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Children with autism spectrum disorders (ASD) who exhibit chronic gastrointestinal (GI) symptoms and marked fluctuation of behavioral symptoms exhibit distinct innate immune abnormalities and transcriptional profiles of peripheral blood (PB) monocytes

Harumi Jyonouchi [a,*], Lee Geng [a], Deanna L. Streck [b], Gokce A. Toruner [b]

[a] Division of Allergy/Immunology and Infection Diseases, Department of Pediatrics, University of Medicine and Dentistry of New Jersey (UMDNJ)-New Jersey Medical School (NJMS), 185 South Orange Ave, Newark, NJ, United States
[b] Institute of Genomic Medicine, Department of Pediatrics, University of Medicine and Dentistry of New Jersey (UMDNJ)-New Jersey Medical School (NJMS), 185 South Orange Ave, Newark, NJ, United States

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ABSTRACT

Innate/adaptive immune responses and transcript profiles of peripheral blood monocytes were studied in ASD children who exhibit fluctuating behavioral symptoms following infection and other immune insults (ASD/Inf, N = 30). The ASD/Inf children with persistent gastrointestinal symptoms (ASD/Inf + GI, N = 19), revealed less production of proinflammatory and counter-regulatory cytokines with stimuli of innate immunity and marked changes in transcript profiles of monocytes as compared to ASD/no-Inf (N = 28) and normal (N = 26) controls. This included a 4–5 fold up-regulation of chemokines (CCL2 and CCL7), consistent with the production of more CCL2 by ASD/Inf + GI cells. These results indicate dysregulated innate immune defense in the ASD/Inf + GI children, rendering them more vulnerable to common microbial infection/dysbiosis and possibly subsequent behavioral changes.

1. Introduction

Except for a small subset of subjects with known gene mutations, ASD is now considered a behavioral syndrome caused by multiple gene mutations influenced by various environmental factors (Bale et al., 2010; Rudan, 2010; Toro et al., 2010). As in any disease involving multiple genetic and environmental factors, clinical phenotypes of ASD vary considerably, in addition to there being a high frequency of co-morbidities. This makes it very difficult to dissect the pathogenesis of ASD. However, despite the involvement of markedly variable genetic and environmental factors, ASD children suffer from similar behavioral symptoms, indicating that multiple factors may affect common signaling pathways. Thus one approach to elucidate ASD pathogenesis is to focus on well characterized, distinct subsets of the disease with identified known gene mutations. Studies focusing on children with known mutations or genetic defects (fragile X-syndrome, tuberous sclerosis, 22q11.2 microdeletion syndrome, and Rett syndrome) have yielded important information, shedding light on molecular mechanisms that can be applied to ASD children without such genetic defects (Curatolo et al., 2010; Marchetto et al., 2010; Schendel et al., 2010; Wang et al., 2010).

However, such approaches are not suitable for identifying the effects of novel genes and unappreciated involvement of pathways, or genotype–phenotype association in ASD children without known genetic defects. Recent genetic evidence also indicates the effects of multiple genes not specific for autism, but affecting onset/development of neuropsychiatric disorders in early life (Rudan, 2010; Toro et al., 2010). Approaches such as genome wide association studies (GWAS) on single nucleotide polymorphisms (SNP) and copy number variation (CNV) were instrumental in these studies (Cook and Scherer, 2008;

Abbreviations: α-LA, α-lactoalbumin; β-LG, β-lactoglobulin; AC, allergic conjunctivitis; AR, allergic rhinitis; ASD, autism spectrum disorder; ASD-IS, ASD-immune subtype; BMDM cells, bone marrow derived microglial cells; CNS, central nervous system; CNV, copy number variation; CRS, chronic rhinosinusitis; CVID, common variable immunodeficiency; FA, food allergy; FP, food protein; FPIES, food protein induced enterocolitis syndrome; GI, gastrointestinal; GWAS, genome wide association studies; IBD, inflammatory bowel disease; IL, interleukin; IVIG, intravenous immunoglobulin; MS, multiple sclerosis; NJMS, New Jersey Medical School; PB, peripheral blood; PBMCs, peripheral blood mononuclear cells; ROM, recurrent otitis media; SD, standard deviation; SNP, single nucleotide polymorphism; SPAD, specific polysaccharide antibody deficiency; TLR, toll-like receptor; TNF, tumor necrosis factor; sTNFRII, soluble TNF-receptor II; TGF-β, transforming growth factor-β; UMDNJ, University of Medicine and Dentistry of New Jersey.

* Corresponding author at: Division of Allergy/Immunology and Infection Diseases, Department of Pediatrics, UMDNJ-NJMS, 185 South Orange Ave. F507A, MSB, Newark, NJ 07101-1709, USA. Tel.: +1 973 972 1414; fax: +1 973 972 6443.
E-mail address: jyanouha@umdnj.edu (H. Jyonouchi).

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El-Fishawy and State, 2010). However, these approaches seem to be limited in their power to predict contributions of rare genetic defects or assessment of total risk variance (Rudan, 2010). An alternative approach may be to focus on a subset of ASD children with distinct clinical symptoms and/or co-morbid conditions, but no known genetic risk factors.

In our Pediatric Allergy/Immunology Clinic, we evaluated a number of ASD children who were referred for evaluation of immune functions secondary to symptoms indicating immunodysregulation. These symptoms include severe adverse reactions to multiple medications, recurrent infection not responding to the first-line treatment [mainly recurrent otitis media (ROM) and chronic rhinosinusitis (CRS)], chronic gastrointestinal (GI) symptoms, and food allergy (FA). Among these ASD children, we identified a unique subset characterized by markedly fluctuating behavioral symptoms and repeated loss of once acquired cognitive skills, occurring after immune insults (typically viral infection) (Jyonouchi et al., 2008). Moreover, these patients revealed aberrant innate immune responses with certain stimuli of innate immunity (Jyonouchi et al., 2008). It is of note that such abnormalities were not observed in non-ASD children with recurrent infection (CRS and OM) or FA (Jyonouchi, 2009; Jyonouchi et al., 2005b, 2007).

In our previous study, innate immune responses were assessed by overnight stimulation of peripheral blood mononuclear cells (PBMCs) with agonists of toll-like receptors (TLRs). In this setting, the main PB cells responding to TLR agonists are monocytes. PB monocytes are heterogeneous, consisting of classical monocytes ($CD14^{++}$, $CD16^{-}$ cells) and non-classical (or alternatively activated) monocytes ($CD14^{+}$, $CD16^{+}$ cells) (Serbina et al., 2008; Parihar et al., 2010). Classical monocytes make up the majority of PB monocytes. Although the half-life of dormant PB monocytes is short, upon inflammatory stimuli, they escape apoptosis and are recruited to the site of inflammation via chemokines (CCL2 and CCL7) where they differentiate into tissue macrophages (Serbina et al., 2008; Parihar et al., 2010). PB monocytes recruited to the central nervous system (CNS) develop into exogenously derived microglial cells or bone marrow derived microglial (BMDM) cells, which have been shown to play a major role in CNS inflammation (Djukic et al., 2006; Rodriguez et al., 2007; Davoust et al., 2008; Ransohoff and Cardona, 2010).

Given these previous findings, we hypothesized that altered transcript profiles in PB monocytes in association with aberrant innate immune responses are detectable in this subset of ASD children who exhibit fluctuating behavioral symptoms/cognitive skills following immune insults (ASD/Inf). To test our hypothesis, we determined transcript profiles of PB monocytes in comparison with innate immune responses in the ASD/Inf children. Control subjects included both ASD children without such characteristics as described above (ASD/no-Inf) and normal controls. Unexpectedly, our results revealed that the ASD/Inf children with severe GI symptoms (ASD/Inf + GI) but not those without GI symptoms (ASD/Inf-no GI) exhibit distinct innate immune abnormalities in association with significantly altered transcript profiles of PB monocytes.

2. Materials and methods

2.1. The study subjects

The study subjects were recruited following the protocol approved by our institutional review board at University of Medicine and Dentistry of New Jersey (UMDNJ)—New Jersey Medical School (NJMS). The blood samples were obtained after obtainment of the signed parental consent forms. A signed assent form was also obtained from the study subjects older than 7 years of age, if the subject was judged of being capable of signing/understanding the assent form by the parents. In most cases, obtainment of blood samples was coincided with the medically indicated blood work to minimize the frequency of venipuncture. Blood samples from mothers of the study subjects were also obtained, if the mothers consented.

All the study subjects and their mothers were recruited in the Pediatric Subspecialty Clinic where multiple subspecialty clinics operate including cardiology, endocrinology, genetics, developmental pediatrics, allergy/immunology, gastroenterology, general pediatrics, pulmonology, and nephrology. All the subjects were examined prior to the venipuncture to ensure that there was no evidence of active infection or acute illnesses. Demographic information of all the study subjects is summarized in Tables 1 and 2.

2.1.1. ASD diagnosis

In both ASD/Inf and ASD/no-Inf children, ASD diagnosis was made or ascertained by DSM-IV (Diagnostic and Statistical Manual of Mental Disorders IV) criteria, ADI-R (Autism Diagnostic Interview-Revised), and/or ADOS (Autism Diagnostic Observational Schedules). All the ASD children recruited to this study were those with established autism diagnosis from established autism diagnostic centers including ours at UMDNJ. The ASD/Inf children were defined by having at least 3 occurrences of changes in behavioral symptoms and/or loss of cognitive skills documented following infection such as viral syndrome and history of physician diagnosed recurrent ear infection, sinusitis, and other deep seeded infection. These occurrences were documented independently by caretakers, teachers, and therapists.

2.1.2. Diagnosis of atopic disorders

Allergic rhinitis (AR) and allergic conjunctivitis (AC) were diagnosed with positive skin prick test reactivity and/or presence of allergen-specific IgE accompanied by clinical features consistent with AR and AC (Butrus and Portela, 2005; Nassef et al., 2006) Asthma diagnosis was based on NIH guideline criteria (2007). Asthma without skin test reactivity and/or allergen-specific IgE antibody was categorized as non-atopic asthma (Nassef et al., 2006).

2.1.3. Diagnosis of food protein induced enterocolitis syndrome (FPIES)

FPIES to common food proteins (FP) including cow's milk protein, wheat, and soy was diagnosed with the following criteria: 1) presence of objective GI symptoms (diarrhea, loose stool, and constipation) which resolved with avoidance of causative FPs, 2) delayed (more than 6 h) onset of GI symptoms following exposure to offending FPs after resolution of GI symptoms, and 3) cellular immune reactivity to offending FPs defined as the production of more than 1 standard deviation (SD) + control mean value of TNF-α and/or IL-12 by PBMCs with stimuli of causative FPs (Jyonouchi et al., 2005a). Diagnoses of other GI conditions were ascertained by reviewing medical charts and previous laboratory findings.

2.2. Cytokine production assays

PBMCs were isolated by Ficoll–Hypaque density gradient centrifugation. Innate immune responses were assessed by incubating PBMCs (10^6 cells/ml) overnight with TLR4 agonsit (LPS; 0.1 μg/ml, GIBCO-BRL, Gaithersburg, MD), TLR2/6 agonist (zymosan; 50 μg/ml, Sigma-Aldrich, St. Luis, Mo), TLR3 agonist (Poly I:C, Poly I:C, 0.1 μg/ml, Sigma-Aldrich), TLR 5 agonist (flagellin, 0.1 μg/ml, InvivoGen, San Diego, CA),TLR7/8

Table 1
Demographics of the pediatric study subjects.

	ASD/Inf	ASD/no-Inf	Normal controls
Subject number	N = 30	N = 28	N = 26
Age (year): Median (range)	7.5 (3.0–15.6)	5.9 (3.0–17.9)	8.2 (3.0–16.8)
Sex (male:female)	27:3	23:6	22 :4
Ethnicity	2 AA[a], 3 Asian, 23 W 2 mixed	4 AA, 3 Asian, 22 W	1 AA, 2 Asian, 23 W

[a] Abbreviations used: AA (African Americans), W (Caucasians).