of AML1 (RUNX1) in adult mouse hematopoiesis. *Nat Genet, 40*(1), 51-60. doi: 10.1038/ng.2007.7

Hughes, V. (2012). Microglia: The constant gardeners. *Nature, 485*(7400), 570-572. doi: 10.1038/485570a

Imai, Y., Ibata, I., Ito, D., Ohsawa, K., & Kohsaka, S. (1996). A novel gene iba1 in the major histocompatibility complex class III region encoding an EF hand protein expressed in a monocytic lineage. *Biochem Biophys Res Commun, 224*(3), 855-862. doi: 10.1006/bbrc.1996.1112

Janzer, R. C., & Raff, M. C. (1987). Astrocytes induce blood-brain barrier properties in endothelial cells. *Nature, 325*(6101), 253-257. doi: 10.1038/325253a0

Ji, K., Akgul, G., Wollmuth, L. P., & Tsirka, S. E. (2013). Microglia actively regulate the number of functional synapses. *PLoS One, 8*(2), e56293. doi: 10.1371/journal.pone.0056293

Ji, R., Meng, L., Li, Q., & Lu, Q. (2015). TAM receptor deficiency affects adult hippocampal neurogenesis. *Metab Brain Dis, 30*(3), 633-644. doi: 10.1007/s11011-014-9636-y

Ji, R., Tian, S., Lu, H. J., Lu, Q., Zheng, Y., Wang, X., . . . Lu, Q. (2013). TAM receptors affect adult brain neurogenesis by negative regulation of microglial cell activation. *J Immunol, 191*(12), 6165-6177. doi: 10.4049/jimmunol.1302229

Jonsson, T., Stefansson, H., Steinberg, S., Jonsdottir, I., Jonsson, P. V., Snaedal, J., . . . Stefansson, K. (2013). Variant of TREM2 associated with the risk of Alzheimer's disease. *N Engl J Med, 368*(2), 107-116. doi: 10.1056/NEJMoa1211103

Kelemen, S. E., & Autieri, M. V. (2005). Expression of allograft inflammatory factor-1 in T lymphocytes: a role in T-lymphocyte activation and proliferative arteriopathies. *Am J Pathol, 167*(2), 619-626. doi: 10.1016/S0002-9440(10)63003-9

Kierdorf, K., Erny, D., Goldmann, T., Sander, V., Schulz, C., Perdiguero, E. G., . . . Prinz, M. (2013). Microglia emerge from erythromyeloid precursors via Pu.1- and Irf8-dependent pathways. *Nat Neurosci, 16*(3), 273-280. doi: 10.1038/nn.3318

Kleinberger, G., Yamanishi, Y., Suarez-Calvet, M., Czirr, E., Lohmann, E., Cuyvers, E., . . . Haass, C. (2014). TREM2 mutations implicated in neurodegeneration impair cell surface transport and phagocytosis. *Sci Transl Med, 6*(243), 243ra286. doi: 10.1126/scitranslmed.3009093

Kodavanti, P. R., Royland, J. E., Richards, J. E., Besas, J., & Macphail, R. C. (2011). Toluene effects on oxidative stress in brain regions of young-adult, middle-age, and senescent Brown Norway rats. *Toxicol Appl Pharmacol, 256*(3), 386-398. doi: 10.1016/j.taap.2011.04.012

Kodavanti, U. P., Schladweiler, M. C., Ledbetter, A. D., Watkinson, W. P., Campen, M. J., Winsett, D. W., . . . Costa, D. L. (2000). The spontaneously hypertensive rat as a model of human cardiovascular disease: evidence of exacerbated cardiopulmonary injury and oxidative stress from inhaled emission particulate matter. *Toxicol Appl Pharmacol, 164*(3), 250-263. doi: 10.1006/taap.2000.8899

Kopatz, J., Beutner, C., Welle, K., Bodea, L. G., Reinhardt, J., Claude, J., . . . Neumann, H. (2013). Siglec-h on activated microglia for recognition and engulfment of glioma cells. *Glia, 61*(7), 1122-1133. doi: 10.1002/glia.22501

Lai, C., & Lemke, G. (1991). An extended family of protein-tyrosine kinase genes differentially expressed in the vertebrate nervous system. *Neuron, 6*(5), 691-704.

Lauber, K., Blumenthal, S. G., Waibel, M., & Wesselborg, S. (2004). Clearance of apoptotic cells: getting rid of the corpses. *Mol Cell, 14*(3), 277-287.

Lawson, L. J., Perry, V. H., & Gordon, S. (1992). Turnover of resident microglia in the normal adult mouse brain. *Neuroscience, 48*(2), 405-415.

Lehnardt, S. (2010). Innate immunity and neuroinflammation in the CNS: the role of microglia in Toll-like receptor-mediated neuronal injury. *Glia, 58*(3), 253-263. doi: 10.1002/glia.20928

Levesque, S., Taetzsch, T., Lull, M. E., Kodavanti, U., Stadler, K., Wagner, A., . . . Block, M. L. (2011). Diesel exhaust activates and primes microglia: air pollution, neuroinflammation, and regulation of dopaminergic neurotoxicity. *Environ Health Perspect, 119*(8), 1149-1155. doi: 10.1289/ehp.1002986

Li, E., Noda, M., Doi, Y., Parajuli, B., Kawanokuchi, J., Sonobe, Y., . . . Suzumura, A. (2012). The neuroprotective effects of milk fat globule-EGF factor 8 against oligomeric amyloid beta toxicity. *J Neuroinflammation, 9*, 148. doi: 10.1186/1742-2094-9-148

Linnartz, B., Wang, Y., & Neumann, H. (2010). Microglial immunoreceptor tyrosine-based activation and inhibition motif signaling in neuroinflammation. *Int J Alzheimers Dis, 2010*. doi: 10.4061/2010/587463

Lutsik, B. D., Stekhnovich, I. V., & Lutsik, A. D. (1991). [Use of lectins in histochemical examination of microglia of the human brain in bacterial meningoencephalitis]. *Zh Nevropatol Psikhiatr Im S S Korsakova, 91*(2), 41-44.

McKercher, S. R., Torbett, B. E., Anderson, K. L., Henkel, G. W., Vestal, D. J., Baribault, H., . . . Maki, R. A. (1996). Targeted disruption of the PU.1 gene results in multiple hematopoietic abnormalities. *EMBO J, 15*(20), 5647-5658.

Melchior, B., Garcia, A. E., Hsiung, B. K., Lo, K. M., Doose, J. M., Thrash, J. C., . . . 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. doi: 10.1042/AN20100010

Minten, C., Terry, R., Deffrasnes, C., King, N. J., & Campbell, I. L. (2012). IFN regulatory factor 8 is a key constitutive determinant of the morphological and molecular properties of microglia in the CNS. *PLoS One, 7*(11), e49851. doi: 10.1371/journal.pone.0049851

Mossadegh-Keller, N., Sarrazin, S., Kandalla, P. K., Espinosa, L., Stanley, E. R., Nutt, S. L., . . . Sieweke, M. H. (2013). M-CSF instructs myeloid lineage fate in single haematopoietic stem cells. *Nature, 497*(7448), 239-243. doi: 10.1038/nature12026

Muffat, J., Li, Y., Yuan, B., Mitalipova, M., Omer, A., Corcoran, S., . . . Jaenisch, R. (2016). Efficient derivation of microglia-like cells from human pluripotent stem cells. *Nat Med, 22*(11), 1358-1367. doi: 10.1038/nm.4189

Mulholland, George W., et al. "Measurement of 100 nm and 60 nm particle standards by

differential mobility analysis." *Journal of research of the National Institute of Standards and Technology* 111.4 (2006): 257.

Nimmerjahn, A., Kirchhoff, F., & Helmchen, F. (2005). Resting microglial cells are highly dynamic surveillants of brain parenchyma in vivo. *Science, 308*(5726), 1314-1318. doi: 10.1126/science.1110647

Ojo, J. O., Rezaie, P., Gabbott, P. L., & Stewart, M. G. (2015). Impact of age-related neuroglial cell responses on hippocampal deterioration. *Front Aging Neurosci, 7*, 57. doi: 10.3389/fnagi.2015.00057

Olson, J. K., & Miller, S. D. (2004). Microglia initiate central nervous system innate and adaptive immune responses through multiple TLRs. *J Immunol, 173*(6), 3916-3924.

Olson, M. C., Scott, E. W., Hack, A. A., Su, G. H., Tenen, D. G., Singh, H., & Simon, M. C. (1995). PU. 1 is not essential for early myeloid gene expression but is required for terminal myeloid differentiation. *Immunity, 3*(6), 703-714.

Otero, K., Shinohara, M., Zhao, H., Cella, M., Gilfillan, S., Colucci, A., . . . Colonna, M. (2012). TREM2 and beta-catenin regulate bone homeostasis by controlling the rate of osteoclastogenesis. *J Immunol, 188*(6), 2612-2621. doi: 10.4049/jimmunol.1102836

Pandya, H., Shen, M. J., Ichikawa, D. M., Sedlock, A. B., Choi, Y., Johnson, K. R., . . . Park, J. K. (2017). Differentiation of human and murine induced pluripotent stem cells to microglia-like cells. *Nat Neurosci, 20*(5), 753-759. doi: 10.1038/nn.4534

Paolicelli, R. C., Bolasco, G., Pagani, F., Maggi, L., Scianni, M., Panzanelli, P., . . . Gross, C. T. (2011). Synaptic pruning by microglia is necessary for normal brain development. *Science, 333*(6048), 1456-1458. doi: 10.1126/science.1202529

Perry, V. H., Matyszak, M. K., & Fearn, S. (1993). Altered antigen expression of microglia in the aged rodent CNS. *Glia, 7*(1), 60-67. doi: 10.1002/glia.440070111

Pham, T. H., Minderjahn, J., Schmidl, C., Hoffmeister, H., Schmidhofer, S., Chen, W., . . . Rehli, M. (2013). Mechanisms of in vivo binding site selection of the hematopoietic master transcription factor PU.1. *Nucleic Acids Res, 41*(13), 6391-6402. doi: 10.1093/nar/gkt355

Puntambekar, S. S., Davis, D. S., Hawel, L., 3rd, 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 Behav Immun, 25*(4), 629-639. doi: 10.1016/j.bbi.2010.12.016

Ransohoff, R. M., Kivisakk, P., & Kidd, G. (2003). Three or more routes for leukocyte migration into the central nervous system. *Nat Rev Immunol, 3*(7), 569-581. doi: 10.1038/nri1130

Rogers, J., Luber-Narod, J., Styren, S. D., & Civin, W. H. (1988). Expression of immune system-associated antigens by cells of the human central nervous system: relationship to the pathology of Alzheimer's disease. *Neurobiol Aging, 9*(4), 339-349.

Roumier, A., Bechade, C., Poncer, J. C., Smalla, K. H., Tomasello, E., Vivier, E., . . . Bessis, A. (2004). Impaired synaptic function in the microglial KARAP/DAP12-deficient mouse. *J Neurosci, 24*(50), 11421-11428. doi: 10.1523/JNEUROSCI.2251-04.2004

Russell, D. G., & Yates, R. M. (2007). TLR signalling and phagosome maturation: an alternative viewpoint. *Cell Microbiol, 9*(4), 849-850. doi: 10.1111/j.1462-5822.2007.00920.x

Satoh, J., Kawana, N., Yamamoto, Y., Ishida, T., Saito, Y., & Arima, K. (2013). A survey of TREM2 antibodies reveals neuronal but not microglial staining in formalin-fixed paraffin-embedded postmortem Alzheimer's brain tissues. *Alzheimers Res Ther, 5*(4), 30. doi: 10.1186/alzrt184

Savage, J. C., Jay, T., Goduni, E., Quigley, C., Mariani, M. M., Malm, T., . . . Landreth, G. E. (2015). Nuclear receptors license phagocytosis by trem2+ myeloid cells in mouse models of Alzheimer's disease. *J Neurosci, 35*(16), 6532-6543. doi: 10.1523/JNEUROSCI.4586-14.2015

Sawada, M., Sawada, H., & Nagatsu, T. (2008). Effects of aging on neuroprotective and neurotoxic properties of microglia in neurodegenerative diseases. *Neurodegener Dis, 5*(3-4), 254-256. doi: 10.1159/000113717

Schafer, D. P., Lehrman, E. K., Kautzman, A. G., Koyama, R., Mardinly, A. R., Yamasaki, R., . . . Stevens, B. (2012). Microglia sculpt postnatal neural circuits in an activity and complement-dependent manner. *Neuron, 74*(4), 691-705. doi: 10.1016/j.neuron.2012.03.026

Scheffel, J., Regen, T., Van Rossum, D., Seifert, S., Ribes, S., Nau, R., . . . Hanisch, U. K. (2012). Toll-like receptor activation reveals developmental reorganization and unmasks responder subsets of microglia. *Glia, 60*(12), 1930-1943. doi: 10.1002/glia.22409

Schilling, M., Besselmann, M., Muller, M., Strecker, J. K., Ringelstein, E. B., & Kiefer, R. (2005). Predominant phagocytic activity of resident microglia over hematogenous macrophages following transient focal cerebral ischemia: an investigation using green fluorescent protein transgenic bone marrow chimeric mice. *Exp Neurol, 196*(2), 290-297. doi: 10.1016/j.expneurol.2005.08.004

Schmid, C. D., Melchior, B., Masek, K., Puntambekar, S. S., Danielson, P. E., Lo, D. D., . . . Carson, M. J. (2009). Differential gene expression in LPS/IFNgamma activated microglia and macrophages: in vitro versus in vivo. *J Neurochem, 109 Suppl 1*, 117-125. doi: 10.1111/j.1471-4159.2009.05984.x

Scott, R. S., McMahon, E. J., Pop, S. M., Reap, E. A., Caricchio, R., Cohen, P. L., . . . Matsushima, G. K. (2001). Phagocytosis and clearance of apoptotic cells is mediated by MER. *Nature, 411*(6834), 207-211. doi: 10.1038/35075603

Sharif, O., Gawish, R., Warszawska, J. M., Martins, R., Lakovits, K., Hladik, A., . . . Knapp, S. (2014). The triggering receptor expressed on myeloid cells 2 inhibits complement component 1q effector mechanisms and exerts detrimental effects during pneumococcal pneumonia. *PLoS Pathog, 10*(6), e1004167. doi: 10.1371/journal.ppat.1004167

Shemer, A., Erny, D., Jung, S., & Prinz, M. (2015). Microglia Plasticity During Health and Disease: An Immunological Perspective. *Trends Immunol, 36*(10), 614-624. doi: 10.1016/j.it.2015.08.003

Sheng, J. G., Griffin, W. S., Royston, M. C., & Mrak, R. E. (1998). Distribution of interleukin-1-immunoreactive microglia in cerebral cortical layers: implications for neuritic plaque formation in Alzheimer's disease. *Neuropathol Appl Neurobiol, 24*(4), 278-283.

Smith, A. M., Gibbons, H. M., Oldfield, R. L., Bergin, P. M., Mee, E. W., Faull, R. L., & Dragunow, M. (2013). The transcription factor PU.1 is critical for viability and function of human brain microglia. *Glia, 61*(6), 929-942. doi: 10.1002/glia.22486

Soulage, C., Perrin, D., Cottet-Emard, J. M., Pequignot, J., Dalmaz, Y., & Pequignot, J. M. (2004). Central and peripheral changes in catecholamine biosynthesis and turnover in rats after a short period of ozone exposure. *Neurochem Int, 45*(7), 979-986. doi: 10.1016/j.neuint.2004.06.015

Spanaus, K. S., Nadal, D., Pfister, H. W., Seebach, J., Widmer, U., Frei, K., . . . Fontana, A. (1997). C-X-C and C-C chemokines are expressed in the cerebrospinal fluid in bacterial meningitis and mediate chemotactic activity on peripheral blood-derived polymorphonuclear and mononuclear cells in vitro. *J Immunol, 158*(4), 1956-1964.

Spindler, K. R., & Hsu, T. H. (2012). Viral disruption of the blood-brain barrier. *Trends Microbiol, 20*(6), 282-290. doi: 10.1016/j.tim.2012.03.009

Stevens, B., Allen, N. J., Vazquez, L. E., Howell, G. R., Christopherson, K. S., Nouri, N., . . . Barres, B. A. (2007). The classical complement cascade mediates CNS synapse elimination. *Cell, 131*(6), 1164-1178. doi: 10.1016/j.cell.2007.10.036

Stitt, T. N., Conn, G., Gore, M., Lai, C., Bruno, J., Radziejewski, C., . . . et al. (1995). The anticoagulation factor protein S and its relative, Gas6, are ligands for the Tyro 3/Axl family of receptor tyrosine kinases. *Cell, 80*(4), 661-670.

Streit, W. J., & Xue, Q. S. (2014). Human CNS immune senescence and neurodegeneration. *Curr Opin Immunol, 29*, 93-96. doi: 10.1016/j.coi.2014.05.005

Strowig, T., Henao-Mejia, J., Elinav, E., & Flavell, R. (2012). Inflammasomes in health and disease. *Nature, 481*(7381), 278-286. doi: 10.1038/nature10759

Sumi, N., Nishioku, T., Takata, F., Matsumoto, J., Watanabe, T., Shuto, H., . . . Kataoka, Y. (2010). Lipopolysaccharide-activated microglia induce dysfunction of the blood-brain barrier in rat microvascular endothelial cells co-cultured with microglia. *Cell Mol Neurobiol, 30*(2), 247-253. doi: 10.1007/s10571-009-9446-7

Takahashi, K., Rochford, C. D., & Neumann, H. (2005). Clearance of apoptotic neurons without inflammation by microglial triggering receptor expressed on myeloid cells-2. *J Exp Med, 201*(4), 647-657. doi: 10.1084/jem.20041611

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. *Neurochem Res, 34*(1), 38-45. doi: 10.1007/s11064-008-9657-1

Tremblay, M. E., Lowery, R. L., & Majewska, A. K. (2010). Microglial interactions with synapses are modulated by visual experience. *PLoS Biol, 8*(11), e1000527. doi: 10.1371/journal.pbio.1000527

Turnbull, I. R., Gilfillan, S., Cella, M., Aoshi, T., Miller, M., Piccio, L., . . . Colonna, M. (2006). Cutting edge: TREM-2 attenuates macrophage activation. *J Immunol, 177*(6), 3520-3524.

Wake, H., Moorhouse, A. J., Jinno, S., Kohsaka, S., & Nabekura, J. (2009). Resting microglia directly monitor the functional state of synapses in vivo and determine the fate of ischemic terminals. *J Neurosci, 29*(13), 3974-3980. doi: 10.1523/JNEUROSCI.4363-08.2009

Wakselman, S., Bechade, C., Roumier, A., Bernard, D., Triller, A., & Bessis, A. (2008). Developmental neuronal death in hippocampus requires the microglial CD11b integrin and DAP12 immunoreceptor. *J Neurosci, 28*(32), 8138-8143. doi: 10.1523/JNEUROSCI.1006-08.2008

Walton, M. R., Gibbons, H., MacGibbon, G. A., Sirimanne, E., Saura, J., Gluckman, P. D., & Dragunow, M. (2000). PU.1 expression in microglia. *J Neuroimmunol, 104*(2), 109-115.

Wang SC, and Flagan RC (1990) Scanning electrical mobility spectrometer. *Aerosol Science and Technology* 13: 230-240.

Wang, Y., Ulland, T. K., Ulrich, J. D., Song, W., Tzaferis, J. A., Hole, J. T., . . . Colonna, M. (2016). TREM2-mediated early microglial response limits diffusion and toxicity of amyloid plaques. *J Exp Med, 213*(5), 667-675. doi: 10.1084/jem.20151948

Wirenfeldt, M., Dissing-Olesen, L., Anne Babcock, A., Nielsen, M., Meldgaard, M., Zimmer, J., . . . Finsen, B. (2007). Population control of resident and immigrant microglia by mitosis and apoptosis. *Am J Pathol, 171*(2), 617-631. doi: 10.2353/ajpath.2007.061044

Wu, Y., Singh, S., Georgescu, M. M., & Birge, R. B. (2005). A role for Mer tyrosine kinase in alphavbeta5 integrin-mediated phagocytosis of apoptotic cells. *J Cell Sci, 118*(Pt 3), 539-553. doi: 10.1242/jcs.01632

Wynne, A. M., Henry, C. J., & Godbout, J. P. (2009). Immune and behavioral consequences of microglial reactivity in the aged brain. *Integr Comp Biol, 49*(3), 254-266. doi: 10.1093/icb/icp009

Xiang, X., Werner, G., Bohrmann, B., Liesz, A., Mazaheri, F., Capell, A., . . . Haass, C. (2016). TREM2 deficiency reduces the efficacy of immunotherapeutic amyloid clearance. *EMBO Mol Med, 8*(9), 992-1004. doi: 10.15252/emmm.201606370

Yamagata, T., Muroya, K., Mukasa, T., Igarashi, H., Momoi, M., Tsukahara, T., . . . Momoi, T. (1995). Hepatocyte growth factor specifically expressed in microglia activated Ras in the neurons, similar to the action of neurotrophic

factors. *Biochem Biophys Res Commun, 210*(1), 231-237. doi: 10.1006/bbrc.1995.1651

Zhang, Y., Venkitaramani, D. V., Gladding, C. M., Zhang, Y., Kurup, P., Molnar, E., . . . Lombroso, P. J. (2008). The tyrosine phosphatase STEP mediates AMPA receptor endocytosis after metabotropic glutamate receptor stimulation. *J Neurosci, 28*(42), 10561-10566. doi: 10.1523/JNEUROSCI.2666-08.2008

Zietlow, R., Dunnett, S. B., & Fawcett, J. W. (1999). The effect of microglia on embryonic dopaminergic neuronal survival in vitro: diffusible signals from neurons and glia change microglia from neurotoxic to neuroprotective. *Eur J Neurosci, 11*(5), 1657-1667.

Zipser, B. D., Johanson, C. E., Gonzalez, L., Berzin, T. M., Tavares, R., Hulette, C. M., . . . Stopa, E. G. (2007). Microvascular injury and blood-brain barrier leakage in Alzheimer's disease. *Neurobiol Aging, 28*(7), 977-986. doi: 10.1016/j.neurobiolaging.2006.05.016