5-2011

Cytokine genetics and expression : implications of an immunogenetic pathogenesis in autism spectrum disorders.

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CYTOKINE GENETICS AND EXPRESSION: IMPLICATIONS OF AN IMMUNOGENETIC PATHOGENESIS IN AUTISM SPECTRUM DISORDERS

by

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A Dissertation
Submitted to the Faculty of the
School of Medicine of the University of Louisville
in Partial Fulfillment of the Requirements
for the degree of

Doctor of Philosophy

Department of Anatomical Sciences and Neurobiology
University of Louisville
Louisville, Kentucky

May 2011

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A Dissertation Approved on

May 3, 2011

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DEDICATION

This dissertation is dedicated to my parents

Michael William Mott

and

Denise Carey Mott

who always support me without question and no matter what the cost.

ACKNOWLEDGEMENTS

I would like to thank Manuel Casanova, M.D., for his guidance as my mentor and his dedication to autism research. My writing and thinking has improved immeasurably as a result of his input and influence. I would like to thank Fabian Crespo, Ph.D., for his incredible patience teaching me techniques in the lab, and Christopher Tillquist, Ph.D., for teaching me population genetics. I would like to thank Andy Switala for helping me with my statistical analyses, and Will Tucker for teaching me how to use Luminex® technology. I would like to thank Lonnie Sears, Ph.D., for helping with my SNP project and training me to administer the ADI-R. I would like to thank Jane Pickett, Ph.D., at the Autism Tissue Program for supplying our lab with the post-mortem brain specimens used for my transcriptional analysis. I would like to thank P. Gail Williams, M.D., and the nurses at the Kosair Pediatric Research Unit for collecting blood samples for my glutathione analysis. I would like to thank the members of the anatomy teaching team for their support and guidance, particularly Raymond Ho, Ph.D., Nobuyuki Kuwabara, Ph.D., Jennifer Brueckner-Collins, Ph.D., and Nicole Herring, Ph.D. Also, I would like to thank my family and friends who supported me when times were rough, fed me when I was too busy to feed myself, and helped me maintain my sanity throughout this process: Michael and Denise Mott, Travis Mott, Bonnie Patterson, Linda and Mike Ianke, Peter Sloane, Melissa Veatch, Gina Collecchia, Clayton Marshall, Chris Nevitt, Mike Foster, Claudio Gonzalez, Pearce Shanks, Patrick Ormerod, Steve McCoy, and Alison Brotzge.

ABSTRACT

CYTOKINE GENETICS AND EXPRESSION: IMPLICATIONS OF AN IMMUNOGENETIC PATHOGENESIS IN AUTISM SPECTRUM DISORDERS

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May 3, 2011

Autism Spectrum Disorder (ASD) is an idiopathic pervasive neurodevelopmental disorder associated with various neuropathologies and immunological dysfunctions. Cytokines are regulatory proteins that facilitate communication between the immune and central nervous systems and mediate inflammation, immunity, and hemopoiesis. Previous literature demonstrates that cytokine expression is altered systemically and in the central nervous system of individuals diagnosed with ASD when compared to matched neurotypical controls. Here it is proposed that cytokines are crucial mediators in autism pathogenesis. The central hypothesis of this research posits that an underlying genetic susceptibility in cytokine genes is triggered by environmental exigencies (e.g., stress, infection, ultrasound, hypoxia, pollutant or chemical exposure) during prenatal development. This hypothesis proposes that the convergence of these scenarios during vulnerable periods of neurodevelopment ultimately culminates in the autism phenotype. To test whether cytokines are crucial mediators in autism pathogenesis, the DNA sequences of 22 single nucleotide polymorphisms (SNPs) within 13 cytokine genes were genotyped in a cohort of autistic patients and controls. Three SNP frequencies for both

pro-inflammatory [IL1R(+1970)] and anti-inflammatory [IL4(-590) and IL4(-33)] cytokine genes were found to be significantly associated with autism incidence. Next, cytokine mRNA profiles were investigated in post-mortem cortical tissue of eight autistic subjects and eight matched controls. Transcriptional profiling of cytokine genes in five post-mortem cortical regions corresponding to Brodmann Areas 4, 9, 17, 22 and 46 indicated heterogeneous expression of cytokine (TNF-α, IL-6, TGFβ-1, IL-1β) and chemokine (IL-8) transcripts in autistic subjects, but these alterations did not reach statistical significance or reflect values of cortical cytokine translational patterns established in previous literature. Finally, it was shown that systemic cytokine translational expression in the blood plasma of children diagnosed with autism disorder was not modulated with intravenous glutathione administration. These findings indicate that cytokines play an important role in ASD pathogenesis and reveal possible molecular mechanisms that warrant future investigation in etiological research. They also show that the antioxidant agent glutathione, which ostensibly alters cytokine expression at the intracellular level, does not affect systemic cytokine expression or ameliorate behavioral outcome when administered exogenously.

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CHAPTER I

Introduction

Autism: Defining the Clinical Phenotype

When the term 'autism' was first introduced in 1912 by Swiss psychiatrist Eugen Bleuler (1857-1939), it was used to describe schizophrenic behavior. Etymologically, the word autism is derived from the Greek terms *autos*, or self, and *-ismos*, a suffix denoting an action or state. Bleuler observed several clinical manifestations of autism in his schizophrenic patients, referring to their dementia as a "detachment from reality with relative and absolute predominance of the inner life" (Parnas et al., 2002). Schizophrenics were often withdrawn and socially inaccessible, and seemed to live in a world of their own. Bleuler attempted to integrate autism into the diagnostic criteria for schizophrenia. This goal, however, was never realized as autistic behavior is only present in a subset of individuals with the disorder, and thus is not considered a core feature of the schizophrenic pathology. Instead, autism would come to define a pervasive developmental disorder demonstrated in pediatric populations only a few years after Bleuler's death.

As the father of pediatric psychiatry, Leo Kanner (1894-1981) could also be considered the father of autism research. After his emigration from Austria in 1924, Kanner soon founded the Johns Hopkins Children's Psychiatric Clinic. In 1938, he began

studying a group of extremely detached children who had either been diagnosed with childhood schizophrenia or were considered emotionally disturbed. Five years later, Kanner published his seminal article "Autistic disturbances of affective contact," in which he proposed that this group of 11 children, composed of eight boys and three girls who were all under the age of 11 years old, suffered from a unique syndrome that seemed to afflict children of highly intelligent parents. He described them as "happiest when left alone," "like in a shell," with an "anxiously obsessive desire for sameness" and language that contained innumerable verbal rituals and echolalia which was "not meant to have communicative value" (Kanner, 1943). Although many of these children had been diagnosed as 'feebleminded,' Kanner ascertained that they were not cognitively impaired; on the contrary, they were endowed with excellent vocabularies and uncanny memory for past events as well as rote memory for precise and complex patterns and sequences. Kanner concluded that they were suffering from what he termed "early infantile autism," and that while it behaviorally resembled many features found in schizophrenia, this condition had an inherently distinct symptomatology.

At the same time, Hans Asperger (1906-1980), a Viennese pediatrician, noticed a similar pattern of behavior in four boys referred to him at the University of Vienna Children's Hospital. In 1944 he published his Habilitation, or second doctoral thesis, "Autistic psychopathy in childhood," in which he identified behavioral deficits and abilities in these children that clearly resembled the characteristics Kanner had recently qualified. Although he described "autistic psychopathy" only one year after Kanner's article was published, Asperger's contribution remained largely unknown outside of German literature until it was translated into English in 1991. Once translated, it was

clear that Asperger's accounts echoed Kanner's. While Asperger focused on milder manifestations of autism in less affected children, he emphasized that this disorder had a distinct range of deficits, and was most easily recognizable when it appeared concomitantly with mental retardation. In his thesis, Asperger described isolated and "particularly interesting and highly recognizable" children who suffered from "severe and characteristic difficulties in social integration," extreme clumsiness, "emotional poverty," stereotypic movements, "strikingly odd" eye gaze, "a paucity of facial and gestural expression," and stilted, idiosyncratic language that "feels unnatural" due to their unusual diction (Asperger, 1991).

Both Kanner and Asperger are considered pioneers of autism research because they were the first to define the disorder as a distinct and recognizable clinical entity in the field of pediatric psychiatry. It is certainly interesting that two Austrian physicians on different continents independently and simultaneously discovered the same disorder and gave it the same descriptive label. Both authors described their patients as socially isolated often with aggressive and destructive behavioral problems, clumsy in gait and gross motor performance, and impaired with language deficits that included the reversal of pronouns, the tendency to invent words and the use idiosyncratic language. Kanner and Asperger noted that non-verbal aspects of communication were also impaired, which included poor eye contact, lack of expressive gestures and odd vocal intonation during speech. Additionally, they each recognized several other features, namely deficient imaginative play, repetitive patterns of activities that included stereotyped play and body movements, fixation on objects and a dislike of environmental change, odd responses to sensory stimuli, hypersensitivity to noise, and special abilities which usually involved

rote memory skills. While their behavioral observations were strikingly similar, their interpretations of the underlying cause of autistic behavior were very different. Kanner was preoccupied with attributes he thought were shared by parents of autistic children, namely a "maternal lack of genuine warmth" that led to the conception of 'refrigerator mothers,' which predominated autism etiological theory in the 1950s (Kanner, 1949). Asperger, however, considered autism an inherited personality disorder and emphasized that it had a genetic basis. Both authors noted that these autistic behaviors were much more common in boys than in girls.

Comparisons of what later became known as Kanner's early infantile autism and Asperger's syndrome yielded significant disparities between the two otherwise very similar conditions. Kanner's autism could be apparent in infants as early as six months of age, whereas Asperger contended that his syndrome was rarely diagnosed until the third year of life or later. While both physicians agreed that deficiencies in non-verbal communication and social interaction were distinguishing features of the disorder, language skills and acquisition differed significantly between them. Kanner's autism was associated with markedly delayed speech onset, abnormal speech or selective mutism, while children suffering with Asperger's syndrome could learn good use of grammar and vocabulary, although the content was often impoverished and inappropriate in social context and complex meanings were poorly understood (Wing, 1981). Asperger's accounts maintained that his patients often learned to speak before walking, and that walking occurred later than normal, while Kanner's observations indicated that walking developed normally or even earlier than average, while speech developed later or not at all (Van Krevelen, 1971). These distinctive characteristics between Kanner's early

infantile autism and Asperger's syndrome led investigators to question whether the two conditions were simply varieties of the same underlying abnormality or if they were in fact two separate entities.

Four decades after the initial publication of their seminal articles, research and clinical findings suggested that a clear distinction between Kanner's autism and Asperger's syndrome did not exist. While Kanner never acknowledged Asperger's work, Asperger recognized that there were several similarities between his syndrome and Kanner's early infantile autism. Subsequent authors agreed that the two conditions could be differentiated, but that they were more alike than different, and thus could not be considered mutually exclusive diagnostic categories (Van Krevelen, 1971; Wolff & Barlow, 1979). Their dissimilarities were interpreted as variations in severity of the same clinical behavioral pattern, where individuals with Asperger's syndrome showed fewer signs and were less severely affected than those with Kanner's autism. Investigators recognized hallmark behavioral discrepancies between the two similar disorders:

> *"Children who do not talk or who parrot speech and use strange idiosyncratic phrases, who line up toys in long rows, who are oblivious to other people, who remember meaningless facts – these will rightly conjure up Kanner's memory. Children and adults who are socially inept but often socially interested, who are articulate yet strangely ineloquent, who are gauche and impractical, who are specialists in unusual fields – these will always evoke Hans Asperger's name."* (Frith, 1991)

Although Kanner's autism and Asperger's syndrome had somewhat different profiles of cognitive, language and motor functions, it was posited that they could be considered subclassifications on the same 'autistic continuum' (Wing, 1988). Lending further support to this argument, the same individual who was considered autistic in early life

could be diagnosed with Asperger's syndrome in adolescence and adulthood (Wing & Gould, 1979).

Currently, the Diagnostic and Statistical Manual for Mental Disorders (DSM) defines autism as a pervasive developmental disorder which manifests during the first three years of life and is qualified by a triad of impairments in reciprocal social interactions, the development of communication, and severely restricted, stereotyped and repetitive patterns of interests and behaviors (American Psychiatric Association, 2000). To account for the variations of deficiencies and abilities that are present in affected individuals, the DSM considers autism a spectrum disorder, where subtypes are categorized for diagnostic purposes. The three subtypes that compose the autism spectrum include: autistic disorder, Asperger's disorder, and pervasive developmental disorder not otherwise specified (PDD-NOS).

Autistic disorder is defined as classic autism, essentially the same condition identified by Kanner. Qualitative impairments in social interaction are often manifested as marked impairments in eye-to-eye gaze or communicative gestures, inappropriate or limited facial expressions, failure to develop peer relationships at the appropriate developmental level, a lack of spontaneous seeking to share enjoyment or interests with others, and a lack of social or emotional reciprocity. Communicative impairments often include a delay in or lack of spoken language, impairment in the ability to initiate or sustain a conversation, and stereotyped or repetitive use of language or idiosyncratic language. Manifestations of restricted and repetitive patterns of interests or behaviors frequently include an encompassing preoccupation with a subject of interest that is abnormal in intensity, an inflexible adherence to routines or rituals, stereotyped and

repetitive motor mannerisms (e.g. hand flapping or twisting or complex whole-body movements) and a preoccupation with parts of objects. In order for a subject to be diagnosed with autistic disorder, a total of six of the abovementioned items must be present with at least two in the category of social interaction, and one per category of communication and restricted/repetitive behaviors or interests.

Criteria for diagnosis of Asperger's disorder and PDD-NOS are very similar to those of autistic disorder, although behavioral differences are apparent. In Asperger's disorder, qualitative impairments in social interaction and manifestations of restricted and repetitive behaviors or interests are similar to those that typify autistic disorder; however, no clinically significant general delay in language or cognitive development is present. PDD-NOS is considered 'atypical autism', where individuals do not meet criteria for either autistic or Asperger's disorder but do present with severe or pervasive impairments in social interaction, verbal or non-verbal communication, or have the restricted or repetitive behaviors or interests previously mentioned. Although these subjects exhibit autistic symptomatologies, these are usually atypical, subthreshold, or occur at a late age of onset and thus cannot be classified as either autistic or Asperger's disorder. For a complete and thorough review of diagnostic criteria for each subgroup contained with the autism spectrum, please refer to the appendix.

As the abovementioned DSM criteria demonstrates, diagnosing autism spectrum disorders (ASDs) can be challenging since it is solely based on behavioral and developmental observation. Research indicates that at age two, an ASD diagnosis can be considered very reliable (C. Lord et al., 2006). The American Academy of Pediatrics recommends that pediatricians screen children for ASDs at 18 months and 24 months of

age. There are a variety of screening tools available for health care professionals to assess the general development of ASDs. Many incorporate parent reports to increase provider observation of the child's development and promote parent awareness of developmental milestones and disabilities. Examples of parent questionnaires or parent-completed screening tools used to diagnose developmental delays include the Ages and Stages Questionnaires, Communication and Symbolic Behavior Scales, Parents' Evaluation of Developmental Status, Modified Checklist for Autism in Toddlers, and the Screening Tool for Autism in Young Toddlers. Additionally, specialists often screen for ASD diagnosis using one of the following assessments: the Autism Diagnostic Interview-Revised (ADI-R), Autism Diagnostic Observation Schedule-Generic (ADOS-G), Childhood Autism Rating Scale (CARS), and the Gilliam Autism Rating Scale-Second Edition (GARS-2). Pediatric specialists rely on parent interviews and direct observation of the child using standardized autism assessments specific for cognitive and adaptive functioning. While each of these tests has a different method of evaluation, studies have shown that their screening measures have sensitivities and specificities greater than 70% (Committee on Children and Disabilities, 2001).

The reliability of parent-completed screening tools in addition to inter-test diagnostic agreeability and validity represent significant limitations in the consistency of ASD classification assessment. Early intervention programs improve long-term function in children diagnosed with an ASD (D. Dawson & Osterling, 1997). Thus, conflicting diagnoses from different professionals can have significant academic, behavioral and social consequences for the individual child. Evidence indicates that the ADI-R and ADOS-G are the most accurate and reliable assessments available for identifying autism;

however, most evaluators do not use these measurements due to the fact that they are more expensive and require more time and training to administer (C. Lord et al., 2000; M. E. Williams et al., 2009). Cultural and socio-economic status can delay access to diagnostic services available to affected families, make assessment difficult, and result in diagnostic errors (Mandell et al., 2002). An examination of the practices used to evaluate ASDs found that different service systems, measured at public schools, programs evaluating eligibility for developmental disabilities services, and hospital-based programs agreed on ASD diagnosis in the same individual only 45% of the time (M. E. Williams et al., 2009). These discrepancies in diagnosis may be a reflection that professionals do not follow best practice guidelines for assessment of ASDs, and carry significant implications regarding ASD diagnostic criteria measured using different tools by different professionals.

Epidemiology of ASD

The first epidemiological study of autism surveyed 78,000 urban British children aged 8-10 years old between 1963-64, and estimated a prevalence rate of 4.5 per 10,000 (Lotter, 1966). At that time, Kanner's definition of early infantile autism provided the only behaviorally defined criteria for ascertaining the presence of 'autistic conditions.' Thus, this survey's estimate of prevalence may not represent the most accurate estimate of a syndrome poorly defined relative to current standards. Since then, numerous studies have been performed attempting to estimate prevalence of ASDs in countries world-wide, including Australia, Canada, China, Denmark, Finland, France, Germany, Iceland, India, Indonesia, Iran, Ireland, Israel, Japan, Norway, Oman, Singapore, South Africa, Spain,

Sweden, the United Arab Emirates, the United Kingdom, and the United States. Comparison between studies is challenging due to variations in diagnostic assessment tools used and well as changes in diagnostic criteria defined in successive publications of the DSM and the International Classification of Diseases (ICD). Subsequently, it is difficult to assess changes in prevalence rates of ASD over time and in different countries, and even more challenging to extrapolate whether the actual incidence of ASD is related to ethnicity, geographic location, or year of evaluation.

A review of 22 non-overlapping epidemiological studies conducted in 12 countries between the years of 1966-1998 reported a prevalence estimate of 4.4 per 10,000 people diagnosed with autism (Fombonne, 1999). This study found a significant correlation between prevalence rate and year of publication, indicating an increase in prevalence estimates in the 1990s compared to previous decades. The author suggests this increase reflects "an improved recognition and detection of autism together with a broadening of the diagnostic concept and definitions," rather than a true increase in incidence of the disorder (Fombonne, 1999). This review also found a clear association between autism and mental retardation, a significant gender bias where males were approximately 4 times more likely to be affected than females, an association with autism and rare or genetic medical conditions, and no association between autism and social class or parental education. Interestingly, all of the studies surveyed also identified a larger group of children who did not meet diagnostic criteria for autism but qualified for a pervasive developmental disorder.

In contrast to autism studies, very few epidemiological inquiries regarding Asperger's syndrome were investigated until several years later. The first study,

performed in Sweden in 1977, originally estimated a prevalence for Asperger syndrome in seven-year-old children to be 26 per 10,000 (C. Gillberg et al., 1982). Also in the 1970s, British investigators estimated that 1.1 per 10,000 children under the age of 15 years old in London who had been diagnosed as autistic early in life were later diagnosed with Asperger's syndrome (Wing & Gould, 1979). In 1985, another Swedish study estimated the population frequency of Asperger's syndrome in 10,500 children aged 7-16 years old to be 8 per 10,000 (I. C. Gillberg & Gillberg, 1989). Years later, the same Swedish group studied 1,519 7-16 year old children in the same area and estimated the prevalence of Asperger's syndrome to be 28.5 per 10,000; however, their confidence intervals were very large and thus this data should be considered imprecise (Ehlers & Gillberg, 1993). While these early studies suggested that the prevalence of Asperger's syndrome was higher than that for autism, recent findings indicate that prevalence rates of Asperger's syndrome are actually significantly lower (Chakrabarti & Fombonne, 2001; Ellefsen et al., 2007; Kadesjo et al., 1999; Powell et al., 2000; Sponheim & Skjeldal, 1998).

Follow up epidemiological studies of ASD estimated autism prevalence rates for surveys published between 1966-1991 to be 4.4/10,000 while these rates increased to 9-11/10,000 between the years of 1992-2001, with the most recent estimation being 20/10,000 within the last decade (Fombonne, 2001, 2009). In the 1990s, average prevalence estimates for PDD-NOS were approximately 15/10,000 and the rate of Asperger's syndrome was consistently lower, at 2.5/10,000 (Fombonne, 2001). Within the last decade these values have increased to 30/10,000 for PDD-NOS and 6/10,000 for Asperger's syndrome (Fombonne, 2009). These apparent increases in ASD prevalence

rates cannot be directly attributed to increases in incidence of the disorder, however. Research suggests that variability in prevalence of ASDs across studies can be explained by differences in diagnostic criteria, case-finding method and issues of study design (Posserud et al., 2009; J. G. Williams et al., 2006). Referral trends over time are affected by heightened public awareness, availability of diagnostic services, changes in referral patterns, decreasing age at diagnosis, and changes in diagnostic concepts and definitions. Thus, there are multiple confounding factors in ASD prevalence estimate investigations and comparison between studies is problematic. Ideally, repeated surveys that use the same diagnostic tools, performed in the same geographic locations at different periods of time would yield the most accurate prevalence estimates of ASD.

Although evaluating ASD prevalence over time is challenging, epidemiological evidence collected within the last decade suggests that incidence of the disorder is increasing. The Centers for Disease Control and Prevention (CDC) conducted a surveillance study in 2006, which estimated that 1% or one child in every 110 aged 8 years old in the United States is currently diagnosed with an ASD (Centers for Disease Control and Prevention, 2009). Compared to data they collected in 2002, this represented at 57% increase in the average prevalence of ASD measured at the same locations throughout the country (Centers for Disease Control and Prevention, 2007). These sites included areas in Alabama, Arizona, Colorado, Georgia, Maryland, Missouri, North Carolina, Pennsylvania, South Carolina, and Wisconsin. Every site except Colorado reported a significant increase in identified ASD prevalence within all major sex, racial/ethnic and cognitive functioning categories (Centers for Disease Control and Prevention, 2009). Interestingly, these increases were more attributable to autism

diagnosis rather than broader ASD diagnoses (e.g., PDD-NOS or Asperger's syndrome). ASD prevalence estimates of 1% in the United States match recent estimates provided by groups in the United Kingdom and Canada (Baird et al., 2006; Baron-Cohen et al., 2009; Lazoff et al., 2010). The most recent estimate of ASD prevalence was performed in 2007 by the Health Resources and Services Administration's Maternal and Child Health Bureau. This report surveyed over 78,000 children, and found that 1.1% or one child out of 91 in the United States aged 3-17 years old is currently diagnosed with an ASD (Kogan et al., 2009). This study also found a male-to-female ratio of approximately 4:1, and higher ASD prevalence among single mothers and non-Hispanic white children. One limitation of this study that should be mentioned, however, is that it was based on parent-reports of diagnosis, which were not externally validated.

Comparative analysis indicates that ASD prevalence estimates are associated with geographic region. Studies performed outside North America and Europe show significantly lower prevalence estimates for ASDs (Al-Farsi et al., 2011; Honda et al., 2005; Zhang & Ji, 2005). One study compared estimates reported for children born between the years of 1994-1999 within Denmark and Western Australia, and found that overall ASD prevalence rates were significantly higher in Denmark (Parner et al., 2011). It is likely that these differences are due to diagnostic assessment tools and service availability associated with each country. Children in Western Australia are not routinely screened for developmental disabilities as they are in Denmark. Assessments and services are also free in Denmark, whereas individual families are financially responsible for the same services in Western Australia. Studies comparing ASD prevalence rates between countries are only just beginning, and preliminary results indicate that significant

differences exist (Zaroff & Uhm, 2011). Interpretation of this analysis will prove complicated because in addition to differences in diagnostic assessment tools and services, investigators must also take into consideration the effects of culture on information processing specifically regarding social interaction, a core feature in ASD symptomatology. It is also possible that environmental and/or geographical differences could account for disparities in prevalence estimates between countries. Increased prevalence rates indicate that environmental influence plays a particularly significant role in the incidence of ASD, and that research into viable environmental etiologies is warranted.

Regardless of differences in prevalence rates across countries and ethnicities, evidence suggests that ASD incidence may be increasing. One fact is becoming progressively clearer: pervasive developmental disorders are not rare and should be considered an urgent public health concern. Future epidemiological studies should aim to be longitudinal in their analysis of the same geographic location, and make sure to use the same screening tools and diagnostic criteria for assessment. Additionally, population studies performed in different countries should aim to standardize assessment tools in order to make inter-study comparison more feasible. Until then, estimates of ASD prevalence according to birth date and geographic location will prove difficult, and few conclusions can be made regarding changes in incidence rates through time.

It is estimated that American society spends $35 billion annually in direct and indirect costs associated with autism treatment (Ganz, 2006). Direct costs are based on medical and nonmedical expenditures. Direct medical expenses include physician, emergency and other professional services, behavioral therapies, prescription

medications, equipment and other supplies, and medically related travel and time costs. Special education, childcare, home and vehicle modifications, and supported employment services are examples of direct nonmedical expenses. Indirect costs are qualified as loss of productivity measured in income associated with individuals with autism and their caregivers, due to missed work hours, switching to jobs that are lower paying but more flexible, and loss of employment. Age-specific autism data show that direct medical costs are highest for the first five years of life where behavioral therapies are the largest contributor, and that adult care represents the largest lifetime direct cost (Ganz, 2007). Compared to lifetime estimates of direct medical costs for typical Americans, this means that individuals with autism spend twice as much, and spend it at a much younger age (Alemayehu & Warner, 2004). Clearly these financial burdens are daunting, not only for the families of affected individuals, but also for society in general. Taken together, studies that suggest increased incidence combined with significant annual individual and societal cost estimates indicate that etiologic and preventative research in ASD is imperative.

Neuroanatomical and Neuropathological Features

Magnetic resonance imaging (MRI) and postmortem neuropathological studies indicate that ASD is characterized by dynamic age-specific structural and functional abnormalities. Developmental heterochronicity, wherein various brain regions grow at different rates compared to controls, is a defining anatomical feature associated with the disorder (Carper & Courchesne, 2005; Carper et al., 2002; Courchesne et al., 2001; Sparks et al., 2002). Topographic change caused by developmental heterochronicity

likely produces the cytoarchitectural alterations that delineate a distinct neuropathology in autism. The extent of deviation from the normal trajectory of brain development may be used as a diagnostic tool of subtype specificity in the future (Courchesne et al., 2003). Impairments in both local and global connectivity have been described, with an over-development of local connectivity networks at the expense of long distance connectivity (Casanova et al., 2006; Courchesne & Pierce, 2005). The developmental abnormalities that typify ASD, outlined below, are sources of structural and functional change that generate the clinical phenotype.

Young children with ASD are macrocephalic and macroencephalic, two features which manifest prior to and during the time clinical diagnosis is ascertained. Longitudinal studies have shown that at birth, head circumferences of ASD children are significantly smaller than those of neurotypical children, but that by 6-14 months of age ASD head circumferences are significantly larger (Courchesne et al., 2003). Head circumference values are considered an accurate index of brain size, and MRI-based studies reveal that autistic children have significantly increased brain volumes compared to controls (Bartholomeusz et al., 2002; Piven et al., 1996). Brain volumes of autistic children increase the most between the ages of 2 and 4.5 years, where cerebellar and cerebral white matter (WM) accounts for the majority of growth (Courchesne et al., 2001). This macroencephaly does appear to have regional specificity, but volumetric studies are inconsistent in their findings. For example, one study indicated that the greatest increase in WM volume takes place in the frontal lobes, and the least in the occipital lobes (Carper et al., 2002). Still others report that the greatest generalized volume increase occurs in the occipital and parietal lobes (Filipek, 1996; Piven et al., 1996). Brain growth acceleration

precedes and is associated with the onset of clinical symptoms, and the specific pattern of growth reflects the severity of ASD diagnosis (Courchesne et al., 2003; G. Dawson et al., 2007; Dementieva et al., 2005). ASD brain growth trajectories appear to decelerate after the first year of life, plateau during adolescence, and be comparable to controls in adulthood (Redclay & Courchesne, 2005). The functional consequences of abnormal brain development observed in autism explain many of the behavioral characteristics that define the disorder (I. L. Cohen, 2007). Although adult brain sizes are comparable between autistic subjects and matched controls, a core neuropathology remains present, and indicates that functional connectivity in different regions is diminished.

MRI-based studies reveal multiple distinguishing neuranatomical features associated with autism, and the foremost of these involves a significant increase in WM volume. While WM makes up less than a third of total cerebral volume, it accounts for 65% of the volume increase reported in the brain tissue of autistic subjects over controls (Herbert et al., 2003). After adjusting for total brain volume, autistic subjects maintain a significantly greater WM volume compared to age-matched controls, which suggests that this WM volume increase is a specific feature associated with autism rather than a reflection of macrocephaly (Bigler et al., 2010; Herbert et al., 2003). When cerebral WM areas were parcellated into an outer zone of radiate WM composed of intrahemispheric corticocortical connections and an inner zone of bridging and sagittal compartments, the outer radiate WM was increased in all cerebral lobes with frontal lobe predominance while the inner zone WM showed no increase in autism (Herbert et al., 2004). Relative reduction in various areas of the corpus callosum is a consistent finding in autism when compared to controls (Hardan et al., 2000; Hardan et al., 2009; Piven et al., 1997). As

with findings in overall brain enlargement, WM concentrations are not as large compared to controls in older autistic individuals (Chung et al., 2004; Waiter et al., 2005). The overgrowth of specific WM regions is part of a pathologic process that disrupts the development of normal brain structure and function in autism, although the underlying molecular mechanisms by which this occurs are currently not understood.

Volumetric analyses indicate that abnormalities in multiple cortical and subcortical structures are associated with autism. The limbic system, known for its role in emotion, memory and motivation, is consistently affected. Several studies report reductions in the size of the amygdala and hippocampus in autistic subjects, as well as poorer performance on neuropsychological tasks associated with each structure (Aylward et al., 1999; Herbert et al., 2003; Loveland et al., 2008; Saitoh et al., 2001). Still other studies report that children with autism have larger hippocampi compared to controls, while the amygdala is enlarged in only young autistic children (Schumann et al., 2004). While these limbic structures may be larger in autistic children, they have been found to be smaller in adults when compared to controls (Aylward et al., 1999). Thalamic abnormalities have also been observed, and include increased cell packing density, decreased cell size, and an overall decrease in thalamic volume in autistic individuals (Hardan et al., 2006; Schultz et al., 1999; Tsatsanis et al., 2003). Increased parieto-temporal lobe and cerebellar hemisphere volumes are associated with autism (Brambilla et al., 2003). Overgrowth of the frontal and temporal lobes and amygdala are synchronized with the abnormally accelerated brain growth that occurs between the ages of 2-4 years in autistic children (Carper et al., 2002; Hazlett et al., 2005; Sparks et al., 2002). While cortical thinning typically occurs with age, this process appears to be

accelerated in autistic individuals (Hustler et al., 2007). According to voxel-based MRI analysis, gray matter is reduced in a regionally specific manner and CSF total volume is significantly increased in autistic patients when compared to controls (McAlonan et al., 2005). Other studies report gray matter volumetric increases in specific areas in autism (Rojas et al., 2006). While some MRI studies have produced discordant findings, they reveal a deregulation of brain growth in early childhood in autism, wherein growth trajectories are atypical and regionalized.

The first studies to reveal neuropathological changes in autism were performed in the 1980s by numerous groups (Bauman & Kemper, 1985; Courchesne et al., 1988; Gaffney et al., 1987; Ritvo et al., 1986). Five neuropathological features, in particular, are associated with autistic disorder[1]: increased brain weight and WM volume during childhood, reduced neuronal size and increased cell packing density in the forebrain limbic system, reduced numbers of Purkinje cells in the cerebellum, age-related changes in cell size and numbers in the nucleus of the diagonal band of Broca, deep cerebellar nuclei and inferior olive, and malformations of cerebral cortex and brainstem (Bauman & Kemper, 2005). The most consistent finding across postmortem studies in autism is the significant decrease in cerebellar Purkinje cells when compared to controls (Arin et al., 1991; Bailey et al., 1998; Ritvo et al., 1986). Purkinje cells are also smaller in size in autism compared to age and sex-matched controls (Fatemi et al., 2002). Abnormalities in size and number of neurons in the fastigeal, globose and emboliform nuclei have also

[1] As a cautionary note, postmortem neuropathological studies in autism are based on reports of a very small number of brains. Due to the broad age spectrum as well as clinical diversity of ASD, the pattern of neuropathological changes is incomplete and inconsistent. Thus, the morphological markers and neuropathological diagnostic criteria of autism have not been established.

been shown, and appear to change with age (Bauman & Kemper, 1994). What follows is a summary of the collection of neuropathological features in autism that have been observed by different groups.

Cortical and sub-cortical morphological abnormalities associated with autism primarily involve the limbic system. Histological studies have shown that the hippocampus of autistic individuals has reduced cell size and simplified dendritic branching compared to age-matched controls (Raymond et al., 1996). Evidence of neuronal cell size and packing density in autistic amygdale is contradictory. While some studies report a reduction in neuronal size and increased cell packing densities, others find no significant difference in cell size but do report significantly fewer neurons associated with the amygdala of autistic patients (Bauman & Kemper, 1990; Schumann & Amaral, 2006). Cell packing density was reportedly increased in the hypothalamus and mamillary body (Bauman & Kemper, 1985). Smaller neurons have been reported in the basal ganglia and cerebellum of 4- to 7-year-old children with autism, specifically in Purkinje cells, the dentate nucleus, amygdala, nucleus accumbens, caudate and putamen, with corrections in size by adulthood (Wegiel et al., 2008). A generalized reduction in density of axons and dendrites in the autistic brain has also been proposed (Guerin et al., 1996). These studies indicate that a delay of neuronal growth evidenced by cortical dysgenesis, which is brain structure-specific, occurs in autism and undergoes modification during the life span.

Several brainstem and cerebellar morphological abnormalities have been revealed in neuropathological studies on autism. Neurons in the inferior olivary nucleus appear more abundant in autism, but their size varies with age in that they are enlarged in

children younger than 12 years old but smaller in adults over 21 when compared to age-matched controls (Anderson et al., 1993; Kemper & Bauman, 2002). The pons, medulla, and midbrain midsagittal areas are all smaller in autistic subjects, and the pons seems to develop more rapidly in autism compared to controls (Hashimoto et al., 1995). As mentioned previously, the cerebellum is the most consistent site of neural abnormality in autism. Alterations in Purkinje cell density and number appears to be more prominent in specific regions (Arin et al., 1991). Hyerplasia and hypoplasia in cerebellar vermal regions is evident (Courchesne et al., 1994). These studies indicate atrophy of the neocerebellar cortex, with regionally specific marked loss of Purkinje cells.

Neocortical minicolumns, the basic architectonic and functional units of the human brain that organize neurons in cortical space, are smaller, more numerous, and less compact in autistic patients compared to controls (Buxhoeveden & Casanova, 2002; Casanova et al., 2002; Casanova et al., 2006). While this minicolumnar pathology has been observed bilaterally in Brodmann cortical areas 3, 4, 9, 17, 21 and 22, the narrowest minicolumns are found in the dorsolateral prefrontal cortex of autistic subjects (Casanova & Trippe, 2009; Casanova et al., 2006). Reduction in size of neocortical neurons and their nuclei is likely an indicator of reduced or impaired functional connectivity between distant cortical regions with a bias toward local rather than global information processing (Casanova et al., 2006; Just et al., 2004; Koshino et al., 2005). Reductions in corpus callosum and gyral window size confirm that a constrained cortical network of connections exists, which favors short-range corticocortical fibers at the expense of long-range commissural fibers (Casanova et al., 2009). Malformations of cortical development have been observed in disorders caused by abnormalities of cell proliferation, apoptosis,

cell migration, cortical organization and axon pathfinding (Hevner, 2007). Thus, the minicolumnar abnormalities seen in autistic individuals suggest that the cause of the underlying pathology likely occurs during fetal or very early postnatal development.

Studies of clinicopathological correlations in autism reveal a link between several domains of functional deficits and primary neuropathology. One of the core features of ASD symptomatology involves deficient verbal abilities related to the understanding of semantics and social pragmatics (Wetherby et al., 1998). Studies of language-related neocortex reveal reduced neuronal density in Wernicke's area (BA 22) and gyrus angularis (BA 39) and increased glial cell density in both of these regions as well as in Broca's area (BA 44) in autistic subjects when compared to controls (Lopez-Hurtado & Prieto, 2008). Investigators hypothesize that structural alterations in language-related cortical areas contributes to the communication impairment in autism.

Another core feature of ASD behavior includes abnormalities in social reciprocity, eye contact, and facial expression. It has been established that patients with autism have deficits in face processing, perception and recognition (Grelotti et al., 2001; Joseph & Tanaka, 2003). Functional magnetic resonance imaging (fMRI) studies have shown that the fusiform gyrus, which is involved in face-processing, is hypoactive in autistic patients (Pierce et al., 2004). It is believed that this hypoactivation is associated with the failure of autistic subjects to make direct eye contact (Dalton et al., 2005). Neuropathological studies reveal reduced neuronal number and volume in the fusiform gyrus and suggest that an underdevelopment of connections between primary visual cortex (BA 17) and the fusiform gyrus may contribute to abnormal face perception in autism (van Kooten et al., 2008).

Impairments in gross and fine motor function as well repetitive and stereotyped behaviors are common findings in patients with autism. It has been proposed that sensorimotor deficits may be associated with pathological changes in the basal ganglia and cerebellum (Bailey et al., 1998; Sears et al., 1999). A positive correlation between caudate volume and repetitive behavior scores has been reported in autism (Hollander et al., 2005). Cerebellar findings including a decrease in the number of GABAergic Purkinje cells and increased feed-forward inhibition from basket cells indicate altered inhibition of cerebellar nuclei which could directly affect cerebellocortical output and lead to changes in motor behavior and cognition (Arin et al., 1991; Yip et al., 2008).

A particular range of cognitive deficits in autism demonstrate that performance IQ is generally higher than verbal IQ and that comprehension is usually low on intelligence tests (Siegel et al., 1996). These cognitive deficits are likely related to abnormalities in the memory and limbic systems. Size reductions in the hippocampal formation and amygdala in autism have been reported, as well as reduced complexity of dendritic arbors in the hippocampus (Aylward et al., 1999; Bauman & Kemper, 1985). The anterior cingulate gyrus is reduced in volume and positron emission tomography (PET) activity in subjects with autism is decreased (Haznedar et al., 1997). The caudate nucleus is involved in learning, short- and long-term memory, planning and problem solving, thus observed caudate volume changes in autistic children may also explain cognitive deficits that typify autism (Fuh & Wang, 1995; Poldrack et al., 1999; Schmidtke et al., 2002).

Neuroanatomical and neuropathological studies have revealed an atypical pattern of development in ASD. The brains of autistic subjects are generally larger during the onset of clinical symptoms when compared to controls, where WM contributes

disproportionately to brain volume enlargement in a regionally specific manner. Developmental heterochronicity is a defining feature of ASD, however inconsistencies in findings make cross-study comparison difficult. Multiple factors account for these inconsistencies, most notably among them conflicting subject diagnostic and exclusionary criteria. Statistical problems also exist in data collection, due to small sample size, confounding factors such as comorbidity with other disorders, IQ, postmortem interval, cause of death and medication history. While discordant findings exist, it is clear that characteristic neuropathologies are associated with the core symptoms of ASD. Image analysis can be used to distinguish subjects with autistic disorder, Asperger's syndrome or PDD-NOS from controls, which may yield significant diagnostic applications (Akshoomoff et al., 2004; El-Baz et al., 2007). Generalized processing abnormalities related to a constrained neural network underlie the observed and defining behaviors found in ASD and lead to the idea of autism as a neural information processing disorder (Gustafsson, 1997; Happe et al., 2001; Herbert, 2005). Precisely how these neurobiological abnormalities relate to the behavioral phenotype is currently under investigation. Taken together, these findings suggest that while abnormalities seen in the brains of autistic individuals represent an ongoing neuropathology that continues to change through adulthood, this process has a neurodevelopmental component that may be prenatal in origin.

Concept of Inflammation

The immune system serves as a sentinel against foreign invasion, injury, and aberrant cell growth, and propagates the appropriate response necessary to eliminate

foreign antigens, facilitate tissue repair, and maintain homeostasis. The immune response can be divided into two basic components: innate immunity and adaptive immunity. Innate immunity is often referred to as the first line of defense in the host immune system because it is stimulated by common structures shared by groups of microbes. Once microbial presence is recognized, a programmed immunological response is initiated. In the case of a pathogen invasion, for example, innate immune cells recognize pathogen-derived molecular patterns (PAMPS) and molecular patterns from injured tissue via the pattern recognition receptors (PRRs) in their cell membranes. Toll-like receptors (TLRs) are a class of PRRs that are well defined and one of the most conserved components of the innate immune system. The principal components of the innate immune system include physical and chemical barriers, phagocytic cells, natural killer (NK) cells, blood proteins, and cytokines (Abbas et al., 2007). The adaptive immune system is sometimes referred to as the acquired or specific immune system, due to its ability to distinguish between closely related antigens, and the fact that its protective responses are acquired with experience. The main components of the adaptive immune system include lymphocytes and their secreted products, including antibodies. There are two types of adaptive immune responses: humoral and cell-mediated. Humoral immunity is mediated by antibodies, which are produced by B lymphocytes. These antibodies recognize and target extracellular microbes for elimination by effector cells and molecules (e.g., the complement system). Cell-mediated immunity is mediated by T lymphocytes and their products, which include cytokines. These cells recognize and target infected host cells for elimination, thus protecting the host from intracellular microbial infection. Phylogenetically, the innate immune system is older than the adaptive immune system,

which has become increasingly more specialized in more highly evolved species. Both innate and adaptive immune systems function cooperatively in order to maintain an integrated system of host defense.

Inflammation is a cellular reaction of immunity, triggered by injury or infection, which is characterized by vasodilation along with the concurrent recruitment of local immune and chemical mediators acting in concert to eliminate infectious agents and restore homeostasis. Inflammation can result from either innate or adaptive immune system responses. Although it is commonly considered a protective mechanism used to control injury or infection and promote tissue repair, inflammation can also cause tissue damage and disease. In the classical pathogen-initiated inflammatory response, tissue-resident macrophages and mast cells recognize signs of infection and produce a variety of inflammatory mediators; including cytokines and chemokines. Cytokines are a group of regulatory proteins that play vital roles in cellular communication during both the innate and adaptive immune responses. Chemokines are a family of structurally homologous small cytokines that stimulate leukocyte movement and regulate their migration from blood into tissue. Both cytokines and chemokines serve as mediators of inflammation, immunity, and hemopoiesis. When resident immune cells encounter pathogens in the local area, they release cytokines that activate and recruit circulating leukocytes to the site of infection. Recruitment, and thereby the acute-phase immune response, is possible due to the fact that cytokines cause the postcapillary venules in the activated endothelium of local blood vessels to become selectively permeable to leukocyte (mostly neutrophil) extravasation (Medzhitov, 2008). This selectivity is based on the distinct expression of a combination of adhesion molecules and chemokine receptors that exist on neutrophils

and monocytes. In this way, systemic immune cells are allowed access to the infected area so that they can recognize and ingest bound microbes by the process of phagocytosis. Once ingested, the phagosome fuses with lysosomes, creating the phagolysosome. It is within the phagolysosome that microbicidal mechanisms occur. Activated neutrophils or macrophages ultimately destroy microbes with the proteolytic enzymes, reactive nitrogen intermediates, or highly reactive oxidizing agents that are contained in their phagolysosomes.

In an acute inflammatory response, once neutrophils reach the site of injury or infection, they kill any pathogen or antigen-presenting cell (APC) with which they come into contact by releasing the toxic contents of their granules (Nathan, 2002, 2006). If the acute response is successful, the pathogen is destroyed and a tissue repair phase is initiated by anti-inflammatory lipoxins, which recruit monocytes to the area that produce another cytokine: transforming growth factor-β (TGF-β). Together they serve to remove dead cells and begin the tissue remodeling process (Serhan & Savill, 2005). If neutrophils are unable to destroy the pathogen, they are replaced with macrophages and T cells, and chronic inflammation may occur in which granulomas are produced from layers of macrophages and T lymphocytes that cluster together and form walls in order to contain infection and protect the host (Abbas et al., 2007). The mechanisms of infection-based inflammation are well defined compared to inflammation caused by other conditions, particularly chronic inflammatory states. Systemic chronic inflammation seems to be caused by different instigators and characterized by different physiologic mechanisms that are not related to those seen in the acute inflammatory response. For this reason, it has been proposed that the standard view of inflammation as a reaction to infection or

injury should be expanded to account for such inflammatory processes (Medzhitov, 2008).

Resident CNS Immune Cells and Their Role in Neuroinflammation

Neuroinflammation is characterized by the activation of resident immune CNS cells, the expression of proinflammatory mediators, and the infiltration of various cellular components from the peripheral immune system. Although it was once considered an immune-privileged site because of the existence of the blood brain barrier (BBB), accumulating evidence shows that the CNS is capable of immune and inflammatory responses reminiscent of those seen systemically (Rivest, 2009). While pathogen-specific immune responses are limited because the lymphatic system does not extend into the brain, rapid responses to immune challenge occur nonetheless. In response to injury, infection, or tissue dysfunction, the CNS is capable of mounting an appropriate response involving either the innate or adaptive immune elements discussed previously. In addition to recruiting cells from the peripheral immune system, the CNS has its own resident immunological cells and immune signaling pathways. In fact, the human nervous system has a greater range of distinct cell types than any other organ system in the body. Although cell types are different in the CNS, they perform similar functions. Review of the major glial cell types and their immunological roles provides a basis of comparison between inflammation exhibited systemically versus that seen in the CNS.

The term glia originates from the Greek word meaning "glue," as nineteenth century scientists assumed that these cells held the nervous system together. While there is no evidence to support that glial cells bind neurons, they do serve several significant

functions in the developing and adult CNS. They support neurons, provide the brain with structure, produce myelin to insulate nerve cell axons, scavenge for debris following neuronal injury or infection, guide neuronal migration and direct process outgrowth, regulate the properties of the pre-synaptic terminal, and control neuronal metabolism by regulating the substances that reach neurons and supplying the necessary metabolites to axons. Glial cells can be divided into two major classes: microglia and macroglia. Microglia are physiologically and embryologically unrelated to other CNS cell types. Derived from mesencyme, they invade the brain in perinatal stages of development or following brain injury in adulthood. Thus it is not surprising that microglia function as phagocytes that respond to injury, infection and disease in the CNS. There are two types of macroglial cells in the CNS: oligodendrocytes and astrocytes. These cells arise from ectoderm during embryologic development. In white matter, oligodendrocytes envelop several axonal internodes simultaneously, thus insulating these neuronal processes and modulating the rate of nerve signal propagation. In gray matter, oligodendrocytes surround and support neuronal cell bodies. Astrocytes are the most numerous of the glial cells, named for their star-shaped morphology. Although they have several immunological functions, astrocytes are best known for their constitutive role as an element of the BBB. Recent research regarding glial cell dysfunction reveals a common mechanism implicated in the etiology of neurodegenerative and neurodevelopmental disease.

Microglial cells are considered the resident macrophages of the CNS, as they are the primary effector cells that respond to changes in the microenvironment and the principle mediators of inflammation. As the first line of defense against neural infection,

microglial cells are responsible for the innate immune response in the brain. Because of their function in general maintenance, phagocytosis of cellular debris, and expression of toll-like receptors (TLRs), which are types of PRRs that recognize the presence of pathogens in their local environment, microglial cells are easily relatable to the macrophages and dendritic cells seen systemically (Beyer et al., 2000). They differ, however, in that they have lower expression of various cell surface markers, which makes them less effective at recognizing antigens or executing phagocytosis (Davoust et al., 2008). In healthy brain tissue, microglia are considered to be in a resting state with a small cell soma and elaborate branching processes that extend and retract as they monitor an area approximately 30-50μm wide (Raivich, 2005). The motility of microglial processes is so highly specialized that it provides them with the remarkable ability to completely scan the neural parenchyma every few hours with little process overlap (Nimmerjahn et al., 2005). These microglial processes are in direct contact with astrocytes, neurons, and blood vessels, providing the brain with a dynamic and efficient immune surveillance system whose components are able to interact with each other.

Microglia become activated in response to brain injury or various immunological stimulants, which induces a significant morphological change from a ramified to an amoeboid cellular shape (Frank-Cannon et al., 2009; Kreuzberg, 1996). This amoeboid shape allows microglia to perform the same functions as macrophages, namely the ability to phagocytose foreign matter, serve as APCs, produce inflammatory mediators in the form of cytokines and chemokines, and recruit T cells to the site of injury. Damaged tissue, infectious agents, and PAMPs are recognized by microglial PRRs, which trigger the rapid cellular activation and release of several soluble factors that can be pro- or anti-

inflammatory in nature and may also be cytotoxic (Liao et al., 2004; Liu et al., 2002; S. C. Morgan et al., 2004; Moss & Bates, 2001). Microglia are capable of proliferation (microgliosis) upon activation, a condition associated with several pathological conditions (Streit et al., 1999). Meningeal inflammation evidenced by diffuse microglial activation can be particularly detrimental to brain tissue, and is associated with demyelination, substantial neuron, astrocyte and oligodendrocyte loss, and pronounced cortical thinning (Magliozzi et al., 2010). Conversely, research indicates that the proinflammatory response is essential in the promotion of brain and spinal cord tissue repair following injury, specifically in remyelination, axonal regeneration, and locomotor recovery (Arnett et al., 2001; Boivin et al., 2007; Kigerl et al., 2007). Thus it seems that microglia can have either neurotoxic and neuroprotective roles in the inflammatory process, depending on the type of stimulus introduced or the stage of disease progression. If microglial activation persists, the permeability of the BBB may become compromised, allowing increased infiltration of peripheral macrophages, which propagate the neuroinflammatory scenario (Schmid et al., 2009).

As the most abundant glial cell population in the CNS, astrocytes outnumber neurons ten-fold, and they play a pivotal role in maintaining CNS homeostasis and neuronal function (Dong & Benveniste, 2001; Pekney & Nilsson, 2005). Astrocytes contact CNS-resident immune cells and blood vessels through their long processes, which terminate in end-feet. They maintain an extensive network of finely branched processes that, like microglia, occupy contiguous non-overlapping domains (Bushon et al., 2002). Astrocytes interact extensively with microglia, and can exert both pro- and anti-inflammatory effects on them (Farina et al., 2007; Min et al., 2006). While some

important functional components of their end-feet include bringing nutrients to neuronal cells, participating in synaptic function and plasticity, and maintaining an appropriate chemical environment for neuronal signaling, astrocytes are better known for the influence of their perivascular end-feet. When their end-feet contact microvessels within the CNS, astrocytes cause vessel endothelial cells to form tight junctions, which restrict systemic access to the CNS by forming a glia limitans between brain parenchyma and the vascular system: the BBB (Bechmann et al., 2007). Astrocytes are also highly permeable to potassium and maintain the extracellular potassium ion concentration between neurons. These functions protect neurons from excess firing that could interfere with cell signaling. In addition to maintaining the integrity of the BBB and protecting neurons from surfeit potassium influx, astrocytes detoxify excess excitatory amino acids, metabolize neurotrophic factors, and secrete proinflammatory cytokines and chemokines (Bauer et al., 2001; Nedergaard et al., 2002; Prat et al., 2001). Like microglial cells, astrocytes express basal levels of TLRs, where TLR3 is most predominantly expressed (Bsibsi et al., 2006; Farina et al., 2005). Importantly, astrocytes interact with and modulate the actions of other cell types such as microglia and monocytes, and even down-regulate their activation (Andjelkovic et al., 2000; Kostianovsky et al., 2008).

Like microglia, astrocytes are also responsible for propagating the neuroinflammatory scenerio. There is evidence that in addition to forming the BBB, astrocytes up-regulate many BBB features in response to particular exposures within their microenvironment (Haseloff et al., 2005; Hayashi et al., 1997; Sobue et al., 1999). This process can have detrimental ramifications. Several CNS pathologies involve disturbances of BBB function and astrocyte-endothelial cooperation including stroke,

bacterial infection, multiple sclerosis, HIV, Alzheimer's disease, Parkinson's disease, epilepsy, and brain tumors (Abbott et al., 2006). Reactive astrogliosis occurs in response to infection, trauma, ischemia, and neurodegenerative disease, which causes molecular and morphologic changes in astrocytes, and may also cause scar formation (Eddleston & Mucke, 1993; Maragakis & Rothstein, 2006; Pekney & Nilsson, 2005). Glial scar formation occurs in response to extreme tissue damage and inflammation when astrocyte proliferation and processes overlap to form barriers that prevent axon regeneration and inflammatory cell infiltration in an effort to protect healthy tissue (Bush et al., 1999; Herrmann et al., 2008). In this way, glial scars can be neuroprotective by forming potent cell migration barriers that separate areas of intense inflammation from healthy tissue, and prevent the infiltration of inflammatory cells or infectious agents (Sofroniew, 2005). Recent research indicates that astrocytes enhance neutrophil survival and regulate neutrophil function, a process that may be considered neuroprotective since necrotic neutrophils are neurotoxic and enhancing their phagocytotic abilities is protective against microbial infection (Xie et al., 2010). It is becoming increasingly clear that different specific signaling mechanisms trigger different functional changes in reactive astrocytes in a context-dependent manner, which can impact neural function, regulation of blood flow, synaptic function and plasticity (Sofroniew, 2009).

Resident oligodendrocytes and neuronal cells are also capable of participating in the innate immune response of the CNS. Cellular apoptosis, necrosis, and inflammation are associated with white-matter damage in response to CNS injury (Raghupathi, 2004). Pericytes, perivascular macrophages and neurons have been shown to contribute to BBB induction (Ramsauer et al., 2002; Schiera et al., 2003; Zenker et al., 2003). In addition to

microglial cells, other types of CNS macrophages exist of mesodermal origin, including perivascular, meningeal, and chroid plexus macrophages. Recent studies have found that, like astrocytes and microglial cells, perivascular and chroid plexus macrophages also express TLRs (Crack & Bray, 2007; Laflamme & Rivest, 2001). There is ample evidence to suggest that almost all CNS cell types participate in the pathophysiology of CNS trauma and immune activation as a byproduct of being a component in a complex neural network that can have both beneficial and detrimental effects depending on environmental context. No doubt the discovery of new neuroimmunomodulatory mechanisms will continue to evolve the neuroinflammatory paradigm.

Resident CNS immune cells share many similarities with systemic immune cells, in that they express various PRRs, communicate using the same chemical mediators such as cytokines, and modulate inflammatory behavior through a complex cellular network. Embryologically, most neuroimmune cells are derived from different tissue than systemic immune cells, with the exception of microglia. Although these cells are physiologically different, they perform similar functions as their systemic counterparts. Acute neuroinflammatory responses, like systemic ones, are usually short-lived and considered beneficial to the CNS because they repair damaged tissue and prevent further injury. Chronic neuroinflammation, however, is detrimental and damaging to the nervous system. It is characterized by long term activation of microglial cells, increased expression of inflammatory mediators, and increased oxidative and nitrosative stress (Tansey et al., 2007). Chronic neuroinflammation has been implicated in the etiology of various neurodegenerative disorders, including multiple sclerosis, Alzheimer's disease, Parkinson's disease, and Huntington's disease (Block & Hong, 2005; E. G. McGeer &

McGeer, 2007). Here, it is proposed that neuroinflammatory processes play a role in the etiology and pathophysiology of ASD.

Cytokine Polymorphisms: Implications in CNS Disease

Cytokines are a diverse group of soluble regulatory proteins that function as mediators in many physiological processes, including inflammation, immunity and hemopoiesis. As mediators, cytokines regulate the migration, activation, proliferation, differentiation, and function of the many types of cells involved in inflammatory and immune responses. While they are essential to maintaining protective immunity, cytokines have been implicated in autoimmune diseases and diseases with a chronic inflammatory component (Borish & Steinke, 2003).

Cytokines are usually very complex in their activities. Most cytokines are highly pleiotropic and act on many different cell types exerting different effects. Different cytokines can have redundant effects, or their activities can be either synergistic or antagonistic depending on the target cells. Normally, cytokines do not act alone, but are produced in a cascade fashion, and it is their combined effect that determines the type of response. The responsiveness of a cell to a particular cytokine is determined by the expression of specific receptors for that cytokine. The binding of the cytokine to its receptor then triggers the activation of a variety of signaling mechanisms that lead to changes in at the level of gene expression, resulting in transient- or chronic-altered cellular proliferation, differentiation, and/or function.

Cytokines play an essential role in the regulation of inflammatory responses and are involved in the regulation of both innate and acquired immunities. The cytokines that

function in innate immunity and inflammation are normally produced by cells of the monocitic and myeloid lineages and several other types of cells in response to microbial, chemical, physical, and other types of inflammatory stimuli. The main goal of cytokines in these responses is the localization and elimination of the instigating insult, orchestrating the recruitment of both cells and molecules (accomplished through increased vascular permeability and leukocyte infiltration) at the local site and a variety of systemic responses that include fever, acute-phase protein synthesis and the mobilization of leukocytes from the bone marrow. Among the main cytokines involved in these responses are tumor necrosis factor α (TNFα), interleukin (IL)-1, IL-6, IL-12, type I interferons (IFN; IFNα and IFNβ), and chemokines, a family of cytokines that function to mobilize and attract different types of leukocytes to sites of inflammation. These cytokines are said to have proinflammatory activity. If cytokines are secreted in excess as a result of an overwhelming infection, or in cases where the insult cannot be easily eliminated or the stimulus for cytokine secretion persists, leading to chronic inflammation, these same cytokines can have pathologic effects leading to the damage of healthy cells and tissues. Because of its potentially serious consequences, the immune system has mechanisms to prevent excessive inflammation, including cytokines with anti-inflammatory activity. These cytokines include transforming growth factor β (TGFβ) and IL-10, which antagonize many of the effects of the proinflammatory cytokines mentioned above. It should be kept in mind, however, that certain cytokines can have both pro- and anti-inflammatory effects depending on different factors such as the cell and tissue type and the kinetics of release.

In acquired immunity, cytokines play an equally important role. In general, the type of cytokines produced during an immune response determines the effector mechanisms that predominate and major expression patterns have been characterized (Mosmann & Coffman, 1989). For example, different subsets of helper ($CD4^+$) T cells that differ in both their cytokine secretion patterns and the effector mechanisms that they induce have been identified. Cells belonging to the Th1 subset secrete IFNγ, IL-2 and TNF α/β and are primarily involved in cellular immunity mechanisms and delayed-type hypersensitivity reactions; cells of the Th2 subset secrete IL-4, IL-5, IL-10 and IL-13 and are primarily involved in humoral mechanisms and allergic-type reactions; and cells of the newly described Th17 subset secrete IL-17, IL-22 and a variety of other proinflammatory cytokines (Harrington et al., 2006). Th17 cells are thought to be involved not only in immune responses to extracellular bacteria but also in autoimmune diseases. Th1 and Th2 cells affect one another: Th1 cells trigger macrophage activation using IFNγ, which inhibits the proliferation of Th2 cells, and Th2 cells secrete IL-10, which inhibits the secretion of IFNγ by Th1 cells. In keeping with the need for balance in the immune system, a different subset of T cells exists, namely T-regulatory cells, which act as negative regulators of the activities of other subsets. These cells act, in part, through the secretion of the anti-inflammatory cytokines TGFβ and IL-10 (Bettelli et al., 2006).

Both in innate and acquired immunity, the maintenance of a balance between pro- and anti-inflammatory cytokines or among the different $CD4^+$ T-cell subsets and their cytokines is essential for homeostasis and the proper function of the immune system. Disruptions in the balance can have pathologic implications resulting in excessive

inflammation and tissue damage, increased susceptibility to infectious agents, and/or the emergence of autoimmune conditions (Ollier, 2004). Abnormal levels of various cytokines have been described in many diseases, such as autoimmune hepatitis, rheumatoid arthritis, asthma, systemic lupus erythematosus, inflammatory bowel disease, and some brain disorders like schizophrenia and Alzheimer's disease (Kronfol & Remick, 2000; Theoharides et al., 2004; Vitkovic et al., 2000). Given that cytokines are key components in the homeostatic mechanisms regulating the immune system, it is not surprising that variations in their structure at the genetic or protein level or their production level have been associated with disease processes and the susceptibility to infections.

Cytokines and their receptors are encoded by highly polymorphic genes. These polymorphisms are responsible for the observed inter-individual differences in cytokine production and they likely impact the immune response (Hollegaard & Bidwell, 2006; Keen, 2002b; Warle et al., 2003). Cytokine genes and their receptor genes are highly conserved in their exon sequences, while the majority of polymorphisms occur in the non-translated regions of the gene, located within the promoter, the introns, or the untranslated-3' regions (Keen, 2002a). Cytokine polymorphisms continue to be discovered as mutation detection techniques improve to map the extent of cytokine polymorphisms. Cytokines that were once thought to be non-polymorphic, such as IL-2, IL-8, and IL-12 are now being shown to have single nucleotide polymorphisms (SNPs), often within the 5' promoter regions (Keen, 2002b). Polymorphisms that occur in promoter regions impact the levels of protein expression in several ways. Polymorphisms within the 3' and 5' regulatory sequences can affect transcription factors. Intronic

polymorphisms can affect mRNA splicing or the binding of enhancers and silencers (Keen, 2002b). As the amount of data on cytokine polymorphisms increases and becomes available, there are a growing number of studies that show an effect of cytokine gene polymorphism on immune disease susceptibility, severity, and outcome (Keen, 2002a).

Most cytokines can be synthesized and released within the central nervous system and while they are mainly produced by microglia and astroglia, neurons also produce cytokines under certain conditions. Although cytokines are usually secreted in response to specific stimuli, the low-level expression of specific cytokines appears to be maintained in blood vessels within the brain (Licinio et al., 1998; Vitkovic et al., 2000). Within the CNS, just as within the systemic immune system, cytokines are regulated in cascades through feedback loops, and cytokine receptors have been detected in the brain (Kronfol & Remick, 2000). Besides providing communication between neural cells, specific cytokines have a significant role in signaling the brain to produce neurochemical, neuroendocrine, neuroimmune, and behavioral changes (Maes et al., 1995). There is suggestive evidence that this signaling is part of the comprehensive mechanism to mobilize resources to combat physical and physiological stress in an attempt to maintain relative homeostasis. Because cytokines are associated with central neurotransmitters and cytokine regulation is affected by stress, many studies have investigated the possible role of cytokines in psychiatric disorders. These studies have demonstrated the role of abnormal levels of cytokines in major depression, Alzheimer's disease and schizophrenia (Hanson & Gottesman, 2005; Hopkins, 2007; Maes et al., 1995; P. McGeer & McGeer, 2001a, 2001b). Analysis of cytokine genetics in autism, however, is yet uninvestigated.

Evidence for Systemic Immune Dysfunction in ASD

In addition to behavioral and neurological impairments, increasing evidence substantiates that immune abnormalities are also prevalent in patients diagnosed with ASD. Epidemiological studies demonstrate that ASD is strongly associated with various familial autoimmune diseases. Mothers and relatives of autistic patients are significantly more likely to have autoimmune diseases such as multiple sclerosis, rheumatoid arthritis, and type 2 diabetes, than are family members of healthy controls (Comi et al., 1999). Interestingly, it has also been shown that the frequency of autoimmune diseases including hypothyroidism and rheumatic fever is significantly increased in families that have a child diagnosed with ASD in comparison with families that have a child with an autoimmune disorder (Sweeten et al., 2003a). A medical record study found that mothers who suffer from asthma or allergies during their second trimester are at the highest risk of having a child with ASD (Croen et al., 2005). A recent Danish study indicated that parental autoimmune conditions, specifically maternal ulcerative colitis and paternal type 1 diabetes, are significantly associated with the incidence of infantile autism (Mouridsen et al., 2007). While there is no definitive autoantibody pattern in ASD, enhanced autoimmunity has been reported in several cases, leading some investigators to speculate whether autism should be considered an autoimmune disease (Ashwood & Van de Water, 2004).

Several systemic immune aberrations have been demonstrated in patients with ASD, lending further support to the hypothesis that immune factors play a role in its pathogenesis. Changes in the numbers and activities of various immune cells indicate that both cell-mediated and humoral immunity are impaired in ASD. Autistic children

demonstrate significantly higher monocyte counts in blood plasma when compared to healthy controls, suggesting that their immune system is activated (Sweeten et al., 2003b). It has been reported that 45% of children with autism have significantly decreased NK cell activity compared to controls, indicating that the innate immune system's ability to kill virus-infected or damaged cells is diminished (Vojdani et al., 2008). Significantly increased serum concentrations of albumin, gamma globulin, and immunoglobulins of the IgG subclass have been reported in children with autism, suggesting an enhanced susceptibility to viral infection (Croonenberghs et al., 2002b). Upon stimulation with various agents, peripheral blood mononuclear cells (PBMC) of ASD patients produce significantly higher amounts of proinflammatory cytokines than controls, yet another demonstration of aberrant innate immune responses associated with the disorder (Jyonouchi et al., 2001). Plasma levels of active TGF-β1 are significantly lower in ASD children when compared to both typically developing children and children with developmental disabilities, suggesting that immune responses in autism may, in part, be regulated by this cytokine (Ashwood et al., 2008).

Anomalous T cell function and numbers have been reported in autism, but specific findings have not been reproducible. Adaptive immune responses stimulated by exposure to specific antigens are often driven by T helper (Th) cells, which are classified into two or more distinct functional types based on the cytokines that they produce and which ultimately determine their effector functions (Abbas et al., 1996). Among these, Th1 cells are stimulated mainly by intracellular pathogens and promote the activation and microbicidal activities of phagocytes by producing cytokines such as IFN-γ. Th2 cells are stimulated by chronic parasitic infections or allergens, and promote the production of IgE

antibodies by B cells and the activation of eosinophils by producing cytokines such as IL-4 and IL-5. Elevated levels of both Th1 and Th2 cytokines have been reported in children with ASD, which suggest that an imbalance of Th1 or Th2 cytokines may play a role in its pathogenesis. Evidence of Th system shifts from Th1 to increased Th2 activity is supported by decreases in intracellular IL-2 and IFN-γ production and increases in IL-4 production (Gupta et al., 1998). A study of PBMC in children with ASD found that they produced significantly higher levels of the Th2 cytokines IL-4, IL-5 and IL-13 than controls at baseline, suggesting that the immune response appears to be predominantly Th2 in origin (Molloy et al., 2006). However, other investigators have found significantly elevated plasma production of IL-2, IL-12 and IFN-γ, suggesting that autism involves activation of the Th1 type of immune response (Singh, 1996). Studies of whole blood cultures from children with ASD have also shown increased levels of IFN-γ production, supporting a Th1 response (Croonenberghs et al., 2002a). These preliminary findings have not been replicated in subsequent studies perhaps due to differences in methodology, ASD classification criteria, co-morbidity presence, case-control matching criteria, immunization status, or co-administration of psychotropic medications.

Although the evidence is controversial, gastrointestinal symptoms and an inflammatory mucosal pathology have been demonstrated in a subset of children with ASD as well (Horvath et al., 1999; Wakefield et al., 2000). While prevalence estimates from population-based studies are lacking, epidemiological studies report that approximately 20% of ASD children have GI symptoms (Fombonne et al., 2001; Taylor et al., 2002). Intestinal pathologies include ileo-colic lymphoid nodular hyperplasia, enterocolitis, gastritis and esophagitis (Furlano et al., 2001; Torrente et al., 2002;

Wakefield et al., 2000)[2]. Gluten and casein-free diets have proven to improve behavior in ASD children (Knivesberg et al., 2002). There is evidence that a dysregulated proinflammatory cytokine profile and reduced level of regulatory cytokines is present in the peripheral blood and intestinal mucosa of children with ASD who have gastrointestinal symptoms (Ashwood & Wakefield, 2006). This data suggests that ASD pathogenesis may result from a primary mucosal immunopathology during development that leads to the secondary systemic and CNS immunopathologies seen in these patients.

How the immune dysfunctions seen in ASD are related to the neuropathies that typify this disorder is not yet understood, but it may have significant implications regarding disease pathogenesis and treatment. The apparent association between ASD diagnosis and familial autoimmune disease prevalence suggests that common genes and/or environmental factors may contribute to both immune dysfunction and developmental brain pathologies. Immune responses measured in children with ASD have yielded contradictory results, and these studies suffer from inherent limitations. An estimated 46% of children with ASD are treated with psychotropic medications that are known to increase cytokine levels, which could serve as a significant confounding factor in the baseline measurements of these immune mediators (Aman et al., 2003; Haack et al., 1999; Pollmacher et al., 2000). Additionally, the developmental progression with regards to the immune system is unclear since no longitudinal studies have been performed, thus it is not known how the cytokine responses of ASD children change over time compared to neurotypical children. Further investigations into the immune

[2] Due to issues pertaining to patient collection bias and possible conflicts of interest regarding data interpretation, the majority of the authors on the Wakefield et al., 2000 paper retracted this article from public record in January 2010.

regulatory mechanisms in ASD are clearly warranted, and will likely yield therapeutic applications.

Evidence for CNS Immune Dysfunction in ASD

Increasing evidence indicates that in addition to systemic immune abnormalities, an atypical CNS immune profile is also present in ASD patients. Autistic patients have significantly higher levels of circulating anti-brain autoantibodies when compared to controls; including antibodies to myelin basic protein, cerebellar neurofillaments, neuron-axon filament proteins, caudate nucleus, serotonin receptor, and brain endothelial cells (Connolloy et al., 1999; Plioplys et al., 1989; Singh & Rivas, 2004; Singh et al., 1997a; Singh et al., 1997b; Singh et al., 1993). It has also been shown that glial fibrillary acidic protein is significantly elevated in the CSF of children with ASD, which indicates an ongoing process of reactive astrogliosis (Rosengren et al., 1992). Whether or not the presence of anti-brain antibodies and reactive astrogliosis are primary or secondary factors in the pathophysiology of ASD is yet to be determined, but these studies suggest that localized inflammation of the central nervous system may be a contributing factor.

While only a few studies have been performed regarding neuroinflammation in ASD, they demonstrate an active and ongoing neuroinflammatory process in the cerebral cortex, white matter, and cerebellum. Immunohistochemical studies of brain tissue from ASD patients reveal marked microglial and astroglial activation in the anterior cingulate gyrus and midfrontal gyrus, with the most prominent activation in the granular cell layer and white matter of the cerebellum (Vargas et al., 2005). The dorsolateral prefrontal cortex of autistic individuals demonstrates significant increases in white matter microglial

somal volume and gray matter microglial density that does not correlate with age and appears to be heterogeneous (J. T. Morgan et al., 2010). The anti-inflammatory cytokine TGF-β1 is significantly increased in cortical and cerebellar regions, and the largest spectrum of increases in pro-inflammatory cytokines is present in the anterior cingulate gyrus of autistic individuals (Vargas et al., 2005). Of particular interest from this study are one chemokine, macrophage chemoattractant protein-1 (MCP-1), and one cytokine, TGF-β1. MCP-1 is produced by activated astrocytes, mediates the infiltration of monocytes and macrophages to areas of injury, and its elevation in brain tissue is associated with various diseases characterized by neuroinflammation (Henkel et al., 2004; Kelder et al., 1998; Mahad & Ransohoff, 2003). TGF-β1 is an anti-inflammatory cytokine, with key roles in tissue remodeling and repair following injury, that suppresses the immune response by inhibiting T-cell proliferation and maturation and down-regulating the expression of MHC II receptors (Letterio & Roberts, 1998). Reactive astrocytes were found to be the main source of cytokines in the brains of autistic subjects (Vargas et al., 2005). Significant increases in several proinflammatory cytokines (TNF-α, IL-6, GM-CSF, IFN-γ, and IL-8) have been demonstrated in the frontal cortex of patients with ASD when compared to controls, with no concomitant increase in the Th2 cytokines (IL-4, IL-5 and IL-10) analyzed (Li et al., 2009). It has been proposed that the presence of activated neuroglia and proinflammatory cytokines in the CNS of autistic subjects may reflect an abnormal persistence of fetal patterns of development in response to genetic and/or environmental factors that disturb neurodevelopment and produce the

neurocytoarchitectural changes and CNS dysfunction that typifies ASD[3] (Pardo & Eberhart, 2007).

Consistent and significantly higher levels of activated immune markers have also been measured in the CSF of ASD subjects, indicating an overall pro-inflammatory profile. CSF from autistic patients shows a significant increase in MCP-1, IL-6, IFNγ and IL-8 when compared to controls (Vargas et al., 2005). In a pilot study of autistic children with developmental regression, comparative analysis of CSF to concurrent cytokine serum levels in autistic patients demonstrates a significant elevation of TNF-α expression in CSF, with an averaged ratio of 53:1 compared to a 1:1 ratio for control subjects (Chez et al., 2007). These results suggest that TNF-α in CSF may serve as a potential marker for the pathological process involved in autistic regression. Evidence indicates that levels of soluble tumor necrosis factor receptor II are significantly elevated in the CSF of children with autism, which do not reflect serum expression levels in the same individuals either (Zimmerman et al., 2005). While CSF studies of immune mediators in autism are still in their infancy, these findings can be interpreted as indicators of chronic neuroinflammation.

In order to better understand the molecular changes associated with ASD neuropathology, investigators have assessed the immune transcriptome in the cerebral

[3] A cautionary note in reference to immunocytochemical studies of postmortem brain tissue from autistic subjects: a majority of the patients studied in the Vargas et al., 2005, Li et al., 2009, and Morgan et al., 2010 died of hypoxic lesions that were ischemic in origin including near death experiences associated with drowning. Ischemic reperfusion injuries of this kind result in inflammation and oxidative damage primarily targeting white matter, and are defined neuropathologically by astrogliosis and microgliosis. Therefore, it cannot be determined whether the neuroinflammation present in these tissues represents a core pathology associated with ASD or are secondary reactions of this tissue associated with the cause of death.

cortex of postmortem brain specimens. Expression profiling of the superior temporal gyrus reveals that transcriptome variability is generally increased in autistic subjects, indicating that the disorder is heterogeneous even at the molecular level (Garbett et al., 2008). Cytokine and chemokine transcriptional analysis in various cortical regions reveals a heterogeneous regulation of expression of these proteins when compared to controls (Mott et al., unpublished data). Given that cytokines mediate inflammatory responses in the CNS and that their heterogeneous expression in cortical tissue is associated with ASD pathology, comparative analysis of polymorphic sites in cytokine genes may produce significant results and implicate them in the pathogenesis of this neurodevelopmental disorder.

Given the abovementioned neuroimmune evidence and the neuropathological features that typify this disorder, it has been proposed that a critical period of pathogenesis occurs during fetal brain development or in the first year of life which results in the ASD phenotype (Pardo, 2008; Pardo & Eberhart, 2007). The dominant features of ASD neuropathology include abnormalities in mini-columnar organization and subcortical white matter, disorganization of cortical neurons, and brain growth abnormalities (Bauman & Kemper, 2005; Casanova, 2007). The neurobiological and behavioral features that characterize ASD could be attributed to a pathogenic process that disturbs neurodevelopment during crucial periods pre- or perinatally. Recent studies show that maternal autoantibodies are present in the serum of mothers of patients with autism, indicating that maternal immunological factors may also play a role in the pathogenesis of ASD (Braunschweig et al., 2007; Singer et al., 2008; Zimmerman et al., 2007). Glial neuroimmune responses are associated with pathogenic mechanisms of

neurodegeneration in several neurological disorders, and appear to be involved in processes that lead to neuronal dysfunction. In the future, microglial activation, reactive astrogliosis and increased expression of specific cytokines in the CNS may provide early markers for ASD and allow clinical diagnosis within the first year of life, even before obvious clinical features manifest. In the meantime, immune studies in autism should focus on elucidating the underlying neuroimmunopathology responsible for the chronic neuroinflammation that seems to characterize at least a subset of subjects with ASD.

Etiological Evidence in an Idiopathic Disorder

Although there is no known cause of ASD, unequivocal evidence indicates that there is a strong genetic component. Several genetic disorders are consistently associated with autism, including Angelman syndrome, Rett's syndrome, tuberous sclerosis, fragile X syndrome, and Down syndrome (D. Cohen et al., 2005; Harris et al., 2008; Lowenthal et al., 2007). Whether autism and these syndromes share underlying pathophysiological genetic networks has yet to be determined, but current research in this area is underway. Twin and family studies demonstrate that autism is one of the most highly heritable complex neuropsychiatric conditions. In the late 1960s it was noted that the recurrence rate of autism in a given family was 50-100 times greater than that expected by chance (Rutter, 1968). A more recent study estimated that parents of a child diagnosed with ASD are 25 times more likely to produce affected siblings compared to controls (L. B. Lord et al., 1991). While it is difficult to interpret recurrence rates of autism due to the phenomenon of genetic 'stoppage' wherein families choose not to have more children once an ASD diagnosis is obtained, studies report significantly higher sibling recurrence

rates in ASD families compared to the general population (Rutter et al., 1999; Slager et al., 2001). Additionally, siblings and parents of ASD patients are more likely than controls to show cognitive and behavioral features reminiscent of those seen in the broader autism phenotype (Bishop et al., 2004; Bolton et al., 1994; Costantino et al., 2006). These findings indicate that ASD traits are not only genetically transmitted, but also highly heritable.

Average concordance rates of autism in monozygotic (MZ) twin pairs are consistently and significantly higher than those found in dizygotic (DZ) twin pairs (Bailey et al., 1995; S. Folstein & Rutter, 1977; Le Couteur et al., 1996; Lichtenstein et al., 2010; Ritvo et al., 1985; Rosenberg et al., 2009; Steffenburg et al., 1989). Recent research estimates average concordance rates of autism in MZ twin pairs to be 70-80% while DZ twin pair rates are 0-10% (S. E. Folstein & Rosen-Sheidley, 2001). Even studies investigating concordance rates in more generalized ASDs report significantly higher MZ concordances compared to those for DZ twins (Lichtenstein et al., 2010; Rosenberg et al., 2009; Taniai et al., 2008). While some investigators postulated that twining could be a risk factor in the development of ASD, subsequent studies do not support this hypothesis (Betancur et al., 2002; Croen et al., 2002; Hallmayer et al., 2002). Multivariate twin studies suggest that the three core behavioral symptoms of the autism 'triad' are fractionable and highly heritable individually, such that different autistic aspects of behavior may have distinct genetic origins (Happe & Ronald, 2008; Happe et al., 2006; Ronald & Hoekstra, 2011). The question of whether genetic influences can be ascertained in a disorder defined by symptomatic heterogeneity is substantial, and represents a significant limitation in cross-study comparison. Future research will likely

reveal that different genetic causal pathways result in different ASD subtypes and symptoms.

Little is known about the genes underlying autism susceptibility, and the majority of studies focus on linkage analysis and candidate gene identification. It has been estimated that genetic abnormalities account for up to 20% of ASDs, and include chromosomal abnormalities, copy number variations (CNVs), single-gene disorders, and rare point mutations (Abrahams & Geschwind, 2008; Marshall et al., 2008). Examples of chromosomal abnormalities identified in ASD include autosomal aneuploidies, sex chromosome anomalies, deletions, duplications, translocations, inversions and marker chromosomes (C. Gillberg, 1998). One of the most common sites for chromosomal abnormalities in autism is located on chromosome 15q11-13, where duplications of maternal origin are frequent (S. E. Folstein & Rosen-Sheidley, 2001). The most consistent evidence for linkage, however, occurs on chromosome 7q22-32, according to meta-analysis (Trikalinos et al., 2006). Other studies also implicate autism loci on chromosome 7, as well as chromosomes 3, 4, and 11 (Schellenberg et al., 2006). Despite the significantly different sex ratio present in autism, few studies suggest linkage on the X chromosome (Auranen et al., 2002; Shao et al., 2002). It has been proposed that linkage analyses have yielded inconsistent findings due to limitations inherent to this analytic approach in a complex genetic model where it is likely that multiple risk alleles confer susceptibility to the disorder (Losh et al., 2008).

Conversely, genome wide association scans (GWAS) and screening for CNVs provide a more powerful approach in comparing genetic risk factors between cases and controls in disorders of complex etiologic origin. This analytic approach is capable of

scanning thousands of samples and applies algorithms that compare the frequencies of single alleles or multimarker haplotypes between disease and control cohorts. Inherited CNVs have been found in 50% of ASD subjects, as compared to control estimates of 5-10% (Itsara et al., 2009; Zhao et al., 2007). CNVs may include insertions, deletions, and duplications of DNA, as measured in oligonucleotides and single nucleotide polymorphisms (SNPs). While a unified genetic theory for ASD based on the observed high frequencies of CNVs fits the prevalence estimates previously discussed, it does not explain the sex-dependent penetrance in which males are four times more likely to be affected (W. T. Brown, 2010; Zhao et al., 2007). GWAS have identified several genes and SNPs that are associated with autism disorder on chromosomes 2, 6, 7, 15, and 17; although subsequent studies have been unable to replicate many of these findings (Freitag, 2007).

Discordant findings in GWAS are likely due to many the same confounding factors that make comparison between epidemiological studies in ASD difficult. Many of the original GWAS studies had small sample sizes and thus may not be considered reliable in detecting true autism loci. Additionally, false-positive signals are always a possibility even in studies with large sample sizes. Perhaps the largest contributing factors are disparities in diagnostic definition or pedigree ascertainment between studies. Even cohorts that use similar diagnostic criteria may have somewhat different characteristics. Examples include gender composition, language, developmental regression, IQ distribution, family size, and ethnic origins. Future GWAS studies of large sample size that follow rigorous diagnostic protocols and replicate distributions of cohort

characteristics will minimize heterogeneity and provide the most accurate linkage analysis.

Like most neuropsychiatric disorders, ASD is a syndrome of complex genetic origin. The genetic traits that have been characterized do not exhibit classic Mendelian dominant or recessive modes of inheritance associated with a single gene locus. Likewise, the wide phenotypic variability found in ASD cannot be attributed to simple modes of inheritance. Contemporary etiologic theory proposes a genetic model in which multiple genes interact with one another to produce the autism phenotype. Thus, ASD may be considered a polygenetic disorder whereby several genes act synergistically. Early studies supported a multi-locus model of inheritance, where at least three epistatic (interacting) loci were predicted (Pickles et al., 1995). More recent estimations based on GWAS predict as many as 10-20 different genes interact in ASD pathogenesis (Risch et al., 1999; Spence, 2004). GWAS and cytogenetic analyses serve to narrow down genetic regions of interest, but they are not hypothesis-driven and do not account for clinical and empirical evidence, which is essential in elucidating candidate genes that may predispose individuals to disease. While it is apparent that genes strongly impact the likelihood of developing ASD, no definitive genetic pattern has been identified although a multitude of candidate genes have been implicated (Muhle et al., 2004). Replications of these findings have been inconsistent, however, likely due to the heterogeneity of the ASD phenotypes investigated.

Given the neuropathological features and immunological dysfunctions that are found in ASD subjects, genes that are known to affect neurodevelopment and immune function are prime candidates for analysis. Several studies have linked autism with

immune-based genes (Lee et al., 2006; Torres et al., 2006; Warren et al., 1996; Warren et al., 1992). One group found a genetic linkage with autism for a variant of the MET receptor tyrosine kinase, which is involved in both neurobehavioral and immune function (Campbell et al., 2006). It is possible that the convergence of multiple combinations of genotypes may result in different ASD phenotypes, further complicating analytic interpretations. Thus, the phenotypic traits of chosen cohorts must be clearly and narrowly described when investigating candidate genes of interest. While the interaction of multiple genetic factors has become increasingly evident as a causative function in ASD pathogenesis, genetic influence cannot explain the increased prevalence indicated by epidemiological studies.

Given that the autism heritability estimate is approximately 80% based on the abovementioned twin studies, environmental factors must represent another etiologic component in ASD pathogenesis. It has been proposed that MZ twin discordances can be attributed to epigenetic changes, somatic mutations or chorionic environmental influences (Bohm & Stewart, 2009; Bruder et al., 2008; Cheung et al., 2008; Kaminsky et al., 2009). Two of the most salient environmental factors associated with ASD are prenatal maternal exposures and postnatal birth complications. Perinatal obstetric complications represent a putative risk factor for the development of ASD (Kolevzon et al., 2007; Ronald et al., 2010). Types of obstetric complications associated with autism include prematurity, low birth weight, respiratory distress syndrome, rhesus incompatibility, resuscitation, infection, anemia, and trauma (Bolton et al., 1997). Maternal use of various agents, including thalidomide, valproic acid, misoprostol, and ethanol have been associated with ASD (Aronson et al., 1997; Bandim et al., 2003; Christianson et al., 1994; Moore et al.,

2000; Nanson, 1992; Stromland et al., 1994; G. Williams et al., 2001). Congenital conditions associated with autism include hypothyroidism, cytomegalovirus infection, and rubella infection (Chess, 1971; Rovet & Ehrlich, 2000; Stubbs et al., 1984). Although the measles-mumps-rubella (MMR) vaccine received considerable attention in its relation to the development of ASD, subsequent studies refute any evidence to substantiate that MMR vaccination is a risk factor for ASD development (Dales et al., 2001; Kaye et al., 2001; Madsen et al., 2002; Taylor et al., 1999).

Intrauterine and perinatal exposure to infectious agents and immune factors have been implicated as primary mediators of CNS damage in several neuropsychiatric conditions including schizophrenia, bipolar disorder, cerebral palsy and autism (Miller et al., 2005; Nelson et al., 1998; Rodier & Hyman, 1998; Yolken & Torrey, 1995). It has been proposed that ASD pathogenesis may be attributed to environmental toxicity experienced during fetal development or early postnatal life (Deth & Muratore, 2010; Palmer et al., 2009; Windham et al., 2006). Xenobiotic risk factors associated with autism include the heavy metals mercury, lead, and cadmium, as well as arsenic, maternal smoking, alcohol, cocaine abuse, and certain pesticides (Ashwood et al., 2006; Kern et al., 2007; London, 2000; Mutter et al., 2005). Specifically, maternal seafood ingestion and dental amalgams could provide primary sources for *in utero* mercury exposure, while mercuric or aluminum vaccine preservatives may serve as potential postnatal sources (Deth & Muratore, 2010). Although thimerosal was considered to play a causative role in autism development, removal of this preservative from infant vaccines in 2001 did not result in a change in prevalence of the disorder (Schechter & Grether, 2008). Pesticides are known to have severe neurotoxic effects, and can cross the blood-brain-barrier and

placenta. One epidemiological study reported an association between maternal residential proximity to areas of pesticide application during gestation and incidence of ASD (Roberts et al., 2007). Another found an association between ASD and the use of household pesticides, with the strongest effect during the second trimester (Hertz-Picciotto et al., 2008). Of particular interest is the fact that estrogen and progesterone have been shown to be neuroprotective against inflammatory damage, suggesting that sex hormonal status may be a critical mediating factor involved in xenobiotically induced inflammatory brain damage (Kipp et al., 2007). Gender-based differences in developmental immunotoxicity have been evidenced in animal studies of *in utero* exposure to heavy metals (Bunn et al., 2001). These studies obviously have significant implications regarding the gender bias in ASD and suggest that it may be linked to specific prenatal teratogenic exposures.

Maternal infection resulting in neurological damage to the offspring in a temporally dependent manner associated with gestational development has been proposed in ASD (Depino, 2006; Meyer et al., 2006). Animal models of ASD, while controversial, report behavioral and neuropathological features reminiscent of the ASD phenotype in humans and may be useful models of etiologic investigation. Maternal respiratory viral infections produce autistic neuropathological and behavioral features in rodent models (Patterson, 2002; Shi et al., 2007). Interestingly, the same group found that a single administration of the cytokine IL-6 during a specific period of murine gestation produced the same 'autistic' phenotype (S. E. Smith et al., 2007). Maternal infections have also been shown to influence the offspring immune system. For example, stimulation of pro-inflammatory cytokine production in rats during pregnancy resulted in innate immune

dysfunction in the offspring (Hodyl et al., 2007). Early life immune insult is likely a significant component in ASD pathogenesis, but little is known regarding the precise developmental period(s) of immune vulnerability or the array of environmental toxins, infections or conditions that produce observable dysfunctional outcomes.

Recent evidence indicates that prenatal stress is associated with ASD. Mothers of children diagnosed with autism disorder report significantly more stressful life events during their pregnancies when compared to controls (Beversdorf et al., 2005). Prenatal stress caused by natural disasters measured in tropical storms and hurricanes from 1980-1995 was significantly associated with autism prevalence, which increased in a dose-response fashion with storm severity (Kinney et al., 2008a). Furthermore, the results of this study indicated that fetuses exposed during gestational months 5-6 and 8-9 had significantly greater risk of developing ASD compared to children exposed during other gestational periods. This group outlines multiple mechanisms by which prenatal stress could result in the altered neurodevelopmental trajectory that typifies ASD. Examples include neuroinflammatory effects, fetal hypoxia caused by a reduction in uterine or placental circulation, maternal stress hormones crossing the placenta and altering the development of the hypothalamic-pituitary-adrenal axis, complications of pregnancy and/or delivery, and epigenetic effects on gene expression involved in the stress response (Kinney et al., 2008b). Prenatal stress and fetal exposure to elevations of maternal stress hormones such as testosterone during critical periods of gestation could represent a risk factor for ASD and explain the gender differences in prevalence (Knickmeyer & Baron-Cohen, 2006).

Since the interaction between genes and environment guide development, gene-environment interactions likely play etiologic roles in ASD pathogenesis. It has been proposed that the majority of ASD cases are caused by the combination of environmental factors and multiple susceptibility genes (Cederlund & Gillberg, 2004; Glasson et al., 2004). The actual environmental insult, considered here to be of immunologic origin, likely precedes the first neurological sign of ASD. Possible models to explain early life immune insult and the neuropathologic/immunologic features of ASD have been proposed elsewhere (Dietert & Dietert, 2008). Given a particular genotype, an individual may be genetically predisposed to developing ASD based on particular pre- or perinatal environmental exigencies. The confluence of genetic predisposition in an epistatic multilocus model and a second "hit" from an environmental or immunogenetic risk factor could account for the pathogenesis and etiologic heterogeneity of ASD.

Molecular pathways critical for normal neuronal and cortical organization as well as immune function signify the most likely influential targets in a disorder typified by dysfunction in these areas. Specifically, the HGF/MET pathway is associated with multifunctional roles involved in these systems, and recent evidence demonstrates a strong association of a SNP in the promoter region of the MET gene with ASD, as well as altered levels of mRNA in proteins associated with this pathway in the brain tissue of affected individuals (Campbell et al., 2007; Campbell et al., 2006). Other genes implicated in the ASD pathophysiology have been reviewed, and include those encoding reelin, various neurotrophins, serotonin-related genes, GABAergic receptors, neuroligins, SHANK3, and HOXA1 (Pardo & Eberhart, 2007). These genes are associated with the neurobiological causes of autism, but cannot account for the immunological aspects of

the disorder. Thus the search for distinguishing genetic characteristics related to ASD and how these interact with prenatal environmental triggers to produce the ASD phenotype remains an avenue for continued research.

While it is evident that ASDs have a strong genetic component, they have a complex and poorly understood pattern of inheritance, likely due to the heterogeneous nature of the disorder. Because GWAS have failed to reveal strong genetic candidates due to conflicting results, it is probable that multiple rare genetic variants are involved. Future linkage analyses must aim to describe endophenotypes in ASD in order to create more narrow behavioral and pathological profiles and thereby more homogenous and comparable samples. Current etiologic theory predicts that the confluence of genetic predisposition at multiple interacting sites along with the presence of prenatal environmental risk factors represents the most feasible model of ASD pathogenesis. During which stage of development these events are likely to occur is yet undetermined. Studies investigating prenatal windows of vulnerability in autism contain evidence to support environmental risk at every trimester of gestation. While one study suggested that maternal stress factors prior to the 32nd week of gestation played significant roles in ASD incidence, still other studies suggest second and third-trimester stress and infectious risk factors are critical (Beversdorf et al., 2005; Kinney et al., 2008a; Yamashita et al., 2003). Determining specific periods of developmental vulnerability along with identifying the teratogenic insults that elicit the immunologic and pathologic manifestations of the ASD phenotype will play essential roles in future prevention of the disorder. Equally as important will be the identification of islets of genetic susceptibility associated with

ASD, which will have significant therapeutic implications. Once these etiological attributes are described, true prevention and treatment can begin.

Investigating Cytokine Polymorphisms and Expression in ASD: Central Hypothesis and Specific Aims

Current literature demonstrates abnormalities in cytokine expression levels in ASD subjects when compared to controls. Independent study results, however, are contradictory in their findings regarding characterization of these differences (see Table 1 below). One potential reason for discrepancies between studies could be associated with comparisons of young ASD patients with adult controls, or sample cohorts that have a wide range of ages within patient and control groups. It has been established that cytokine expression profiles change with age in a tissue-specific manner (Csiszar et al., 2003; Sack et al., 1998). Thus, studies that do not compare age-matched cohorts when investigating cytokine expression profiles face significant confounding factors associated with age related changes in immune mediators. Contradictory results in different autistic samples could also confirm the presence of different endophenotypes in ASD based on cytokine genotypes. It is reasonable to infer that the combination of these different genotypes may bias a characteristic immune response, constitutively affecting the Th1/Th2/Th17 balance. In the context of an acute inflammatory episode, such a bias may tip the balance toward a more vigorous and potentially deleterious acute-phase immune response or induce a state of chronic inflammation.

Table 1. Cytokine expression patterns in autistic patients compared to controls: an inter-study comparison

Cytokine	Plasma	Whole blood	PBMC	CSF	Brain
IL-1β	⇑(1) NS(5) ↑(6)		NS(7) ⇑(8)		NS(13)
IL-1RA	NS(3)	⇑(3)	NS(8)	↑(11)	
IL-2			NS(7) ↑(14)		
IL-4			⇑(14)		NS(13)
IL-6	⇑(1) NS(3) NS(4) ↑(6) NS(11)	↑(3)	NS(7) ⇑(8)	⇑(12)	↑(12) ⇑(13)
IL-8	⇑(1)				⇑(13)
IL-10	NS(6)	NS(3)	NS(7) ⇓(10) NS(14)		↑(12) NS(13)
IL-12	⇑(1) ⇑(4)		NS(7)		
IFNγ	⇑(4) NS(5)	⇑(3)	NS(7) ↑(14)	⇑(14)	⇑(13)
TNFα	NS(4) NS(5)	↑(3)	NS(7) ⇑(8) ⇑(10)		⇑(13)
TGFβ-1	⇓(2) ⇓(9)				⇑(12)

⇑⇓ Significantly increased/decreased level ($p \leq 0.05$), ↑Trend toward increased level that does not reach significance, NS No significant difference. PBMC: Peripheral blood mononuclear cells, CSF: cerebrospinal fluid.

Study Index:

1. (Ashwood et al., 2011)
2. (Okada et al., 2007)
3. (Croonenberghs et al., 2002a)
4. (Singh, 1996)
5. (Sweeten et al., 2004)
6. (Emanuele et al., 2010)
7. (Saresella et al., 2009)
8. (Jyonouchi et al., 2001)
9. (Ashwood et al., 2008)
10. (Ashwood & Wakefield, 2006)
11. (Zimmerman et al., 2005)
12. (Vargas et al., 2005)
13. (Li et al., 2009)
14. (Molloy et al., 2006)

There is currently not enough evidence to determine if the abnormal immune response present in ASD is a contributing factor for or a simple by-product of the disease. However, growing evidence demonstrates that ASD may be caused by the combination of genetic susceptibility and specific environmental insults that occur during neurodevelopment such as particular infections or antigens in the diet; and a proinflammatory scenario associated with abnormal cytokine production would exacerbate the whole process. It is possible that there are phenotypes of the immune system predisposed to stronger or weaker inflammatory immune responses, chronic inflammation or autoimmunity, and that these phenotypes can manifest from several different combinations of genotypes in different cytokine genes with variable expression. The combinations of specific genotypes of multiple cytokines may therefore be useful as markers for specific autistic endophenotypes.

Immune activation during pregnancy can impact offspring neurodevelopment with far-reaching behavioral sequelae. Cytokine levels are altered in human pregnancies complicated with infection and maternally generated cytokines can cross the placenta and enter fetal circulation (Depino, 2006; Zaretsky et al., 2004). Thus, it is possible that a maternal abnormal immune response linked to infection or injury during pregnancy may be one contributing factor for altered neurological development in the fetus. The immune activation of pregnant mice or rats using either lipopolysaccharide (a proxy for bacterial infection) or polyriboinosinic polyribocytidylic acid (a proxy for viral infection) results in modified cytokine expression in the maternal-fetal tandem pair (Gilmore et al., 2005; Urakubo et al., 2001). Furthermore, the timing of the insult during gestation is important with regard to the ultimate neurological and behavioral impact (S. E. Smith et al., 2007).

A recent study of maternal infection requiring hospitalization during pregnancy found that first trimester viral infection and second trimester bacterial infection were significantly associated with ASD diagnosis (Altadottir et al., 2010). It is inferred here that the timing and etiological origin of immune activation potentiates different neurological and behavioral fetal results, and that variability due to cytokine expression polymorphisms could either exacerbate or attenuate the degree of both maternal and fetal immune activations.

If specific cytokine genotypes associated with abnormal cytokine expression are linked to autism, this could be understood as a genetic predisposition to ASD. Given expression polymorphisms in certain key cytokine genes, some overall immune phenotypes will be predisposed for stronger or weaker immune activation, contributing to the etiology or emergence of autism. Beyond the concept of cause and effect, it is proposed here that specific genetic cytokine make up may be a risk factor that will exacerbate any immune response triggered by multiple prenatal environmental factors leading to the oxidative stress and neuropathological abnormalities present in this disease. Maternal and/or fetal immune activation may permanently alter the fetal Th1/Th2/Th17 balance, predisposing the fetus to a lifetime of chronic inflammatory or autoimmunity issues.

The central hypothesis investigated herein purports that autism pathogenesis is caused by the combination of genetic susceptibility in cytokine genes and a second "hit" prenatal infectious scenario that occurs during specific periods of neurodevelopment. The questions this research aims to answer are:

1.) Do cytokine genes differ between autistic subjects and controls?

2.) Does cytokine transcriptional expression differ significantly from that of controls in the cerebral cortex of autistic patients?

3.) Can cytokine expression profiles be modified systemically in autism?

Specific experimental aims were designed to address each question, and are relayed below.

Experimental aim 1 was to characterize different SNPs of selected candidate genes encoding cytokines in autistic patients and controls. Based on previous investigations that convey abnormal expression levels of cytokines in ASD, it is hypothesized that there will exist significant differences between cytokine SNPs in an autism cohort compared to neurotypical controls. A positive association with ASD for cytokine SNPs could be interpreted as genetic susceptibility and represent a significant risk factor for ASD pathogenesis.

Experimental aim 2 was to characterize the transcription (mRNA) pattern for chosen cytokines in postmortem cortical brain tissue of autistic patients and compare these to control values. Previous investigations of cytokine translational (protein) profiles have revealed significant differences in cytokine expression between autistic subjects and controls (see Table 1). The assumption, based on molecular dogma, is that investigated transcriptional patterns will reflect translational patterns already established. If cytokine mRNA patterns are not significantly different between autistic and control cohorts, this would indicate that post-transcriptional modification of cytokine genes my play a role in ASD pathogenesis. This would open new investigative areas of etiologic research in ASD focused on molecular mechanisms of immunogenetic regulation.

Experimental aim 3 was to characterize the cytokine translational expression patterns in autistic subjects treated with placebo or glutathione. Systemic oxidative stress has been documented in autistic children (Chauhan & Chauhan, 2006; James et al., 2004). The tripeptide glutathione is the primary determinant of redox status in human cells (Schafer & Buettner, 2001). Plasma levels of glutathione are abnormally decreased in autistic subjects (James et al., 2004). Glutathione has significant impact on a multitude of cellular processes involved in immune signaling. It is known to modulate cell differentiation, proliferation, antigen presentation, regulate apoptosis, and enhance lymphocyte proliferation. Disturbances in glutathione homeostasis are implicated in the etiology and progression of neurodegenerative diseases (Ballatori et al., 2009). It is proposed here that intravenous administration of glutathione in autistic subjects will alter systemic cytokine expression and ameliorate behavioral symptoms. If cytokine expression levels are significantly altered (and reflect expression patterns seen in neurotypical subjects) in response to glutathione administration, this could represent a metabolic treatment for autism with potential therapeutic applications.

The focus of this research is to determine if cytokine genetic and expressional profiles can be linked, in a causative fashion, to the pathophysiology of ASD. Because of their role in prenatal neurodevelopment and immune signaling pathways, cytokines offer an associative link between the abnormal immunological profiles and neuropathological features present in this disorder. Indications of a genetic susceptibility in cytokine genes linked to abnormal expression profiles would provide significant etiologic evidence in this idiopathic disease. In addition, metabolic treatment that regulates cytokine expression may yield substantial therapeutic implications.

CHAPTER II

Cytokine Polymorphisms in Autism

Introduction

Autism is an idiopathic neurodevelopmental disorder that typically appears during the first three years of life. It is diagnosed on the basis of impairments in cognition, social interaction, and communication (American Psychiatric Association, 2000). Autism spectrum disorder (ASD) comprises a broader category of neurodevelopmental disorders including autistic disorder, Asperger's disorder and pervasive developmental disorder not otherwise specified (PDD-NOS). An epidemiological survey performed in 2007-2008 found that 1.1% of children in the United States aged 3 to 17 years old are currently diagnosed with ASD, and males are four times more likely to be diagnosed than females (Kogan et al., 2009). Though the etiology remains largely unclear, ASD is a highly heritable disorder (85%) that affects monozygotic and dizygotic twins twelve and four times greater, respectively, than the general population (Greenberg et al., 2001; Spence, 2004). In the largest and most recent study of proband-ascertained twin pairs, it was found that pairwise ASD concordance was 31% for dizygotic and 88% for monozygotic twins; and this data also suggests that zygosity and sex may contribute to ASD heritability (Rosenberg et al., 2009). Family studies have shown that parents of an affected child are more likely than control parents to demonstrate cognitive or behavioral

features that are reminiscent of those found in ASD, though fail to achieve diagnosis (Losh et al., 2008).

Genome-wide association studies have rendered some relevant findings for possible genetic risk factors, but lack consistency; that is, none of the single nucleotide polymorphisms (SNPs) or genomic regions correlated with ASD has been replicated across studies. This apparent lack of consistency may be due to several reasons. How different studies clinically conceptualize ASD endophenotypes may rule out the possibility of consistency. It has been shown that analysis of different core deficits results in associations with multiple different regions of the genome (Schellenberg et al., 2006). Rare variants that impinge on common neurodevelopmental pathways may lead to convergent diagnosis (Bucan et al., 2009). At issue is whether ASD endophenotypes truly represent a spectrum of neurological behavior and deficits with a common underlying pathology or are rather a collection of similar behaviors of heterogeneous genetic and/or developmental origin. Association studies assume environmental homogeneity, but it is likely that ASD is the result of combinations of variable genetic susceptibilities and pertinent environmental exposures.

Recent studies suggest a strong immunological component may contribute to the pathogenesis of ASD. An increased prevalence of autoimmune disorders has been reported among first– and second–degree relatives of children with ASD when compared to controls; even when compared to families of children with autoimmune diseases (Sweeten et al., 2003a). Interestingly, in detailed analysis of ASD subtypes in Sweeten et al., it was found that an increased frequency of autoimmunity existed in the autism and Asperger's disorder families when compared to those of children with autoimmune

diseases—but not in the PDD–NOS families. Research demonstrates that some autistic children have perturbed cellular and humoral immunity (Gupta, 2000; Plioplys et al., 1994; Saresella et al., 2009). Brain protein auto–antibodies have been found in sera from autistic children, implying an autoimmune response is present in this disorder in the individuals studied (Singh et al., 2002; Singh et al., 1998). Research from the same group maintains that a major subset of autism, so called Autoimmune Autistic Disorder, is caused by a neuro-autoimmune pathogenesis triggered by viral infection (Singh, 2009). Increased neuroglial response, characterized by astroglial and microglial activation, has been demonstrated in the post-mortem brain tissue of autistics (Vargas et al., 2005). Such neuroglial activation indicates an immune response of the central nervous system (CNS) representing an overall neuroinflammatory scenario which, argued here, may typify this disorder in some individuals. This is relevant for the current study, as cases of PDD-NOS and cases diagnosed with co-morbid autoimmunity were excluded. Obviously, neuroinflammation can occur in the absence of an auto–immune response.

The immune system affects the CNS primarily through cytokines and chemokines; diverse groups of regulatory proteins that mediate inflammation, immunity, and hemopoiesis. Levels of the proinflammatory cytokines TNF-α and IL-6 are significantly increased in brain tissue of some ASD patients when compared to controls (Li et al., 2009). Increased levels of proinflammatory cytokines TNF-α, IL1-β, IL-2, IL-6, and IFN-γ are present in the peripheral blood mononuclear cells (PBMC) of some children with ASD (Jyonouchi et al., 2001; Molloy et al., 2006). A neuro–inflammatory process is shown in the cerebral cortex, cerebellum, and CSF of some autistic individuals, and cytokine expression profiling indicates that macrophage chemo-attractant protein

MCP-1 and TGF-β1 are the most up–regulated cytokines in these brain tissue samples (Vargas et al., 2005).

Polymorphisms in the regulatory regions of cytokine genes effect expression levels and have been shown to play a role in a variety of diseases including asthma, autoimmune diseases, periodontal diseases, diabetes, Alzheimer's disease, and coronary heart disease (Yucesoy et al., 2003). It is known that cytokine gene polymorphisms can alter immune disease susceptibility, severity, and outcome (Keen, 2002b). Since some polymorphisms directly impact protein expression, and some ASD is characterized by an abnormal immune response demonstrated in cytokine expression systemically and in the CNS, it is reasonable to conjecture that polymorphisms in cytokine genes are correlated with ASD pathogenesis. Here, a model that involves immune activation and inflammatory processes is proposed, not one necessarily contingent on autoimmunity.

To investigate the co–occurrence of cytokine polymorphisms and ASD, this study was carried out to ascertain frequency–based differences between two ASD endophenotypes and population controls for polymorphisms among a set of eleven cytokine genes (*IL1A, IL1B, IFNG, TGFB1, TNF, IL2, IL4, IL6, IL12B, and IL10*), two cytokine receptors (*IL1R1, IL4R*), and one cytokine receptor antagonist (*IL1RN*) (see Table 2 for more details)[4]. These genes represent cytokines from both the innate and adaptive immune response systems. The results of this study may offer insight into immune activation and its role in the pathogenesis of ASD.

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[4] Note: If referring to the protein, a hyphen is used; if referring to a gene, no hyphen is used. For example, IL4 is the gene name, while IL-4 refers to the cytokine protein. Also, cytokine names use the Greek nomenclature, while gene names do not. For example, IFNG refers to the interferon gamma gene, while IFN-γ indicates the cytokine.

Table 2. Cytokine SNP name, chromosome locus and RS number

Gene	Polymorphism*	Chromosome locus	dbSNP ID
IL1A	−889 C →T	2q14	rs1800587
IL1B	−511 C →T	2q14	rs16944
	+3962 C →T		rs1143634
IL1R1	+1970 C →T	2q12	rs2234650
IL1RN	mspa1 11100 C →T	2q14	rs315952
IL2	−330 T →G	4q26-q27	rs2069762
	+166 G →T		rs2069763
IL4	−1098 T →G	5q31.1	rs2243248
	−590 C →T		rs2243250
	−33 T →C		rs2070874
IL4R	+1902 G →A	16p12.1-p11.2	rs1801275
IL6	−174 G →C	7p21	rs1800795
	nt565 G →A		rs1800797
IL10	−1082 A →G	1q31-q32	rs1800896
	−819 C →T		rs1800871
	−592 C →A		rs1800872
IL12B	−1188 A →C	5q31.1-q33.1	rs3212227
IFNG	+874 T →A	12q24.1	rs2430561
TGFB1	codon 10 C →T	19q13	rs1800470
	codon 25 G →C		rs1800471
TNF	−308 G →A	6p21.3	rs1800629
	−238 G →A		rs361525

*Alleles to the left of the arrow are ancestral and those to the right are derived

SNP information was retrieved from the dbSNP (build 119, http://www.ncbi.nlm.nih.gov/SNP; accessed March 2011)

Materials and Methods

ASD Samples and Sources

DNA samples of ASD participants came from two sources: 23 participants were from Coriell Cell Repositories (http://ccr.coriell.org/autism/) and 26 autistic/Asperger's participants were recruited for the study by Dr. Lonnie Sears in the Department of Pediatrics at the University of Louisville Hospital. All ASD samples fit the diagnostic

criteria of the Diagnostic and Statistical Manual-IV, as confirmed by the Autism Diagnostic Interview–Revised (C. Lord et al., 1994). Of the 49 ASD patients, 37 were autistic, six were autistic with co–morbid mental retardation, and six were diagnosed with Asperger's disorder. Of the six female ASD patients, two were diagnosed as autistic co–morbid with mental retardation, two were autistic, and two were diagnosed with Asperger's disorder. Potential participants with autism were excluded if they had any patent genetic disorder such as fragile–X syndrome or tuberous sclerosis; evidence of seizure disorder or autoimmunity also precluded participation. Exclusions were based on medical and neurological histories and examination. This study was approved by the Institutional Review Board of the University of Louisville (IRB 284.07).

Control Samples and Sources

DNA samples were obtained from amalgamated population surveys (Middleton et al., 2003). Data was verified in the original articles prior to inclusion in the present study. Populations were chosen to reflect a general European sample given the constraints of populations available. Specifically, population data was chosen from Germany, Ireland, England, Italy, and several regional samples of Caucasians in the United States. Not surprisingly, complete data for all cytokines reported for the ASD participants were not available for all population controls (see Table 3 for details).

Table 3. Allele and genotype frequencies at 22 SNP loci in ASD participants and controls

Gene	SNP	Sample size		Minor allele frequency (%)		Genotype frequency (%)								
		Control	ASD	Control	ASD		Control	ASD		Control	ASD		Control	ASD
IL1A	−889 C →T	976	39	29.7	29.5	CC	48.5	53.8	CT	43.6	33.3	TT	7.9	12.8
IL1B	−511 C →T	1241	48	34.4	35.4	CC	42.1	37.5	CT	47.0	54.2	TT	10.9	8.3
	+3962 C →T	540	47	24.4	26.6	CC	57.4	53.2	CT	36.5	40.4	TT	6.1	6.4
IL1R1	+1970 C →T	540	49	33.1	45.9	CC	43.5	28.6	CT	46.9	51.0	TT	9.6	20.4
IL1RN	mspa1 11100 T →C	540	49	27.9	33.7	TT	51.3	38.8	TC	41.7	55.1	CC	7.0	6.1
IL2	−330 T →G	816	47	30.4	27.7	TT	48.0	53.2	TG	43.1	38.3	GG	8.8	8.5
	+166 G →T	540	47	30.2	31.9	GG	49.4	48.9	GT	40.7	38.3	TT	9.8	12.8
IL4	−1098 T →G	540	45	7.7	10.0	TT	85.0	80.0	TG	14.6	20.0	GG	0.4	0.0
	−590 C →T	1505	46	13.7	20.7	CC	74.6	67.4	CT	23.6	23.9	TT	1.9	8.7
	−33 C →T	540	44	12.8	20.5	CC	75.7	68.2	CT	23.0	22.7	TT	1.3	9.1
IL4R	+1902 A→G	540	49	21.3	23.5	AA	62.0	59.2	AG	33.3	34.7	GG	4.6	6.1
IL6	−174 G →C	540	49	39.7	40.8	GG	37.0	34.7	GA	46.5	49.0	AA	16.5	16.3
	nt565 G →A	2505	49	39.9	39.8	GG	35.8	34.7	GC	48.5	51.9	CC	15.7	14.3
IL10	−1082 A→G	2650	48	47.9	49.0	AA	26.8	16.7	AG	50.6	68.8	GG	22.6	14.6
	−819 C →T	1172	48	22.5	18.8	CC	60.0	64.6	CT	35.0	33.3	TT	5.0	2.1
	−592 C→A	2407	48	23.4	21.9	CC	59.0	62.5	CA	35.1	31.3	AA	5.8	6.3
IL12B	−1188 A →C	631	43	20.7	26.7	AA	62.6	51.2	AC	33.4	44.2	CC	4.0	4.7
IFNG	+874 T →A	351	47	47.7	40.4	AA	25.4	34.0	AT	53.8	51.1	TT	20.8	14.9
TGFB1	codon 10 T →C	890	46	39.7	43.5	TT	35.5	28.3	TC	49.7	56.5	CC	14.8	15.2
	codon 25 G →C	888	43	7.7	10.5	GG	85.7	81.4	GC	13.3	16.3	CC	1.0	2.3
TNF	−308 G →A	2360	49	16.2	20.4	GG	70.1	63.3	GA	27.4	32.7	AA	2.5	4.1
	−238 G →A	540	49	6.9	6.1	GG	86.7	87.8	GA	13.0	12.2	AA	0.4	0.0

DNA Isolation and Amplification

Samples collected using buccal swabs at the University of Louisville were isolated using a standard phenol-chloroform extraction and ethanol precipitation. The Cytokine Genotyping PCR Kit (Invitrogen, Carlsbad, CA) was used to ascertain genotypes reported in Table 2.

Statistical Analysis

Genotype, minor allele frequencies, and tests of Hardy–Weinberg equilibrium were calculated using Excel. While the SNP data for the ASD participants could be linked within individuals, the controls were based on population data. Therefore, each SNP was tested individually with logistic regression in R (R Development, 2011). Genotype was encoded using orthogonal polynomials, such that the linear coefficient could be interpreted as the log–odds ratio as in the Cochran–Armitage test, assuming the alleles are co–dominant. The quadratic term accounted for any deviation from co–dominance. Statistical significance was corrected for multiple comparisons with a false discovery rate of $q^* = 0.1$ according to the method of Benjamini and Hochberg (Benjamini & Hochberg, 1995).

Results

The distributions of allele and genotype frequencies at 22 SNP loci in ASD participants and controls are found in Table 3. When carefully examining the table, several items of interest are apparent. The IL1 family is found closely clustered on

chromosome 2q13. This may be of potential interest from the perspective of population genomics. A review of the table frequencies of minor alleles and genotypes reveals relative congruency across the five IL1 loci except for *IL1R1*. The difference between the minor allele frequency for cases versus controls is rather large, and the relative frequencies of the different homozygotes vary. The fact that the minor allele frequency for this one locus among the family of IL1 loci differs between cases and controls and not at the other four IL1 loci suggests a possible association between *IL1R1* SNPs and ASD. Scanning down the minor allele frequencies, two SNPs (-590 and -33) for *IL4* seem to vary between cases and controls, and similarly differ in one of the homozygotes. This pattern of differences implies that the *IL4* -590 T and -33 T constitute a linked haplotype; though this was unable to be tested due to the use of population controls. Of particular interest are the results for *IL10* -1082. There is very little difference between the minor allele frequencies for cases and controls, but consideration of the genotype frequencies suggests that the cases may be out of Hardy–Weinberg equilibrium.

Tests for Hardy-Weinberg Equilibrium

Tests of Hardy–Weinberg equilibrium are found in Table 4. While two SNPs, *IL4* -33 and *IL10* -1082, are statistically significant at $p < .05$ and thus not considered in Hardy-Weinberg equilibrium; none of the results are statistically significant when the Benjamini–Hochberg correction is applied. Although this is true, the results of *IL10* -1082 are brought to the attention of the reader. IL-10 is one of the more important anti-inflammatory cytokines; it has been associated with several diseases likely due to immune activation, and *IL10* -1082 is the locus that exerts the greatest control of gene

expression. There was a lower frequency of the higher expression *IL10* -1082 GG genotype among ASD patients in this study. Though not statistically significant in this data set following a multiple-test correction, a possible relationship between *IL10* -1082 and ASD should be further explored.

Risk Estimates for Genotype Association

The estimates of odds ratios for the association of ASD and individual SNPs show that genotype frequencies for the proinflammatory *IL1R1* +1970 and anti–inflammatory *IL4* -590 and *IL4* -33 alleles are significantly different in ASD patients compared to controls even after the Benjamini–Hochberg correction (Table 5). All three of these SNPs are negatively associated with ASD; that is, they are not protective. None of the tests for the linear coefficient of the overall trend of the genotypes (e.g., the Cochran–Armitage test) was significant. Due to sample zeros, the quadratic term was excluded from the models for *IL4* -1098 and *TNF* -238: None of the autistics were homozygous for the minor allele at these loci, and maximum likelihood estimates of the parameters would diverge to ∞ were both the linear and quadratic terms included.

Table 4. Hardy-Weinberg table for raw data

Gene	SNP	Sample size		Chi-Square		P-Value	
		Control	Autistic	Control	ASD	Control	ASD
IL1A	−889 C →T	976	39	1.898677548	1.530522007	0.168226476	0.216034228
IL1B	−511 C →T	1241	48	2.124070384	1.643534762	0.145000787	0.199841262
	+3962 C →T	540	47	0.046411601	0.056275809	0.829429339	0.812482065
IL1R1	+1970 C →T	540	49	1.930570968	0.035469831	0.16469607	0.850614552
IL1RN	mspa1 11100 T →C	540	49	0.764843801	2.681770178	0.381816907	0.101502877
IL2	−330 T →G	816	47	0.323264051	0.085809257	0.569652641	0.769573999
	+166 G →T	540	47	0.597612045	0.667976972	0.439490599	0.413757818
IL4	−1098 T →G	540	45	0.397807863	0.555555556	0.528223545	0.456056544
	−590 C →T	1505	46	0.008231917	3.371032001	0.927707203	0.066352195
	−33 C →T	540	44	0.496365467	4.021224476	0.481101474	0.044931087
IL4R	+1902 A →G	540	49	0.012818436	0.054920288	0.909857305	0.814712666
IL6	−174 G →C	540	49	0.460495721	0.010078319	0.497392389	0.920034048
	nt565 G →A	2505	49	0.294291928	0.266799465	0.587483201	0.605486845
IL10	−1082 A →G	2650	48	0.503812276	6.77187697	0.477829815	0.00926054
	−819 C →T	1172	48	0.015056333	0.414465515	0.902341299	0.519711645
	−592 C →A	2407	48	0.881695966	0.362081494	0.347737594	0.547352494
IL12B	−1188 A →C	631	43	0.19007596	0.685462593	0.662853404	0.407712023
IFNG	+874 T →A	351	47	2.150933539	0.173143771	0.142483281	0.67733282
TGFB1	codon 10 T →C	890	46	1.319108458	1.031284593	0.250752305	0.309857384
	codon 25 G →C	888	43	3.060845721	0.716507246	0.080199936	0.397291992
TNF	−308 G →A	2360	49	0.198168534	0.001172724	0.656203231	0.972681736
	−238 G →A	540	49	0.075516242	0.206787635	0.783468218	0.649296959

Table 5. Results of the logistic regression analysis

Gene	SNP	Linear trend (odds ratio)			P_Q
		MLE	CI	P	
IL1A	−889 C→T	1.21	[0.69, 1.93]	0.458	0.138
IL1B	−511 C→T	0.93	[0.50, 1.53]	0.790	0.333
	+3962 C→T	1.06	[0.51, 1.85]	0.851	0.766
IL1R1	+1970 C→T	1.80	[1.15, 2.76]	0.00795 •	0.793
IL1RN	mspa1 11100 T→C	1.07	[0.51, 1.89]	0.827	0.200
IL2	−330 T→G	0.93	[0.50, 1.53]	0.803	0.679
	+166 G→T	1.15	[0.68, 1.79]	0.571	0.584
IL4	−1098 T→G	1.35	[0.60, 2.72]	0.426	n.t.
	−590 C→T	2.27	[1.21, 3.77]	0.00362 •	0.0897
	−33 C→T	2.79	[1.40, 5.23]	0.00171 •	0.0446
IL4R	+1902 A→G	1.18	[0.56, 2.07]	0.610	0.852
IL6	−174 G→C	0.97	[0.60, 1.48]	0.891	0.709
	nt565 G→A	1.03	[0.65, 1.57]	0.901	0.772
IL10	−1082 A→G	1.02	[0.60, 1.71]	0.939	0.0152
	−819 C→T	0.62	[0.15, 1.36]	0.351	0.534
	−592 C→A	1.01	[0.49, 1.70]	0.981	0.651
IL12B	−1188 A→C	1.20	[0.47, 2.30]	0.637	0.508
IFNG	+874 T→A	0.73	[0.44, 1.15]	0.190	0.918
TGFB1	codon 10 T→C	1.14	[0.69, 1.79]	0.597	0.462
	codon 25 G→C	1.55	[0.36, 3.66]	0.409	0.778
TNF	−308 G→A	1.36	[0.54, 2.51]	0.411	0.951
	−238 G→A	0.88	[0.33, 1.95]	0.782	n.t.

MLE: maximum likelihood estimate; CI: 95 % confidence interval; P_Q: P-value for the quadratic term; n.t.: not testable; •: statistically significant at $q^* = 0.1$.

Discussion

This is the first study to examine the association between ASD and cytokine polymorphisms. Previous research has focused on the measurement of cytokine levels in the brain, CSF, and blood rather than genotyping the polymorphic sites that are putatively responsible for relative inter-individual levels of circulating cytokine concentrations. The present study demonstrates positive association with ASD for cytokine polymorphisms *IL4* -590T, *IL4* -33T, and *IL1R1* +1970T.

The Issue of Multiple Comparisons

The fundamental issue with using something like a cytokine panel, where many SNPs are scored simultaneously, is the theoretical statistical concern of multiple comparisons. If one considers that the probability of obtaining a statistically significant result by chance is $1- 0.95^{K}$, where K is the number of tests conducted, appropriately interpreting the results of multiple comparisons can quickly become an issue. In this particular case, 22 SNPs in 13 genes were tested. Considering only the 13 genes, there is roughly a 50% chance of obtaining a significant result by chance. In order to correct for multiple comparisons, the method of Benjamini and Hochberg was used. However, statistically correcting for multiple comparisons, while important, can obviate some important data, and further fails to account for the importance of population history and the hazards of operating without a model of biologic plausibility.

Consider two possible models for the contribution of particular genes (or SNPs in those genes) influencing inflammation and the etiology of ASD. One model is that independent spontaneous mutations are occurring in individuals in any of multiple genes

contributing to the initiation or maintenance of inflammation. This model presumes that the regulation of inflammation can be broken in multiple ways; all leading to inflammation contributing to the suite of behaviors that are clinically distributed along the autistic spectrum. Arguably, it would be unlikely that in a small group of autistics the same SNP would be shared among sufficient individuals to achieve statistical significance[5]. The consequence of using this model is that while it might be proven that inflammation is a common contributing factor to the etiology of autism, it would be difficult to prove the genetic contributions of particular SNPs.

Another possible model is that autistics share immune gene SNPs in common as a function of population structure and shared ancestry. In this model, theoretical constraints would preclude historically negative selection coefficients, and the contribution of particular genes to the etiology of autism would emerge only in conducive environmental circumstances. Environmental factors interacting with genetic variability may be the *in utero* influence of industrial pollutants or exposure to infectious disease. Admittedly, a logical weakness of this model is that if individuals are sharing SNPs in inflammation genes they would also be sharing SNPs in genes not related to inflammation; and the problem of multiple comparisons becomes once again significant. This model does, however, allow the possibility that when considering the contribution of the immune system to the etiology of ASD, some cytokine genes (and their SNPs) would be more likely than other cytokine genes to be implicated depending on their roles in the immune activation process. Herein it is argued that IL4 and IL1R1 are two genes playing

[5] However, certain regions of the genome do seem particularly susceptible to mutation. An example is IL10, which lies in a region of high recombination, and has a relatively large number of SNPs in its promoter region.

fundamental roles in the immune activation process, and putatively in the etiology or progression of ASD.

IL-4 and IL-1R functions in the immune system and CNS

Of interest are the specific functions of IL-4 and IL-1R within the immune system and CNS. IL-4 activates cytotoxic immunity in response to virus-infected cells, and drives T cell differentiation toward the Th2 phenotype. It is released in allergic and inflammatory responses, and rescues T lymphocytes from apoptosis (Vella et al., 1997). IL-4 enhances the antigen-presenting capacity of B cells by stimulating MHC class II molecules, B7, CD40, surface IgM, and low-affinity IgE receptor expression (Borish & Steinke, 2003). Research with adult murine neural progenitor cells shows that IL-4-activated microglia preferentially induce oligodendrogenesis over neurogenesis (Butovsky et al., 2006). This study also demonstrates that both neuro- and oligodendrogenesis activated by microglia are blocked in the presence of LPS (a proxy for bacterial infection). These results are of particular interest, given that microglia are activated in various brain regions of autistic patients where enlarged cerebral white matter volumes are also present and recent evidence indicates an association between increasing radiate white matter volume and impairment (Courchesne et al., 2001; Herbert et al., 2004; Mostofsky et al., 2007). IL-4 production in peripheral blood mononuclear cells of autistic children is significantly higher than that of normal controls (Molloy et al., 2006). Increased IL-4 levels are present in the sera of patients with asthma, and it was recently established that a SNP in the IL-4 gene confers susceptibility to the disease (Amirzargar et al., 2009). Therefore, evidence of the increased IL-4 production,

chronically activated microglial cells, elevated white matter volumes, and significantly different genotypic frequencies in the IL4 promotor region which are capable of effecting protein expression levels suggests that this cytokine may relay susceptibility to autism.

In the brain, IL-1 plays a role in long-term potentiation, and in mouse and rat studies it has been demonstrated that most IL-1 receptors are found in the hippocampus, a region that is morphologically enlarged in autistic individuals (Schumann et al., 2004). Rodent studies demonstrate that IL-1 is induced in the hippocampus during the learning process, and that exogenously administered IL-1 increases memory function while blockage of IL-1 signaling impairs memory function (Yirmiya & Goshen, 2011). In the immune system, IL-1 plays a significant role in the induction of Th17 cells, which have been shown to provide protection against bacterial infection in mice (Acosta-Rodriguez et al., 2007). While studies of IL-1 expression in the CNS of autistic individuals do not yield significant differences when compared to controls, systemic evidence indicates that increased IL-1 levels are present in the plasma, whole blood, and peripheral blood mononuclear cells of autistic patients (Ashwood et al., 2011; Croonenberghs et al., 2002a; Jyonouchi et al., 2001). Micro-array evidence suggests that the gene pair IL-1R1/IL1-R2 plays a crucial role in mediating dendritic cell functions during the primary immune response (Zhong et al., 2009). If polymorphisms in the IL1R1 gene cause deficient dendritic cell functioning in the systemic immune system, this would impair the antigen presenting abilities of these cells and weaken communication between the innate and adaptive immune systems. Functionally, autistic patients demonstrate impaired performance on neuropsychological tasks involving hippocampal circuits when compared to controls (Loveland et al., 2008). While IL-1 expression has not been investigated in the

hippocampus of autistic individuals, future research in this area may link IL-1 levels in this region to behavioral impairment. Given the findings of this study, which demonstrate significantly different genotypic frequencies in the IL1R gene of autistic individuals, this cytokine may play significant roles in the pathogenesis of ASD.

Conclusion

The original impetus for this research project was to investigate the possible association of various cytokine SNPs with autism. It has been reasonably well–established that inflammation, at some level, is involved in the etiology and progression of ASD. The thirteen genes in the genotyping panel used in this study are arguably some of more significant genes involved in the initiation and/or mediation of inflammation. Much remains to be learned about the intricacies of the expression dynamics of the cytokine network. Much more remains to be learned about the pleiotropic nature of cytokine expression at different times in the life history of the organism and in response to different kinds of immune challenges.

ASD is a complex disease with a pathogenesis that involves the genetic variation of a number of contributing genes in addition to the action of environmental factors. Changes in gene expression, coordination, and patterns of interactions of cytokine gene products likely influence the development of ASD, but the mechanisms remain to be revealed. Simultaneously, epigenetic factors play contributing roles by affecting the network of interaction of critical genes and their proteins. This is the first reported study to examine the association between ASD and cytokine polymorphisms. SNPs in cytokine genes and their promoters may alter cytokine expression and cause an overall imbalance

in the regulation of the immune system, resulting in an inflammatory profile seen both in the central nervous system and systemically in autistic individuals. The present study demonstrates positive association with ASD for cytokine polymorphisms *IL4* (-590T), *IL4* (-33T) and *IL1R1* (+1970T), and adds to the growing literature that a dysregulated immune response may be central to the pathogenesis of certain endophenotypes within ASD.

CHAPTER III

Cytokine Transcriptional Expression in the Cerebral Cortex of Autistic Subjects

Introduction

Autism is a pervasive neurodevelopmental disorder typically manifesting in the first three years of life, and diagnosis depends on a triad of impairments in social interaction, communication and cognition (American Psychiatric Association, 2000). Autism is idiopathic, although evidence indicates a complex etiology with an underlying genetic susceptibility likely acting in concert with immunological dysfunctions and unidentified environmental exposures (Ashwood et al., 2006; Persico & Bourgeron, 2006). Post-mortem studies reveal neurobiological abnormalities in cytoarchitectural and neuronal organization, suggesting altered neuronal maturation and/or defective cortical organization may play roles in the pathogenesis of autism (Bauman & Kemper, 2003; Casanova et al., 2002; Pardo & Eberhart, 2007). Evidence indicates that maternal immune activation during embryonic development could account for these neuropathological abnormalities and this activation may be cytokine dependent (Shi et al., 2007; Shi et al., 2009; S. E. Smith et al., 2007). Cytokines are regulatory proteins that mediate inflammation, immunity and hemopoiesis, and facilitate communication between the immune and central nervous systems (CNS) (Borish & Steinke, 2003). Patients with

autism have altered cytokine levels in plasma, peripheral blood mononuclear cells, whole blood supernatant, cerebrospinal fluid, and brain tissue (Croonenberghs et al., 2002a; Jyonouchi et al., 2001; Li et al., 2009; Molloy et al., 2006; Vargas et al., 2005).

To investigate neuroinflammatory mechanisms, the transcription of four cytokines (TGFβ-1, IL-1β, IL-6, and TNFα) and one chemokine (IL-8) was analyzed in five regions of the cerebral cortex of postmortem brains using reverse transcriptase-initiated real-time PCR on eight autistic-control pairs. This technique permits monitoring product formation, and allows precise quantification of baseline mRNA. Sample size is limited by RNA quality and tissue availability but is sufficient to uncover robust changes that may characterize the neuropathology typifying autism. This study reveals differential transcription of cytokine genes in various cortical regions of autistic brains compared to neurotypical controls, and proposes novel molecular mechanisms yet uninvestigated which may be involved in the pathogenesis of ASD.

Materials and Methods

Human Brain Samples

Autistic and control brain specimens were obtained through The Autism Tissue Program (ATP) (http://www.brainbank.org) from the Harvard Brain Tissue Resource Center, supported in part by PHS grant R-240MH068855. Formaldehyde-fixed sections of Brodmann Areas (BA) 4, 9, 17, 22 and 46 from eight individuals with autism and eight control individuals were analyzed. Cortical areas correspond to primary motor cortex (BA 4), dorsolateral prefrontal cortex (BA 9 and 46), primary visual cortex (BA 17), and superior temporal gyrus/Wernicke's area (BA 22). Review of the ATP clinical database

resulted in the 16 matched subjects presented in this study (Table 6), and no significant difference in mean age or postmortem interval (PMI) was present between groups. Tissue recovery followed an institutionally approved informed consent procedure and was coordinated nationally by the ATP and NIMH/NINDS Harvard Brain Tissue Resource Center.

Table 6. Autistic and control samples

Diagnosis	ATP ID#	Sex	Age	PMI	Cause of Death
Control	AN12552	M	56	24	Multiple Injuries
Control	AN03334	M	36	26.02	Possible Pulmonary Embolism
Control	AN08678	M	40	25.25	Hepatic Encephalopathy
Control	AN12137	M	31	32.92	Asphyxia
Control	AN19760	M	28	23.25	Unknown
Control	AN05475	M	39	UK	Heart Attack
Control	AM07932	M	17	6.5	Accident
Control	AN04432	M	22	12	Central Hepatic Laceration
Control MEAN			33.6	21.4	
Autism	AN08792	M	30	20.3	Gastrointestinal bleeding
Autism	AN00493	M	27	8.3	Drowning
Autism	AN01093	M	56	19.48	Anoxic Encephalopathy
Autism	AN06746	M	44	30.8	Acute Myocardial Infarction
Autism	AN00764	M	20	23.7	Accident
Autism	AN01971	M	39	31.5	Hospitalized with Pneumonia
Autism	AN01613	M	29	UK	Heart Attack
Autism	AN11989	M	30	16.06	Congestive Heart Failure
Autism MEAN			34.4	21.4	

PMI: Post-mortem interval (hours), UK: Unknown. Autism Tissue Program identifier is depicted by ATP ID#. The age and postmortem interval were not significantly different across groups (two-tailed t-test = 0.9 and 0.99, respectively).

Sample Preparation

Tissue was dissected for gray matter specimens approximately 40-80 μg, and total RNA was isolated using the Ambion Recoverall™ Total Nucleic Acid Isolation Kit for FFPE Samples (Applied Biosystems, Foster City, CA) according to the manufacturer's protocol. The integrity of RNA was assessed on an ND-1000 Spectrophotometer V3.3 (Nanodrop Technologies, Wilmington, DE).

Two-step RT-PCR

Total RNA extractions were reverse transcribed into cDNA using the TaqMan Reverse Transcription Reagent Kit (Applied Biosystems) and primed with the standard random hexamer according to the protocol. The reverse transcription (RT) reaction was performed with a DNA Thermal Cycler 480 (PerkinElmer, Fremont, CA) as follows: 25°C for 10 min, 48°C for 30 min and 95°C for 5 min. Real-Time PCR was performed in low-transmissiveness 96-well reaction plates (AbiPrism; Applied Biosystems) using SYBR® Green PCR Master Mix (Applied Biosystems) as the reporter. All reactions were conducted as follows: 50°C for 2 min, 95°C for 10 min, 95°C for 15 s (40 cycles), 60°C for 1 min (40 cycles), 95°C for 15 s, 60°C for 30 s, and 95°C for 15 s with a 7300 Real-Time PCR System (Applied Biosystems). Amplified cellular GAPDH from all samples served as a housekeeping gene to normalize expression and monitor reproducibility. Primers were synthesized by Alpha DNA (Montreal, Quebec) and their sequences follow:

TGFβ-1:

Forward: 5'-CAACAATTCCTGGCGATACCT-3'

Reverse: 5'-GCTAAGGCGAAAGCCCTCAAT-3'

IL-8:

Forward: 5'-CAAAGAACTGAGAGTGATTGAGAGTG-3'

Reverse: 5'-CCCTACAACAGACCCACACA-3'

IL-1β:

Forward: 5'-TCTACACCAATGCCCAACTG-3'

Reverse: 5'-AGCGAATGACAGAGGGTTTC-3'

TNFα:

Forward: 5'-AGGCGGTGCTTGTTCCTCA-3'

Reverse: 5'-GTTCGAGAAGATGATCTGACTGCC-3'

IL-6:

Forward: 5'-CACACAGACAGCCACTCACCTC-3'

Reverse: 5'-CTGCCAGTGCCTCTTTGCTG-3'

GAPDH:

Forward: 5'-GAGTCAACGGATTTGGTCGT-3'

Reverse: 5'-TGGAAGATGGTGATGGGATT-3'

Qualitative Analysis

The instrument on-board software 7300 System SDS (Applied Biosystems) generated output data, which was transferred to an MS Excel spreadsheet for analysis. The software generated a log-linear calibration graph which plotted primer copy number

from run-off transcript for each sample's mRNA against the number of cycles it took for each reaction product to exceed a preset florescence threshold (C_T). To obtain quantitative measurements, C_T values for each sample were performed in duplicate and compared with those obtained in the standard curve fitted to points of the calibration graph. All readings were standardized to the amplification values obtained for the GAPDH housekeeping gene: $\Delta C_{T,x} = C_{T,x} - C_{T,GAPDH}$ where x is the target gene. Thus, the number of each transcript is expressed relative to GAPDH. Relative change in expression of gene x between the autistic and neurotypical case is $\Delta\Delta C_{T,x} = \Delta C_{T,x}^{\text{Autism}} - \Delta C_{T,x}^{\text{Control}}$, and the corresponding fold change in expression was calculated using $2^{-\Delta\Delta C_{T,x}}$ (Fu et al., 2006).

Statistical Analysis

The relative expression of gene x (x = *TNF*, *IL6*, *TGFB1*, *IL1B*, or *IL8*) in the tissue sample from participant i, Brodmann area j was analyzed using the mixed-effects linear model

$$\Delta C_{T,x}^{(i,j)} = \beta_{0,x} + \beta_{1,x} D_i + \boldsymbol{\beta}_{2,x}^{T} \mathbf{R}_j + \gamma_{i,x} + \varepsilon_{ij,x}$$

D is a dummy variable encoding diagnostic category ($D_i = 0.5$ for autistic i, and $D_i = -0.5$ otherwise). Encoding ensures that the coefficient $\beta_{1,x}$ is an estimate of $\Delta\Delta C_{T,x}$ over all five Brodmann areas. $\mathbf{R}_j$ is a dummy variable encoding BA j; the $\gamma_{i,x}$ are independent, identically distributed random intercepts with zero mean; and the $\varepsilon_{ij,x}$ are independent, identically distributed residuals.

Prior to fitting, preliminary analysis with the LIBRA toolbox (Verboven & Hubert, 2005) robustly examined the raw C_T data for outliers. The linear models were fit using R software packages NLME (Pinheiro et al., 2010) and contrast (Kuhn et al., 2010). Goodness of fit was established by comparing standardized residuals to a standard normal distribution using the Kolmogorov-Smirnov D statistic.

The mixed effects linear model used here accounts for the correlation between measurements of different Brodmann areas within the same brain. Five univariate Wilcoxon tests would implicitly assume that those different measurements were independent, necessitating a correction for multiple comparisons. The linear model, on the other hand, assumes a particular (normal) distribution of observed data within groups. Preliminary non-parametric tests for outliers in the data, as well as post-hoc tests of the residuals of the fit indicate that this assumption is not validated. Thus, the mixed effects linear model was considered the best choice for this data analysis and applied accordingly.

Results

The investigated control and autistic brains showed no significant difference in age ($t_{14} = 0.13$, two-tailed $p = 0.900$) or post-mortem interval ($t_{12} = 0.006$, $p = 0.995$) between autistic and comparison subgroups. Preliminary analysis detected no statistical outliers among the C_T values. Post hoc test of the residuals found no significant deviation from the expected standard normal distribution, with the greatest Kolmogorov-Smirnov $D = 0.127$ and its associated $p = 0.214$, in the model fit for *TNF*. Average $\Delta\Delta C_{T,x}$ was not significantly different from zero for any of the target genes: *TNF* ($F_{1,14} = 0.207$, $p =$

0.656), *IL6* ($F_{1,14}$ = 0.308, p = 0.587), *TGFB1* ($F_{1,14}$ = 0.024, p = 0.879), *IL1B* ($F_{1,14}$ = 0.665, p = 0.428) or *IL8* ($F_{1,14}$ = 0.074, p = 0.789). Relative expression of all five target genes also did not vary significantly by Brodmann area, nor was there any significant variation of $\Delta\Delta C_{T,x}$ with Brodmann area. Mean transcriptional expression levels for autistic and control samples and fold change values by cortical region are shown in Table 7 below.

Table 7. Differential gene expression in autistic patients

Target Gene	BA[a]	Relative expression[b] Aut.	C.C.	Fold change Mean	95 % CI
	4	−10.9	−10.8	1.14	[0.66, 1.97]
	9	−11.0	−10.8	1.19	[0.69, 2.04]
IL6	17	−11.1	−10.8	1.22	[0.70, 2.11]
	22	−11.0	−11.0	1.04	[0.60, 1.81]
	46	−11.1	−10.8	1.17	[0.68, 2.02]
	4	0.136	0.258	1.09	[0.44, 2.71]
	9	0.258	0.266	1.01	[0.41, 2.49]
TGFB1	17	0.440	0.260	0.88	[0.35, 2.21]
	22	0.168	−0.015	0.88	[0.35, 2.22]
	46	0.420	0.113	0.81	[0.32, 2.02]
	4	−1.20	−1.32	0.92	[0.31, 2.68]
	9	−1.17	−1.33	0.89	[0.31, 2.60]
IL8	17	−1.19	−1.28	0.93	[0.32, 2.74]
	22	−1.16	−1.51	0.78	[0.27, 2.30]
	46	−1.07	−1.44	0.77	[0.26, 2.27]
	4	−4.25	−4.03	1.17	[0.70, 1.93]
	9	−4.26	−4.09	1.12	[0.68, 1.84]
TNF	17	−4.38	−4.06	1.25	[0.75, 2.08]
	22	−4.32	−4.33	0.99	[0.59, 1.68]
	46	−4.05	−3.92	1.09	[0.66, 1.82]
	4	0.96	2.21	2.37	[0.54, 10.31]
	9	1.17	2.00	1.78	[0.41, 7.65]
IL1B	17	1.29	1.98	1.61	[0.37, 7.06]
	22	0.92	1.63	1.64	[0.37, 7.28]
	46	1.14	1.95	1.75	[0.40, 7.66]

a. Brodmann area
b. $C_{T,target} - C_{T,GAPDH}$; estimated from the fixed effects of the linear models; Aut = autism; C.C. = comparison clients

Discussion

Results indicate altered cytokine expression in the cortex of autistic patients, where gene transcripts TNF-α, IL-6, TGFβ-1, IL-1β, and IL-8 are heterogeneously expressed in different cortical regions when compared to controls. BA 22 showed a differential pattern of expression in which gene transcript levels were down-regulated compared to controls for all transcripts except IL-1β, which appeared up-regulated. IL-1β showed the most up-regulation throughout all five cortical regions, with BA 4 showing a two-fold increase compared to control samples. Though sample size was limited and no statistical significance was determined, these findings suggest that transcriptional changes may be related to the neuropathology present in autism.

The CNS immune profile is only beginning to be revealed in autistic patients, where studies demonstrate an active and ongoing neuroinflammatory process in the cerebrospinal fluid, cerebral cortex, white matter and cerebellum (Chez et al., 2007; J. T. Morgan et al., 2010; Vargas et al., 2005). Very few studies have investigated cytokine expression profiles within the brain tissue of autistic subjects. TGFβ-1 was found to be significantly increased in cortical and cerebellar tissue when compared to controls, while proinflammatory cytokines including IL-6 were significantly increased in the anterior cingulate gyrus (Vargas et al., 2005). Significant increases in several proinflammatory cytokines, including IL-6, IL-8, IFNγ, and TNFα, have also been demonstrated in the frontal cortex of ASD patients (Li et al., 2009). These postmortem studies indicate that cytokine expression at the translational level is significantly different in various cortical

regions of autistic subjects when compared to neurotypical controls[6]. Interestingly, although we analyze different cortical regions than the abovementioned translational studies, our transcriptional results did not indicate significant differences in cytokine transcripts of autistic subjects when compared to matched controls. These contradictory results suggest that post-transcriptional regulatory mechanisms may play a role in ASD pathogenesis. Indeed, investigations of post-transcriptional gene regulatory networks in patients with ASD suggest that epigenetic factors, including dysregulation of microRNA expression, may contribute to observed alterations in gene expression and lead to the pathophysiological conditions that underlie autism (Sarachana et al., 2010).

Immune transcriptome alterations in the temporal cortex of ASD patients have been previously identified, and microarray studies reveal that greater transcript variability is present in the brains of autistic subjects when compared to controls (Garbett et al., 2008). Whether this variability is a reflection of the heterogeneity of the disorder or an inherent characteristic related to the core neuropathology is not known, but represents a serious limitation when drawing conclusions in studies of gene expression in the brain tissue of autistic subjects. Another confounding factor that should be considered when reviewing results of gene expression in the postmortem human brain involves agonal conditions associated with the samples provided. A majority of the patients contained in

[6] Investigators should be cautious when interpreting these results. Multiple investigators have reported that brain samples from the Autism Tissue Program show extensive degradation, which was discovered after several research groups had already published studies using the same tissue. Brain banks need to focus their efforts on establishing quality assessment measures prior to distributing brain samples to research laboratories. In the meantime, investigators should be cautious drawing conclusions from their findings, and perform quality control assessments on tissue to determine whether their evidence represents a core pathology associated with ASD or rather is a remnant of poor tissue quality.

the ATP database died of hypoxic lesions that were ischemic in origin including near death experiences associated with drowning. Ischemic reperfusion injuries of this kind result in inflammation and oxidative damage primarily targeting white matter, and are defined neuropathologically by astrogliosis and microgliosis. Thus, there is a distinct possibility that the cytokine profiles relayed in Vargas et al., 2005, Li et al., 2009, Morgan et al., 2010, and in the current study are attributed to conditions associated with near death experiences rather than the autism neuropathology. There is evidence that hypoxia has significant effects on RNA integrity in postmortem brain samples (Tomita et al., 2004), and as three of our samples died of injuries that were hypoxic in origin, this should be considered a limitation in the present study. Future studies regarding neuroinflammatory mechanisms in autism should match samples not only based on age, gender and PMI, but also on agonal conditions in order to determine if gene expression profiles truly are associated with autism or are simply remnants of the cause of death.

In conclusion, multiple studies demonstrate that an altered immune profile is present in the brain tissue of autistic individuals. While only a few studies have attempted to characterize the cytokine translational profile within various cortical regions, they have all revealed significant alterations in cytokine expression in autism. This is the first study to report on cytokine transcriptional expression in autism cortical tissue. Surprisingly, transcriptional results did not reflect those of translational results reported by other groups. This finding has led the investigators herein to conclude that post-transcriptional regulatory mechanisms may be putatively responsible for the observed differences in translational cytokine expression reported by other groups. The increased expression variability among ASD subjects indicates that the disorder may be heterogeneous on the

molecular level, further supporting the idea that the interplay between the environment and genetics are significant etiological components in this multifactorial disorder.

CHAPTER IV

Intravenous Glutathione Administration and Cytokine Expression Analysis in the Plasma of Autistic Children

Introduction

Autism spectrum disorders (ASD) are complex neurodevelopmental disabilities defined on the basis of a triad of behavioral impairments in three domains: socialization, language, and stereotyped behaviors (American Psychiatric Association, 2000). Accumulating evidence over the last few decades indicates that immune dysregulation is another key feature of the ASD pathology. Gastro-intestinal (GI) abnormalities, including inflammation, are frequently reported in children with autism, however the prevalence of these GI symptoms is inconsistent and varies widely across studies (Horvath & Perman, 2002; Molloy & Manning-Courtney, 2003; Taylor et al., 2002; Valicenti-McDermott et al., 2006). As a result, many non-traditional treatments have been proposed for autistic patients, including heavy metal detoxification and nutritional therapies (Aman, 2005). No evidence-based studies support these kinds of interventions in the treatment of autism, although anecdotal reports claim that they are effective (Christison & Ivany, 2006; Levy & Hyman, 2003). Systemic and CNS inflammation is present in patients diagnosed with autism, and persist despite oral or intravenous steroid or immunoglobulin therapies

(Handen et al., 2009). Thus, it is undetermined whether there is a pathophysiological relationship between autism and GI abnormalities or if these are just unrelated co-existing conditions that affect a subset of patients. Regardless, there is clearly some force that is driving the ongoing inflammatory response in ASD.

Oxidative stress, defined as an imbalance between the production and manifestation of reactive oxygen species (ROS) and the body's ability to detoxify reactive intermediates or repair the damage caused by them, has been demonstrated in the peripheral tissues of children with autism (Chauhan & Chauhan, 2006; Keller & Persico, 2003). Increased nitric oxide levels in red blood cells and higher antioxidant enzyme activity have been reported in autistic patients (Sogut et al., 2003). Chemically, oxidative stress is associated with increased production of oxidizing species or a significant decrease in the capability of antioxidant defenses, such as glutathione (Schafer & Buettner, 2001). The cys-containing tripeptide glutathione is the primary determinant of redox status in all human cells because it is a thiol-containing compound that is critical for heavy metal detoxification and elimination. Significantly lower levels of glutathione peroxidase, an antioxidant enzyme, and plasma glutathione, as well as higher ratios of oxidized glutathione to reduced glutathione, have been reported in autistic children and indicate that a disruption in antioxidant defense mechanisms is associated with the disorder (James et al., 2004; James et al., 2006; Yorbik et al., 2002).

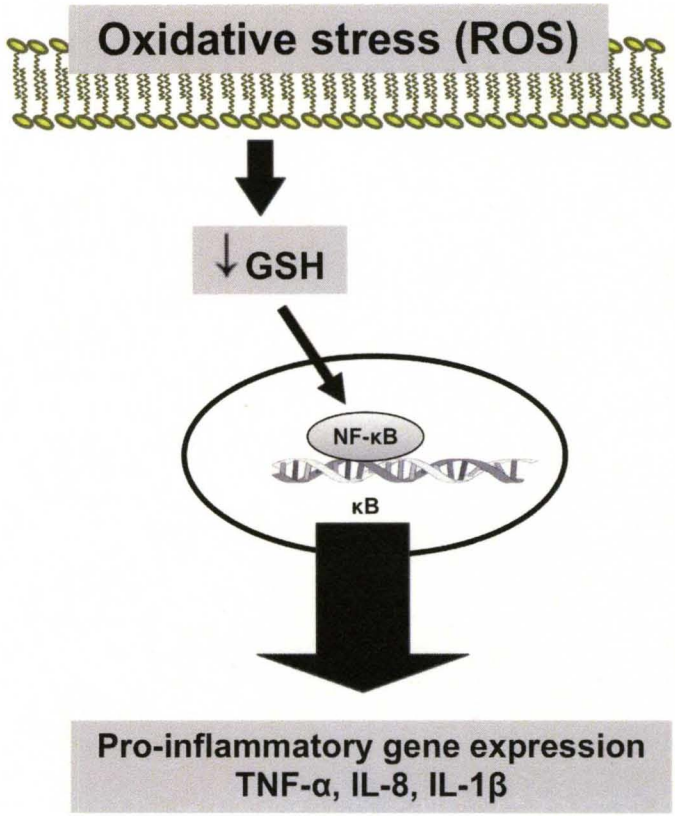
Oxidative stress occurs in the brain tissue of various neurodegenerative diseases including Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, Huntington's disease, and stroke (Beal, 2002; Neumann et al., 2008; M. A. Smith et al., 1994; Takeda et al., 2000). The brain is particularly vulnerable to oxidative stress during

early development because it has low levels of antioxidants, high energy requirements, and a high fat and iron content (Juurlink & Paterson, 1998). Astrocytes serve as reservoirs for glutathione within the brain, and provide the cysteine necessary for thiol metabolism in human neuronal cells. There is growing evidence supporting the role of oxidative stress in the brain tissue of autistic subjects (Sajdel-Sulkowska et al., 2008). Postmortem immunohistochemical studies have demonstrated that oxidative damage, evidenced by lipid modification, is localized primarily in the white matter of patients with autism, suggesting that axons may be the sites of primary oxidative damage within the brain of these individuals (Evans et al., 2008). This evidence is particularly relevant to the global underconnectivity deficits demonstrated in ASD (Casanova et al., 2009), and may account for these white matter changes from a molecular perspective.

Glutathione is the main intracellular antioxidant and protects neurons from oxidative stress (Siesjo et al., 1980). Thus, impaired glutathione production contributes to oxidative stress, which could delay the clearance of heavy metals or xenobiotics. Glutathione deficiency has been associated with Parkinson's disease, schizophrenia, ADHD, HIV, and inflammatory bowel disease (Do et al., 2000; Dvorakova et al., 2006; Iantomasi et al., 1994; Jenner, 1993; Kalebic et al., 1991). Although studies of glutathione expression in postmortem brain tissue of autistic subjects remain to be performed, systemic studies clearly indicate dramatically lower glutathione in ASD, which suggests that autistic children may have more difficulty resisting infection, resolving inflammation, and detoxifying environmental contaminants (James et al., 2006). The control of intracellular redox status is vital to proper cellular function via transcription factor-regulated intracellular signaling pathways that culminate in the

transcription of proinflammatory cytokines (see Figure 1 below). During oxidative stress and inflammation, transcription factors including NF-κB are activated and lead to the up-regulation of proinflammatory genes (Arrigo, 1999; Mercurio & Manning, 1999). The redox status of glutathione in particular is essential for the transcriptional regulation of these proinflammatory genes, and increasing intracellular glutathione has been shown to decrease the release of cytokines and chemokines by decreasing NF-κB activation (Rahman et al., 2005). Specifically, the NF-κB family of transcription factors is known to activate the expression of IL-1β, TNF-α, IL-6 and IL-8 (Akira & Kishimoto, 1997; Gotoh & Cooper, 1998; Rahman & MacNee, 1998). Thus, given that intracellular glutathione levels are low in ASD subjects (James et al., 2006), it is expected that an over abundance of proinflammatory cytokines will be expressed systemically. Indeed, several studies demonstrate that proinflammatory cytokine levels are significantly increased in the plasma and whole blood of autistic individuals (Ashwood et al., 2011; Croonenberghs et al., 2002a; Singh, 1996). In addition, increases in specific cytokine levels in autistic children have been associated with more impaired communication and aberrant behaviors (Ashwood et al., 2011). In this study, it is proposed that peripheral administration of exogenous glutathione in autistic children will result in a decrease of the expression of proinflammatory cytokines in plasma and improvements in behavioral outcome.

Figure 1. Oxidative stress alters the intracellular concentrations of glutathione (GSH), which modulates NF-κB activation leading to the transcription of proinflammatory cytokines.



It is challenging to determine how the immune-brain connection is responsible for creating and maintaining the ongoing CNS inflammatory response and abnormal metabolic profiles in autism. Exposures to environmentally toxic agents, disturbances in redox homeostasis and perturbations in immune balance have all been proposed as contributing factors to the pathophysiology of ASD. The cause of oxidative species in autism is not known but could be a result of exposure to environmental toxins such as heavy metals or infections during early development. It has been shown that chronic methylmercury exposure in primate cortex leads to a large increase in activated microglia, suggesting that heavy metal exposure may not only play an etiologic role in

oxidative stress but also in microglia-based neuroinflammation which is evident in autism (Charleston et al., 1994; Vargas et al., 2005). Additionally, thimerosal and inorganic mercury lower glutathione levels in a dose-response fashion in cultured human neuronal cells (Deth & Muratore, 2010). It is important to keep in mind that the production of ROS occurs in the course of normal physiological processes, however oxidative stress occurs when an overabundance of ROS causes cellular damage. The most important intracellular determinant of redox status is glutathione, and depleted levels of glutathione in patients with autism are an indication of oxidative stress (James et al., 2004). It is proposed here that weekly exogenous administration of glutathione will decrease the expression of proinflammatory cytokines measured in the plasma of children with ASD, and be correlated with behavioral improvement. In this way, modification of systemic glutathione levels and thereby cytokine expression may offer molecular therapeutic mechanisms in children diagnosed with ASD.

Materials and Methods

Subjects

Participants were enrolled in the study through the Kosair Charities Pediatric Research Unit at Kosair Children's Hospital in Louisville, Kentucky. Children with autism and severe behavior problems between the ages of 5 and 17 years old were invited to participate in this research study through the Department of Pediatrics at the University of Louisville Health Sciences Center, Weisskopf Child Evaluation Center, and Kosair Children's Hospital. Participants included 19 children with ASD (mean age 8.1, 17 males). This study was approved by the University of Louisville institutional review

board (GLUTATHIONE-KCPCRU-01; HSPPO #08.0220) and complied with all requirements regarding human subjects. Parents gave informed consent. Prior to study inclusion, the Autism Diagnostic Interview-Revised (ADI-R) was administered to confirm that each patient met diagnostic criteria. Rating scales were administered at designated times throughout the study per the protocol schedule to determine changes in behavior with treatment. The Clinical Global Impressions Improvement (CGI-I) Scale was completed to assess change in response to treatment. This assessment has been used extensively in psychiatric treatment studies and has been shown to have good reliability and validity.

Study Design

This was placebo-controlled and double-blind study, in which parents, study doctors and research nurses did not know when each child received which treatment. Patient participation lasted up to five months and included 18 study visits (including the screening visit). Once diagnosis was confirmed, a pre-drug phase screening visit was scheduled during which time written consent and a baseline blood specimen (~1 teaspoon) was obtained. Subjects were randomized to receive either glutathione or placebo (normal saline) intravenously over 15 minutes on a weekly basis. Randomization ensured that subjects received either glutathione or placebo in the first 2-month block and then crossed over to the alternate treatment in the second 2-month block. There was one week between the glutathione and placebo treatments during which time subjects did not receive any study drug.

During weeks 1-8, subjects received weekly injections of glutathione or placebo (whichever they were randomized to during that 2-month block). At week 9, the subject did not receive study drug and a blood specimen (~1 teaspoon) was collected. The subject crossed over to the other treatment during weeks 10-17 and at week 18 the subject did not receive study drug and a final blood specimen (~1 teaspoon) was obtained. Subject and sample information is contained in Table 8 below.

Table 8. Subject and sample information for glutathione study

Subject #	Gender	Age	Collection Type	Collection Date
002	M	10	Placebo	10/09/09
003	M	9	Glutathione	9/25/09
004	M	6	Glutathione	09/04/09
			Placebo	11/06/09
005	M	7	Glutathione	10/09/09
			Placebo	12/11/09
006	F	6	Baseline	08/28/09
			Placebo	11/06/09
			Glutathione	01/08/10
009	M	9	Baseline	10/23/09
			Placebo	12/31/09
			Glutathione	03/05/10
010	M	10	Baseline	11/06/09
			Placebo	01/15/10
			Glutathione	03/19/10
013	M	7	Baseline	01/29/10
			Placebo	04/09/10
			Glutathione	06/11/10
014	M	7	Baseline	02/05/10
			Glutathione	04/16/10
			Placebo	06/18/10
015	F	11	Baseline	02/12/10
016	M	8	Baseline	04/02/10
			Placebo	06/11/10
			Glutathione	08/13/10
017	M	11	Baseline	04/16/10
			Glutathione	07/01/10
			Placebo	09/03/10
018	M	6	Baseline	04/23/10
			Placebo	07/09/10
			Glutathione	08/27/10
020	M	6	Baseline	09/17/10
			Placebo	11/26/10
			Glutathione	01/28/10
021	M	8	Baseline	10/29/10
			Placebo	01/07/11
022	M	6	Baseline	02/18/11
023	M	7	Baseline	12/03/10
024	M	11	Baseline	02/25/11
025	M	9	Baseline	02/25/11

Sample preparation

For each subject, peripheral blood was collected in acid-citrate dextrose Vacutainers (BD Biosciences; San Jose, CA), centrifuged at 2,000 rpm for 20 minutes at room temperature, and the plasma harvested. Plasma was aliquoted and stored at -20 °C at the Kosair Charities Pediatric Clinical Research Unit facility. Plasma samples were transplanted on ice every 2 months to the Molecular Anthropology and Population Studies (MAPS) laboratory at the University of Louisville Health Sciences Center where they were stored at -20 °C until cytokine analysis.

Multiplex Analysis

Quantification of cytokines in the plasma were determined using human multiplexing bead immunoassays (Invitrogen, Carlsbad, CA) that are based on sandwich immunoassays that utilize Luminex® fluorescent-bead-based technology. Plasma samples were run in concordance with the human cytokine 10-plex assay (Invitrogen) as recommended by the manufacturer. The following cytokines were measured: Interleukin-1β (IL-1β), IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α), and granulocyte macrophage-colony stimulating factor (GM-CSF). Briefly, all samples were clarified by centrifugation (1,000 g for 10 min) prior to analysis. 50 μL of plasma was diluted 1:1 in human cytokine 10-plex assay diluent and incubated with antibody-coupled beads for two hours at room temperature on an orbital shaker at 500-600 rpm. Between each step the complexes were washed twice in wash buffer (Invitrogen) and aspirated using a vacuum manifold. The beads were then incubated with a biotinylated detector antibody for one hour before incubation with R-

Phycoerythrin (streptavidin-RPE) for 30 minutes. Finally, the complexes were resuspended in 100 μL of wash buffer and analyzed using a flow-based Luminex® 100™ device (Luminex Corporation, Austin, TX). Sample cytokine concentrations were calculated using a standard curve derived from the known reference cytokine concentrations supplied by the manufacturer. A five-parameter algorithm model with a weighted function ($1/y^2$) was used to calculate final concentrations and values are expressed in pg/mL.

Statistical Analysis

Sample cytokine concentrations in pg/mL were estimated by inverting the response curves, which were calculated using samples of known concentration provided by the manufacturer. Mean MFI (maximum fluorescence intensity) for a given concentration was modeled using a five-parameter logistic curve in the form recommended by Liao and Liu (Liao & Liu, 2009):

$$\text{MFI} = D + \frac{A - D}{[1 + (2^{1/g} - 1)(\frac{x}{C^*})^{b^g}]}$$

The parameters were estimated by maximum likelihood, assuming that the observed MFI followed a Poisson distribution. Cytokine concentrations between glutathione and placebo phases were tested using the Wilcoxon signed rank test for matched pairs. In all there were 11 matched pairs because one subject, #003, had glutathione data but no placebo data. The null hypothesis for each cytokine was that there would be no difference in concentration between placebo and glutathione phases. The alternative hypotheses were that concentrations of pro-inflammatory cytokines would be reduced under

glutathione treatment, and that other cytokine levels would differ in either direction from their placebo values. P-values were corrected for multiple comparisons using the false discovery rate (Benjamini & Hochberg, 1995). All calculations were performed using R software version 2.10.0 (R Development, 2011).

Results

Cytokine levels for nine of the ten cytokines investigated did not differ significantly between ASD patients who received placebo or glutathione administration when the Benjamini Hochberg correction was applied (Table 9). Median plasma cytokine levels were not calculable for GM-CSF as they were below the sensitivity limit of detection by the Luminex® 100™ device. CGI-I test scores indicated that no change was evaluated in most (8/12) autistic subjects in response to treatment with glutathione, and two subject's behavior actually worsened with glutathione treatment (Table 10). One subject, #009, reportedly had minimal improvement to little change in behavior with glutathione administration and another subject, #004, had minimal to much improvement in behavior with glutathione administration. Only two subjects had much improved behavior reported on the CGI-I scale, and both of these values were associated with placebo administration.

Table 9. Comparison of median plasma cytokine levels in children with ASD at baseline, placebo and glutathione blood draws

Cytokine[†]	Baseline (n=15)	Placebo (n=13)	Glutathione (n=12)	P-value[§] (n=11)
IFN-γ	1.009[*]	1.009	1.129	0.584
IL-1β	0.829	0.097	0.995	0.6637
IL-2	0.427	0.868	0.321	0.4755
IL-4	6.486	6.486	6.332	0.8311
IL-5	0.120	0.120	0.122	0.0144
IL-6	1.781	1.416	1.689	0.4492
IL-8	5.940	7.737	8.339	0.7676
IL-10	0.550	0.632	0.865	0.3326
TNFα	1.032	1.842	0.821	0.6499

[*] All values are measured in pg/mL.

[†] GM-CSF concentrations are not reported as they were below the sensitivity limit of detection.

[§] P-values were calculated using the Wilcoxon rank test for matched pairs between glutathione and placebo groups. When corrected for multiple comparisons using the false discovery rate, none of these P-values reached statistical significance at $p \leq 0.05$.

Table 10. Comparison of treatment and CGI-I scores

Subject #	Treatment	CGI-I Score*
002	Placebo	2
003	Glutathione	4
004	Glutathione	2.5
	Placebo	2
005	Glutathione	4
	Placebo	4
006	Placebo	4
	Glutathione	4
009	Placebo	3
	Glutathione	3.5
010	Placebo	4
	Glutathione	4
013	Placebo	2
	Glutathione	5.5
014	Glutathione	4
	Placebo	4
016	Placebo	4
	Glutathione	4
017	Glutathione	4
	Placebo	4
018	Placebo	5
	Glutathione	5
020	Placebo	4
	Glutathione	4
021	Placebo	4

* Compared to the patient's condition prior to initiation of treatment, this patient's condition is rated as: 1=very much improved; 2=much improved; 3=minimally improved; 4=no change from baseline; 5=minimally worse; 6= much worse; 7=very much worse

Discussion

The etiology of ASD remains unknown, but accumulating evidence indicates that there is an association between the ASD pathology and immune dysfunction. Peripherally, altered T cell function, increased natural killer and monocyte cell activation, and altered immunoglobulin profiles have been demonstrated in autistic individuals (Ashwood & Wakefield, 2006; Croonenberghs et al., 2002a; Enstrom et al., 2009; Saresella et al., 2009; Sweeten et al., 2004). Neuroinflammation is another characteristic finding in autism (Vargas et al., 2005). Oxidative damage, evidenced by plasma glutathione deficiency, is also present in individuals with ASD (James et al., 2004). The mechanisms linking immune and neuropathological dysfunction in ASD are still unclear, but evidence indicates that specific cytokines are capable of affecting neurodevelopment and consequently, behavior. Cytokines and cytokine receptors in the CNS modulate neural differentiation and plasticity. IL-6 is known to alter neuron proliferation and survival, cortical neuron dendrite development, neural activity and long-term potentiation (Gadient & Patterson, 1999; Juttler et al., 2002; Mehler & Kessler, 1998). Other cytokines, including IL-1β and TNF-α, are associated with neurite growth, oligodendrocyte toxicity, and regulation of homeostatic synaptic plasticity in the hippocampus (Barker et al., 2001; Cacci et al., 2008; Munoz-Fernandez & Fresno, 1998). Taken together, these findings suggest that cytokine dysregulation during the process of neurodevelopment may have significant biological effects on neuronal development and activity, which would negatively affect behavior.

It is likely that dysfunctional immune activity related to cytokines may affect the core features of ASD. Significant elevation of IL-1β, IL-6, IL-8 and IL-12p40 cytokine

levels have been reported in the plasma of ASD children, and those with a regressive form of ASD exhibited higher levels compared to children with non-regressive ASD (Ashwood et al., 2011). Furthermore, impairments in behavior were more pronounced as certain cytokine levels increased, such that IL-4 levels were associated with greater impairments in non-verbal communication, and significant associations were observed between increased IL-6, IL-8, and IL-1β levels and aberrant behaviors assessed by the Aberrant Behavior Checklist (Ashwood et al., 2011). Systemic modulation of cytokine expression, therefore, may provide therapeutic applications for ASD children with severe behavioral problems. Given that increasing intracellular glutathione levels has shown to decrease the release of proinflammatory cytokines, exogenous glutathione treatment offers a possible molecular therapy capable of putatively decreasing the expression of those cytokines associated with aberrant behaviors, and ultimately resulting in behavioral improvement in affected individuals.

In the present study, no significant changes in cytokine levels were reported in the plasma of ASD children in response to treatment with glutathione. CGI-I scores assessed throughout the study indicated that no significant changes in behavior were reported in response to glutathione treatment. Glutathione is synthesized intracellularly from glutamate, cysteine and glycine and is located largely within the cell (Voet & Voet, 2004). Exogenous administration of glutathione has been shown to have a very short half life in human plasma, with rapid elimination and total clearance within approximately 10 minutes (Wendel & Cikryt, 1980). Thus, one possibility as to why cytokine expression levels were not affected by exogenous glutathione administration could be that the tripeptide was simply unable to permeate cell membranes due to it being metabolized so

quickly within the bloodstream. Repleting glutathione levels with precursors of its synthesis, such as N-acetyl-cysteine (NAC) or 2-oxothiazolidine-4-carboxylic acid may prove to be more applicable treatments for increasing intracellular glutathione levels in ASD as cysteine precursors are more cell-permeable, and may even be given orally (Ghezzi, 2011). Modification of intracellular glutathione levels should still be considered a possible molecular therapy capable of regulating systemic proinflammatory cytokine expression and behavioral outcome in autistic children but not with exogenous administration of glutathione itself. Instead, treatment should focus on precursors of glutathione synthesis that are more permeable and metabolized more slowly, so that changes in intracellular glutathione levels may have more long-lasting effects on NF-κB activation, proinflammatory cytokine expression, and ultimately behavior.

CHAPTER V

Discussion

Review of Findings

Previous investigations demonstrate that abnormalities in cytokine translational expression levels both systemically and in the CNS are one component of the immune dysfunction that characterizes a subset of subjects with ASD. Some of these results are contradictory (see Table 1) and could be a reflection of discrepancies between studies in terms of classification and inclusionary criteria, methodology, co-morbidity presence, immunization status, or co-administration of psychotropic medications. Contradictory evidence could also confirm the presence of yet undetermined immune derived endophenotypes within ASD. Immune activation during pregnancy alters cytokine expression in maternal serum, the placenta, and fetal brain (Urakubo et al., 2001; Gillmore et al., 2005; Depino, 2006). Evidence for CNS and immune dysfunction involving cytokines in ASD led to the hypothesis purported here: that autism pathogenesis is caused by the combination of genetic susceptibility in cytokine genes and a second "hit" prenatal infectious scenario that occurs during specific periods of development. The primary questions of each investigation are as follows:

1.) Do cytokine genes differ between autistic subjects and controls?

2.) Does cytokine transcriptional expression reflect previously qualified values of translational expression in the cerebral cortex of autistic patients?

3.) Can cytokine expression profiles and behavior be modified systemically with glutathione administration in children with ASD?

The findings of each investigation are reviewed below.

SNP Investigation

Polymorphisms within the regulatory regions of cytokine genes are known to effect expression levels and play a role in a variety of diseases. Thus, experimental aim 1 was to characterize different SNPs of selected candidate genes encoding cytokines in autistic patients and controls to determine if any cytokine SNPs are associated with ASD. Twenty-two single nucleotide polymorphisms (SNPs) in 11 cytokine genes (*IL1A, IL1B, IFNG, TGFB1, TNF, IL2, IL4, IL6, IL12B, IL10*), two cytokine receptors (*IL1R1, IL4R*) and one cytokine receptor antagonist (*IL1RN*) were analyzed. These genes were chosen because they represent cytokines from both the innate and adaptive immune responses. ASD DNA samples were obtained through an online cell repository and recruited for the study at the University of Louisville Hospital. Control samples were obtained though amalgamated population surveys available in an online database. Tests for Hardy-Weinberg equilibrium in both ASD and control cohorts indicated no statistically significant p-values following a multiple-test correction, indicating that the two groups were genetically comparable at the investigated locations. Results indicated that proinflammatory *IL1R1* +1970T, and anti-inflammatory *IL4* -590T and -33T alleles are significantly different in ASD patients compared to controls. This analysis suggests that these cytokine SNPs may confer susceptibility to the disorder.

Transcription Investigation

Recent research indicates that a neuroinflammatory scenario is present in individuals with autism, evidenced by abnormal cytokine translational expression in various cortical regions (Li et al., 2009; Vargas et al., 2005). To investigate whether a neuroinflammatory scenario is present at the transcriptional level, the mRNA profiles of four cytokines (TGFβ-1, IL-1β, IL-6 and TNF-α) and one chemokine (IL-8) were analyzed in five regions of the cerebral cortex (BA 4, 9, 17, 22, and 46) of postmortem brains in eight autistic-control pairs. Autistic and control brain specimens were obtained through the Autism Tissue Program and formaldehyde-fixed sections corresponded to primary motor (BA 4), dorsolateral prefrontal (BA 9 and 46), primary visual (BA 17) and superior temporal/Wernicke's (BA 22) cortical areas. No significant difference was found in mean age or postmortem interval between autistic and control samples, indicating that these groups were comparable. RNA extraction and reverse transcription reactions were followed by real-time PCR analysis, where GAPDH was used as the housekeeping gene. Statistical analysis used a mixed effects linear model to account for correlation between measurements of different Brodmann areas within the same brain, and results indicated that relative expression of all five target genes did not vary significantly by cortical location between autistic and control groups. Thus, these transcriptional results do not indicate that a neuroinflammatory scenario is present at the transcriptional level. Because increased levels of proinflammatory cytokines have been evidenced at the translational level (see Table 1), the investigators herein concluded that post-transcriptional regulatory mechanisms may be putatively responsible for the observed differences in translational cytokine expression and warrant future study.

Glutathione Investigation

Oxidative stress, evidenced by depleted levels of plasma glutathione, indicate that a disruption in antioxidant defense mechanisms is associated with ASD (James et al., 2004). During oxidative stress and inflammation, transcription factors including NF-κB are activated and lead to the up-regulation of proinflammatory cytokines. Increases in several proinflammatory cytokines have been demonstrated in the plasma of ASD children, and specific cytokine levels have been associated with more impaired communication and aberrant behaviors (Ashwood et al., 2011). It has been demonstrated that increasing intracellular glutathione decreases the activation of NF-κB and results in a decrease in the release of proinflammatory cytokines and chemokines (Rahman et al., 2005). To investigate whether cytokine expression can be modified systemically and demonstrate a correlation with behavioral change, exogenous glutathione was administered weekly in children with ASD and their plasma analyzed after a 2-month treatment block. ASD children were recruited for this study through Kosair Charities Pediatric Research Unit at Kosair Children's Hospital. This study was double-blind and placebo controlled. Multiplex analysis of ten cytokines (GM-CSF, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IFN-γ and TNF-α) was performed using a Luminex® 100 device and statistical analysis was performed using the Mann Whitney Wilcoxon test. Results indicated that there was no significant difference in median cytokine concentrations for nine of the ten cytokines investigated between ASD patients who received placebo or glutathione administration. The median plasma cytokine concentrations of GM-CSF were not calculable as they were below the sensitivity limit of detection by the Luminex® 100™ device. CGI-I test scores indicated that little to no change was evaluated in the

majority of autistic subjects in response to treatment with glutathione. As exogenous administration of glutathione has been shown to have a very short half-life in human plasma, it was concluded that cytokine expression levels were not affected by glutathione administration because the tripeptide was simply unable to permeate cell membranes due to its quick systemic elimination. Repleting glutathione levels with precursors of its synthesis, such as N-acetyl-cysteine (NAC) or 2-oxothiazolidine-4-carboxylic acid may prove to be more applicable treatments for increasing intracellular glutathione levels in ASD, as these cysteine precursors are more cell-permeable (Ghezzi, 2011). This study demonstrates that exogenous administration of glutathione is not sufficient to change cytokine expression or aberrant behavior in ASD children. Changes in intracellular glutathione levels mediated by cysteine precursors may have more long-lasting effects on NF-κB activation, proinflammatory cytokine expression, and behavioral improvement.

Limitations of Findings

There are inherent statistical issues with analyzing multiple polymorphic sites within a genetic association study. One critical assumption in calculations of genotypic frequency within a small sample cohort is that the estimated allele frequencies exactly equal the underlying allele frequencies in the population being considered. In analyzing a cytokine panel that contains 13 genes, there is roughly a 50% chance of obtaining a significant result by chance. In order to correct for multiple comparisons, the Benjamini Hochberg method was used. One limitation in the SNP study, therefore, is the possibility that some important data may have been obviated during the application of the Benjamini Hochberg correction. Another significant limitation was the number of ASD samples

available for comparison. Generally, statistical power increases with the number of individuals studied in genotypic investigations, and ideal genotype studies involve hundreds, if not thousands, of individuals (Purcell et al., 2003). This investigation was limited by the 49 ASD subjects that could be recruited from the University of Louisville Hospital for participation during the duration of the study. Future investigations of cytokine genetics in ASD should analyze sample sizes that include much larger sample cohorts. Perhaps the most significant limitation contained in the SNP study is the assumption that the contribution of particular cytokine SNPs influences inflammation and the etiology of ASD equally. Just as ASD is heterogeneous, so too may be the etiological mechanisms that confer susceptibility to the disorder. In other words, it is possible that specific SNPs may be associated with the ASD subtypes or even with comorbidities that so often occur within these subtypes. Thus, future research into any genotype analysis regarding ASD should correlate subtype classification with SNP frequency for the most robust conclusions.

Two significant limitations in the analysis of cytokine transcriptional profiles included agonal conditions associated with the samples investigated, as well as issues of tissue quality provided by the brain bank. These issues affect all immunocytochemical studies of postmortem brain tissue from autistic subjects as investigators receive their tissue from the same brain banks. Several of the autistic patients studied here and elsewhere in the literature died of hypoxic lesions that were ischemic in origin. Ischemic reperfusion injuries of this kind result in inflammation and the oxidative damage that have been interpreted as characterizing an "autistic" neuropathology. Due to these issues of agonal conditions associated with the samples available, it has not been definitively

demonstrated that the neuroinflammation (and cytokine expression) present in these tissues represents a core pathology associated with ASD or are rather secondary reactions of this tissue associated with the cause of death. Additionally, multiple investigators who received samples from the Autism Tissue Program have reported extensive degradation in their tissue. While RNA quantification was performed prior to analysis in this investigation, brain banks should focus their efforts on establishing quality assessment measures prior to distributing tissue to research laboratories. Otherwise, remnants of poor tissue quality may be inferred as pathological findings inherent to the ASD phenotype.

While there are several advantages of Luminex® analysis, there are also several major problems. Baseline levels and detected levels of cytokines and other proteins reported in studies that use Luminex technology are not the same as those observed by ELISA techniques, or of other immunoassay kits (Loo et al., 2011; Richens et al., 2010). Cytokine concentrations reported in the study of glutathione treatment did not match those reported by other groups, and this is likely attributable to issues associated with the specific kit being used for analysis. Different manufacturers use different buffering, antibodies, and antibody sensitivities, and design their systems to start at higher or lower baselines with more or less dynamic ranges in order to fit multiple assays into one kit. Thus, cytokine levels cannot be compared across different manufacturers. Additionally, under physiological conditions cytokines are generally low and many patients simply do not have detectable cytokines in their plasma (de Jager et al., 2009). GM-CSF was not detectable in any of the plasma analyzed in the glutathione study. Although the cytokine concentrations reported in this study did not match concentrations measured by other groups, the baseline trends were similar and the reliability of the behavioral results is

unequivocal. Exogenous glutathione administration does not ameliorate aberrant behavior in autistic children and does not warrant therapeutic application in future research, although modification of intracellular glutathione via the use of precursor molecules may modify cytokine expression systemically in ASD children and result in improved behavioral outcome.

Infection and inflammation during neurodevelopment: Common mechanisms in collateral neuropathological damage

The fetal and neonatal immune system has significant influences on the developing CNS, and immune dysfunction during critical periods of development carries serious neuropathological implications. Increasing evidence suggests that progressive CNS damage is mediated by immune mechanisms, which may be contributing factors of susceptibility in Alzheimer's disease, Parkinson's disease, and schizophrenia (Long-Smith et al., 2009; Porcellini et al., 2010; Teixeira et al., 2008). Review of current research regarding neurodegeneration in the presence of neuroinflammation has led some investigators to postulate that inflammatory and neurodegenerative pathologies of the CNS share common molecular mechanisms (Zipp & Aktas, 2006). A classic example to substantiate this theory can be found in studies of multiple sclerosis (MS). As a demyelinating disease, previous research focused on oligodendrocyte and white matter dysfunction. Subsequently, a century of histopathological study ignored the significance of neuronal damage in MS, and it was not until the 1990s that early axonal pathologies in the brains of MS patients were correlated with the degree of inflammation within a lesion (Trapp et al., 1998). Since then, infiltration of immune mediators into brain parenchyma

has proven to have severely deleterious effects on neuronal tissue, as evidenced by Wallerian degeneration in MS and neuronal apoptosis in bacterial meningitis and HIV encephalopathy (Evangelou et al., 2000; Kaul et al., 2001; Nau & Bruck, 2002). Neuronal damage was considered an indirect consequence of immune cell invasion since immune cells did not usually have neuronal antigens and supposedly had no affinity for neurons since they did not express major histocompatibility complex molecules. However, recent evidence illustrates that activated immune cells actually do have the capacity to directly target neurons and induce apoptosis in the inflamed brain (Giuliani et al., 2003).

Perinatal exposure to infectious agents is linked to the pathogenesis of neuropsychiatric disorders, but the mechanisms responsible for triggering the interaction of the developing immune system and CNS which result in neurodevelopmental disturbance have yet to be discovered. Epidemiological studies indicate that pregnant women exposed to second-trimester respiratory infection have a significantly increased risk for giving birth to a child that will develop schizophrenia (A. S. Brown, 2006). Recent epidemiological research also indicates that early prenatal viral infection is associated with ASD incidence (Altadottir et al., 2010). Mouse models of intrauterine infection/inflammation cause cognitive deficits and neurodegeneration in their offspring (Golan et al., 2005). Inflammatory responses in the fetus and neonate have been shown to contribute to cerebral white matter damage (Rezaie & Dean, 2002). Regarding autism, several pre- and perinatal environmental exposures have been investigated using animal models.

Although the use of animal models in investigations of neuropsychiatric disease is controversial, measurements of behavioral, functional, anatomical, and histological

characteristics parallel those found in humans and thus warrant review. Neonatal infection with borna disease virus has been proposed as a model of neurodevelopmental damage in rats that causes cytoarchitectural features and behavioral abnormalities similar to those observed in children with autism (Hornig et al., 1999). It has been proposed that genetic susceptibility and exposure to a maternal immune response (rather than direct infection of the fetus) are key players in the development of the autism phenotype (Patterson, 2005). Offspring of pregnant mice who are injected with poly(I:C) to mimic viral infection during early gestation show cerebellar pathologies consistent with those seen in autism (Shi et al., 2009). Systemic lipopolysaccharide (LPS) administration, a commonly used experimental method designed to mimic bacterial infection, is known to induce a peripheral inflammatory response that crosses the BBB and affects cognitive function, dendritic structure, neuronal and glial proliferation, and in some cases produces brain lesions (Hagberg & Mallard, 2005). There is substantial evidence to suggest that proinflammatory cytokines, particularly IL-6, mediate the effects of maternal immune activation on fetal brain development (S. E. Smith et al., 2007). This should come as no surprise, since it has been established that IL-6 effects brain development, the balance between neurogenesis and gliogenesis, learning, memory, and the CNS response to injury and disease (Bauer et al., 2007; He et al., 2005). Although the precise mechanisms of fetal brain activation are yet undetermined, evidence indicates that immune mediators have a significant role in the pathogenesis of autism and other neuropsychiatric disorders.

Neuroglial cells have significant roles in pre- and postnatal neurodevelopment, thus prenatal neuroimmune disruption mediated by these cells can have profound neurological ramifications. Microglia and astroglia, for example, are involved in cortical

organization, neuroaxonal guidance and synaptic plasticity (Fields & Stevens-Graham, 2002). Astrocytes promote neuronal survival by releasing growth factors and controlling uptake and removal of excitotoxic neurotransmitters from the synaptic microenvironment (Nedergaard et al., 2002). Astrocytes also produce several soluble factors that promote synaptogenesis (Barres, 2008). In a study of human fetal astrocytes, TLR3 was the only TLR with consistent expression in the resting state, which is known to bind double stranded RNA in the presence of a viral infection (Farina et al., 2005). Microglia have roles in late embryonic brain development and early postnatal brain maturation by modulating axon pathfinding and inducing neuronal apoptosis, phagocytosis, synapse refinement, and innate immunity (Chamak et al., 1994; Deverman & Patterson, 2009). Embryonic microglia also secrete factors that are angiogenic, and depletion of microglia during neonatal development reduces vascularization (Checchin et al., 2006). All neural and glial cell types in the developing CNS use cytokines for paracrine and autocrine signaling. Since cytokines also serve as peripheral immune regulators, it follows that neurodevelopmental processes are vulnerable to disruption by immune dysregulation occurring prenatally in the form of maternal infection.

While neuronal damage is a characterizing feature of neuroinflammatory disease, it is unclear whether neuroinflammation is a consequence of neurodegeneration or *vice versa*. Molecular imaging techniques will be required to reveal the precise mechanisms shared by each pathological condition. Although various neurological diseases differ considerably etiologically and pathologically, many share crucial inflammatory processes and immunological mechanisms that cause brain damage. Future research that targets these processes will focus on the interface of immune response and neuronal

homeostasis, and elucidate novel therapeutic applications for both neurodegenerative and neurodevelopmental disorders.

Conclusions

ASD remains a pervasive idiopathic neurodevelopmental disorder with a characteristic neuropathology that is associated with specific behavioral deficits. While accumulating evidence suggests that genetic and immunological components have significant pathogenic roles in the development of ASD, inheritance patterns and immune mechanisms are complex and difficult to interpret in a disease defined by heterogeneity. In addition to the assorted behavioral manifestations on the spectrum, endophenotypes of ASD based on genetic or immunologic markers may be associated with symptom severity in subsequent studies. The notion that prenatal factors are involved in autism is currently only speculative but has significant implications. Maternal and/or fetal immune activation during critical periods of development may permanently alter the fetal immunological balance and predispose the fetus to a lifetime of chronic inflammation both peripherally and in the CNS. Collaboration will be imperative in future studies, which should be performed on larger cohorts of ASD patients and aim to rectify inclusionary criteria for both patient and control cases. Well-designed demonstrations of environmental influence on neurodevelopmental trajectories will provide coherent and compelling molecular mechanisms by which genetic susceptibility and prenatal immune challenge contribute to the etiology of autism. Longitudinal studies that begin prior to diagnosis would provide ideal means for understanding whether changes in cytokine expression play an etiologic role or have predictive potential in ASD, or whether they are purely phenomenological.

The investigations contained herein add to the growing research that implicates cytokines in the pathogenesis of ASD. These novel findings indicate that SNPs in the cytokine genes *IL1R1* +1970, and *IL4* -590 and -33 are significantly different in a cohort of ASD patients when compared to controls, and suggest that the T allele at each of these locations may confer susceptibility to ASD. In the first investigation of cytokine transcriptional expression in the cortical tissue of autistic subjects, it was found that cytokine mRNA expression does not differ significantly in the cortex of autistic patients when compared to controls. Given that translational expression patterns do indicate significant alterations in cytokine expression at the protein level, this work suggests that post-transcriptional regulatory mechanisms may be responsible for altered translational cytokine profiles in cortical tissue of autistic individuals. This was the first study to investigate the use of exogenous glutathione as a therapeutic application in modifying systemic cytokine expression and ameliorating aberrant behaviors associated with ASD. Results indicated that exogenous glutathione administration is not sufficient for systemic modification of cytokine expression and that, contrary to anecdotal reports, glutathione does not improve aberrant behaviors in ASD subjects. Given the immunological and neuropathological dysfunctions that characterize ASD, cytokines provide a molecular pathway whereby basic cellular processes in both systems may be permanently altered during prenatal development and ultimately culminate in the autistic phenotype, and the results presented herein indicate that cytokine studies in ASD merit further investigation.

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APPENDIX

Diagnostic Criteria for Autism Spectrum Disorder according to the American Psychiatric Association's Diagnostic and Statistical Manual-IV, Text Revision (DSM-IV-TR).

Diagnostic Criteria for 299.00 Autistic Disorder

A. Six or more items from (1), (2), and (3), with at least two from (1), and one each from (2) and (3):

1. qualitative impairment in social interaction, as manifested by at least two of the following:

a. marked impairment in the use of multiple nonverbal behaviors such as eye-to-eye gaze, facial expression, body postures, and gestures to regulate social interaction

b. failure to develop peer relationships appropriate to developmental level

c. a lack of spontaneous seeking to share enjoyment, interests, or achievements with other people (e.g., by a lack of showing, bringing, or pointing out objects of interest)

d. lack of social or emotional reciprocity

2. qualitative impairments in communication as manifested by at least one of the following:

a. delay in, or total lack of, the development of spoken language (not accompanied by an attempt to compensate through alternative modes of communication such as gesture or mime)

b. in individuals with adequate speech, marked impairment in the ability to initiate or sustain a conversation with others

c. stereotyped and repetitive use of language or idiosyncratic language

d. lack of varied, spontaneous make-believe play or social imitative play appropriate to developmental level

3. restricted repetitive and stereotyped patterns of behavior, interests,

and activities, as manifested by at least one of the following:

a. encompassing preoccupation with one or more stereotyped and restricted patterns of interest that is abnormal either in intensity or focus

b. apparently inflexible adherence to specific, nonfunctional routines or rituals

c. stereotyped and repetitive motor manners (e.g., hand or finger flapping or twisting, or complex whole-body movements)

d. persistent preoccupation with parts of objects

B. Delays or abnormal functioning in at least one of the following areas, with onset prior to age 3 years: (1) social interaction, (2) language as used in social communication, or (3) symbolic or imaginative play.

C. The disturbance is not better accounted for by Rett's Disorder or Childhood Disintegrative Disorder.

Diagnostic Criteria for 299.80 Asperger's Disorder

A. Qualitative impairment in social interaction, as manifested by at least two of the following:

1. marked impairment in the use of multiple nonverbal behaviors such as eye-to eye gaze, facial expression, body postures, and gestures to regulate social interaction

2. failure to develop peer relationships appropriate to developmental level

3. a lack of spontaneous seeking to share enjoyment, interests, or achievements with other people (e.g., by a lack of showing, bringing, or pointing out objects of interest to other people)

4. lack of social or emotional reciprocity

B. Restricted repetitive and stereotyped patterns of behavior, interests and activities, as manifested by at least one of the following:

1. encompassing preoccupation with one or more stereotyped and restricted patterns of interest that is abnormal either in intensity of focus

2. apparently inflexible adherence to specific, nonfunctional routines or rituals

3. stereotyped and repetitive motor mannerisms (e.g., hand or finger

flapping or twisting, or complex whole-body movements)

4. persistent preoccupation with parts of objects

C. The disturbance causes clinically significant impairment in social, occupational, or other important areas of functioning.

D. There is no clinically significant general delay in language (e.g., single words used by age 2 years, communicative phrases used by age 3 years).

E. There is no clinically significant delay in cognitive development or in the development of age-appropriate self-help skills, adaptive behavior (other than in social interaction), and curiosity about the environment in childhood.

F. Criteria are not met for another specific Pervasive Developmental Disorder or Schizophrenia.

Diagnostic Criteria for 299.80 Pervasive Developmental Disorder Not Otherwise Specified (Including Atypical Autism)

This category should be used when there is a severe and pervasive impairment in the development of reciprocal social interaction associated with impairment in either verbal or nonverbal communication skills or with the presence of stereotyped behavior, interests, and activities, but the criteria are not met for a specific Pervasive Developmental Disorder, Schizophrenia, Schizotypal Personality Disorder, or Avoidant Personality Disorder. For example, this category includes "atypical autism" - presentations that do not meet the criteria for Autistic Disorder because of late age at onset, atypical symptomatology, or subthreshold symptomatology, or all of these.

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WORK EXPERIENCE

University of Louisville School of Medicine, *Tutor,* Medical Gross Anatomy
September 2007 – present

University of Louisville School of Medicine, *Graduate Teaching Assistant,* Neuroanatomy
October 2008 – December 2008
October 2009 – December 2009

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University of Louisville School of Medicine, *Graduate Teaching Assistant*, Dental Gross Anatomy

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University of Louisville School of Medicine, *Graduate Teaching Assistant*, Dental Head and Neck and Neuroanatomy

January 2008 – March 2008
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University of Louisville Department of Psychiatry and Behavioral Sciences, *Laboratory Technician*, Molecular Anthropology and Population Studies Laboratory

June 2006 – present

University of Chicago Department of Organismal Biology and Anatomy, *Laboratory Technician*, Ramirez Laboratory

September 2005 – June 2006

University of Chicago Hospitals Department of Radiation Oncology, *Death Database Organizer*

September 2005 – June 2006

University of Florida Whitney Laboratory for Marine Bioscience, *REU Intern,* Ono Laboratory

June 2005 – September 2005

University of Chicago Department of Ecology and Evolution, *Laboratory Technician,* Coyne Laboratory

June 2004 – September 2004

PROFESSIONAL DEVELOPMENT

Dine and Discover: Teaching Strategies
Avoiding Slackers, Dictators and Other Problems: How to Structure and Assess Team Projects that Promote Effective Student Teamwork, 2011

Delphi Center for Teaching and Learning: Understanding Today's Learners: Meet Generation NeXt, 2011

Delphi Center for Teaching and Learning: Teaching for Lasting Change: A Pedagogy of Formation for the Health Professions, 2011

Understanding How Students Learn: "I'm just not good at this stuff" and Other Dangerous Ideas: How to talk to Students about Determinants of Classroom Performance, 2011

Understanding How Students Learn: Guiding Students in Thinking About Their Own Thinking: Using Metacognition to Improve Student Learning, 2011

ACTIVITIES

National Safe Place, *Neuroanatomy Consultant for Teen Brain Tutorials*
September 2010-present

School of Medicine Graduate Council, *Graduate Student Representative*
July 2010-June 2011

Medical Reserve Corps, *Non-medical volunteer*
May 2009 – present

School of Medicine Academic Grievance Committee, *Graduate Student Representative*
August 2009-July 2010
July 2010-June 2011

School of Medicine Faculty Forum, *Graduate Student Representative*
July 2008-June 2009

School of Medicine Medical Council, *Graduate Student Representative*
July 2007-June 2008

Gorilla Forest at The Louisville Zoo, *Primate Behaviorist, volunteer zookeeper*
June 2006 – June 2010

AWARDS

International Meeting for Autism Research Travel Award, Autism Research Institute 2010

Graduate Student Council Travel Award, University of Louisville 2010

Brain Tissue Recipient, Autism Tissue Program, Harvard Brain Tissue Resource Center, McLean Hospital, Belmont Massachusetts, 2010

Graduate Student Council Travel Award, University of Louisville 2009

Louisville Zoo Area Award for Outstanding Volunteer Service to Gorilla Forest, 2008

Graduate Student Council Travel Award, University of Louisville 2007

Integrated Programs in Biomedical Sciences (IPIBS) Doctoral Fellowship, University of Louisville 2006

REU (Research Experience for Undergraduate) Internship, Whitney Laboratory for Marine Bioscience 2005

MISCELLANEOUS TRAINING EXPERIENCE

Health Care and Code of Ethical Conduct Ethics Training, University of Louisville, 2010

Kentucky Health Emergency Listing of Professionals for Surge/Medical Reserve Corps Incident Command System and Family Disaster Plans Training, 2009

Autism Diagnostic Interview-Revised (ADI-R) Training, University of Louisville Department of Pediatrics Weisskopf Child Evaluation Center, 2008

Department of Environmental Health and Safety Bloodborne Pathogens Training, University of Louisville 2007

HIPAA and Research Fundamentals Training Course, University of Louisville 2007

CITI Course in the Protection of Human Research Subjects, Biomedical Investigator Course, University of Louisville 2006

PROFESSIONAL MEMBERSHIPS

International Society for Autism Research, Student member, 2009-present

Association for Women in Science, Student member, 2008-present

American Society for Neuroscience, Student member, 2007-present

Louisville Chapter of the Society for Neuroscience, Student member, 2007-present

PUBLICATIONS

Research Papers:

Mott M, Casanova MF, Tillquist CR, Fernandez-Botran GR, Sears L, Switala A, Crespo F. Transcriptional Analysis of cytokines in the cerebral cortex of autistic patients. Journal of Neuroimmunology. In preparation.

Mott M, Crespo FA, Casanova M, Switala A, Fernandez-Botran GR, Sears L, Tillquist CR. Cytokine polymorphisms in autism: immunogenetic implications of pathogenesis. Brain Behavior and Immunity. In preparation.

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