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Immune Endophenotypes in Children With Autism Spectrum Disorder

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ABSTRACT

BACKGROUND: Autism spectrum disorder (ASD) is characterized by social communication deficits and restricted, repetitive patterns of behavior. Varied immunological findings have been reported in children with ASD. To address the question of heterogeneity in immune responses, we sought to examine the diversity of immune profiles within a representative cohort of boys with ASD.

METHODS: Peripheral blood mononuclear cells from male children with ASD ($n = 50$) and from typically developing age-matched male control subjects ($n = 16$) were stimulated with either lipopolysaccharide or phytohemagglutinin. Cytokine production was assessed after stimulation. The ASD study population was clustered into subgroups based on immune responses and assessed for behavioral outcomes.

RESULTS: Children with ASD who had a proinflammatory profile based on lipopolysaccharide stimulation were more developmentally impaired as assessed by the Mullen Scales of Early Learning. They also had greater impairments in social affect as measured by the Autism Diagnostic Observation Schedule. These children also displayed more frequent sleep disturbances and episodes of aggression. Similarly, children with ASD and a more activated T cell cytokine profile after phytohemagglutinin stimulation were more developmentally impaired as measured by the Mullen Scales of Early Learning.

CONCLUSIONS: Children with ASD may be phenotypically characterized based upon their immune profile. Those showing either an innate proinflammatory response or increased T cell activation/skewing display a more impaired behavioral profile than children with noninflamed or non-T cell activated immune profiles. These data suggest that there may be several possible immune subphenotypes within the ASD population that correlate with more severe behavioral impairments.

Keywords: Autism spectrum disorder, Behavior, Children, Cluster analysis, Cytokine, Endophenotyping, Immune

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Autism spectrum disorder (ASD) is a heterogeneous neurodevelopmental disorder characterized by stereotyped interests, repetitive behaviors, and impairments in social communication. Currently 1 in 68 children have been identified as having ASD (1,2). Despite the high prevalence of ASD, the etiology(ies) and pathogenesis remain poorly understood. Numerous published findings have identified widespread differences in the immune systems of children with ASD, both at the systemic and cellular levels (3). These differences have been associated with impairments in the core features of ASD, as well as other aberrant behaviors, decreased adaptability, and more impaired cognition in children with ASD (4,5). Neuroimmune interactions begin during early neurodevelopment and continue throughout life, with the immune system supporting many aspects of neuronal function. Dysregulation in the immune system could increase sensitivity to altered neurologic function from a variety of sources, particularly during early development (6). Importantly, altered immune function in the brain of individuals with ASD is suggested by evidence of neuroglial activation and neuroinflammation in the central nervous system (7), as well as the presence of antibodies reactive to neuronal tissue (8–12) in a subset of children with ASD. These immune differences may be related to numerous genetic associations linked with immune functions in ASD, such as linkages to the major histocompatibility complex genes (13–15). Consequently, research that identifies the biological basis of the immune dysfunction in children with autism may facilitate early detection, prevention, and/or treatment.

Among the immunological findings in ASD, reports of increased proinflammatory markers remain the most consistently observed. These include increased levels of proinflammatory cytokines, such as interleukin (IL)-1β, tumor necrosis factor alpha (TNFα), and IL-6, and chemokines, such as monocyte chemoattractant protein-1 (MCP-1), in plasma, cerebrospinal fluid, and postmortem brain tissue (4,16–19). As noted, many of these studies highlight a connection between immune dysregulation and more impaired behaviors (5). These findings suggest a proinflammatory immune profile is prevalent within the ASD population, or at least in a

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subgroup of the population, where immune activation may be linked to more impaired behavioral symptomology.

In this study, we utilized a cohort of children that consisted of children primarily from Northern California with a broad presentation of ASD severity and extent of comorbid features along with age-matched and geographically matched typically developing (TD) control subjects. We hypothesized that within this well-defined cohort of children, a subgroup could be identified based on immune function that would be associated with a characteristic behavioral profile. To determine this relationship, children in the study received a battery of cognitive and behavioral assessments along with a number of physiological measures including neurological and immunological evaluations. Immune measures were used to cluster children into subgroups and behaviors were assessed between resulting groups.

METHODS AND MATERIALS

Subjects

Participants were enrolled through the Autism Phenome Project study conducted at the University of California Davis M.I.N.D. Institute. The study protocols including recruitment and behavioral assessments have been previously described in detail (20,21). Because of the gender disparities in ASD, we chose to focus on male subjects to eliminate issues of gender differences. In brief, following clinical evaluation for diagnostic confirmation, participants were placed in one of two groups: 1) children with a confirmed diagnosis of ASD ($n = 50$, median age 3.21 [interquartile range 2.80–3.52 years]); or 2) children who were confirmed as typically developing control subjects ($n = 16$, median age 2.80 [interquartile range 2.47–3.14 years]). Final diagnosis of ASD was confirmed by the Autism Diagnostic Interview-Revised (ADI-R) (22) and the Autism Diagnostic Observation Schedule (ADOS) (23). The ADOS and ADI-R consist of standardized, semi-structured interviews and diagnostic algorithms from the DSM-IV-TR (24), with definitions of autism from ICD-10 (25). Inclusion criteria for the TD children included scores on the Lifetime Edition of the Social Communication Questionnaire (26), a screening tool for ASD, below the recommended screening cutoff for young children of 11 and developmental scores within 2 standard deviations of the mean on all subscales of the Mullen Scales of Early Learning (MSEL) (27). Exclusion criteria for TD control subjects included a diagnosis of intellectual disability, pervasive developmental disorder, or specific language impairment or any known developmental, neurological, or genetic disorder. The TD control subjects were recruited through interactions with school districts and other outreach mechanisms. The exclusion criteria for all subjects consisted of the presence of Fragile X syndrome or other serious neurological (e.g., seizures), psychiatric (e.g., bipolar disorder), or known medical conditions including autoimmune disease, inflammatory bowel disease, or celiac disease. The administration of all diagnostic instruments was carried out by experienced clinicians at the M.I.N.D. Institute.

All children enrolled in the study were assessed for developmental performance using MSEL. The MSEL has components for visual reception, fine motor, receptive language, and expressive language. Standardized developmental quotients (DQs) were calculated for each subscale of the MSEL by dividing each child's age-equivalent score by their chronological age at the time of testing and multiplying by 100 to provide a consistent metric for cognitive measures and to accommodate floor effects. The Child Behavior Checklist (28) was provided by parents of children in the study and consisted of questions designed to measure behavioral issues in children, including sleep, hyperactivity, and aggression.

Participant groups did not differ significantly for age. All children were free of any major immune modifying medications and in good health and free of fever (temperature $< 100.5^{\circ}$F) at the time of blood draw. All participants were native English speakers, ambulatory, and had no suspected vision or hearing problems. All subjects were screened via parental interview for current and past physical illness. This study was approved by the institutional review boards at the University of California, Davis. Informed consent was obtained before participation.

Cell Isolation

Peripheral blood (8.5 mL) was collected in an acid-citrate-dextrose Vacutainer tube (BD Biosciences, San Jose, California) and processed within 12 hours of collection. Blood was centrifuged for 10 minutes at 2300 rpm, and plasma was drawn off and stored at -80°C. The blood pellet was mixed 1:1 with Hanks Balanced Salt Solution (Gibco, Gaithersburg, Maryland) without ionized calcium or ionized magnesium. The diluted blood was then layered, carefully, over a Ficoll-Paque gradient (Pharmacia Biotech, Piscataway, New Jersey) and centrifuged at 1700 rpm for 30 minutes at room temperature. Peripheral blood mononuclear cells (PBMC) were then harvested from the interface layer and washed twice with Hanks Balanced Salt Solution. Viability was determined by trypan blue exclusion. Cells were then resuspended at a final concentration of $.5 \times 10^6$ cells/mL in tissue culture medium consisting of RPMI-1640 (Gibco) supplemented with 10% low endotoxin heat inactivated fetal bovine serum (Omega Scientific, Tarzana, California), 100 IU/mL penicillin, and 100 IU/mL streptomycin (Sigma, St Louis, Missouri).

Cellular Stimulations

Isolated PBMC were stimulated for 48 hours in RPMI-1640 media with 10% fetal bovine serum (Gibco), 1% penicillin and streptomycin alone, or with the addition of either 1.0 μg/mL lipopolysaccharide (LPS) (*Escherichia coli* serotype 0111:B4; Sigma) or 8 μg/mL phytohemagglutinin (PHA) (Sigma) and cultured at 37°C with 5% carbon dioxide. After this period, cells were collected and spun at 2000 rpm for 10 minutes and supernatants were collected and stored at -80°C until analyzed by Luminex multiplexing technology. Stimulations (48 hours) were used to relate this work to previous work by our group.

Luminex Multiplex Analysis

Supernatants from LPS stimulated cultures were quantified for granulocyte-macrophage colony-stimulating factor, IL-1β, IL-6, IL-10, IL-12(p40), MCP-1 (chemokine [C-C motif] ligand 2), and TNFα, and supernatant PHA-stimulated cultures were