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Characterization of Pro-inflammatory and Anti-inflammatory Microglia in the Anterior Cingulate Cortex in Autism Spectrum Disorder

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by

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ABSTRACT

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Autism spectrum disorder (ASD) is associated with functional abnormalities of the anterior cingulate cortex (ACC), a brain area that mediates social behavior. Given evidence of a role of inflammation in ASD, markers of pro-inflammatory and anti-inflammatory microglia were studied using postmortem ACC tissues from ASD and age-matched typically developed control donors. Gene expression levels of pro-inflammatory (*CD68, HLA-DRA, IL1B, NOS2, PTGS2*) and anti-inflammatory (*ARG1, IGF1, MRC1, PPARG*) microglial genes were measured using quantitative real-time PCR. Additionally, brain sections were immunohistochemically stained for a microglial marker. Expression levels of *IGF1* were modestly higher, while the expression of *MRC1* was modestly lower in ASD donors when compared to control donors. No other differences in gene expression levels between the two groups of donors were observed. Statistical significance for changes in expression levels *IGF1* and *MRC1* did not survive correction for multiple comparisons. Further research on anti-inflammatory microglial involvement in ASD is warranted.

DEDICATION

I would like to dedicate this thesis to my family: Steve, Nancy, Tanner, and Giuseppina, who have supported my lifelong goals unconditionally. I love you all more than you can imagine.

Dad, thank you for believing in me when I felt like no one else did. You have prepared me for the real world, taught me how to overcome obstacles on my own, and have been an incredible father. Most importantly, thank you for teaching me that age is only a number and I can do anything I set my heart to.

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CHAPTER 1

INTRODUCTION

Autism Spectrum Disorder

Autism spectrum disorder (ASD) is a collection of disorders that encompasses neurodevelopmental disorders such as Autistic Disorder, Asperger Syndrome, and Pervasive Developmental Disorder-Not Otherwise Specified. ASD is associated with deficits in an individual's language and speech, social interaction, and/or motor function. Since the first epidemiological survey in 1966, the prevalence of autism has increased from 1 in 2,000 individuals to approximately 1 in 160 individuals globally (World Health Organization 2013). In the United States, it affects 1 in 68 children with a male to female ratio of 5:1 (Baio 2014). It is argued that improved diagnostic tools and increased awareness of ASD in the last 50 years have had an effect on the increase in ASD prevalence (Vargas et al. 2005; Wing et al. 2011).

The median age of ASD diagnosis is approximately 4.5 years, even though it is possible for a child to be diagnosed as early as the age of 2 (Baio 2014). There are a variety of tools used to diagnose ASD; however, access to these tools still remains a challenge to low income countries. The detection of ASD in the adult population proves to be more difficult than diagnoses of children because it requires alternative diagnostic tools. Early diagnosis of ASD allows for increased time to develop routines and assist in behavioral management of the individual (World Health Organization 2013).

With such a high occurrence rate, the cost of treating and assisting autistic individuals in the United States is astronomical. Diagnosis usually comes early in life and can bring life-long costs. Moreover, if the individual has intellectual disabilities, the cost of care increases because of the need for additional services. No current treatments or pathognomonic markers for ASD

are known to exist currently, vastly increasing the need to understand ASD neuropathology. A thorough understanding of the pathobiology of this disorder also has the potential to contribute to the development of advanced diagnostic tools that could lead to more precise diagnoses.

As a spectrum disorder, the behavioral phenotype of individuals diagnosed with ASD is variable and is often further differentiated. Autistic Disorder is defined by these specific deficits: repetitive motor behavior, impaired social interaction, decreased intellectual ability, and compromised verbal communication skills. Conversely, individuals with Asperger Syndrome have normal language skills and those with Pervasive Developmental Disorder-Not Otherwise Specified have some, but not all of the deficits required for an autism diagnosis (Bill and Geschwind, 2009). It is been proposed that Asperger Syndrome and Pervasive Developmental Disorder-Not Otherwise Specified are less severe forms of Autistic Disorder, even though a clear distinction between the subtypes is sometimes difficult to make, partially due to the lack of biological markers (Worley and Matson 2012). Current revisions to the diagnostic criteria, now DSM-V, have removed these subgroups and have categorized Autistic Disorder, Asperger Syndrome, and Pervasive Developmental Disorder-Not Otherwise Specified as ASD (reviewed by Kim, 2015).

Etiology

Throughout the past century, scientists have debated about the etiology of ASD from two distinct perspectives: (1) ASD is caused by a genetic mutation or abnormality or (2) that ASD is the product of exposure to particular environmental elements. The true pathophysiology of ASD remains unknown, keeping scientists searching for answers. Recent research has provided data supporting both viewpoints, leading the scientific community to conclude that the etiology of

ASD is likely the product of a combination of genetic abnormalities and exposure to some yet unknown environmental insult.

Studies performed on identical and fraternal twins reveal a syndromic link to ASD (Bill and Geschwind, 2009). More recently, research shows that gene mutations contribute to approximately 55% of ASD cases (Hallmayer et al. 2011). Currently, there are 25+ loci that are considered autism susceptibility candidate genes. Additionally, there are rare Mendelian mutations specific to ASD such as a *de novo* (not in parents) copy number variation (CNV) that are being intensely investigated (Bill and Geschwind, 2009). In contrast to single nucleotide polymorphisms (SNPs), a change in one nucleotide in the DNA, CNV is a type of change in the genome that results in the deletion or duplication of a genomic region that is greater than 1,000 nucleotides (Sebat et al. 2007; Geschwind 2008).

Approximately 35% of children diagnosed with ASD have a recognized genetic disorder or a distinguishable chromosomal duplication or deletion. Many of the recognized genetic syndromes associated with ASD originate from single gene mutations linked to the mammalian target of rapamycin (mTOR) pathway, such as Rett's syndrome, fragile X mental retardation 1 (*FMR1*), and tuberous sclerosis complex (*TSC1/2*) (McFadden and Minshew, 2013). The mTOR pathway functions as a regulator of cell development, proliferation, and survival. Many components of the pathway are located near neuronal synapses where they mediate synaptogenesis by regulating the morphology of dendritic spines and synaptic protein synthesis. Mutations in individuals with *FMR1*, *TSC1/2*, and other disorders such as neurofibromatosis 1 (*NFM1*) and phosphatase and tensin homolog (*PTEN*) have been found to lead to an overactive mTOR signaling pathway (reviewed by Sawicka and Zukin 2012). Specifically, in *FMR1*, the upregulated mTOR pathway results in an increase of cap-dependent protein translation. Mice

studies have shown that when there is an increase in the components of the cap-dependent translation initiation complex, the mice display enhanced repetitive behavior (Huber et al. 2015). Based off of the role the mTOR pathway plays in other ASD-like disorders, it has been suggested that persons with ASD may have an irregular mTOR pathway as well (reviewed by Sawicka and Zukin 2012). Although Rett's syndrome, *FMR1*, and *TSC1/2* are associated with ASD, the pathophysiology of each disorder stems from a different genetic abnormality (reviewed by Strathearn, 2009). Because genetic abnormalities do not account for 100% of the cause, it is speculated that in addition to specific gene mutations, environmental risk factors, increased ages of parents, and a general lack of parental nurture may be correlated with an increased risk for ASD (Durkin et al. 2008; Strathearn 2009; World Health Organization 2013).

Additionally, studies have suggested that maternal autoimmune disorders and various teratogens may play a role in the pathogenesis of ASD (Strömland et al. 1994; Atladóttir et al. 2009; Keil et al. 2010; reviewed by Dufour-Rainfray et al. 2011; Stevens et al. 2013; Chen et al. 2016). A Danish study of 3325 children who had been diagnosed with ASD showed an increased risk when the mother had a history of an autoimmune disorder such as celiac disease or rheumatoid arthritis. The researchers also concluded that there is a potential genetic link between maternal and/or paternal type 1 diabetes (T1D) based on the parental history of T1D and the number children with ASD (Atladóttir et al. 2009). Epidemiological studies have also reported a correlation between the exposure of a fetus to teratogens such as valproic acid (antiepileptic drug), thalidomide (sedative), and misoprostol (drug for gastric ulcers) and a diagnosis of ASD (reviewed by Dufour-Rainfray et al. 2011). Furthermore, ethanol consumption by pregnant women not only causes abnormalities in the child such as fetal alcohol syndrome, but researchers are hypothesizing it may have teratogenic effects on cellular regulation and may

cause abnormal gene expression via epigenetic modifications. Researchers are currently attempting to determine what effect exposure time has on these teratogenic defects (reviewed by Dufour-Rainfray et al. 2011). While these studies do not encompass all of the components of the pathophysiology of the disorder, they do show the complexity of the etiology of ASD.

Anterior Cingulate Cortex and ASD

The central nervous system is composed of: gray matter, where neuronal cell bodies and glia are located, and white matter, which houses primarily neuronal axons and glia. In the gray matter, information is processed from stimuli and signals are transmitted through short- and long-range neuronal axons. Gray matter in the cerebral cortex envelops the brain in sulci and gyri, while white matter is immediately adjacent to the inner layer of the cortex. Specialized cells in white matter, oligodendrocytes, provide insulation for neuronal axons that pass through the area, facilitating the transmission of signals along axons. Microglial cells and macroglial cells (including oligodendrocytes and astrocytes) are abundantly present in the white matter, while there are very few neuronal cell bodies found in white matter. Divided into two hemispheres, four lobes, the spinal cord, and complex brain areas, the central nervous system is able to rapidly communicate between areas of different functions via the transmission of signals through neurons, ultimately sending information to each region of the body.

The cingulate cortex is located in the medial portion of the cerebral hemispheres and is a part of the limbic system, making it a major component in emotional processes. The cingulate cortex is divided into two sections: the anterior cingulate cortex (ACC) and the posterior cingulate cortex (Vogt et al. 1992; Know your Brain 2015). It is primarily hypothesized that the cingulate cortex, as a whole, processes cognitive and emotional, sensory, and motor information

(reviewed by Bush, Luu, & Posner, 2000). Secondly, the cingulate cortex assimilates information from different networks including motivation, cognition, emotion, and evaluation of error. The anterior cingulate cortex, also referred to as Brodmann area 24 (BA24), has been recognized as a brain area that displays abnormalities in individuals with ASD. The ACC subserves many behavioral functions, demonstrated in studies using neuroimaging and electrical recordings, and influences activity in other various brain regions to regulate endocrine, motor, cognitive, and visceral responses (reviewed by Bush, Luu, & Posner, 2000). Differentiated into many distinct sub-regions, the ACC assists in tasks that range from basic to complex. During complex tasks, the ACC becomes activated in combination with the prefrontal cortex, suggesting that it is essential in higher-order thinking (Margulies et al. 2007).

A meta-analysis, including 21 research publications, reported that the ACC is a brain area that demonstrates abnormalities in individuals with ASD (DeRamus and Kana 2015). MRI comparisons revealed decreased ACC gray matter volume in ASD individuals when compared to typically developed controls (Greimel et al. 2013). Several studies have utilized functional magnetic resonance imaging (fMRI), useful for the inspection of neural structures, to demonstrate decreased synchronization of critical cortical regions during complex task performance when comparing individuals with ASD to typically developed control subjects. This functional decrease was specifically localized to connectivity between the frontal (anterior cingulate and other prefrontal cortices) and more posterior cortical regions (reviewed by McFadden and Minshew, 2013). Additionally, fMRI studies have demonstrated hypoactivation of the ACC during visual stimulation, attentional and cognitive processing, and during social response tasks in ASD subjects (reviewed by Gomot et al. 2006; Silk et al. 2006; Kohls et al. 2013; Urbain et al. 2015). A separate meta-analysis of 24 studies examining social processes

concluded that there was hypoactivation in the perigenual ACC of ASD subjects solely during social tasks (Di Martino et al. 2009). While the meta analyses and fMRI studies agree, inconsistences in methods, analyses, and subject criteria (i.e. only subjects with Asperger's syndrome, inclusion of subjects on medications, or comorbidities), make it challenging to compare the findings of these studies (Dichter et al. 2009). It is likely that the subjects of some ASD studies are mostly individuals with moderate- to highly-functional ASD, potentially excluding the individuals with severe ASD who lack verbal skills, whose disruptive behaviors prevent participation, and those who are unable to interact with researchers. The exclusion of these individuals would depend on the type of analysis performed. From this, it is simple to see how difficult it can be to obtain reliable results from ASD studies.

Mainly, imaging methods demonstrating disruption of the function of the anterior cingulate cortex in ASD are interpreted as gray matter deficits; however, white matter deficits have also been implicated in ASD. White matter pathology is typically examined *in vivo* by diffusion tensor imaging, a technique that measures the amount of diffusion throughout the brain matter. Under normal conditions in the brain, white matter is less permissive of the diffusion of water in comparison to gray matter. In ASD subjects, increased diffusion of water through the white matter has been detected in comparison to control subjects, leading researchers to hypothesize that ASD brains have surplus interstitial space in white matter (Groen et al. 2011). Several studies have indicated an increase in white matter volume and a decrease in the structural integrity of white matter in individuals with ASD, abnormalities that may contribute to motor impairment and disruption in cognitive processing in ASD (Noriuchi et al. 2010; Groen et al. 2011; Ingalhalikar et al. 2011). White matter pathology, as indicated in these imaging studies,

implies that cells found in the white matter are abnormal and/or there are structural changes in neuronal axons coursing through white matter.

Thus far, most of the published research on the cellular pathology of ASD has focused on neurons. Previously, we investigated the possible role of macroglia in white matter ASD pathology and abnormal gene expression changes of macroglial markers *MOG* and *GFAP* (Crawford et al. 2015). Other researchers have hypothesized that microglia play a role in the pathophysiology of ASD (reviewed by Takano 2015). Microglia are of particular interest because they are the immune cell of the CNS and play a role in synaptic maturation during development. It has been reported that children with ASD suffer from chronic neuroinflammation, which could potentially lead to neuronal cell death and a loss of synaptic connections from upregulated inflammatory mediators (reviewed by Pardo et al. 2005; Zimmerman et al. 2005; reviewed by Rodriguez and Kern 2011). In addition to a possible link between inflammation in the CNS and neural network deficits, Vargas and coworkers found that microglia are consistently activated, suggesting microglia are continually working to protect the CNS from injury or threat of damage, in all brain regions in ASD subjects (Vargas et al. 2005; reviewed by Edmonson et al. 2016). The microglial responses in ASD individuals were comparable to responses seen in other neurodegenerative disorders, such as Parkinson's disease and Alzheimer's disease (Vargas et al. 2005). Suzuki and coworkers found evidence of microglia involvement in ASD, reporting an increase in a microglial binding ligand in multiple brain regions including the ACC in young adults with ASD (Suzuki et al. 2013). These early findings support the investigation of the alleged role of microglia in the ACC in ASD pathology.

Microglia

In addition to the evidence supporting microglia's possible role in white matter pathology in ASD, microglia have been linked to autism through various other mechanisms. Located throughout the brain and spinal cord, microglial cells are responsible for immune system and central nervous system (CNS) protection against pathogenic factors (reviewed by Saijo & Glass, 2011). Microglia, the macrophages of the CNS, originate in the yolk-sac, arising from erythromyeloid progenitors (EMPs), and migrate to the brain during early fetal development (reviewed by Casano & Peri, 2015; Saijo & Glass, 2011). In contrast, neurons and macroglia originate from the neuroectoderm (Eglitis and Mezey 1997; Glees 2005).

Forms

Ramified. Structured similarly to astrocytes, each microglial cell has multiple branching processes that extend in all directions. In the ramified state, also termed the 'resting' or 'inactivated' form, these microglial branches continuously survey their surroundings in the CNS and have temporary contact with local synaptic structures (reviewed by Saijo & Glass, 2011; Tremblay, Lowery, & Majewska, 2010). Ramified microglia represent the quickest moving structures in the brain with an average velocity of 5.44 ± 2.33 μm/min. The rapid velocity of microglial processes allow for swift surveillance and immediate detection of injury and the initiation of an active response, eventually prompting complete microglial activation (Lee et al. 2008).

Activated. When injury or the threat of damage to the CNS is present, ramified microglia are able to rapidly modify their morphology, function, and gene expression to provide protection.

This highly regulated progression of alteration is defined as microglial activation. The once extended branching processes retract, giving the cell the appearance of an amoeba. With the microglia no longer focused on surveillance, they promptly migrate to the site of infection or injury via chemotactic gradients (reviewed by Kettenmann et al. 2011).

General Functions

During early CNS development and in adulthood, microglia function as the "maid," removing accumulating apoptotic neurons and reducing inflammation. Microglia are also involved in the creation of synapses in the postnatal brain, a process termed synaptogenesis. In the adult brain, microglia serve as the "guards," ready to protect, as well as the homeostatic regulators of the CNS (reviewed by Kettenmann et al. 2011). Abnormalities detected in nearby neuronal synapses initiate synaptic remodeling by microglia, which is necessary for maturation and homeostasis. During the removal of damaged synapses, microglia release cytokines, reactive oxygen species (ROS), and growth factors (Kettenmann et al. 2013). The phagocytic and synaptic pruning abilities of microglia are suggested to be imperative for normal brain development and neurogenesis (reviewed by Saijo & Glass, 2011). In transgenic mice models, when a microglia-expressed chemokine receptor gene was knocked out, microglia were found in reduced cell density when compared to wild-type mice. In addition to decreased microglial density, reduced synaptic pruning and circuit maturation was observed. These changes in the knockout mice suggest that alterations were due to decreased microglial activity (Paolicelli et al. 2011).

Apoptotic Signaling

A study by Sieger et al., using transgenic calcium reporter zebrafish, has helped researchers understand the mechanism by which microglia are precisely-guided to areas of apoptotic neurons. Targeted laser ablations of neurons were performed in the brains of the zebrafish and rapid Ca^{2+} gradient waves were established around the ablation site. Sieger et. al. determined that through a possible ATP gradient created by Ca^{2+} signaling, microglia were being guided to specific apoptotic neuronal sites (Sieger et al. 2012). Additionally, reversible exposure of phosphatidylserine on the surface of neurons is stimulated by an increase in Ca^{2+} or the release of ROS by lipopolysaccharides (LPS) from activated microglia (Neher et al. 2011; Brown and Neher 2012). Chemotactic signals released by apoptotic neurons and exposed phosphatidylserine residues on the neuronal surface are indicative of phagocytic readiness for microglia (reviewed by Casano & Peri, 2015).

Microglia mainly phagocytize apoptotic neurons in order to promote a reduction of inflammation and synaptic remodeling. Conversely, cells that are not undergoing apoptosis can also be phagocytized by microglia. Phosphatidylserine, normally found on the inside of healthy cells, can also be exposed on the surface of viable neurons if they become stressed. The exposure of phosphatidylserine is recognized by microglia and can result in the phagocytosis of the viable neuron. This response by microglia is considered primary phagocytosis, while secondary phagocytosis would follow apoptosis or necrosis, and has been coined ‘phagoptosis’ (Brown and Neher 2012). The potential effects of phagoptosis in a chronically inflamed brain area suggest that the abnormal phagocytosis of viable neurons by microglia may contribute to neurodegeneration and subsequent disease.

Activation Pathways

When the CNS undergoes a damaging event, microglia are immediate responders to the site of damage. Comparable to a macrophage response outside of the CNS, microglia may be activated through two distinct pathways, which are just beginning to be characterized in the literature (Shechter et al. 2013). Microglia can demonstrate a pro-inflammatory (M1) phenotype, otherwise known as the "classically activated" phenotype. Activated by interferon gamma (IFNϒ) and toll-like receptors, pro-inflammatory microglial cells produce cytokines and assume phagocytic roles that promote defense mechanisms and digest neurons. Conversely, the anti-inflammatory (M2) microglial phenotype is also referred to as "alternatively activated" microglia. M2 microglia are mainly activated in the presence of IL-4 and facilitate CNS healing by participating in phagocytosis, neuronal remodeling, and tissue regeneration (**Table 1**) (Gordon 2003; Arnold et al. 2007; Kigerl et al. 2009).

A signaling pathway directs information from the cellular surface of the inactivated microglial cell to its nucleus, where specific genes are activated that determine the resulting activated phenotype of the cell. The main difference in phenotypes that can occur is dependent on the isoform of galectin that interacts with the microglial cell. For differentiation to the M1 phenotype, galectin-3 is released by activated microglia, binds, and activates the microglial toll-like receptor 4 (TLR4). This activation creates a chain of further pro-inflammatory microglial activation (Burguillos et al. 2015). Conversely, the binding of galectin-1 to CD45 on the microglial surface inhibits the production of pro-inflammatory mediators, upregulating the anti-inflammatory phenotype through modulation of the CREB, NF-κB, and p38-MAPK pathways (Starossom et al. 2012). Additionally, the transcription of distinct pro-inflammatory or anti-inflammatory markers have been found to be regulated by second messengers, such as cAMP

and Ca^{2+}, and miRNAs, that can promote one phenotype by downregulating the expression of the other (Schebesch et al. 1997; Martinez-Nunez et al. 2011).

With the main goal of pro-inflammatory microglia being to protect the CNS against invading pathogens, the upregulation of M1 receptors and cytokines assist with the defense mechanisms. Communication with pro-inflammatory microglia and other immune cells is possible through the presentation of the human leukocyte antigen-antigen D related (HLA-DR), FcϒR, and CD86 (Taylor et al. 2005). Additionally, the production of IL-12, ROS, and inducible nitric oxide synthase (iNOS) assist with the M1 phenotype classification (Mantovani et al. 2004; Kigerl et al. 2009).

Contrarily, the main purpose of the anti-inflammatory microglial mediators and receptors is to promote repair, downregulate inflammation, and to encourage healthy CNS functions. Some of the best characterized anti-inflammatory markers include the mannose receptor CD206, a heparin-binding lectin Ym1, FIZZ1, and the enzyme arginase 1 (ARG 1), which has the ability to decrease the production of iNOS (Stein et al. 1992; Corraliza et al. 1995; Hung et al. 2002; Raes et al. 2002).

The anti-inflammatory phenotype is diverse; it can be broken down into subtypes. The M2a subtype mainly focuses on suppressing inflammation and is induced by IL-4 and IL-13, leading to the upregulation of ARG 1 (Stein et al. 1992). The exposure to IL-10, TGF-β, or glucocorticoids allows for the M2c subtype, which appears to be involved in healing damaged tissues after the pro-inflammatory phenotype is downregulated (**Table 1**) (Mantovani et al. 2004). The least understood subtype is the M2b classification, which slightly resembles the pro-inflammatory phenotype by lacking the anti-inflammatory markers previously discussed (Mosser and Edwards 2008). The M2b microglia have a response most similar to the anti-inflammatory

microglia in general, thus are listed as an M2 subtype. The lack of full understanding the M2 subtypes should yield caution to researchers attempting to study these subtypes separately, and encourages viewing the M2 subtype as a spectrum within the anti-inflammatory phenotype (Hanisch 2013).

Table 1. Characteristics of microglial phenotypes and subtypes

Phenotype/Subtype	Polarization	Cytokines Released	Markers	Functions
M1	IFNγ and LPS-TLR4 signaling	IL-1, IL-6, IL-12, IL-15, IL-23, and TNFα	CD16, CD32, CD86, MHCII, and iNOS	Phagocytosis for defense against pathogens, release of NO, and neuron digestion
M2a	IL-4 or IL-13	IGF 1, IL-10, TGFβ, PDGF, IL-1Ra, and fibronectin 1	CD163, CD204, CD206, ARG 1, Fizz1, and YM1	Inflammation suppression, tissue repair, and immune response against parasites
M2b	FcγR, IL1B, or LPS-signaling	IL-1β, IL-6, IL-10, and TNFα	CD86, CD163, MHCII, IL-10 (high), IL-12 (low), SPHK1	Pro-inflammatory and anti-inflammatory functions
M2c	IL-10 or TGFβ	Unknown	CD163, CD204, CD206, ARG 1	Healing functions and debris scavenging

Adapted from "Repertoire of microglial and macrophage responses after spinal cord injury," S. David & A. Kroner, 2011, *Nature Reviews. Neuroscience*, *12*(7), 388–99.

Following injury to the CNS, the pro-inflammatory response can be maintained during the subacute and chronic phases, upholding a neurotoxic environment for the site of injury. The anti-inflammatory response is limited to the subacute phase (Kigerl et al. 2009). In a study performed by Kigerl et al., following a spinal cord injury, iNOS levels were increased drastically

within the first three days of injury, a primary M1 response. During the monitored one-month time period after the injury, M1 surface receptors CD86, CD16, and CD32 increased over time (Kigerl et al. 2009). Anti-inflammatory microglia were upregulated initially, with IL-4 receptor expression levels increasing more than twofold after 72 hours post injury, but M2 gene expression returned to pre-injury levels by the seventh day. The decrease in the anti-inflammatory phenotype suggests that chemokines and cytokines may cause existing microglia to differentiate into the pro-inflammatory phenotype (Kigerl et al. 2009).

Microglia and ASD

Neuroinflammatory processes caused by chronically activated microglia can contribute to the loss of synaptic connections and can cause neuronal death. The normal response to inflammation is initiated by the pro-inflammatory microglial phenotype to encourage an immune response against invaders and to engulf apoptotic neurons (Soehnlein and Lindbom 2010). The response is then altered to an anti-inflammatory response, where angiogenesis is promoted and cellular debris is cleared (Varin and Gordon 2009). When the pro-inflammatory response continues rampantly, it can result in overproduction of inflammatory cytokines and ROS that further induce tissue damage and cellular death (Kigerl et al. 2009).

Microglial dysfunction, shown in a mouse model of Rett syndrome, leads to neural circuitry dysfunction and abnormal behavior. When microglial cells functioning abnormally, the CNS becomes crowded with damaged cells, that impairs neural function (Derecki et al. 2012). Additionally, when there is a lack of synaptic pruning, there is increased connectivity and superfluous inputs between the synapses, also causing deficits in motor learning and associated memory (Paolicelli et al. 2011). Neural function impairment and abnormal immune responses

are distinct characteristics of ASD and have led scientists to hypothesize that there is a connection between ASD and microglial responses.

During chronic neuroinflammation, the inflammatory reaction by microglia begins with the typical pro-inflammatory response. The typical pro-inflammatory response is eventually overtaken by the pro-inflammatory response, but during chronic neuroinflammation, the pro-inflammatory response becomes self-generating and does not cease. A destructive inflammatory cycle is created, increasing inflammation and microglial activation further. The general shift away from anti-inflammatory reparative processes has been hypothesized to be caused by a failed anti-inflammatory microglial response (Amor et al. 2010; Rao et al. 2012). This failed response might be due to decreased numbers of anti-inflammatory microglia or decreased production of neuroprotective factors produced by the M2 phenotype.

Several studies have investigated the possibility that individuals with ASD suffer from chronic neuroinflammation related to over-active microglia. One study in particular, performed by Vargas and colleagues, examined the activation of microglia and astroglia in brains and cerebral spinal fluid of autistic subjects. They found activation of astroglia and microglia, along with active neuroinflammation in the white matter of the cerebral cortex and cerebellum. These glial responses were proposed to be neuroinflammatory reactions of the CNS innate immune system with microglial activation being the leading cellular response (Vargas et al. 2005).

In general, the majority of the studies that have studied microglial activation in various other disorders have not specifically studied the different microglial phenotypes. While Vargas et al. studied pro-inflammatory microglial cytokines and their putative role in chronic neuroinflammation in ASD, this group did not study the anti-inflammatory microglial phenotype. The paucity of data regarding the role of the different types of microglia in ASD drove the

present investigation of both the pro-inflammatory and the anti-inflammatory microglial phenotypes in ASD.

CHAPTER 2

METHODS

Brain Tissue

Flash-frozen postmortem BA24 tissue blocks from thirteen ASD donors and thirteen typically developed control donors were acquired from Autism BrainNet (formerly Autism Tissue Program, Harvard Brain Tissue Resource Center, Belmont, MA) and Neurobiobank (formerly NICHD Brain and Tissue Bank for Developmental Disorders, Baltimore, MD). This study was reviewed by the Institutional Review Board of East Tennessee State University, who determined that it does not constitute human research under the Department of Health and Human Services exemption 45 CFR 46.101(b) relating to the use of publicly available unidentifiable pathology specimens. ASD and control donors were matched prior to experimentation by gender, age, and RNA quality. Age and RNA quality were matched as closely as possible with a difference of no more than three years in age and one RNA integrity number for ASD and control donor pairs (**Table 2**).

ASD donors were diagnosed by the Autism Diagnostic Interview-Revised (ADI-R) and met diagnostic criteria outlined in the Diagnostic and Statistical Manual (DSM) IV for autistic disorder. For the protection of the identity of the ASD and control subjects, causes of death were not incorporated in Table 2. Control donors died by drowning (3 donors), asphyxia (2 donors), heart attack (2 donors), unspecified injuries (2 donors), dilated cardiomyopathy (1 donor), motor vehicle accident (1 donor), pneumonia (1 donor), respiratory insufficiency (1 donor), asthma (1 donor), commotio cordis (1 donor), abdominal injuries (1 donor), and unknown cause (1 donor). ASD donors died by asphyxia (2 donors), cardiac arrhythmia (2 donors), acute respiratory distress syndrome (1 donor), bowel obstruction (1 donor), cancer (1 donor), congestive heart

failure (1 donor), diabetic ketoacidosis (1 donor), motor vehicle accident (1 donor), stopped breathing (1 donor), head trauma (1 donor), cardiopulmonary arrest (1 donor), skull fractures (1 donor), complications from seizure disorder (1 donor), and subdural hemorrhage (1 donor).

Table 2. Subject Demographic Information

Controls								
Pair	**ID[a]**	**Age**	**Gender**	**RIN[b]**	**PMI[c] (hours)**	**Toxicology**	**Tissue Preservation**	**Matter Type used for qPCR[d]**
1	AN14757	24	M	7.8	21.33		Frozen	WM[e]
2	AN07176	21	M	7.6	29.91		Frozen	WM, GM[f]
3	AN07444	17	M	6.1	30.75	Sertraline	Frozen	WM
4	5408	6	M	5.8	16		Frozen	WM
4*	4203	7	M	NA	24		Fixed	NA
5	4848	16	M	7.5	15		Frozen	WM, GM
6	5342	22	M	8.0	14		Fixed, Frozen	WM, GM
7	5079	33	M	5.3	16	Ethanol	Frozen	WM, GM
8	M3231M	37	M	4.9	24		Frozen	WM, GM
9	AN12137	31	M	4.5	32.92		Frozen	WM, GM
10	AN03217	19	M	5.3	18.58		Frozen	WM, GM
11	AN00544	17	M	5.8	28.92		Frozen	WM, GM
12	AN17425	16	M	6.8	26.16		Frozen	WM, GM
13	4590	20	M	6.8	19		Fixed, Frozen	WM, GM
14	4670	4	M	6.2	17		Frozen	WM, GM
17	4787	12	M	5.7	15	Singular, Albuterol, Prednisone, Claritin	Frozen	GM
18	1105	16	M	7.8	17		Frozen	GM
	MEAN	**18.71**		**6.37**	**21.50**			
	SEM	**2.21**		**0.27**	**1.52**			

Table 2 (continued)

ASD								
Pair	**ID**	**Age**	**Gender**	**RIN**	**PMI (hours)**	**Toxicology**	**Tissue Preservation**	**Matter Type used for qPCR**
1	AN04166	24	M	8.1	18.51		Frozen	WM
2	AN03935	19	M	7.0	28		Frozen	WM, GM
3	AN02987	15	M	6.7	30.83		Frozen	WM
4	5144	7	M	8.0	3		Fixed, Frozen	WM
5	5302	16	M	4.8	20	Risperdal, Luvox, Clonidine, Insulin	Frozen	WM, GM
6	5176	22	M	5.1	18	Risperdal	Fixed, Frozen	WM, GM
7	5297	33	M	2.5	50	Seroquel, Prozac, Depakote, Geodon	Frozen	WM, GM
8	5027	37	M	4.7	26	Risperdal, Luvox	Frozen	WM, GM
9	AN11989	30	M	5.7	16.06		Frozen	WM, GM
10	AN07817	19	M	4.5	14.83		Frozen	WM, GM
11	AN00764	20	M	5.9	23.66	Minocycline	Frozen	WM, GM
12	AN04682	15	M	5.6	23.23		Frozen	WM, GM
13	4999	20	M	7.0	14		Fixed, Frozen	WM, GM
14	5308	4	M	7.0	21		Frozen	WM, GM
17	5565	12	M	7.0	22		Frozen	GM
18	5403	16	M	6.6	35		Frozen	GM
	MEAN	**19.31**		**6.01**	**22.76**			
	SEM	**2.18**		**0.36**	**2.60**			
	P-value[g]	**0.85**		**0.45**	**0.67**			

4* used as a control match for ASD (ID 5144) for fixed tissue stain only
ID[a]= identification number
RIN[b]= RNA integrity number
PMI[c]= post-mortem interval
qPCR[d]= quantitative real-time polymerase chain reaction
WM[e]= white matter
GM[f]= gray matter
P-value[g]= results of an independent t-test comparing control and ASD groups, statistically significant when $p<0.05$

Tissue Preparation and Sectioning

Frozen BA24 tissue was sectioned at thicknesses of 50 μm and 10 μm at -20°C using a cryostat microtome (Leica CM3050S) for differential gene expression analysis and immunohistochemical identification respectively. Tissue sections were mounted on room temperature (22°C) slides and were desiccated at room temperature for 5 minutes before being stored at -80°C. To avoid cross contamination between subjects, the internal elements of the microtome were cleaned using 100% ethanol. Tissue sections from subject pairs were prepared on the same day to guarantee equal storage time.

Laser Capture Microdissection and Punch-Dissection

Areas 4.5 mm^2 of white matter from 20 μm-thick frozen tissue sections were captured by laser capture microdissection (LCM) using an Arcturus XT (Life Technologies, Grand Island, NY) instrument. An ultraviolet laser cut the white matter sections from the surrounding tissue and these were then placed onto CapSure macrocaps by infrared laser spotting. Additionally, white and gray matter, containing superficial and deep matter, from 50 μm tissue sections were grossly punch-dissected, using a disposable 3.5 mm trephine. The LCM white matter was removed from the cap using lysis buffer incubation at 42°C. Dissected tissues were stored at -80°C and were later homogenized for RNA isolation.

RNA and cDNA Preparation

Total RNA was isolated from the LCM white matter and some of the white matter punches (used to study the expression of CD68 and IL1B) using a Maxwell 16 LEV simplyRNA Tissue Kit (Promega, Madison, WI). A Direct-zol RNA MicroPrep Kit (Zymo Research, Irvine,

CA) was used extract total RNA from the gray matter and remaining white matter punches for the analysis of the expression of the remaining genes. RNA quality was assessed by measuring RIN values with the Bioanalyzer RNA 6000 Nano chip (Agilent Technologies, Santa Carla, CA) and the Agilent 2200 TapeStation (Agilent Technologies, Santa Clara, CA) respectively. Double stranded cDNA was made by reverse transcription of the RNA samples using the Superscript III kit (Life Technologies; Grand Island, NY) that utilized both oligodTs and random hexamer primers during synthesis.

Polymerase Chain Reactions

Five primers were purchased (Qiagen; Valencia, CA) while the remaining seven primers were designed using PrimerQuest software (Integrated DNA Technologies, Coralville, IA) **Table 3**. The gene sequences for the designed primers are listed in Appendix B. For primer temperature and cycle number optimization, end-point polymerase chain reaction (PCR) was performed using a T100 Thermo Cycler (Bio-Rad, Hercules, CA). Each reaction contained SYBR Green Master Mix (Qiagen; Valencia, CA), cDNA template, and gene specific primers.

Gene expression results from LCM white matter and white matter punches were compared by quantitative real-time polymerase chain reaction (qPCR) to determine which tissue isolation technique ultimately yielded sufficient amounts of RNA for the gene expression studies. Once determined, qPCR was performed for all gene expression analyses. Each PCR reaction was performed in triplicate and a standard curve was used to determine the efficiency of reactions. Medians of triplicates were used for statistical analysis to reduce the impact of outliers.

Table 3. Introduction to the reference genes, pro-inflammatory microglial markers, and anti-inflammatory microglial markers used for gene expression and immunohistochemistry

Gene Name	Alias	Protein Name	Brief Description of Function
Reference Genes			
GAPDH	*Glyceraldehyde-3-phosphate dehydrogenase*	GAPDH	Catalyzes an energy-yielding step in glycolysis
TATA (TBP)	*TATA-box binding protein*	TBP	Transcription factor
Pro-inflammatory Microglial Markers			
CD68	*Cluster of differentiation 68*	CD68	Cell surface protein that clears cellular debris, and promotes phagocytosis
HLA-DRA	*Major histocompatibility complex, class II, DR alpha chain*	HLA-DRA	Presents peptide antigens that are able to create an immune response
IL1B	*Interleukin 1 beta*	IL1β	Cytokine mediator in inflammatory responses and involved in cell proliferation, differentiation, and apoptosis
NOS2	*Nitric oxide synthase 2*	iNOS	Enzyme that generates nitric oxide (reactive free radical)
PTGS2	*Prostaglandin-endoperoxide synthase 2 (inducible)/ cyclooxygenase*	COX2	Enzyme responsible for prostanoid biosynthesis involved in inflammation and mitogenesis
Anti-inflammatory Microglial Markers			
ARG1	*Arginase 1*	ARG1	Enzyme that converts arginine into compounds used for wound repair and down-regulates nitric oxide
IGF1	*Insulin like growth factor 1*	IGF1	Ligand that stimulates proliferation of oligodendrocytes (supports myelination of neuronal axons)
MRC1	*Mannose receptor, C type 1*	CD206	Receptor that binds and internalizes mannosylated ligands on potentially pathogenic microorganisms so they can be neutralized by phagocytic engulfment
PPARG	*Peroxisome proliferative activated receptor gamma*	PPARγ	Receptor that inhibits pro-inflammatory gene expression

Statistical Analysis

Expression data for target genes was normalized to reference genes *GAPDH* and *TATA*. Fold changes in the expression of genes of interest comparing ASD to control subjects were obtained using the $2^{-\Delta\Delta Ct}$ method of Livak and Schmittgen (Livak and Schmittgen 2001). For these calculations, the geometric means of Ct values of reference genes were used for normalizations. SPSS (version 22, IBM, New York, NY) was used to identify and remove extreme outliers in data sets based on the outlier labeling rule that utilizes the third and first

quartiles and a multiplying factor of 2.2 (Hoaglin and Iglewicz 1987). Normality tests were run using GraphPad Prism (version 5.0b, GraphPad Software, Inc.) and regressions were performed using SPSS. GraphPad Prism was used to analyze the data using an independent student's t-test. Data that was not normally distributed were analyzed using the Mann-Whitney non-parametric test. Results were considered statistically significant when p-value< 0.05. Statistical results are reported before and after Holm's Bonferroni correction for the number of gene comparisons.

Immunohistochemistry

Pro-inflammatory

For visualization of pro-inflammatory microglial cells, frozen tissue sections, *n=8 pairs*, were immunohistochemically stained for the HLA-DRA pro-inflammatory microglial protein. The slides that were used for analysis were removed from the -80°C freezer and were immediately transferred into -20°C acetone (13 min). Endogenous peroxidase activity in the tissue was neutralized in 0.1M PBS/ 1.5% H_2O_2 (15 min). After the tissue was blocked with 3% BSA (1 h), the sections were incubated with a monoclonal mouse anti-human HLA-DR antigen, alpha-chain clone TAL.1B5 (Dako, Carpinteria, CA) at a dilution of 1:100 (overnight at 4°C). Following primary antibody incubation, the sections were incubated in a corresponding secondary antibody, Vectastain mouse IgG ABC kit (Vector Laboratories Inc, Burlingame, CA) (2 h), and then with avidin-biotinylated horseradish peroxidase complex (1 h). Sections were washed in 0.1M PBS (10 min), then 0.05 M Tris (2 times for 10 min each). The pro-inflammatory microglial cells were then visualized by incubating sections in 50 mL of 0.05M Tris, 0.3% ammonium nickel sulfate, and 3,3'-diaminobenzidine tetrahydrochloride (5 min), then in another 50 mL of the same solution, but also containing 50 µl H_2O_2 (5 min). Between all

remaining incubation steps, excluding between blocking and primary antibody incubation, sections were washed in 0.1M PBS (3 times for 10 min each). Sections were dehydrated in sequential washes of 75%, 95%, and 100% ethanol (30 sec each). The final dehydration step was in xylene (5 min) and the sections were then dried in the hood (5 min) in preparation for analysis. The attempted protocols during the optimization process can be found in sequential order in **Table 4**.

Table 4. Attempted protocols for the HLA-DRA immunohistochemical stain on frozen and fixed tissue

Buffer	Block	Primary Antibody Manufacturer and Dilution	Components for Primary Antibody Incubation	Secondary Antibody Manufacturer and Dilution	Components for Secondary Antibody Incubation	Developer
0.05M TBS	NHS[a]/ 0.05M TBS + 0.2% Triton-X-100 + 2% SA	Dako[b] 1:100	NHS/ 0.05M TBS + 0.2% Triton-X-100 + 2% SA	Vector[c] 1:200	Horse Serum[d]/ 0.05M TBS + 0.2% Triton-X-100	DAB[e]
*0.05M TBS	NHS/ 0.05M TBS + 0.2% Triton-X-100 + 2% SA	Dako 1:100	NHS/ 0.05M TBS + 0.2% Triton-X-100 + 2% SA	Vector 1:200	Horse Serum/ 0.05M TBS + 0.2% Triton-X-100	DAB
0.1M PBS	3% BSA/ 0.1M PBS	Dako 1:100	NHS/ 0.1M PBS	Vector 1:200	Horse Serum/ 0.1M PBS	DAB
0.1M PBS	3% BSA/ 0.1M PBS	Dako 1:100 and 1:200	NHS/ 0.1M PBS	Vector 1:200	Horse Serum/ 0.1M PBS	DAB
0.05M PBS	3% BSA/ 0.05M PBS	Dako 1:100	NHS/ 0.05M PBS	Vector 1:200	Horse Serum/ 0.05M PBS	DAB
0.1M PBS	3% BSA/ 0.1M PBS	Dako 1:100	NHS/ 0.1M PBS	Vector 1:400	Horse Serum/ 0.1M PBS	DAB
**0.1M PBS	3% BSA/ 0.1M PBS + 0.2% Tween 20	Dako 1:100	NHS/ 0.1M PBS + 0.2% Tween 20	Vector 1:400	Horse Serum/ 0.1M PBS + 0.2% Tween 20	DAB
**0.1M PBS	6% BSA/ 0.1M PBS + 0.2% Tween 20	Dako 1:100	NHS/ 0.1M PBS + 0.2% Tween 20	Vector 1:400	***Horse Serum/ 0.1M PBS + 0.2% Tween 20	DAB

Table 4 (continued)

**0.1M PBS	3% BSA/ 0.1M PBS + 0.2% Tween 20; NHS/ 0.1M PBS + 0.2% Tween 20	Dako 1:100	NHS/ 0.1M PBS + 0.2% Tween 20	Vector 1:400	Horse Serum/ 0.1M PBS + 0.2% Tween 20	DAB
* **0.1M PBS	NHS/ 0.1M PBS + 0.1% Tween 20; NHS/ 0.1M PBS + 0.2% Triton-X-100	Dako 1:100	NHS/ 0.1M PBS + 0.1% Tween 20; NHS/ 0.1M PBS + 0.2% Triton-X-100	Vector 1:400	Horse Serum/ 0.1M PBS + 0.1% Tween 20; NHS/ 0.1M PBS + 0.2% Triton-X-100	DAB
**0.1M PBS	NHS/ 0.1M PBS + 0.2% Triton-X-100	Dako 1:100	NHS/ 0.1M PBS + 0.2% Triton-X-100	Vector 1:400	***Horse Serum/ 0.1M PBS + 0.2% Triton-X-100	DAB
**0.1M PBS	3% BSA/ 0.1M PBS + 0.2% Triton-X-100; 3% BSA/NHS/ 0.1M PBS + 0.2% Triton-X-100	None-Negative Optimization	NHS/ 0.1M PBS + 0.2% Triton-X-100	Vector 1:400	Horse Serum/ 0.1M PBS + 0.2% Triton-X-100	DAB
**0.1M PBS	6% BSA/ 0.1M PBS + 0.2% Triton-X-100	None-Negative Optimization	NHS/ 0.1M PBS + 0.2% Triton-X-100	Vector 1:400	Horse Serum/ 0.1M PBS + 0.2% Triton-X-100	DAB
**0.1M PBS	3% BSA/ 0.1M PBS + 0.2% Triton-X-100	None-Negative Optimization	NHS/ 0.1M PBS + 0.2% Triton-X-100; NHS/ 0.1M PBS	Vector 1:400	Horse Serum/ 0.1M PBS + 0.2% Triton-X-100; Horse Serum/ 0.1M PBS	NovaRED[f]
**0.1M PBS	1% BSA/ 0.1M PBS + 0.2% Triton-X-100	Dako 1:100	1% BSA/ 0.1M PBS + 2% Triton-X-100	Jackson AffiniPure[g] 1:400	1% BSA/ 0.1M PBS + 2% Triton-X-100	NovaRED
**0.1M PBS	1% BSA/ 0.1M PBS	Dako 1:100	1% BSA/ 0.1M PBS	Jackson AffiniPure 1:400	1% BSA/ 0.1M PBS	NovaRed
**Wash Buffer[h]	Blocking Buffer[h]	Dako 1:100	Blocking Buffer	Life Technologies[i] 1:3000	Blocking Buffer	DAB/Metal Concentrate[h]
0.1M PBS	0.1M PBS + 0.2% Triton-X-100	Dako 1:100	NHS/ 0.1M PBS + 0.2% Triton-X-100	Vector 1:200	Horse Serum/ 0.1M PBS + 0.2% Triton-X-100	None-Fixed Tissue

*Stain incubated at 37°C instead of 25°C
**Stain performed by Emma E. Pendola under my supervision
***incubated for 2 separate amounts of time
NHS[a]= Normal Horse Serum
Dako[b]=Monoclonal Mouse Anti-Human HLA-DR Antigen, Alpha-Chain Clone TAL.1B5
Vector[c]=Mouse IgG ABC kit used for bright-field microscopy
Horse Serum[d]= component of the Vector Mouse IgG ABC kit

DAB[e]= 3,3'-diaminobenzidine tetrahydrochloride
NovaRED[f]=Vector NovaRED Peroxidase (HRP) Substrate Kit
Jackson AffiniPure[g]= Peroxidase-conjugated AffiniPure Donkey Anti-Mouse IgG used for bright-field microscopy
Wash Buffer, Blocking Buffer, and DAB/Metal Concentrate[h]= components of the Thermo Pierce Peroxidase Detection Reagent Pack
Life Technologies[i]= HRP-Goat Anti-Mouse IgG

Anti-inflammatory

As an attempt to visualize the anti-inflammatory microglial cells, the mannose receptor, CD206, was chosen as the cellular marker. For bright-field and fluorescent microscopy, a Pierce mannose receptor/CD206 antibody (Thermo Fisher, Rockford, IL) and purified mouse anti-human CD206 antibody (BD Pharmigen, San Jose, CA) were used for primary antibody incubations. The secondary antibodies used for bright-field microscopy included: a secondary antibody from a Vectastain mouse IgG ABC kit (Vector Laboratories Inc, Burlingame, CA), an ECL anti-mouse IgG horseradish peroxidase linked whole antibody (GE Healthcare UK Limited, Buckinghamshire, UK) and a peroxidase-conjugated AffiniPure donkey anti-mouse IgG (Jackson Immuno, West Grove, PA). An Alexa Fluor 488-conjugated AffiniPure donkey anti-mouse IgG (Jackson Immuno, West Grove, PA) was used as the secondary antibody for fluorescent microscopy. For bright-field microscopy, all slides were immediately transferred from the -80°C freezer into cold acetone (stored at -20°C) for fixation of the tissue to the slide (rapid stain (r): 5 min; overnight stain (o): 10 min). For fluorescent microscopy, all slides were transferred from the -80°C freezer to a desiccator (5-10 min) prior to fixation by acetone. Endogenous peroxidase activity in the tissue was neutralized in 0.1M PBS/H_2O_2 (bright-field only). After the tissue was blocked (r: 10 min; o: 2 h), the sections were incubated in primary antibody at various dilutions. Following primary antibody incubation (r: 15 min; o: overnight), the sections were incubated in a corresponding secondary antibody (r: 10 min; o: 2 h) and then with avidin-biotinylated horseradish peroxidase complex (r:10 min; o:1 h), excluding the protocols when the Thermo

Pierce Peroxidase Detection Reagent Pack was used. When the Thermo Pierce Pack was not used, sections were washed in the corresponding buffer and were visualized by incubating sections in 50 mL of 0.05M Tris, 0.3% ammonium nickel sulfate, and 3,3'-diaminobenzidine tetrahydrochloride (r and o: 5 min), then in another 50 mL of the same solution, but also containing 50 µl H_2O_2 (r: 5 min; o: 10 min). The Thermo Pierce Pack did not require the previous step, but instead, the sections were incubated in the Thermo Pierce DAB/Metal Concentrate (r: 5 min). Between all remaining incubation steps, excluding between blocking and primary antibody incubation, sections were washed in the corresponding buffer. All sections for bright-field microscopy were dehydrated before analysis sequentially in 75%, 95%, and 100% ethanol (30 sec each) and xylene (5 min), while the sections for fluorescent microscopy were prepared for analysis using a Slowfade Antifade Kit (Invitrogen, Rockford, IL). The components for the intermediate steps of each attempted protocol are listed in **Table 5**.

Table 5. Attempted protocols for the CD206 immunohistochemical stain on frozen tissue

Buffer	Block	Primary Antibody Manufacturer and Dilution	Components for Primary Antibody Incubation	Secondary Antibody Manufacturer and Dilution	Components for Secondary Antibody Incubation	Developer
0.1M PBS	3% BSA/ 0.1M PBS	Thermo Fisher[a] 1:500	NHS/ 0.1M PBS	Vector 1:200	Horse Serum/ 0.1M PBS	DAB
0.1M PBS	3% BSA/ 0.1M PBS	Thermo Fisher 1:250	NHS/ 0.1M PBS	Vector 1:200	Horse Serum/ 0.1M PBS	DAB
0.05M TBS	3% BSA/ 0.1M PBS	Thermo Fisher 1:500	NHS/ 0.1M PBS	Vector 1:200	Horse Serum/ 0.1M PBS	DAB
0.1M PBS	3% BSA/ 0.1M PBS + 0.2% Triton-X-100	Thermo Fisher 1:500	NHS/ 0.1M PBS + 0.2% Triton-X-100	Vector 1:200	Horse Serum/ 0.1M PBS + 0.2% Triton-X-100	DAB
0.1M PBS	3% BSA/ 0.1M PBS + 0.2% Triton-X-100	Thermo Fisher 1:250	NHS/ 0.1M PBS + 0.2% Triton-X-100	Vector 1:200	Horse Serum/ 0.1M PBS + 0.2% Triton-X-100	DAB

Table 5 (continued)

0.1M PBS	3% BSA/ 0.1M PBS + 0.2% Triton-X-100	None-Negative Optimization	1% BSA/ 0.1M PBS + 0.2% Triton-X-100	Vector 1:200	Horse Serum/ 0.1M PBS + 0.2% Triton-X-100	DAB
0.1M PBS	3% BSA/ 0.1M PBS + 0.2% Triton-X-100	None-Negative Optimization	NHS/ 0.1M PBS + 0.2% Triton-X-100	Vector 1:200	Horse Serum/ 0.1M PBS + 0.2% Triton-X-100	DAB
0.1M PBS	3% BSA/ 0.1M PBS + 0.2% Triton-X-100	Thermo Fisher 1:125	NHS/ 0.1M PBS + 0.2% Triton-X-100	Vector 1:200	Horse Serum/ 0.1M PBS + 0.2% Triton-X-100	DAB
0.1M PBS	3% BSA/ 0.1M PBS + 0.2% Triton-X-100	None-Negative Optimization	NHS/ 0.1M PBS + 0.2% Triton-X-100	Jackson AffiniPure[b] 1:200	Horse Serum/ 0.1M PBS + 0.2% Triton-X-100	DAB
0.1M PBS	3% BSA/ 0.1M PBS + 0.2% Triton-X-100	Thermo Fisher 1:125	NHS/ 0.1M PBS + 0.2% Triton-X-100	Jackson AffiniPure 1:200	Horse Serum/ 0.1M PBS + 0.2% Triton-X-100	DAB
0.1M PBS	3% BSA/ 0.1M PBS + 0.2% Triton-X-100	Thermo Fisher 1:250	NHS/ 0.1M PBS + 0.2% Triton-X-100	Vector 1:200	Horse Serum/ 0.1M PBS + 0.2% Triton-X-100	DAB
0.1M PBS	3% BSA/ 0.1M PBS + 0.2% Triton-X-100	Thermo Fisher 1:125	NHS/ 0.1M PBS + 0.2% Triton-X-100	Vector 1:400	Horse Serum/ 0.1M PBS + 0.2% Triton-X-100	DAB
0.1M PBS	3% BSA/ 0.1M PBS + 0.2% Triton-X-100	Thermo Fisher 1:125	NHS/ 0.1M PBS + 0.2% Triton-X-100	Vector 1:400	Horse Serum/ 0.1M PBS + 0.2% Triton-X-100	DAB
0.1M PBS	3% BSA/ 0.1M PBS + 0.2% Triton-X-100	Thermo Fisher 1:125	1% BSA/ 0.1M PBS + 0.2% Triton-X-100	Jackson 1:200	1% BSA/ 0.1M PBS + 0.2% Triton-X-100	Nova RED
0.1M PBS	3% BSA/ 0.1M PBS	Thermo Fisher 1:125	1% BSA/ 0.1M PBS	Vector 1:400	1% BSA/ 0.1M PBS	DAB
0.1M PBS	3% BSA/ 0.1M PBS	Thermo Fisher 1:125	1% BSA/ 0.1M PBS	Jackson AffiniPure 1:200	1% BSA/ 0.1M PBS	Nova RED
0.1M PBS	3% BSA/ 0.1M PBS	Pharmigen[c] 1:500	NHS/ 0.1M PBS	Vector 1:200	Horse Serum/ 0.1M PBS	DAB
0.1M PBS	3% BSA/ 0.1M PBS + 0.2% Triton-X-100	Pharmigen 1:250	NHS/ 0.1M PBS + 0.2% Triton-X-100	Vector 1:200	Horse Serum/ 0.1M PBS + 0.2% Triton-X-100	DAB
0.1M PBS	3% BSA/ 0.1M PBS + 0.2% Triton-X-100	None-Negative Optimization	NHS/ 0.1M PBS + 0.2% Triton-X-100	Vector 1:200	Horse Serum/ 0.1M PBS + 0.2% Triton-X-100	DAB

Table 5 (continued)

0.1M PBS	3% BSA/ 0.1M PBS + 0.2% Triton-X-100	Thermo Fisher 1:250	NHS/ 0.1M PBS + 0.2% Triton-X-100	Vector 1:200	Horse Serum/ 0.1M PBS + 0.2% Triton-X-100	DAB
0.1M PBS	3% BSA/ 0.1M PBS + 0.2% Triton-X-100	Pharmigen 1:125	1% BSA/ 0.1M PBS + 0.2% Triton-X-100	Vector 1:400	1% BSA/ 0.1M PBS + 0.2% Triton-X-100	DAB
0.1M PBS	3% BSA/ 0.1M PBS + 0.2% Triton-X-100	Pharmigen 1:250	NHS/ 0.1M PBS + 0.2% Triton-X-100	Vector 1:200	Horse Serum/ 0.1M PBS + 0.2% Triton-X-100	DAB
0.1M PBS	3% BSA/ 0.1M PBS + 0.2% Triton-X-100	Pharmigen 1:125	NHS/ 0.1M PBS + 0.2% Triton-X-100	Vector 1:400	Horse Serum/ 0.1M PBS + 0.2% Triton-X-100	DAB
0.1M PBS	3% BSA/ 0.1M PBS + 0.2% Triton-X-100	Pharmigen 1:125	1% BSA/ 0.1M PBS + 0.2% Triton-X-100	Jackson AffiniPure 1:200	1% BSA/ 0.1M PBS + 0.2% Triton-X-100	NOVA Red
Wash Buffer	Blocking Buffer	None-Negative Optimization	Blocking Buffer	GE Healthcare UK Limited[d] 1:200	Blocking Buffer	DAB/Metal Concentrate
Wash Buffer	Blocking Buffer	None-Negative Optimization	Blocking Buffer	GE Healthcare UK Limited 1:500 and 1:1000	Blocking Buffer	DAB/Metal Concentrate
Wash Buffer	Blocking Buffer	None-Negative Optimization	Blocking Buffer	Thermo Fisher 1:1000	Blocking Buffer	DAB/Metal Concentrate
Wash Buffer	Blocking Buffer	None-Negative Optimization	Blocking Buffer	None-Negative Optimization	Blocking Buffer	DAB/Metal Concentrate
0.1M PBS	3% BSA/ 0.1M PBS	None-Negative Optimization	NHS/ 0.1M PBS	None-Negative Optimization	Horse Serum/ 0.1M PBS	DAB
Wash Buffer	Blocking Buffer	None-Negative Optimization	Blocking Buffer	None-Negative Optimization	Blocking Buffer	DAB/Metal Concentrate
0.1M PBS	10% Donkey Serum/ 3% BSA/ 0.1M PBS	Thermo Fisher 1:125	1% BSA/ 0.1M PBS	Jackson Immuno[e] 1:500	1% BSA/ 0.1M PBS	NA
0.1M PBS	10% Donkey Serum/ 0.1M PBS + 0.1% Tween 20	Pharmigen 1:75 and 1:250	0.1M PBS + 0.1% Tween 20	Jackson Immuno 1:500	0.1M PBS + 0.1% Tween 20	NA

Thermo Fisher[a]= Pierce Mannose Receptor/CD206 used for bright-field microscopy
Jackson AffiniPure[f]= Peroxidase-conjugated AffiniPure Donkey Anti-Mouse IgG used for bright-field microscopy
Pharmigen[g]=Purified Mouse Anti-Human CD206 used for bright-field microscopy
GE Healthcare UK Limited[i]=ECL Anti-Mouse IgG, Horseradish Peroxidase linked wholeantibody used for bright-field microscopy
Jackson Immuno[j]= Alexa Fluor 488-conjugated AffiniPure Donkey Anti-Mouse IgG used for fluorescence microscopy

Immunohistochemical Analysis

ImageJ software (version 1.44o) was used to calculate the area fraction of HLA-DRA labeled microglial cells. To calculate the amount of HLA-DRA immunoreactivity in a specific area, images of HLA-DRA labeled tissue from control and ASD subjects were converted into an 8-bit image. Ten randomized images were taken for each tissue section and a fixed threshold was determined by taking the average threshold from all slides. Fractional area was calculated by dividing the HLA-DRA positive area (μm^2) in the 8-bit image by the total area of the image frame. The data were analyzed using an independent and dependent student's t-test for ASD and age-matched control donors. Results were considered statistically significant when $p<0.05$.

CHAPTER 3

RESULTS

Subject Demographics

Control and ASD donors were matched by gender, age, and RIN. Donors were matched within 3 years of age and 1 RIN value (Auer et al. 2003). While PMI was not a factor in matching donor pairs, it was analyzed and compared statistically to ensure there was no difference between ASD and matched control donors. There was no significant difference in the average age (p= 0.920; **Figure 1**), RIN values (p= 0.446; **Figure 2**), nor PMI (p= 0.649; **Figure 3**) when comparing ASD to matched control donors.

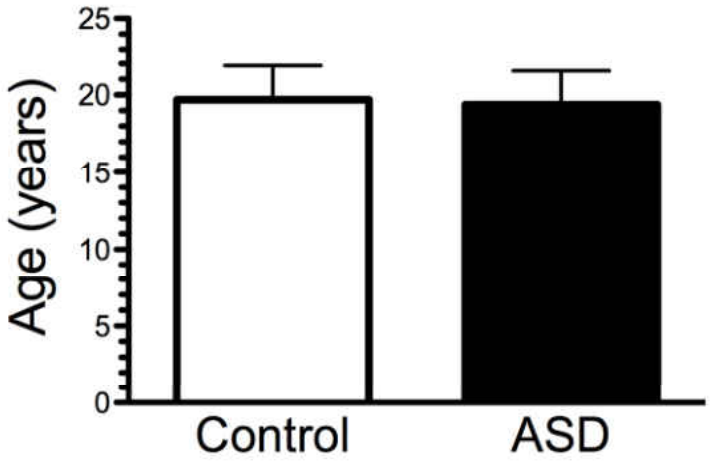

Figure 1. Comparison of average age of ASD and matched control donors (*n*=18 pairs). No statistically significant difference was observed.

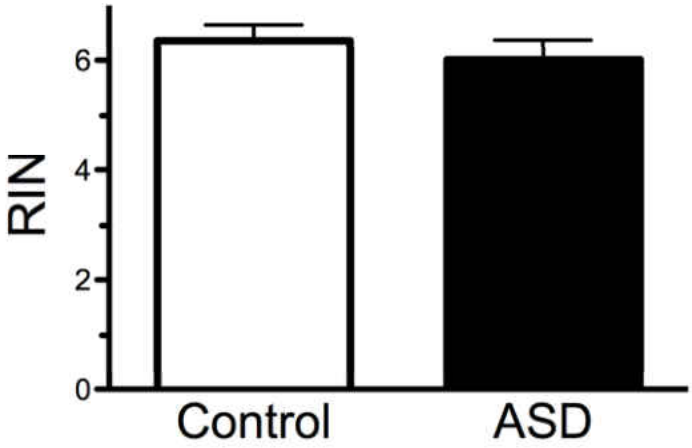

Figure 2. Comparison of average RIN value of ASD and matched control donors (*n*=18 pairs). No statistically significant difference was observed.

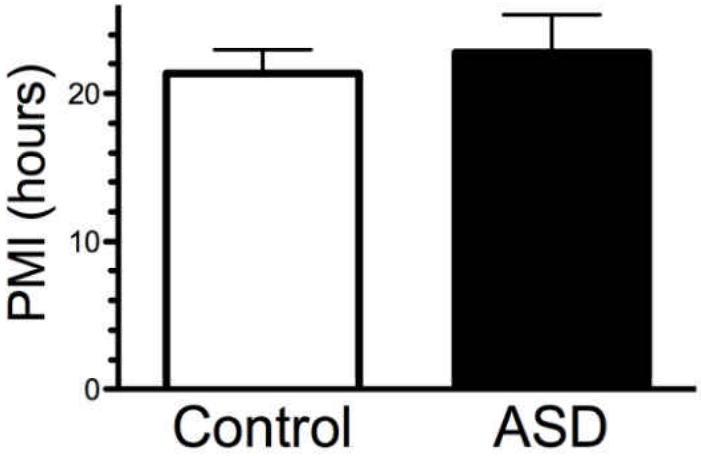

Figure 3. Comparison of average PMI of ASD and matched control donors (*n*=18 pairs). No statistically significant difference was observed.

Gene Expression

White Matter

In initial work, it was determined that gross punch-dissection of human BA24 white matter yielded sufficient amounts of RNA for gene expression studies so that the laser capture microdissection of white matter would not be required. Following the conclusion that punches could be used, expression levels of both pro-inflammatory and anti-inflammatory microglial target genes were measured in punch-dissected white matter from ASD and control donors. Target gene expression data was normalized to two reference genes, *GAPDH* and *TATA*, after it was determined that there was no significant difference in the *GAPDH/TATA* ratio comparing the typically developed control donors and ASD donors (p= 0.088; **Figure 4A**), (p= 0.263; **Figure 4B**).

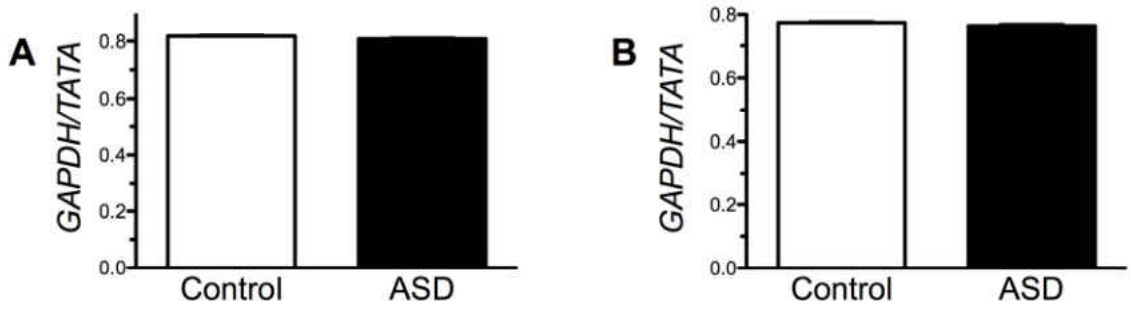

Figure 4. Ratio of housekeeping genes *GAPDH/TATA* used for the normalization of the gene expression data for *CD68*, *IL1B* (**A**, *n*=11 pairs), *ARG1*, *HLA-DRA*, *IGF1*, *MRC1*, *NOS2*, *PPARG*, and *PTGS2* (**B**, *n*= 10 pairs) in white matter of typically developed control and ASD donors. No statistically significant differences were observed when the two groups of donors were compared.

The expression of five pro-inflammatory microglial genes were measured by qPCR in post-mortem BA24 tissue that was punch-dissected from the white matter of individuals with

ASD and matched controls. **Figure 5** displays expression levels of the five pro-inflammatory microglial genes in white matter tissues. There were no statistically significant differences in the expression levels of *CD68* (p= 0.493), *HLA-DRA* (p= 0.995), *IL1B* (p= 0.149), *NOS2* (p= 1.000), nor *PTGS2* (p= 0.657) comparing ASD and control groups.

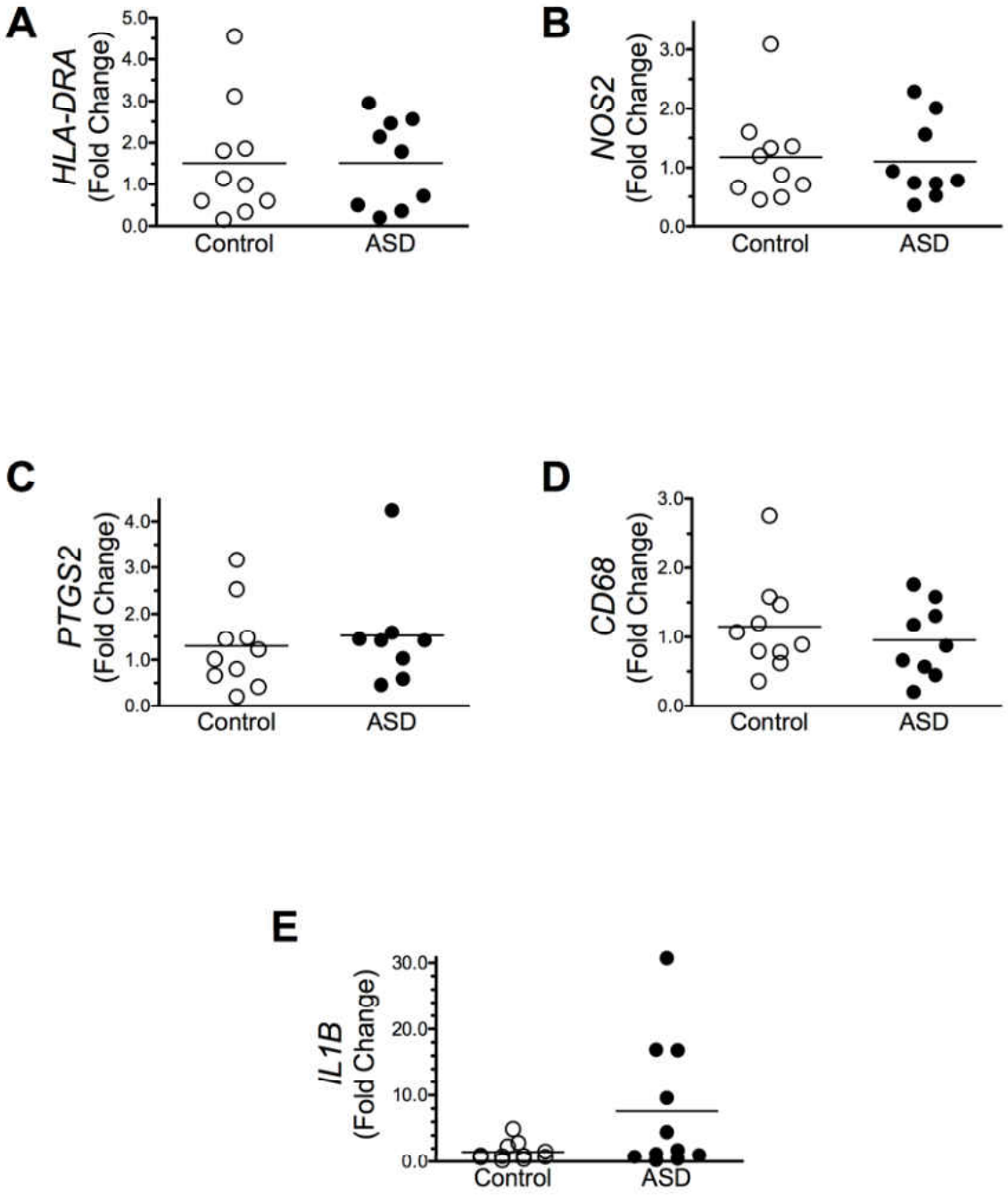

Figure 5. Expression of pro-inflammatory microglial genes *HLA-DRA* (**A**), *NOS2* (**B**), *PTGS2* (**C**), *CD68* (**D**), and *IL1B* (**E**) in white matter from matched pairs of ASD (closed symbols) and control (open symbols) donors (**A**, **B**, and **C**, n=10 pairs; **D** and **E**, n=11 pairs). Gene expression levels were normalized to the geometric mean of stable reference genes (*GAPDH* and *TATA*). Mean values are indicated by horizontal lines. No statistically significant differences were observed when the two groups of donors were compared.

Likewise, analyses of the expression levels of four anti-inflammatory microglial expressed genes also demonstrated no statistically significant differences when comparing the two groups of donors (**Figure 6**); *ARG1* (p= 0.500), *MRC1* (p= 0.553), and *PPARG* (p= 0.336). In contrast, *IGF1* expression levels were significantly higher in ASD donors as compared to control donors (p= 0.014).

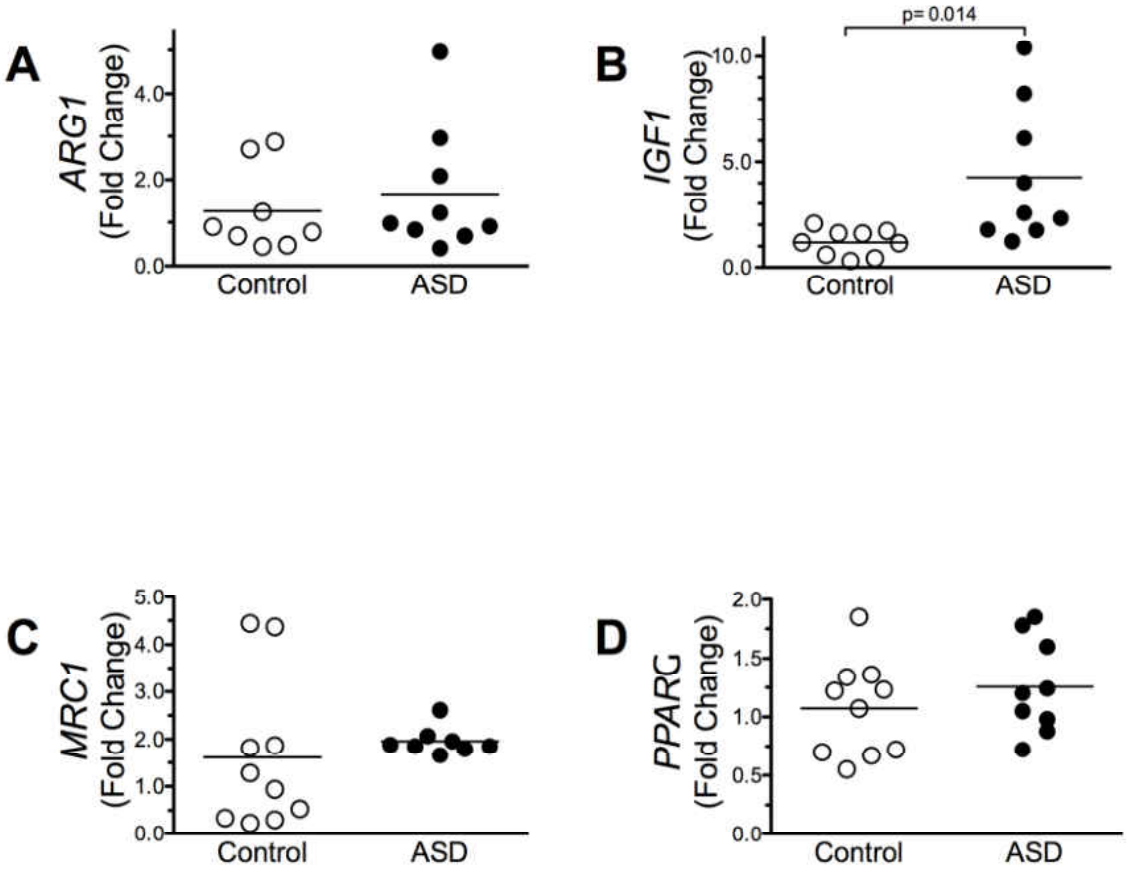

Figure 6. Expression of target anti-inflammatory microglial genes *ARG1* (**A**), *IGF1* (**B**), *MRC1* (**C**), and *PPARG* (**D**) in white matter from matched pairs of typically developed control (open symbols) and ASD (closed symbols) donors (*n*=10 pairs). Gene expression levels were normalized to the geometric mean of stable reference genes (*GAPDH* and *TATA*). Mean values are indicated by horizontal lines and statistical significance is indicated above the data points.

Gray Matter

Target gene expression data was normalized to two reference genes, *GAPDH* and *TATA*, after it was determined that there was no significant difference in the *GAPDH/TATA* ratio when comparing typically developed control donors and ASD donors (p= 0.558; **Figure 7**).

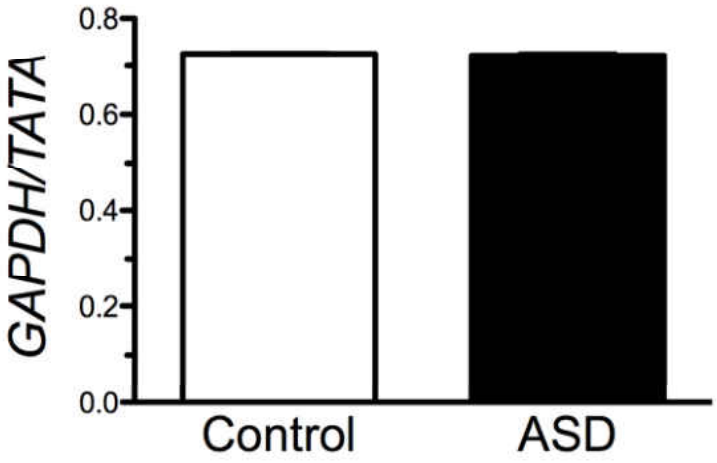

Figure 7. Ratio of housekeeping genes *GAPDH/TATA* used for the normalization of the gene expression data for *HLA-DRA*, *IGF1, IL1B*, and *MRC1* in the gray matter of typically developed control and ASD donors (*n*=13 pairs). No statistically significant difference was observed when the two groups of donors were compared.

Chosen for examination in gray matter was *IGF1* because its expression demonstrated differences between the two study groups in white matter. In addition, pro-inflammatory, *HLA-DRA,* and anti-inflammatory microglial genes, *MRC1*, were selected to parallel the markers used in the immunohistochemistry portion of the study. Although there was no significant difference in *IL1B* expression levels in white matter comparing the two groups of donors, the expression of *IL1B* exhibited a wide range of Ct values in the ASD donors (**Figure 5E**) and was chosen as well.

The expression levels of *MRC1* in gray matter were significantly lower in ASD donors as compared to typically developed control donors (p= 0.034; **Figure 8A**). In contrast to the white matter results, there was no significant difference in the expression of anti-inflammatory *IGF1* in gray matter (p= 0.273; **Figure 8B**). The expression of *HLA-DRA* was similar when comparing ASD to matched control donors (p= 0.301; **Figure 9**).

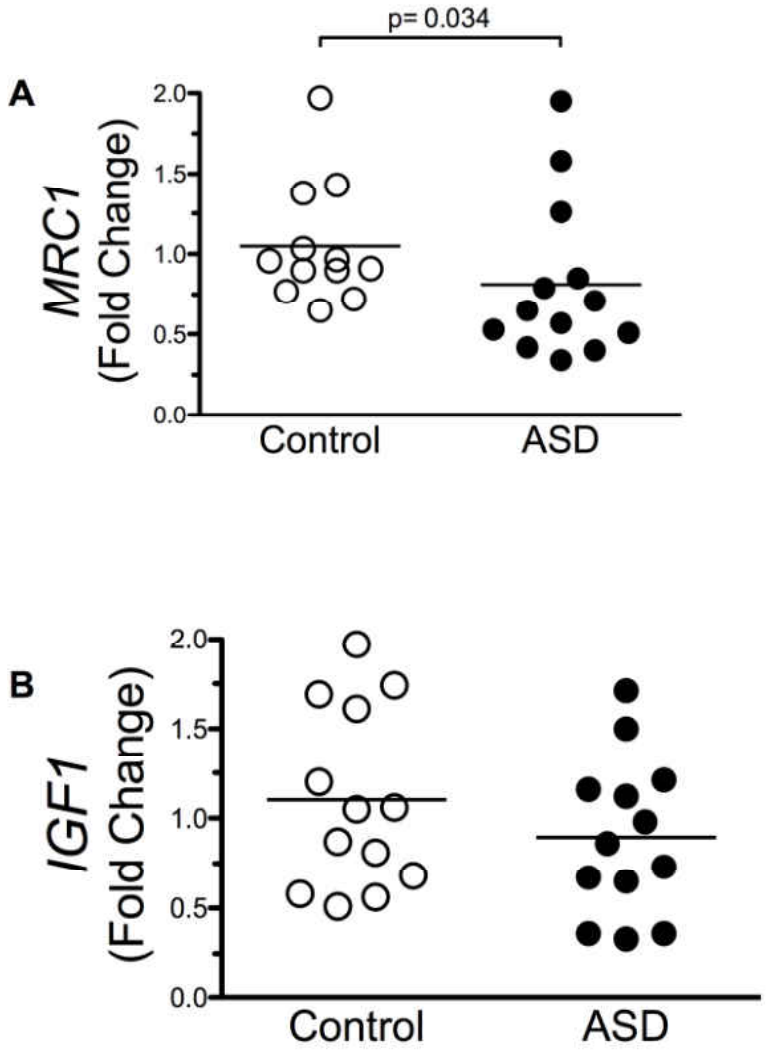

Figure 8. Expression of anti-inflammatory *MRC1* (**A**, *n*= 12 control and 13 ASD donors) and *IGF1* (**B**, *n*=13 pairs) in gray matter in typically developed control (open symbols) and ASD (closed symbols) donors. Gene expression levels were normalized to the geometric mean of stable reference genes (GAPDH and TATA). Mean values are indicated by horizontal lines and statistical significance is indicated above the data points.

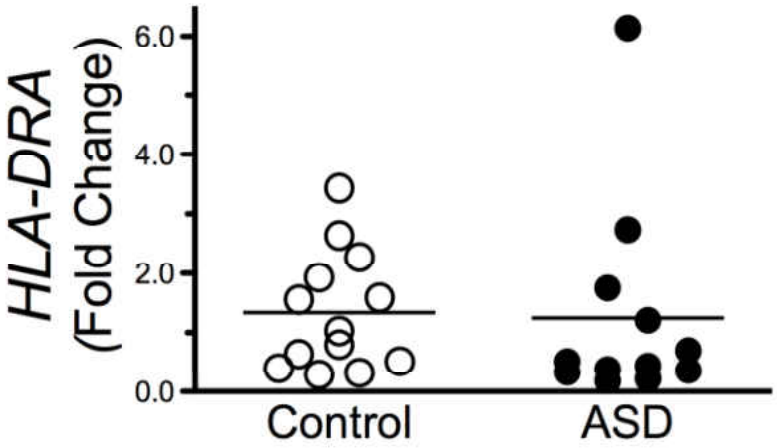

Figure 9. Expression of pro-inflammatory *HLA-DRA* in gray matter in typically developed control (open symbols) and matched ASD (closed symbols) donors (*n*=13 control and 12 ASD donors). Gene expression levels were normalized to the geometric mean of stable reference genes (*GAPDH* and *TATA*). Mean values are indicated by horizontal lines. No statistically significant difference was observed when the two groups of donors were compared.

The expression levels of *IL1B* demonstrated wide variation in comparison to the other gene expressions that were studied, just as occurred in *IL1B* expression levels in white matter (**Figure 5E**). While there was no significant difference in the expression of *IL1B* when comparing ASD to matched control donors, this wide variation of *IL1B* expression fold changes comparing the two groups is interesting. We provide a combination of two figures for *IL1B* expression in gray matter (**Figure 10**); the circular and diamond symbols combined depicts the range of data before outliers were removed by the outlier labeling rule (with the statistical comparison generating a p= 0.719), while the circular symbols alone illustrate the data after 3 outliers were removed (generating a p= 0.951). One outlier was removed from the control data (open diamond symbol), while two outliers were removed from the ASD data set (closed diamond symbols). Hence, removing outliers in the *IL1B* dataset, as was performed for all data sets, resulted in hiding the fact that *IL1B* expression levels were highly variable in ASD subjects.

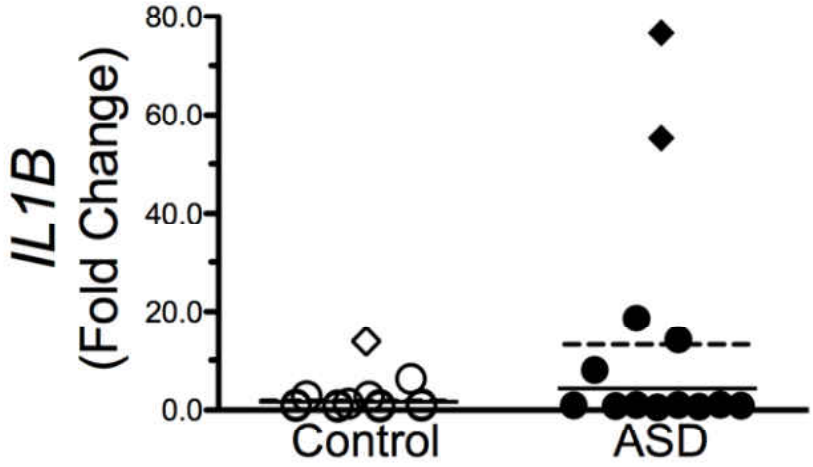

Figure 10. Expression of anti-inflammatory *IL1B* in gray matter pre-removal of outliers with the outlier labeling rule (diamond symbols and dotted horizontal lines) (*n*=13 pairs) and post-removal of the outliers (circular symbols and solid horizontal lines) (*n*=12 control and 11 ASD donors) in typically developed control (open symbols) and ASD (closed symbols) donors. Gene expression levels were normalized to the geometric mean of stable reference genes (*GAPDH* and *TATA*). Mean values are indicated by horizontal lines. No statistically significant differences were observed when the two groups of donors were compared, whether outliers are removed or not.

Statistical Limitations of the Study

Due to the number of statistical comparisons of gene expression levels, it was necessary to report corrected p-values to reduce the chance of a type I error. Hence, the Holm's Bonferroni correction was used to adjust p-values (**Table 6**). After the correction, the group comparison of *IGF1* expression levels in white matter was no longer statistically significant (p'=0.112). Likewise, the comparison of *MRC1* expression levels in gray matter in the two groups also failed to reach statistical significance (p'=0.136).

Table 6. P-values after Holm's Bonferroni correction for multiple independent student's t-tests of gene expression from ACC white and gray matter in ASD and control donors

WHITE MATTER			
Gene	***p-value***	***p'* (Adjusted p-value)**	**Outcome**
IGF1	0.014	0.112	Not significant
IL1B	0.149	1.000	Not significant
PPARG	0.336	1.000	Not significant
CD68	0.493	1.000	Not significant
ARG1	0.500	1.000	Not significant
MRC1	0.553	1.000	Not significant
PTGS2	0.657	1.000	Not significant
HLA-DRA	0.995	1.000	Not significant
NOS2	1.000	1.000	Not significant
GRAY MATTER			
Gene	***p-value***	***p'* (Adjusted p-value)**	**Outcome**
MRC1	0.034	0.136	Not significant
IGF1	0.273	0.819	Not significant
HLA-DRA	0.301	0.819	Not significant
IL1B with outliers	0.719	0.819	Not significant
IL1B outliers removed	0.951	0.951	Not significant

Calculations adapted from "A simple sequential rejective method procedure," S. Holm, 1979, *Scandinavian Journal of Statistics, 6*, 65-70.

Immunohistochemistry

HLA-DRA

Frozen BA24 tissue (20 µm thick) sections were stained using an antibody directed against HLA-DRA. Area fraction (area of positive staining as a fraction of the entire area analyzed) averages were calculated from ten randomly selected white matter areas per subject

(**Figure 11**). No statistically significant difference was found when comparing control and ASD donors (p= 0.250; **Figure 12**; *n*=8 pairs).

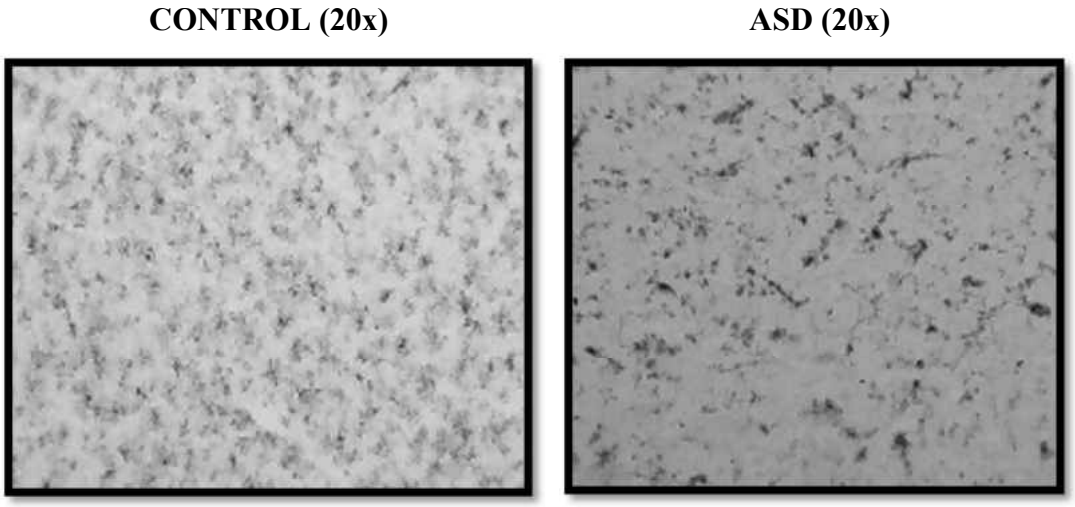

Figure 11. Immunohistochemical stain for the pro-inflammatory microglial marker HLA-DRA in a representative typically developed control donor and paired ASD donor.

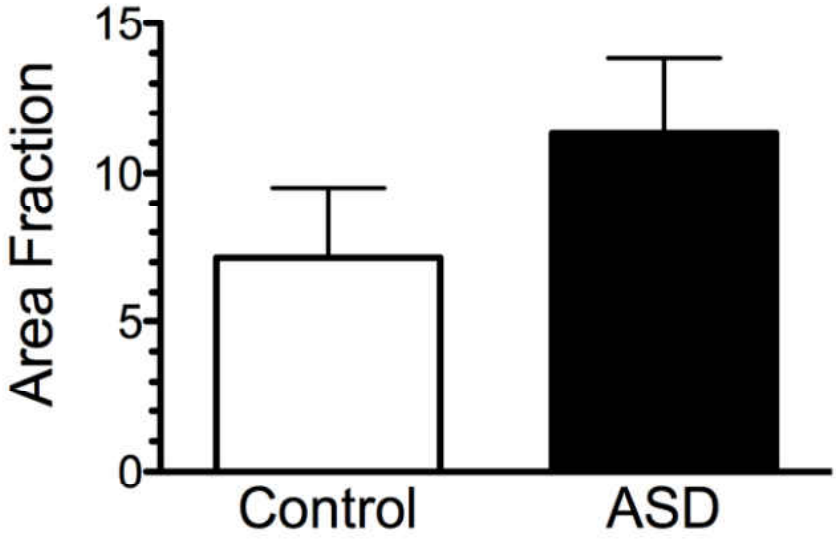

Figure 12. Comparison of area fractions of immunohistochemically stained HLA-DRA in control and ASD donor postmortem tissue (*n*= 8 pairs). No statistically significant difference was observed when the two donor groups were compared.

CD206

For each procedure, excluding the protocols for negative optimization, there was a positive stained and background slide. The positive stained slide was incubated in both primary and secondary antibody, while the background slide was incubated in buffer with only the secondary antibody (minus the primary antibody). The purpose of having both conditions was to verify that what is being stained is not artifactual. During the process of optimization, it appeared that there might be some type of artifact as a result of freezing the tissue. Despite testing multiple primary and secondary antibodies for the mannose receptor, CD206, in the immunohistochemistry experiments, there was no successful identification of this anti-inflammatory microglial protein marker in white matter.

CHAPTER 4

DISCUSSION

ASD is considered a spectrum disorder of atypical connections between brain areas, causing deficits in speech, motor skills, and social interaction. Most research to date has focused on the role of neurons in ASD pathology, while other major cell types in the brain have received far less attention. In the past, our lab has investigated the role of neurons and macroglia (astrocytes and oligodendrocytes) in ASD pathology, but not microglia. As the immune cells for the CNS, microglia protect the brain from pathogenic factors and preserve homeostatic conditions via synaptic pruning and phagocytosis of apoptotic neurons. Microglia are differentiated into two distinct phenotypes: pro-inflammatory and anti-inflammatory. Pro-inflammatory microglia produce cytokines and assume phagocytic roles that promote defense mechanisms and digest neurons. In contrast, anti-inflammatory microglia facilitate CNS healing by participating in phagocytosis, neuronal remodeling, and tissue regeneration. Given these extremely important roles of microglia in the CNS, it is imperative to characterize and determine the potential contributory role of these cells to the pathology of ASD.

Within the field of ASD research, pathology studies using postmortem brain tissues have been mainly limited to examining brain areas as a whole, without considering potential differences in white and gray matter. It is known that microglia reside in both white and gray matter, however; it is still unknown whether there is a difference in the functions of microglia that exist in white as compared to those that occur in gray matter, such as is the case for macroglia (McKay et al. 2007). The present research is the first study to begin to characterize microglial phenotypes at the cellular level in both white and gray matter in ASD. The primary findings of this study demonstrate a significantly high level of expression of insulin-like growth

factor 1 gene, *IGF1,* in ACC white matter and a significantly low level of expression of mannose receptor type 1, *MRC1*, in ACC gray matter from donors with ASD when compared to typically developed controls.

IGF1 is a mitogenic factor imperative for fetal brain development and growth. In the brain, IGF1 encourages the differentiation and maturation of oligodendrocytes, myelination, and neuronal survival. It is also able to act as an opponent to ROS-related processes by inactivating mediators that participate in cell death (reviewed by Homolak et al. 2015). In humans, there is an age-related decline in IGF1 levels and there is increasing evidence that *IGF1* expression is reduced in neurodegenerative disorders such as Alzheimer's disease and Parkinson's disease (reviewed by Bassil et al. 2014). Interestingly, a case study reported that a 15-year-old boy with mental retardation had a homozygous partial deletion of the *IGF1* gene (Woods et al. 1996). Likewise, *IGF1* knockout mice display postnatal lethality, developmental retardation, defects in organ systems, and infertility (Liu et al. 2000). A pilot study involving 9 children with Phelan-McDermid syndrome, a highly penetrant cause of ASD, reported an association between three months of IGF1 treatment and substantial improvement in social impairment and behavior (Kolevzon et al. 2014). Oddly, reduced levels of *IGF1* is associated with an extended lifespan in invertebrates and rodents (Yang et al. 2005). In fact, it is thought that IGF1 antagonists, used to decrease IGF1 signaling, could impede cancerous cell proliferation and the process of aging (reviewed by Bassil et al. 2014). In contrast to the effects of reduced IGF1 action, there is a significant upregulation of *IGF1* in subcortical white matter after traumatic brain injury (TBI) in male mice, suggesting that sustained *IGF1* levels offer neuroprotection after TBI (Madathil et al. 2010). However, *IGF1* overexpression may lead to cell death by speeding up the cell cycle and the process of aging (Yang et al. 2005; reviewed by Bassil et al. 2014). These studies

demonstrate the importance of IGF1 to normal development and aging. It can be hypothesized that tight regulation of *IGF1* expression in humans is essential to prevent morbidities that are linked with conditions of deficient or excessive *IGF1* expression (Yang et al. 2005).

IGF1 binds tightly with the insulin-like growth factor 1 receptor (IGF-1R), triggering the auto-phosphorylation of the intracellular β-subunit kinase domain of IGF-1R. This auto-phosphorylation recruits adaptor proteins and subsequently, activates several pathways such as the MAP kinase and PI3-kinase/Akt pathways (Moloney et al. 2008). Through the MAP kinase signaling pathway, the binding of IGF1 to IGF-1R initiates cell proliferation and differentiation (Conti et al. 2011; Fernandez and Torres-Alemán 2012). Activation of the PI3-kinase pathway inhibits cellular apoptosis, oxidative stress, and inflammation (Conti et al. 2011; Fernandez and Torres-Alemán 2012). Both the MAP kinase and PI3-kinase/Akt pathways activate mTOR, which through increasing the cap-dependent translation initiation complex, can increase mRNA translation that can influence multiple developmental functions (Levitt et al. 2009). Interestingly, Faridar and coworkers (2014) reported increased activation of the MAP kinase pathway in mice with ASD-like social and behavioral deficits (Faridar et al. 2014). The increase in *IGF1* expression in white matter found in the present study could translate to elevated mTOR activation because the mTOR pathway is downstream of IGF1 signaling. If this is occurring, an upregulation of mTOR and its activity in individuals with ASD would confirm a hypothesis made by other researchers that mTOR is in fact upregulated in ASD, causing an increase in unregulated protein synthesis (Sawicka and Zukin 2012; Wang and Doering 2013; Chen et al. 2014).

The high level of *IGF1* expression in ASD donors relative to control donors may reflect a compensatory mechanism of the brain in response to altered neurotransmission in the ACC, to

decreased structural integrity of the ACC white matter, or deficits in cognitive processing by encouraging the proliferation of oligodendrocytes and myelination. Increasing proliferation and differentiation of oligodendrocytes by IGF1 would in turn potentially myelinate neuronal axons, increase synchronization between brain areas, and increase white matter structural integrity. Hence, it is not possible at this point to know whether *IGF1* gene expression changes are casual in the pathology of autism or as a result of cellular processes that are activated to correct deficits that have their root causes in other pathological mechanisms. With the prevalence of myelinating oligodendrocytes in the white matter, it is plausible that the demand for IGF1 in the white matter of ASD individuals may be higher than in the gray matter, consistent with our findings. Additionally, the increase in *IGF1* expression could possibly be compensating for low IGF-1R levels, in which case, the expression of IGF-1R could be a limiting factor in the efficacy of IGF1 actions in the CNS (Madathil and Saatman 2015).

While the liver is the principal source of *circulating* IGF1, IGF1 is also expressed by neurons, microglia, macrophages, and astrocytes in the CNS (Mascotti et al. 1997; Kettenmann and Ransom 2013). The main source of IGF1 in the brain remains a mystery; some report that microglia and macrophages are the main expressers, while others state that neurons predominantly express IGF1 (Suh et al. 2013; reviewed by Madathil and Saatman 2015). Because the main source of IGF1 in the CNS remains controversial and the dissection method that was used in the present work is not specific for a particular cell type, the *IGF1* expression changes found in this study may not be solely expressed by anti-inflammatory microglia. Other prominent cell types within the white matter, such as astrocytes and oligodendrocytes, could be the source of the elevated IGF1 expression. Given the fact that neurons do not normally reside in the white matter, it is highly unlikely the elevated *IGF1* expression in ASD is occurring in

neurons. In fact, we did not observe an elevation of *IGF1* expression in ASD in gray matter, where neurons occur in high numbers.

IGF1 also has the ability to inactivate ROS, suggesting the increase in *IGF1* expression may be indicative of increased pro-inflammatory microglia, and thus ROS, in individuals with ASD. This theory could only be upheld if IGF1 was in fact, being expressed by anti-inflammatory microglia. Moreover, overactive pro-inflammatory microglia in ASD individuals is not the most convincing hypothesis. One would reason that if pro-inflammatory microglia were causing chronic inflammation in individuals with ASD, we should have also seen a significant increase in one or more of the pro-inflammatory genes investigated in this study. This conclusion leads to the possibility that another cell type (but not neurons) in the CNS is responsible for the increased levels of *IGF1*. Despite what is known about IGF1, further research is required in order to determine the cellular source of the elevated *IGF1* expression levels and the potential role those levels play in the pathology of ASD.

Studies that have attempted to characterize anti-inflammatory microglia have reported *MRC1* as an M2 expressed gene (reviewed by Cherry et al. 2014; Benson et al. 2015; Walker et al. 2015). CD206, the protein form of *MRC1*, is a single domain transmembrane receptor that recognizes mannose, fucose, or *N*-acetylglucosamine residues on the surface proteins of various microorganisms such as *C. albicans, Leishmania donovani, Mycobacterium tuberculosis, Pneumocystis carinii,* HIV, and Dengue virus (Gazi and Martinez-Pomares 2009; Kerrigan and Brown 2009). After recognition by CD206, cellular F-actin depolymerizes so that the foreign material can be engulfed by the cell and digested via the endocytic pathway (reviewed by Gazi and Martinez-Pomares 2009). While the full functions of *MRC1* expression by anti-

inflammatory microglia in the brain are still unknown, CD206 is important for endocytosis and pinocytosis (of surrounding fluids in the CNS) (Lively and Schlichter 2012).

In this present study, lower *MRC1* expression levels in gray matter in ASD donors were found as compared to typically developed controls. This finding is novel in ASD research as there are no existing studies reporting the involvement of the mannose receptor in ASD. One study found that the expression of the mannose receptor was lower in mouse serum during the early stages of inflammation, but was upregulated during the later stages of inflammation. During the later stages of inflammation, the mannose receptor clears inflammatory mediators. In fact, some inflammatory inducers have been shown to down-regulate the expression of the mannose receptor, such as LPS and INFγ (Shepherd et al. 1990; Lee et al. 2002). While this is an interesting connection between the literature and our findings, it is important to point out that the findings by Lee and coworkers was in serum and not in brain tissue. In this regard, the lower *MRC1* expression levels found by Lee et al. are most likely expressed by macrophages and not microglia, since microglia are only found in the CNS. Unfortunately, the distinction between macrophages and microglia in the brain is not clear. While microglia are considered the "macrophages of the CNS," macrophages from other locations in the body are able to cross through the blood brain barrier during an inflammatory response. Additionally, most markers that are stated to be expressed by M1 and M2 microglia are also expressed by M1 and M2 macrophages. With regards to our results, we are not able to confidently say that the lower *MRC1* expression levels in gray matter were being expressed by anti-inflammatory microglia. If the ASD donors happen to have elevated levels of pro-inflammatory mediators, it is possible that the lower *MRC1* expression is occurring from macrophages that have migrated into the CNS.

This reinforces the need, in future studies, to isolate anti-inflammatory microglia and study the expression of the differentially expressed genes found in this study.

IL1β (encoded by the gene *IL1B*) is an inflammatory cytokine that can stimulate inflammation in tissues by activating immune cells in early stages of an immune response (reviewed by Goines and Ashwood 2013). Piton and coworkers (2008) found an association between IL1β receptor associated proteins and ASD, while Ashwood and coworkers (2011) found increased plasma IL1β levels in children with ASD (Piton et al. 2008; Ashwood et al. 2011). It has also been reported that in children with ASD, there is excessive production of IL1β in response to LPS (reviewed by Goines and Ashwood 2013). In our study, we found no statistically significant differences in the expression levels of *IL1B* in white nor gray matter from ASD compared to control donors; however, we did find high levels of variability in the expression levels of *IL1B* in the ASD donors in both matter types. While the variability of *IL1B* expression could suggest an increased pro-inflammatory response in some ASD donors, it is important to consider the cause of donor death. A cause of death such as infection or drowning may cause in increase in pro-inflammatory cytokines and ROS, thus may not accurately represent typical cytokine expression levels in ASD (Bierens 2014).

In summary, while we are not able to distinguish the origin of the differential expression levels for *IGF1* and *MRC1*, we are able to conclude that there is an increase of *IGF1* expression and a decrease of *MRC1* expression in the ASD. These findings give strong justification for future investigations of the specific roles of IGF1, CD206, and pro-inflammatory and anti-inflammatory microglia in ASD pathology. The current findings advance ASD research by providing information for the experimental design to study glia pathology, which could ultimately lead to the development of novel therapeutic options and advanced diagnostic tools.

Limitations

While white matter and gray matter were analyzed separately, each homogenate of matter contained many different CNS cell types such as neurons, astrocytes, oligodendrocytes, microglia, etc. As noted above, a distinction between these two types of brain matter is the general lack of neurons in white matter and thus, the enrichment of glia in white matter relative to gray matter. The anti-inflammatory microglial phenotype is difficult to characterize due to the lack of anti-inflammatory microglial specific markers. Specifically, when discussing the significant expression differences of *IGF1* and *MRC1*, it is difficult to decipher the source of these differential expression levels because they are expressed on a wide array of cells. Reported M2 microglial phenotypic polarizers (stimulators) *IL10*, *IL4, IL13, TGF*β, *TNFα*, and *INFγ* are released by and activate a variety of CNS cell types such as neurons, astrocytes, pericytes, dendritic cells, and other immune cells (Feuerstein et al. 1994; Ledeboer et al. 2002; Gottfried-Blackmore et al. 2009; Kovac et al. 2011; Gadani et al. 2012; Chhor et al. 2013; Villapol et al. 2013; Benson et al. 2015). Additionally, *ARG1* has been reported to be expressed by anti-inflammatory microglia in mice, yet it has been debated in the literature to be expressed in humans. Our study showed low levels of *ARG1* expression in ACC white matter and no statistically significant difference comparing ASD and control donors. Similar to *ARG1*, other reported anti-inflammatory microglial makers such as *YM1, FIZZ1, Dectin-1, and CD301* have been observed in animal studies or have been found to be expressed by human M2 macrophages, but the expression of those genes by human M2 microglia is argued (reviewed by Cherry et al. 2014). Other M2 microglial markers *CD163* and *CD204* are known to be expressed by anti-inflammatory microglia, but are also reported to be expressed by additional cell types, such as macrophages and monocytes (Holfelder et al. 2011; Prosniak et al. 2013). While it is necessary

to isolate anti-inflammatory microglia in order to determine its role in ASD pathology, the lack of definitive markers for the M2 phenotype remains a major limitation of this line of research.

The availability of postmortem ASD brain tissues is also limited, preventing us from working with larger sample sizes. For all gene expression data, p-values were corrected for the number of comparisons of dependent variables (gene expressions) using the Holm's Bonferroni correction. Corrected p-values indicated no statistically significant difference between ASD and control donors for all of the genes studied, including *IGF1* and *MRC1* (**Table 6**). We attribute lack of Bonferroni-adjusted significance of *IGF1* and *MRC1* expression levels to our small sample size. The immunohistochemistry portion of this study was originally attempted using frozen BA24 tissue, however; after many attempts with CD206 antibodies, protocol changes, and negative slide (no primary antibody) optimizations, an immunohistochemical stain for the anti-inflammatory mannose receptor was not successful. At the time of the study, we had not received fixed BA24 tissue, so an immunohistochemical stain on fixed tissue was not feasible. Additionally, because of the limited availability of tissues, some ASD and control donors were exposed to medications (**Table 2**) that might potentially influence the outcomes of this study. When donors were matched, variation between pairs was reduced as much as possible.

Due to the lack of understanding regarding the anti-inflammatory microglial phenotype, we decided to first examine four anti-inflammatory microglial markers, in addition to five pro-inflammatory microglial markers, using punch-dissected white and gray matter. While punch-dissections of white and gray matter capture microglia along with various additional cell types present in the tissue, this approach was ideal for the initial investigation of microglia in ASD. Specifically capturing microglia via laser capture microdissection methods were limited by time and financial resources. The decision was made that if significant changes in gene expression

between ASD and control donors were found in this study, we would then proceed to specifically capture microglial cells for further analysis.

Finally, an elevation of the expression levels of a gene does not necessarily translate to an increase in protein levels of the product of that gene. Translation of protein from mRNA is regulated by a variety of factors. Hence, further research will be required to confirm that elevated IGF1 or reduced MRC1 mRNA levels translate to elevated IGF1 or reduced MRC1 protein levels. Regardless of whether there is a like change in protein levels for these genes, it is certain that a difference in gene expression levels (comparing ASD and control donors) implies a pathological process involving these genes in ASD.

Future Studies

While we are not able to confidently say our findings are specific to microglia, the results of this study do provide a basis for future investigations using immunohistochemistry to identify anti-inflammatory microglia and laser capture microdissection to specifically select microglia from white and gray matter BA24 tissue for further gene expression studies. While the literature indicates successful immunohistochemical identification of CD206 in fixed brain tissue, the late arrival of fixed BA24 tissue in our lab did not allow for optimization due to time constraints. Continuation of this study would begin with the immunohistochemical identification of CD206 in fixed brain tissue and then the explicit capture of anti-inflammatory microglia using laser capture microdissection. Once anti-inflammatory microglia are captured, the expression of *IGF1* in white matter and *MRC1* in gray matter can be analyzed to determine if anti-inflammatory microglia are the source of these differential expression levels. The mentioned methods can also be used to capture astrocytes as a secondary investigation in the study.

Increasing the donor sample size and isolating glial cells in ACC white and gray matter are needed to confirm the differential expression levels of *IGF1* and *MRC1* identified in ASD in the present study. This work is currently underway in the laboratory. The gene and protein expression of IGF-1R is also an imperative aspect to examine. If there is differential expression of *IGF-1R* in ASD individuals, it could theoretically affect the efficiency of increased IGF1 levels that are implicated to occur in ASD by the result of this study. Furthermore, studying the individual components of the mTOR pathway (downstream of IGF1 signaling) in individuals with ASD is a necessary step to further ASD research. Identifying potential abnormalities in a specific component(s) of the mTOR pathway could provide clues for the development of therapeutic agents.

REFERENCES

Amor S, Puentes F, Baker D, van der Valk P. 2010. Inflammation in neurodegenerative diseases. Immunology 129:154–69.

Arnold L, Henry A, Poron F, Baba-Amer Y, van Rooijen N, Plonquet A, Gherardi RK, Chazaud B. 2007. Inflammatory monocytes recruited after skeletal muscle injury switch into antiinflammatory macrophages to support myogenesis. J. Exp. Med. 204:1057–69.

Ashwood P, Krakowiak P, Hertz-Picciotto I, Hansen R, Pessah I, Van de Water J. 2011. Elevated plasma cytokines in autism spectrum disorders provide evidence of immune dysfunction and are associated with impaired behavioral outcome. Brain. Behav. Immun. 25:40–5.

Atladóttir HO, Pedersen MG, Thorsen P, Mortensen PB, Deleuran B, Eaton WW, Parner ET. 2009. Association of family history of autoimmune diseases and autism spectrum disorders. Pediatrics 124:687–94.

Auer H, Lyianarachchi S, Newsom D, Klisovic MI, Marcucci uido, Kornacker K. 2003. Chipping away at the chip bias: RNA degradation in microarray analysis. Nat. Genet. 35:292 293.

Baio J. 2014. Prevalence of Autism Spectrum Disorders among Children Aged 8 Years Autism and Developmental Disabilities Monitoring Network, 11 Sites, United States, 2010.

Bassil F, Fernagut P-O, Bezard E, Meissner WG. 2014. Insulin, IGF-1 and GLP-1 signaling in neurodegenerative disorders: Targets for disease modification? Prog. Neurobiol. 118:1 18.

Benson MJ, Manzanero S, Borges K. 2015. Complex alterations in microglial M1/M2 markers during the development of epilepsy in two mouse models. Epilepsia 56:895–905.

Bierens JJLM, editor. 2014. Drowning: Prevention, Rescue, Treatment. Second Edi. Verlag Berlin Heidelberg: Springer.

Bill BR, Geschwind DH. 2009a. Genetic advances in autism: heterogeneity and convergence on shared pathways. Curr. Opin. Genet. Dev. 19:271–278.

Bill BR, Geschwind DH. 2009b. Genetic advances in autism: heterogeneity and convergence on shared pathways. Curr. Opin. Genet. Dev. 19:271–8.

Brown GC, Neher JJ. 2012. Eaten alive! Cell death by primary phagocytosis: "phagoptosis". Trends Biochem. Sci. 37:325–32.

Burguillos MA, Svensson M, Schulte T, Boza-Serrano A, Garcia-Quintanilla A, Kavanagh E, Santiago M, Viceconte N, Oliva-Martin MJ, Osman AM, et al. 2015. Microglia-Secreted Galectin-3 Acts as a Toll-like Receptor 4 Ligand and Contributes to Microglial Activation. Cell Rep. 10:1626–1638.

Bush G, Luu P, Posner MI. 2000. Cognitive and emotional influences in anterior cingulate cortex. Trends Cogn. Sci. 4:215–222.

Casano AM, Peri F. 2015. Microglia: Multitasking Specialists of the Brain. Dev. Cell Rev. 32:9.

Chen J, Alberts I, Li X. 2014. Dysregulation of the IGF-I/PI3K/AKT/mTOR signaling pathway in autism spectrum disorders. Int. J. Dev. Neurosci. 35:35–41.

Chen S-W, Zhong X-S, Jiang L-N, Zheng X-Y, Xiong Y-Q, Ma S-J, Qiu M, Huo S-T, Ge J,

Chen Q. 2016. Maternal autoimmune diseases and the risk of autism spectrum disorders in offspring: A systematic review and meta-analysis. Behav. Brain Res. 296:61–9.

Cherry JD, Olschowka JA, O'Banion MK. 2014. Neuroinflammation and M2 microglia: the good, the bad, and the inflamed. J. Neuroinflammation 11:98.

Chhor V, Le Charpentier T, Lebon S, Oré M-V, Celador IL, Josserand J, Degos V, Jacotot E,

Hagberg H, Sävman K, et al. 2013. Characterization of phenotype markers and neuronotoxic potential of polarised primary microglia in vitro. Brain. Behav. Immun. 32:70–85.

Conti E, Musumeci MB, De Giusti M, Dito E, Mastromarino V, Autore C, Volpe M, Jeschke MG, Barrow RE, Suzuki F, et al. 2011. IGF-1 and atherothrombosis: relevance to pathophysiology and therapy. Clin. Sci. (Lond). 120:377–402.

Corraliza IM, Soler G, Eichmann K, Modolell M. 1995. Arginase induction by suppressors of nitric oxide synthesis (IL-4, IL-10 and PGE2) in murine bone-marrow-derived macrophages. Biochem. Biophys. Res. Commun. 206:667–73.

Crawford JD, Chandley MJ, Szebeni K, Szebeni A, Waters B, Ordway GA. 2015. Elevated GFAP Protein in Anterior Cingulate Cortical White Matter in Males with Autism Spectrum Disorder. Autism Res.:9.

David S, Kroner A. 2011. Repertoire of microglial and macrophage responses after spinal cord injury. Nat. Rev. Neurosci. 12:388–99.

DeRamus TP, Kana RK. 2015. Anatomical likelihood estimation meta-analysis of grey and white matter anomalies in autism spectrum disorders. NeuroImage Clin. 7:525–536.

Derecki NC, Cronk JC, Lu Z, Xu E, Abbott SBG, Guyenet PG, Kipnis J. 2012. Wild-type microglia arrest pathology in a mouse model of Rett syndrome. Nature 484:105–9.

Dichter GS, Felder JN, Bodfish JW. 2009. Autism is characterized by dorsal anterior cingulate hyperactivation during social target detection. Soc. Cogn. Affect. Neurosci. 4:215–226.

Dufour-Rainfray D, Vourc'h P, Tourlet S, Guilloteau D, Chalon S, Andres CR. 2011. Fetal exposure to teratogens: evidence of genes involved in autism. Neurosci. Biobehav. Rev. 35:1254–65.

Durkin MS, Maenner MJ, Newschaffer CJ, Lee L-C, Cunniff CM, Daniels JL, Kirby RS, Leavitt

L, Miller L, Zahorodny W, et al. 2008. Advanced parental age and the risk of autism spectrum disorder. Am. J. Epidemiol. 168:1268–76.

Edmonson CA, Ziats MN, Rennert OM. 2016. A Non-inflammatory Role for Microglia in Autism Spectrum Disorders. Front. Neurol. 7:9.

Eglitis MA, Mezey E. 1997. Hematopoietic cells differentiate into both microglia and macroglia in the brains of adult mice. Proc. Natl. Acad. Sci. U. S. A. 94:4080–5.

Faridar A, Jones-Davis D, Rider E, Li J, Gobius I, Morcom L, Richards LJ, Sen S, Sherr EH, Samuels I, et al. 2014. Mapk/Erk activation in an animal model of social deficits shows a possible link to autism. Mol. Autism 5:57.

Fernandez AM, Torres-Alemán I. 2012. The many faces of insulin-like peptide signalling in the brain. Nat. Rev. Neurosci. 13:225–239.

Feuerstein GZ, Liu T, Barone FC. 1994. Cytokines, inflammation, and brain injury: role of tumor necrosis factor-alpha. Cerebrovasc. Brain Metab. Rev. 6:341–60.

Gadani SP, Cronk JC, Norris GT, Kipnis J. 2012. IL-4 in the Brain: A Cytokine To Remember. J. Immunol. 189:4213–4219.

Gazi U, Martinez-Pomares L. 2009. Influence of the mannose receptor in host immune responses. Immunobiology 214:554–561.

Geschwind DH. 2008. Autism: many genes, common pathways? Cell 135:391–5.

Glees P. 2005. The Human Brain. Cambridge University Press.

Goines PE, Ashwood P. 2013. Cytokine dysregulation in autism spectrum disorders (ASD): possible role of the environment. Neurotoxicol. Teratol. 36:67–81.

Gomot M, Bernard FA, Davis MH, Belmonte MK, Ashwin C, Bullmore ET, Baron-Cohen S. 2006. Change detection in children with autism: an auditory event-related fMRI study.

Neuroimage 29:475–84.

Gordon S. 2003. Alternative activation of macrophages. Nat. Rev. Immunol. 3:23–35.

Gottfried-Blackmore A, Kaunzner UW, Idoyaga J, Felger JC, McEwen BS, Bulloch K. 2009. Acute in vivo exposure to interferon-enables resident brain dendritic cells to become effective antigen presenting cells. Proc. Natl. Acad. Sci. 106:20918–20923.

Greimel E, Nehrkorn B, Schulte-Rüther M, Fink GR, Nickl-Jockschat T, Herpertz-Dahlmann B, Konrad K, Eickhoff SB. 2013. Changes in grey matter development in autism spectrum disorder. Brain Struct. Funct. 218:929–42.

Groen WB, Buitelaar JK, van der Gaag RJ, Zwiers MP. 2011. Pervasive microstructural abnormalities in autism: a DTI study. J. Psychiatry Neurosci. 36:32–40.

Hallmayer J, Cleveland S, Torres A, Phillips J, Cohen B, Torigoe T, Miller J, Fedele A, Collins J, Smith K, et al. 2011. Genetic heritability and shared environmental factors among twin pairs with autism. Arch. Gen. Psychiatry 68:1095–102.

Hanisch U-K. 2013. Functional diversity of microglia - how heterogeneous are they to begin with? Front. Cell. Neurosci. 7:65.

Hoaglin DC, Iglewicz B. 1987. Fine-Tuning Some Resistant Rules for Outlier Labeling. J. Am. Stat. Assoc. 82:1147–1149.

Holfelder K, Schittenhelm J, Trautmann K, Haybaeck J, Meyermann R, Beschorner R. 2011. De novo expression of the hemoglobin scavenger receptor CD163 by activated microglia is not associated with hemorrhages in human brain lesions. Histol. Histopathol. 26:1007–17.

Homolak J, Janeš I, Filipović M. 2015. The role of IGF-1 in neurodegenerative diseases. Gyrus 3:162–167.

Huber KM, Klann E, Costa-Mattioli M, Zukin RS. 2015. Dysregulation of Mammalian Target of Rapamycin Signaling in Mouse Models of Autism. J. Neurosci. 35:13836–13842.

Hung S-I, Chang AC, Kato I, Chang N-CA. 2002. Transient expression of Ym1, a heparin binding lectin, during developmental hematopoiesis and inflammation. J. Leukoc. Biol. 72:72–82.

Ingalhalikar M, Parker D, Bloy L, Roberts TPL, Verma R. 2011. Diffusion based abnormality markers of pathology: toward learned diagnostic prediction of ASD. Neuroimage 57:918–27.

Keil A, Daniels JL, Forssen U, Hultman C, Cnattingius S, Söderberg KC, Feychting M, Sparen P. 2010. Parental autoimmune diseases associated with autism spectrum disorders in offspring. Epidemiology 21:805–8.

Kerrigan AM, Brown GD. 2009. C-type lectins and phagocytosis. Immunobiology 214:562–75.

Kettenmann H, Hanisch U-K, Noda M, Verkhratsky A. 2011. Physiology of microglia. Physiol. Rev. 91:461–553.

Kettenmann H, Kirchhoff F, Verkhratsky A. 2013. Microglia: New Roles for the Synaptic Stripper. Neuron 77:10–18.

Kettenmann H, Ransom BR. 2013. Neuroglia. Third. New York: Oxford University Press.

Kigerl KA, Gensel JC, Ankeny DP, Alexander JK, Donnelly DJ, Popovich PG. 2009. Identification of Two Distinct Macrophage Subsets with Divergent Effects Causing either Neurotoxicity or Regeneration in the Injured Mouse Spinal Cord. J. Neurosci. 29:13435–13444.

Kim SK. 2015. Recent update of autism spectrum disorders. Korean J. Pediatr. 58:8–14.

Know your brain-Cingulate cortex. 2015. Neurosci. Challenged . [accessed 2016 Jun 28].

http://www.neuroscientificallychallenged.com/blog//know-your-brain-cingulate-cortex

Kohls G, Schulte-Rüther M, Nehrkorn B, Müller K, Fink GR, Kamp-Becker I, Herpertz Dahlmann B, Schultz RT, Konrad K. 2013. Reward system dysfunction in autism spectrum disorders. Soc. Cogn. Affect. Neurosci. 8:565–72.

Kolevzon A, Bush L, Wang AT, Halpern D, Frank Y, Grodberg D, Rapaport R, Tavassoli T, Chaplin W, Soorya L, et al. 2014. A pilot controlled trial of insulin-like growth factor-1 in children with Phelan-McDermid syndrome. Mol. Autism 5:54.

Kovac A, Erickson MA, Banks WA, Neuwelt E, Abbott N, Abrey L, Banks W, Blakley B, Davis T, Engelhardt B, et al. 2011. Brain microvascular pericytes are immunoactive in culture: cytokine, chemokine, nitric oxide, and LRP-1 expression in response to lipopolysaccharide. J. Neuroinflammation 8:139.

Ledeboer A, Brevé JJP, Wierinckx A, van der Jagt S, Bristow AF, Leysen JE, Tilders FJH, Van Dam A-M. 2002. Expression and regulation of interleukin-10 and interleukin-10 receptor in rat astroglial and microglial cells. Eur. J. Neurosci. 16:1175–85.

Lee JE, Liang KJ, Fariss RN, Wong WT. 2008. Ex vivo dynamic imaging of retinal microglia using time-lapse confocal microscopy. Invest. Ophthalmol. Vis. Sci. 49:4169–76.

Lee SJ, Evers S, Roeder D, Parlow AF, Risteli J, Risteli L, Lee YC, Feizi T, Langen H, Nussenzweig MC, et al. 2002. Mannose receptor-mediated regulation of serum glycoprotein homeostasis. Science 295:1898–901.

Levitt P, Campbell DB, Newschaffer CJ, Knudsen EI, Thatcher R, Hammock EAD, Levitt P, Dawson G, Beauchaine TP, Strassberg Z, et al. 2009. The genetic and neurobiologic compass points toward common signaling dysfunctions in autism spectrum disorders. J. Clin. Invest. 119:747–754.

Liu JL, Yakar S, LeRoith D. 2000. Conditional knockout of mouse insulin-like growth factor-1 gene using the Cre/loxP system. Proc. Soc. Exp. Biol. Med. 223:344–51.

Livak KJ, Schmittgen TD. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25:402–8.

Lively S, Schlichter LC. 2012. Age-Related Comparisons of Evolution of the Inflammatory Response After Intracerebral Hemorrhage in Rats. Transl. Stroke Res. 3:132–146.

Madathil SK, Evans HN, Saatman KE. 2010. Temporal and regional changes in IGF-1/IGF-1R signaling in the mouse brain after traumatic brain injury. J. Neurotrauma 27:95–107.

Madathil SK, Saatman KE. 2015. IGF-1/IGF-R Signaling in Traumatic Brain Injury: Impact on Cell Survival, Neurogenesis, and Behavioral Outcome. CRC Press/Taylor & Francis.

Mantovani A, Sica A, Sozzani S, Allavena P, Vecchi A, Locati M. 2004. The chemokine system in diverse forms of macrophage activation and polarization. Trends Immunol. 25:677–86.

Margulies DS, Kelly a. MC, Uddin LQ, Biswal BB, Castellanos FX, Milham MP. 2007. Mapping the functional connectivity of anterior cingulate cortex. Neuroimage 37:579–588.

Martinez-Nunez RT, Louafi F, Sanchez-Elsner T. 2011. The interleukin 13 (IL-13) pathway in human macrophages is modulated by microRNA-155 via direct targeting of interleukin 13 receptor alpha1 (IL13Ralpha1). J. Biol. Chem. 286:1786–94.

Di Martino A, Ross K, Uddin LQ, Sklar AB, Castellanos FX, Milham MP. 2009. Functional brain correlates of social and nonsocial processes in autism spectrum disorders: an activation likelihood estimation meta-analysis. Biol. Psychiatry 65:63–74.

Mascotti F, Cá Ceres A, Pfenninger KH, Quiroga S. 1997. Expression and Distribution of IGF-1 Receptors Containing a ▪ -Subunit Variant (▪ gc) in Developing Neurons. J. Neurosci.

17:1447–1459.

McFadden K, Minshew NJ. 2013a. Evidence for dysregulation of axonal growth and guidance in the etiology of ASD. Front. Hum. Neurosci. 7:671.

McFadden K, Minshew NJ. 2013b. Evidence for dysregulation of axonal growth and guidance in the etiology of ASD. Front. Hum. Neurosci. 7:671.

McKay SM, Brooks DJ, Hu P, McLachlan EM, Hauben E, Schwartz M, Popovich P, Jones T, Popovich P, Wei P, et al. 2007. Distinct types of microglial activation in white and grey matter of rat lumbosacral cord after mid-thoracic spinal transection. J. Neuropathol. Exp. Neurol. 66:698–710.

Moloney AM, Griffin RJ, Timmons S, O 'connor R, Ravid R, Neill CO'. 2008. Defects in IGF - 1 receptor , insulin receptor and IRS - 1 / 2 in Alzheimer ' s disease indicate possible resistance to IGF - 1 and insulin signalling.

Mosser DM, Edwards JP. 2008. Exploring the full spectrum of macrophage activation. Nat. Rev. Immunol. 8:958–69.

Neher JJ, Neniskyte U, Zhao J-W, Bal-Price A, Tolkovsky AM, Brown GC. 2011. Inhibition of microglial phagocytosis is sufficient to prevent inflammatory neuronal death. J. Immunol. 186:4973–83.

Noriuchi M, Kikuchi Y, Yoshiura T, Kira R, Shigeto H, Hara T, Tobimatsu S, Kamio Y. 2010. Altered white matter fractional anisotropy and social impairment in children with autism spectrum disorder. Brain Res. 1362:141–9.

Paolicelli RC, Bolasco G, Pagani F, Maggi L, Scianni M, Panzanelli P, Giustetto M, Ferreira TA, Guiducci E, Dumas L, et al. 2011. Synaptic pruning by microglia is necessary for normal brain development. Science 333:1456–8.

Pardo C a, Vargas DL, Zimmerman AW. 2005. Immunity, neuroglia and neuroinflammation in autism. Int. Rev. Psychiatry 17:485–495.

Piton A, Michaud JL, Peng H, Aradhya S, Gauthier J, Mottron L, Champagne N, Lafrenière RG, Hamdan FF, S2D team, et al. 2008. Mutations in the calcium-related gene IL1RAPL1 are associated with autism. Hum. Mol. Genet. 17:3965–74.

Prosniak M, Harshyne LA, Andrews DW, Kenyon LC, Bedelbaeva K, Apanasovich T V, Heber Katz E, Curtis MT, Cotzia P, Hooper DC, et al. 2013. Glioma grade is associated with the accumulation and activity of cells bearing M2 monocyte markers. Clin. Cancer Res. 19:3776–86.

Raes G, Noël W, Beschin A, Brys L, de Baetselier P, Hassanzadeh GHG. 2002. FIZZ1 and Ym as tools to discriminate between differentially activated macrophages. Dev. Immunol. 9:151–9.

Rao JS, Kellom M, Kim H-W, Rapoport SI, Reese EA. 2012. Neuroinflammation and synaptic loss. Neurochem. Res. 37:903–10.

Rodriguez JI, Kern JK. 2011. Evidence of microglial activation in autism and its possible role in brain underconnectivity. Neuron Glia Biol. 7:205–13.

Saijo K, Glass CK. 2011. Microglial cell origin and phenotypes in health and disease. Nat. Rev. Immunol. 11:775–87.

Sawicka K, Zukin RS. 2012. Dysregulation of mTOR Signaling in Neuropsychiatric Disorders: Therapeutic Implications. Neuropsychopharmacology 37:305–306.

Schebesch C, Kodelja V, Muller C, Hakij N, Bisson S, Orfanos CE, Goerdt S. 1997. Alternatively activated macrophages actively inhibit proliferation of peripheral blood lymphocytes and CD4+ T cells in vitro. Immunology 92:478–486.

Sebat J, Lakshmi B, Malhotra D, Troge J, Lese-Martin C, Walsh T, Yamrom B, Yoon S, Krasnitz A, Kendall J, et al. 2007. Strong association of de novo copy number mutations with autism. Science 316:445–9.

Shechter R, Miller O, Yovel G, Rosenzweig N, London A, Ruckh J, Kim K-W, Klein E, Kalchenko V, Bendel P, et al. 2013. Recruitment of Beneficial M2 Macrophages to Injured Spinal Cord Is Orchestrated by Remote Brain Choroid Plexus. Immunity 38:555–569.

Shepherd VL, Abdolrasulnia R, Garrett M, Cowan HB. 1990. Down-regulation of mannose receptor activity in macrophages after treatment with lipopolysaccharide and phorbol esters. J. Immunol. 145:1530–6.

Sieger D, Moritz C, Ziegenhals T, Prykhozhij S, Peri F. 2012. Long-range Ca2+ waves transmit brain-damage signals to microglia. Dev. Cell 22:1138–48.

Silk TJ, Rinehart N, Bradshaw JL, Tonge B, Egan G, O'Boyle MW, Cunnington R. 2006. Visuospatial processing and the function of prefrontal-parietal networks in autism spectrum disorders: a functional MRI study. Am. J. Psychiatry 163:1440–3.

Soehnlein O, Lindbom L. 2010. Phagocyte partnership during the onset and resolution of inflammation. Nat. Rev. Immunol. 10:427–39.

Starossom SC, Mascanfroni ID, Imitola J, Cao L, Raddassi K, Hernandez SF, Bassil R, Croci DO, Cerliani JP, Delacour D, et al. 2012. Galectin-1 deactivates classically activated microglia and protects from inflammation-induced neurodegeneration. Immunity 37:249–63.

Stein M, Keshav S, Harris N, Gordon S. 1992. Interleukin 4 potently enhances murine macrophage mannose receptor activity: a marker of alternative immunologic macrophage

activation. J. Exp. Med. 176:287–92.

Stevens SA, Nash K, Koren G, Rovet J. 2013. Autism characteristics in children with fetal alcohol spectrum disorders. Child Neuropsychol. 19:579–87.

Strathearn L. 2009. The elusive etiology of autism: nature and nurture? Front. Behav. Neurosci. 3:11.

Strömland K, Nordin V, Miller M, Akerström B, Gillberg C. 1994. Autism in thalidomide embryopathy: a population study. Dev. Med. Child Neurol. 36:351–6.

Suh H-S, Zhao M-L, Derico L, Choi N, Lee SC. 2013. Insulin-like growth factor 1 and 2 (IGF1, IGF2) expression in human microglia: differential regulation by inflammatory mediators. J. Neuroinflammation 10:37.

Suzuki K, Sugihara G, Ouchi Y, Nakamura K, Futatsubashi M, Takebayashi K, Yoshihara Y, Omata K, Matsumoto K, Tsuchiya KJ, et al. 2013. Microglial Activation in Young Adults With Autism Spectrum Disorder. JAMA Psychiatry 70:49.

Takano T. 2015. Role of Microglia in Autism: Recent Advances. Dev. Neurosci. 37:195–202.

Taylor PR, Martinez-Pomares L, Stacey M, Lin H-H, Brown GD, Gordon S. 2005. Macrophage receptors and immune recognition. Annu. Rev. Immunol. 23:901–44.

Tremblay M-È, Lowery RL, Majewska AK. 2010. Microglial interactions with synapses are modulated by visual experience. PLoS Biol. 8:e1000527.

Urbain CM, Pang EW, Taylor MJ. 2015. Atypical spatiotemporal signatures of working memory brain processes in autism. Transl. Psychiatry 5:e617.

Vargas DL, Nascimbene C, Krishnan C, Zimmerman AW, Pardo C a. 2005. Neuroglial activation and neuroinflammation in the brain of patients with autism. Ann. Neurol. 57:67–81.

Varin A, Gordon S. 2009. Alternative activation of macrophages: immune function and cellular biology. Immunobiology 214:630–41.

Villapol S, T. T, J. A. 2013. Role of TGF-β Signaling in Neurogenic Regions After Brain Injury. In: Trends in Cell Signaling Pathways in Neuronal Fate Decision. InTech.

Vogt BA, Finch DM, Olson CR. 1992. Functional Heterogeneity in Cingulate Cortex: The Anterior Executive and Posterior Evaluative Regions. Cereb. Cortex 2:435–443.

Walker DG, Lue L-F, McGeer P, Itagaki S, McGeer E, Perry V, Nicoll J, Holmes C, Waller R, Woodroofe M, et al. 2015. Immune phenotypes of microglia in human neurodegenerative disease: challenges to detecting microglial polarization in human brains. Alzheimers. Res. Ther. 7:56.

Wang H, Doering LC. 2013. Reversing autism by targeting downstream mTOR signaling. Front. Cell. Neurosci. 7:28.

Wing L, Gould J, Gillberg C. 2011. Autism spectrum disorders in the DSM-V: better or worse than the DSM-IV? Res. Dev. Disabil. 32:768–73.

Woods KA, Camacho-Hübner C, Savage MO, Clark AJL. 1996. Intrauterine Growth Retardation and Postnatal Growth Failure Associated with Deletion of the Insulin-Like Growth Factor I Gene. N. Engl. J. Med. 335:1363–1367.

World Health Organization. 2013. Autism spectrum disorders & other developmental disorders: From raising awareness to building capacity. Geneva, Switzerland: WHO Press.

Worley JA, Matson JL. 2012. Comparing symptoms of autism spectrum disorders using the current DSM-IV-TR diagnostic criteria and the proposed DSM-V diagnostic criteria. Res. Autism Spectr. Disord. 6:965–970.

Yang J, Anzo M, Cohen P. 2005a. Control of aging and longevity by IGF-I signaling. Exp.

Gerontol. 40:867–72.

Zimmerman AW, Jyonouchi H, Comi AM, Connors SL, Milstien S, Varsou A, Heyes MP. 2005. Cerebrospinal Fluid and Serum Markers of Inflammation in Autism. Pediatr. Neurol. 33:195–201.

APPENDICES

APPENDIX A: ABBREVIATIONS

Abbreviation	Definition
ACC	anterior cingulate cortex
ADI-R	Autism Diagnostic Interview-Revised
ARG1	*arginase 1*
ASD	autism spectrum disorder
BA24	Brodmann area 24
CD68	*cluster of differentiation 68*
CNS	central nervous system
CNV	copy number variation
DAB	3,3'-Diaminobenzidine tetrahydrochloride
DSM	Diagnostic and Statistical Manual
EMP	erythromyeloid progenitor
FMR1	fragile X mental retardation 1
fMRI	functional magnetic resonance imaging
GAPDH	*glyceraldehyde-3-phosphate dehydrogenase*
GM	gray matter
HLA-DRA	*human leukocyte antigen-antigen D related, alpha chain*
ID	identification number
IGF1	*insulin-like growth factor 1*
IGF-1R	Insulin-like growth factor 1 receptor
IL1B	*interleukin 1 beta*
iNOS	inducible nitric oxide synthase
Kb	kilobase
LPS	lipopolysaccharide
M1	pro-inflammatory/classically activated/neurotoxic
M2	anti-inflammatory/alternatively activated/neuroprotective
MRC1	*mannose receptor type 1*
mTOR	mammalian target of rapamycin
NFM1	Neurofibromatosis 1
NHS	normal horse serum
NO	nitric oxide
NOS2	*nitric oxide synthase 2, inducible*
PMI	post-mortem interval
PPARG	*peroxisome proliferator-activated receptor gamma*
PTEN	phosphatase and tensin homolog
PTGS2	*prostaglandin-endoperoxide synthase 2*
qPCR	quantitative real-time polymerase chain reaction
RIN	RNA integrity number

ROS	reactive oxygen species
SNP	single nucleotide polymorphism
TATA	*TATA-box binding protein*
T1D	type 1 diabetes
TLR4	toll-like receptor 4
TSC1/2	tuberous sclerosis complexes 1 and 2
WM	white matter

APPENDIX B: PRIMER SEQUENCES OF REFERENCE AND MARKER GENES

Target or Reference Gene	Genbank Accession Number	Primer Sequence	PCR Product Size (bp)
ARG1 (set A)	NM_001244438, NM_000045	(f) 5'-GGT GAC TCC CTG TAT ATC TGC CAA G-3' (r) 5'-GCC AAT TCC TAG TCT GTC CAC TTC AG-3'	136
CD68	NM_001040059, NM_001251	Not available (Qiagen)	73
GAPDH	NM_002046	(f) 5'-TGC ACC ACC AAC TGC TTA GC-3' (r) 5'-GGC ATG GAC TGT GGT CAT GAG-3'	87
HLA-DRA (set C)	NM_019111	(f) 5'-GTG GAC AAA GCC AAC CTG GAA ATC-3' (r) 5'-GGA CGT TGG GCT CTC TCA GTT C-3'	121
IGF1 (set B)	NM_001111283, NM_001111284, NM_000618	(f) 5'-TCC CTT TCA AGC CAC CCA TTG A-3' (r) 5'-AGT GTG TTT AGC AGC GGG TAC AAG-3'	115
IL1B	NM_000576, XM_006712496	Not available (Qiagen)	117
MRC1	NM_001009567, NM_002438	Not available (Qiagen)	86
NOS2	NM_006554 (1399 bp)	(f) 5'-GGC TGT CGT TGA GAT CAA CAT TGC TGT G-3' (r) 5'-CGG GAC CGG TAT TCA TTC TGC ATG TAC T-3'	123
PPARγ	NR_027850 (1603 bp)	(f) 5'-TCT CAA ACG AGA GTC AGC CT-3' (r) 5'-GAG TGG GAG TGG TCT TCC ATT AC-3'	120
PTGS2 (set B)	XM_006712199 (1229 bp)	(f) 5'-CTC TGG CTA GAC AGC GTA AAC T-3' (r) 5-CCG TAG ATG CTC AGG GAC TTG-3'	143
TATA	NM_001172085, NM_003194	Not available (Qiagen)	132

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