Chapter

Associations between Monocyte Cytokine Profiles and Co-Morbid Conditions in Autism Spectrum Disorders

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Abstract

Autism spectrum disorder (ASD) is a behaviorally defined syndrome with frequent co-morbidities. Evidence indicate a role of innate immunity in ASD pathogenesis. This study addressed whether innate immune abnormalities are associated with ASD co-morbid conditions and/or other clinical co-variables when assessed as changes in monocyte cytokine profiles. This study included 109 ASD (median 11.5 year) and 26 non-ASD subjects (median 11.4 year). Monocyte cytokine profiles were evaluated in association with age/ethnicity, ASD severity, medications, and co-morbidities present in >15% of ASD subjects [gastrointestinal (GI) symptoms, epilepsy, allergic rhinitis, specific antibody deficiency (SAD), and fluctuating behavioral symptoms resembling pediatric acute-onset neuropsychiatric syndrome (PANS)]. ASD severity did not affect frequency of co-morbid conditions. GI symptoms, epilepsy, SAD, and PANS like symptoms revealed associations with changes in production of tumor necrosis factor-α (TNF-α)/soluble TNF-receptor II (sTNFRII), interleukin-1ß (IL-1ß)/IL-6/IL-10, and IL-6, respectively, mostly independent of other co-variables. ASD severity was associated with changes in multiple cytokines but frequently affected by other clinical co-variables. Our findings revealed associations between specific monocyte cytokine profiles and certain co-morbid conditions in ASD subjects, independent of other clinical co-variables. Our findings will aid in assessing treatment options for ASD co-morbidities and their effects on ASD behavioral symptoms.

Keywords: autism spectrum disorder (ASD), co-morbid conditions, innate immune memory (IIM), monocyte cytokines, trained immunity

1. Introduction

Autism spectrum disorder (ASD) is a behavioral defined syndrome except for a small subset of patients with defined gene mutations such as MECP2 (Rett syndrome) and TSC1/TSC2 (tuberous sclerosis) [1, 2]. However, core ASD symptoms may be the results of the effects of various genetic and environmental factors that may also affect organs other than central nervous system (CNS). Consequently, medical conditions affecting other organs may also affect ASD symptoms, partly through pain and discomfort. This makes it difficult to have reliable objective

diagnostic measures universally applicable for ASD children. This assumption is supported by the fact that ASD is characterized by multiple co-morbid medical conditions, with GI symptoms being the most common [3, 4].

Many co-morbid conditions reported in ASD subjects are associated with immune mediated inflammation in pathogenesis; GI symptoms found in ASD subjects have been in part implicated with chronic GI inflammation due to dysregulated gut immune responses to microbiota [5, 6]. Mounting evidence also indicates that many, but not all the ASD subjects show some immune abnormalities that affect almost every arm of the immune system [7, 8]. Moreover, given the fact that the immune system and the CNS interact closely interact [9], a role of neuroinflammation in ASD pathogenesis is highly suspected in a subset of ASD subjects.

In our previous studies, we focused on abnormalities of innate immunity, which plays a major role in the neuro-immune network including stress responses [10–12]. One of the reasons that we focused on innate immune abnormalities is based on findings from one of the most thoroughly studied animal models of ASD, maternal immune activation (MIA). MIA is induced by sterile stimulants of innate immunity in pregnant rodents [13]. MIA generates lasting effects on behavioral symptoms and the immune functions in offspring [13, 14]. It has been puzzling that how innate immunity, which lacks memory for specific antigens, can cause such lasting effects. However, discovery of innate immune memory (IIM) caused by initial stimuli through epigenetic regulations [15, 16], has helped us understand the lasting effects of dysregulated IIM in various inflammatory conditions. In fact, dysregulated IIM is now implicated in the pathogenesis of common neuropsychiatric diseases such as schizophrenia and depression [17, 18]. We found abnormalities of innate immunity in many, but not all the ASD subjects we studied by assessing cytokine profiles from purified monocytes [11].

Given both the considerable amount of abnormalities found in the monocyte cytokine profiles and the high frequency of co-morbid medical conditions in ASD subjects, questions understandably arise concerning the association of co-morbid conditions with changes in monocyte cytokine production. Many ASD subjects are treated with neurotropic medications including selective serotonin re-uptake inhibitors (SSRIs), anti-seizure medications used as mood stabilizers, neuroleptics, and medications for ADHD. These medications may also affect monocyte cytokine profiles. Therefore, this study addressed whether monocyte cytokine profiles differ depending on co-morbid conditions, ASD severity, and other clinical co-variables. The results indicate that co-morbid medical conditions are associated with changes in production of specific cytokines and such associated are not affected by other clinical co-variables.

2. Materials and methods

Study subjects: Study subjects were recruited following the study protocols (#17:53 and #19:53) approved by the institutional review. Signed consent forms were obtained prior to entering the study.

ASD subjects: ASD subjects (N = 109) were recruited from the Pediatric Allergy/Immunology Clinic at SPUH. Diagnosis of ASD was made at various autism diagnostic centers, including ours, based on the Autism Diagnostic Observation Scale (ADOS) and/or Autism Diagnostic Interview-Revisited (ADI-R), as well as other standard measures. ASD subjects were also evaluated for their behavioral symptoms and sleep habits with the Aberrant Behavior Checklist (ABC) [19] and the Children's Sleep Habits Questionnaires (CSHQ) [20], respectively. Information regarding cognitive ability and adaptive skills were obtained from previous school

evaluation records performed within 1 year of enrollment in the study; these results were based on standard measures such as the Woodcock-Johnson III test (for cognitive ability), and Vineland Adaptive Behavior Scale (for adaptive skills) [21].

Non-ASD controls: A total of 26 non-ASD subjects served as controls. These subjects were recruited in the Pediatrics Subspecialty and General Pediatrics Clinics at our institution. These subjects were typically growing and satisfied our exclusion/inclusion criteria.

Demographics of study subjects were summarized in **Table 1**.

Diagnosis of food allergy (FA): IgE mediated FA was diagnosed with reactions to offending food, by affecting the skin, GI, and/or respiratory tract immediately (within 2 hours) after intake with positive prick skin testing (PST) reactivity, and/or presence of food allergen-specific serum IgE. Non IgE mediated FA (NFA) was diagnosed if GI symptoms resolved, following implementation of a restricted diet (i.e., avoidance of offending food), and symptoms recurred with re-exposure to offending food [22]. NFA was also defined as being non-reactive to PFT and negative for serum IgE specific for food allergens [22].

Diagnosis of asthma and AR: AR and allergic conjunctivitis (AC) were diagnosed when subjects had corresponding clinical features along with positive PST reactivity and/or positive serum IgE specific to causative allergens [23, 24]. Asthma was diagnosed following the asthma guidelines from the Expert Panel Report 3 [25].

Diagnosis of Antibody deficiency syndrome: When the subject revealed protective levels of antibodies in less than 11 of 14 serotypes of *Streptococcus pneumonia* after the booster dose of Pneumovax® or PCV13®, he/she was diagnosed with SAD [26]. Antibody levels greater than 1.3 μg/ml were considered protective [26].

Diagnosis of PANS like symptoms and sleep disorders: Pediatric acute-onset neuropsychiatric syndrome (PANS) is a clinical diagnosis [27]. Most ASD subjects recruited to the study were diagnosed with PANS by other physicians. We attempted to validate the diagnosis based on the PANS diagnostic criteria [27]. In some ASD children, pre-existing neuropsychiatric symptoms made it difficult to apply the clinical diagnostic criteria of PANS. In this study, we categorized ASD patients with recurrent worsening behavioral symptoms following immune stimuli (typically microbial infection) more than 2×, not controlled by proper management of triggering stimuli as ASD subjects with PANS like symptoms. Diagnosis of sleep disorders that lasts at least more than 6 weeks is based on parental reports and results of CSHQ.

Sample collection: Venous blood samples were obtained by the physician in this study. We obtained one sample from each non-ASD controls. As for ASD subjects, we obtained multiple blood samples at different time points from select ASD subjects (N = *9*), in order to assess variability of *monocyte* cytokine profiles. If parents

	ASD subjects (N = 109)	**Non ASD controls (N = 26)**
Gender	95 M, 15 F (Female 13.8%)	19 M, 7 F (Female 26.9%)
Age: Average ± SD (years) Median (Min to Max)	12.4 ± 5.9 11.5 (2.2–26.3)	12.2 ± 5.8 11.4 (3–28.8)
Ethnicity	84 W, 5 AA, 19 Asian, 1 Mixed	22 W, 2 AA, 2 Mixed

Abbreviations used: AA; African American, ASD; autism spectrum disorders, SD; standard deviation, W; Caucasian.

Table 1.
Demographics of study subjects.

or study subjects preferred, we applied a topical lidocaine/prilocaine cream (Emla cream®) to the site of venipuncture prior to blood sampling.

Cell cultures: Ficoll–Hypaque density gradient centrifugation was used for separating PBMCs. From PBMCs, PBMo were further purified using a column of magnetic beads labeled with anti-CD3, CD7, CD16, CD19, CD56, CD123, and glycophorin A (monocyte separation kit II – human, MILTENYI BIOTEC, Cambridge, MA, United States). Combination of these antibodies depletes T, B, natural killer, and dendritic cells from PBMCs.

Cytokine production by purified PBMo was induced by incubating cells overnight (2.5×10^5 cells/ml) with a panel of agonists of TLRs. This assay system was designed to reflect the effects of microbial byproducts commonly encountered in real life. Lipopolysaccharide (LPS), a TLR4 agonist, represents a signaling pathway activated in response to a gram negative [G (−)] bacteria. Zymosan, a TLR2/6 agonist, mimics an innate activation signal in response to G (+) bacteria and fungi. CL097, a TLR7/8 agonist, activates innate signaling pathways in response to ssRNA viruses that cause common respiratory infection. Candida heat extract as a source of ß-glucan, a dectin-1 agonist, was used as well as a C-lectin receptor agonist. PBMos were incubated overnight with LPS (0.1 μg/ml, GIBCO-BRL, Gaithersburg, MD, USA), zymosan (50 μg/ml, Sigma-Aldrich, St. Luis, Mo), C097 (water-soluble derivative of imidazoquinoline, 20 μM, InvivoGen, San Diego, CA, USA), and candida heat extract (HCKA, heat killed *Candida albicans* (10^7 cells/ml, InVivogen, San Diego, CA) in RPMI 1640 with additives as previously described [28]. Overnight incubation (16-20 h) was adequate to induce the optimal responses in this setting in previous studies [11]. Cytokine levels in the culture supernatant were then measured.

Levels of CCL2, IL-1β, IL-6, IL-10, IL-12p40, transforming growth factor-ß (TGF-ß), tumor TNF-α, and sTNFRII cytokines were measured by enzyme-linked immuno-sorbent assay (ELISA); 10–100 μl/well supernatants were used for ELISA. The OptEIA™ Reagent Sets (BD Biosciences, San Jose, CA, USA) were used for ELISA of IL-1ß, IL-6, IL-10, IL-12p40, and TNF-α. For CCL2, sTNFRII, and TGF-ß ELISA, reagents were obtained from BD Biosciences and R & D (Minneapolis, MN, USA). IL-23 ELISA kit was purchased from eBiosciences, San Diego, CA. Intra- and inter-variations of cytokine levels were less than 5%.

Statistical analysis: We used a two tailed Mann–Whitney test for comparison of two sets of numerical data. Kruskal-Wallis test was used for comparison of more than 2 sets of numerical data. When assessing differences in frequency between two groups, we used the Fisher exact test. For assessing differences in frequency among multiple groups, we used the Chi-square test and the Likelihood ratio. P value of less than 0.05 was considered nominally significant. Co-variance analysis was done with the use of analysis of variance (ANOVA) for a fixed factor or for a variable factor. NCSS2020 (NCSS, LLC. Kaysville, UT) was used for such statistical analysis.

3. Results

Clinical characteristics: Frequencies of co-morbid conditions among the recruited ASD subjects are summarized in **Table 2.** These results are consistent with the results of our previous studies [12, 29]. Age and gender were not associated with ASD severity (data now shown). Frequencies of co-morbid conditions and the use of neurotropic medications did not differ due to ASD severity in 108 ASD subjects who were verified ASD severity (**Table 3**).

Changes in monocyte cytokine production depending on ASD severity: We then examined whether monocyte cytokine profiles differed with ASD severity

Comorbid conditions	ASD subjects (N = 109)	Controls (N = 26)
GI[1] symptoms	71/109 (65.1%)	0
history of NFA	68/109 (63.4%)	2/26 (7.7%)
Seizure disorders	18/109 (16.5%)	0
Asthma	5/109 (4.6%)	0
Allergic rhinitis	17/109 (15.6%)	0
Specific antibody deficiency	26/109 (23.9%)	0
PANS like symptoms	62/109 (56.9%)	0
Disturbed Sleep	48/109 (44.0%)	0

[1]*Abbreviations used: ASD; autism spectrum disorder, GI; gastrointestinal, NFA; non-IgE mediated food allergy, PANS; pediatric acute-onset neuropsychiatric syndrome.*

Table 2.
Frequency of comorbid conditions in the study subjects.

ASD[1] severity	Level 1	Level 2	Level 3	Chi-Square
Co-morbid conditions				
GI symptoms	14/27[2]	20/32	36/49	p > 0.1
Seizure disorder	1/27	6/32	10/49	p = 0.081
Antibody deficiency	6/27	10/32	10/49	p > 0.1
PANS like symptoms	12/27	19/32	30/49	p > 0.1
Disturbed sleep	8/27	16/32	23/49	p > 0.1
Medications				
SSRIs	6/27	8/32	4/49	p > 0.1
Anti-seizure medications	3/27	9/32	8/49	p > 0.1
ADHD medications	3/27	3/32	3/49	p > 0.1
Neuroleptics	4/27	1/32	9/49	p = 0.079

[1]*Abbreviations used: ASD; autism spectrum disorder, GI; gastrointestinal, NFA; non-IgE mediated food allergy, ADHD: attention deficiency hyperactivity disorder, PANS; pediatric acute-onset neuropsychiatric syndrome, SSRI: selective serotonin reuptake inhibitor.*
[2]*One ASD subject was excluded from this analysis due to lack of validation of ASD severity.*

Table 3.
Frequencies of Co-morbid conditions and medication use did not differ due to ASD severity.

and if such changes were affected by other clinical co-variables. ASD severity was shown to be associated with changes in production of TNF-α, IL-1ß, IL-10, and CCL2, and TNF-α/sTNFRII ratios (**Table 4**). However, production of inflammatory monocyte cytokines (TNF-α and IL-1ß) under several culture conditions is affected by presence of co-morbid conditions and the use of ADHD medications (**Table 4**).

Changes in monocyte cytokine production depending on co-morbid conditions: Since associations between ASD severity and monocyte cytokine profiles were often affected by other co-morbid conditions and medication use (**Table 4**), we also evaluated whether changes in monocyte cytokine profiles in ASD subjects were affected by the presence of co-morbid conditions.

Co-morbid conditions with objective diagnostic measures: We evaluated changes in monocyte cytokine profiles in association with co-morbid conditions which were

Monocyte cytokine production	ASD[5] severity Level 1	ASD severity Level 2	ASD severity Level 3	Kruskal-Wallis test
	N = 33	N = 37	N = 52	
TNF-α (CLO97)[1]	2390.2 ± 1786.9	2720.2 ± 983.3	2073.0 ± 1171.6	$p < 0.01$
IL-10 (CLO97)	1318.1 ± 595.3	918.2 ± 576.1	1009.9 ± 607.2	$p < 0.05$
TNF-α/sTNFRII (CLO97)[2]	3.78 ± 3.34	8.43 ± 10.43	8.73 ± 17.27	$p < 0.05$
TNF-α (ß-glucan)[3]	1816.2 ± 1139.4	2360.4 ± 1249.9	1512.2 ± 883.9	$p < 0.005$
IL-1ß (ß-glucan)[4]	2578.7 ± 922.0	2809.7 ± 984.4	2062.9 ± 949.7	$p < 0.005$
TNF-α (ß-glucan+LPS)[1]	2338.0 ± 259.0	2658.4 ± 984.5	2000.2 ± 916.5	$p < 0.01$
CCL2 (ß-glucan+LPS)	2708.0 ± 2477.4	1758.6 ± 16681	1574.8 ± 1553.1	$p < 0.05$
CCL2 (zymosan)	9105.2 ± 6631.1	7668.4 ± 4973.2	5870.3 ± 4414.4	$p < 0.05$

[1]ANOVA co-variance analysis revealed an association with the use of ADHD medications ($p < 0.02$ and $p < 0.05$ under culture conditions stimulated with CL097 and ß-glucan+LPS, respectively).
[2]ANOVA co-variance analysis revealed an association with GI symptoms ($p < 0.05$).
[3]ANOVA co-variance analysis revealed an association with PANS like symptoms ($p < 0.05$).
[4]ANOVA co-variance analysis revealed an association with Disturbed sleep ($p < 0.05$).
[5]Abbreviations used: CCL2; C-C chemokine ligand 2, IL; interleukin, LPS; lipopolysaccharide, TNF; tumor necrosis factor.

Table 4.
Differences in monocyte cytokine production depending on ASD severity.

evaluated with objective measures as defined in the method section. We found that such co-morbid conditions were observed in more than 15% of our ASD study subjects; these include GI symptoms, seizure disorders, AR, and SAD. Our results revealed that there are significant associations between monocyte cytokine production and ASD co-morbid conditions as described above (**Table 5**). Presence of GI symptoms are mainly associated with changes in TNF-α production and the ratio of TNF-α/sTNFRII ratios under several culture conditions. Co-variance analysis showed that these parameters were mostly not affected by other clinical co-variables including medication use. The exceptions are TNF-α production and TNF-α/sTNFRII ratios under zymosan stimulated cultures, which are affected by ASD severity (**Table 5**). Diagnosis of seizure disorders was the most notably associated with changes in IL-1ß production and IL-1ß/IL-10 ratios under the CL097 stimulated cultures, independent of clinical co-variables that we assessed (**Table 5**). The AR diagnosis is mainly associated with changes in sTNFRII production. The SAD diagnosis is mainly associated with changes in IL-6 and IL-10 production. Most of these cytokine parameters were again not affected by the other clinical variables that we assessed.

Co-morbid conditions based on clinical diagnosis: Although assessment of PANS like symptoms and sleep disorder were diagnosed without objective measures, given the high frequency of these conditions, we also assessed differences in monocyte cytokine parameters in association with these two co-morbid conditions. Significant differences in certain monocyte cytokine parameters were found in the presence of PANS like symptoms and sleep disorders (**Table 6**). PANS like conditions were associated with changes in inflammatory cytokines (IL-6 and IL-1ß), as well as sTNFRII and CCL2. Only IL-1ß production was affected by other clinical covariables. As for sleep disorders, changes in TGF-ß levels were mainly associated with the presence of sleep disorders and changes in TGF-ß productions was independent of clinical co-variables.

Comorbid conditions	ASD[1] with comorbid condition	ASD without comorbid condition	Non-ASD Control	Kruskal-Wallis test
GI symptoms	**N = 81**	**N = 42**	**N = 26**	
TNF-α (LPS)	685.1 ± 744.8[3]	373.0 ± 366.5	474.4 ± 505.8	$p < 0.01$
TNF-α (zymosan)[2]	1498.8 ± 1055.6	1047.6 ± 709.6	1609.7 ± 748.3	$p < 0.01$
TNF-α (ß-glucan)	1961.6 ± 1108.2	1609.7 ± 1135.7	1931.1 ± 948.5	$p = 0.113$
TNF-α/sTNFRII (LPS)	0.69 ± 0.89	0.50 ± 1.27	1.28 ± 3.53	$p < 0.05$
TNF-α/sTNFRII (zymosan)[2]	2.98 ± 2.61	2.67 ± 5.68	3.54 ± 3.61	$p < 0.05$
TNF-α/sTNFRII (ß-glucan)	6.84 ± 6.79	3.88 ± 2.31	13.5 ± 18.0	$p < 0.01$
Seizure disorders	**N = 24**	**N = 99**	**N = 26**	
IL-1ß (CL097)	3732.3 ± 1092.8	4566.4 ± 1357.5	3715.5 ± 1367.9	$p < 0.01$
IL-1ß/IL-10 (CL097)	5.10 ± 7.98	8.98 ± 12.44	3.98 ± 2.53	$p < 0.001$
TNF-α/sTNFRII	3.89 ± 6.74	8.04 ± 13.74	4.31 ± 3.48	$p < 0.02$
(CL097) CCL2	18862 ± 19577	14588 ± 9672	11499 ± 7621	$p < 0.02$
(CL097) IL-1ß (ß-glucan)	1955.9 ± 996.9	2546.4 ± 972.1	21767.0 ± 999.9	$p = 0.056$
Allergic rhinitis	**N = 20**	**N = 103**	**N = 26**	
sTNFRII (LPS)	1538.1 ± 395.5	1284.0 ± 493.7	1172.5 ± 504.2	$p < 0.05$
IL-1ß (CL097)[2]	5038.3 ± 1368.3	4285.8 ± 1315.6	3715.5 ± 1357.9	$p < 0.01$
sTNFRII (ß-glucan)[2]	497.8 ± 225.2	414.1 ± 286.6	340.8 ± 257.7	$p < 0.05$
sTNFRII (LPS + ß-glucan)	488.8 ± 249.4	392.5 ± 297.3	371.6 ± 354.3	$p = 0.07179$
Antibody deficiency	**N = 31**	**N = 92**	**N = 26**	
IL-6 (medium)	2303.5 ± 1935.8	3869.2 ± 2581.629553 ± 18279	3373.3 ± 1562.9	$p < 0.01$
IL-6 (LPS)	19536 ± 9625	6309.2 ± 1974.7	20152 ± 12909	$p < 0.01$
IL-6 (zymosan)[2]	5510.5 ± 2085.6	388.8 ± 380.0	7538.5 ± 9310.0	$p = 0.106$

Comorbid conditions	ASD[1] with comorbid condition	ASD without comorbid condition	Non-ASD Control	Kruskal-Wallis test
IL-1ß (medium)	214.5 ± 253.3	646.6 ± 503.0	270.3 ± 185.9	$p < 0.05$
IL-10 (medium)	328.3 ± 336.4	1471.8 ± 417.9	597.1 ± 355.7	$p < 0.005$
IL-10 (LPS)[2]	1215.8 ± 539.2	620.1 ± 375.3	1200.0 ± 543.7	$p < 0.05$
IL-10 (zymosan)	506.3 ± 399.8	1150.0 ± 605.8	651.4 ± 380.2	$p = 0.128$
IL-10 (CL097)	863.2 ± 570.6	426.4 ± 354.6	1093.0 ± 579.5	$p = 0.07$
IL-12 (zymosan)	302.2 ± 290.3		360.7 ± 425.0	$p = 0.1206$

[1]Abbreviations used: ASD; autism spectrum disorder, GI; gastrointestinal, IL; interleukin, LPS; lipopolysaccharide, TNF; tumor necrosis factor.
[2]Co-variance analysis revealed that changes in TNF-α and TNF-α/sTNFRII (zymosan) production with GI symptoms are affected by ASD severity ($p < 0.05$). Changes in sTNFRII production (ß-glucan) with allergic rhinitis was affected with the use of anti-seizure medications ($p < 0.05$). Changes in production of IL-6 (zymosan) and IL-10 production (LPS) with antibody deficiency was affected with the use of neuroleptics/SSRIs and PANS like symptoms.
[3]The results were expressed as a mean ± SD. Cytokine levels were shown as pg/ml.

Table 5.
Differences in monocyte cytokine production in association with GI symptoms, seizures disorders, allergic rhinitis, and antibody deficiency in ASD subjects.

Comorbid conditions	ASD[1] with comorbid condition	ASD without comorbid condition	Non-ASD Control	Kruskal-Wallis test
PANS like symptoms	**N = 73**	**N = 50**	**N = 2**	
IL-6 (CL097)	6747.6 ± 1939.2[3]	7795.3 ± 1824.6	6325.5 ± 2011.2	$p < 0.005$
IL-6 (ß-glucan)	5502.2 ± 1725.7	6211.1 ± 1731.7	5230.4 ± 1706.7	$p < 0.05$
IL-6 (ß-glucan+LPS)	6505.7 ± 1654.5	7661.9 ± 1943.0	5756.6 ± 1875.3	$p < 0.00001$
IL-1ß (ß-glucan+LPS)[2]	3176.5 ± 1278.1	3706.3 ± 1029.4	2545.7 ± 987.1	$p < 0.001$
sTNFRII (zymosan)	618.2 ± 350.1	723.0 ± 293.6	663.7 ± 368.1	$p = 0.069$
CCL2 (zymosan)	6717.1 ± 5609.0	8055.4 ± 4899.3	6138.5 ± 6351.9	$p < 0.05$
Sleep disorders	**N = 56**	**N = 67**	**N = 26**	
IL-10 (LPS)[2]	1289.6 ± 499.9	1497.9 ± 422.6	1200.0 ± 543.7	$p < 0.05$
sTNFRII (LPS)[2]	1228.6 ± 473.4	1410.4 ± 484.6	1172.5 ± 551.9	$p < 0.05$
TGF-ß (medium)	535.1 ± 469.6	685.1 ± 443.1	398.0 ± 372.1	$p < 0.01$
TGF-ß (LPS)	545.7 ± 498.1	689.8 ± 436.2	372.8 ± 340.1	$p < 0.005$
TGF-ß (zymosan)	429.2 ± 415.4	542.8 ± 356.7	285.9 ± 287.0	$p < 0.001$
TGF-ß (CLO97)	459.3 ± 442.9	613.7 ± 421.0	325.8 ± 299.2	$p < 0.005$
TGF-ß (ß-glucan)	370.4 ± 335.7	518.4 ± 345.7	259.7 ± 280.8	$p < 0.0005$

[1]*Abbreviations used include: IL; interleukin, LPS, lipopolysaccharide, PANS; pediatric acute-onset neuropsychiatric syndrome, TGF, transforming growth factor, TNF; tumor nectrosis factor, sTNFRII; soluble TNF receptor II.*
[2]*Changes in IL-1ß (ß-glucan+LPS) production with PANS like symptoms are affected with ASD severity. Changes in IL-10 and sTNFRII production with LPS was affected by ASD severity ($p < 0.01$) and Seizure disorder ($p < 0.05$). Changes in production of IL-10 (LPS) with sleep disorder is affected with the use of SSRIs ($p < 0.005$), and specific antibody deficiency and PANS like behaviors ($p < 0.05$) by co-variance analysis. sTNFRII production with sleep disoders are affected with the use of SSRIs ($p < 0.005$) by co-variance analysis.*
[3]*The results were expressed as a mean ± SD. Cytokine levels were shown as pg/ml.*

Table 6.
Differences of monocyte cytokine profiles with presence of PANS like symptoms and sleep disorders in ASD subjects.

4. Discussion

ASD subjects suffer from multiple co-morbid conditions. However, we know little about how the presence of co-morbid conditions are associated with ASD pathogenesis. Core ASD symptoms used for diagnosis such as irritability, hyperactivity, self-injurious behaviors, etc. can be affected by discomfort and pain caused by co-morbid medical conditions. In addition, recently, mounting evidence indicates a pathogenetic association between GI symptoms and the onset/progress of ASD [5, 9]. This may also be true for other common co-morbid conditions such as seizure disorders.

Unfortunately, impaired expressive language in ASD subjects make it more difficult to diagnose co-morbid medical conditions. For example, sinus headache caused by untreated sinusitis and AR can aggravate head banging and aggression (pinching others, etc.). Too often, such behaviors are dismissed as just being autistic, and diagnostic and treatment measures for common childhood diseases may not be properly sought in ASD children [30]. Considering the fact current ASD diagnosis is based on behavioral symptoms, the presence of co-morbid medical conditions may hold a key to assess pathogenesis in markedly heterogeneous ASD subjects and their variable behavioral symptoms.

When addressing the importance of co-morbid conditions frequently seen in ASD subjects, the role of immune mediated inflammation likely needs to be

considered as a common denominator. The immune system has long been thought to play a role in neuroinflammation and is implicated with pathogenesis of ASD. One of the most extensively studied animal models of ASD is MIA, in which, ASD like behavioral changes in offspring are induced by sterile immune activation through stimuli of innate immunity given to pregnant rodents [13]. Discovery of IIM [15, 17] shed a light on the lasting effects of sterile, antigen non-specific inflammation generated in the MIA model. IIM is thought to be generated through epigenetic changes [17] and such changes created in fetal and early infancy could make such individuals more susceptible to common inflammatory conditions such as food induced enterocolitis syndrome (FPIES), a condition that were found frequently in ASD subjects in our clinic. Altered IIM skewed to pro-inflammatory responses may cause dysregulated responses to commensal microbiota in the gut, causing chronic GI inflammation, resembling inflammatory bowel diseases (IBD). Such changes in innate immune responses may lead to aberrant responses to respiratory microbes, resulting in altered clinical manifestations, as well. Such dysregulated innate immune responses to immune stimuli can also affect the brain, since many signaling pathways associated with innate immunity have roles in the nervous system [17].

Despite progress of our understanding of IIM, we do not know which innate immune parameters are associated with co-morbid medical conditions and how these parameters are associated with ASD severity. IIM is closely associated with changes in monocyte cytokine profiles [16]. Previously, we have found significant changes in monocyte cytokine profiles in a subset of ASD patients [10, 11]. Therefore, this study addressed whether the specific monocyte cytokine parameters are associated with ASD co-morbid conditions. In this study, we randomly screened monocyte cytokine profiles in ASD subjects recruited to the study. In our clinic, because of the allergy/immunology specialty, we likely recruited more ASD subjects with co-morbid medical conditions. However, we reasoned that such potentially skewed ASD study subjects may make it easier for us to find specific monocyte markers associated with co-morbid conditions.

We found changes in certain monocyte cytokine parameters had an association with ASD severity (**Table 4**). However, parameters associated with inflammatory responses (production of TNF-α and IL-1ß, and TNF-α/sTNFRII ratio) were also found to be affected by other clinical co-variables including GI symptoms, and PANS like behaviors (**Table 4**). This finding seems to support our initial assumption that associations between ASD behavioral symptoms and changes in monocyte cytokine profiles are affected by other clinical co-variables.

Therefore, we decided to assess changes of monocyte cytokine parameters in association with co-morbid conditions frequently found in ASD subjects. We found GI symptoms along with NFA or FPIES like conditions in ASD subjects at high frequency (>60%), which was consistent to our previous studies [10, 12, 29]. Most of the ASD patients with GI symptoms had a history of FPIES like symptoms (**Table 2**). In these patients, we found changes in production of TNF-α and TNF-α/sTNFRII ratios in association with GI symptoms, but to our surprise, we did not find any associations with other inflammatory markers typically associated with neuroinflammation. It may be that GI symptoms are mainly driven TNF-α mediated inflammation in these ASD subjects as seen in patients with IBD [31]. Our finding may indicate the possibility that treatment measures typically used for IBD patients may be applicable for treating GI symptoms in ASD. Interestingly, TNF-α production under zymosan mediated cultures was affected by ASD severity; this may provide further support of the gut-brain axis concept [5, 6].

As for seizure disorders, we found changes in IL-1ß production under the cultures stimulated with ß-glucan and CLO97 in ASD subjects with seizure disorders

(**Table 5**). This association was independent of any other clinical co-variables by co-variance analysis. IL-1ß has been implicated with a major inflammatory component in febrile seizures and is also implicated in the pathogenesis of seizures associated with neuroinflammation [32, 33]. These results may indicate utility of IL-1ß blockers for controlling seizures in ASD subjects, if control is not well achieved by the 1st line anti-seizure medications. This finding is also intriguing because we have found better control of seizures with the use of IL-1ß blockers in some ASD subjects previously [34].

The presence of AR appeared to be associated with an increase in sTNFRII levels which may be indicative of increase in counter-regulatory measures for allergic inflammation. However, since the numbers of AR patients in this study was relatively low, these results need to be validated in future studies.

Our ASD study subjects included a fair number of ASD subjects with SAD (**Table 2**). These ASD subjects revealed lower production of IL-6 and IL-10 under several culture conditions (**Table 5**). Two of these parameters were affected by the presence of PANS like symptoms. This may not be surprising, since in our experience, we often observe a high frequency of SAD in non-ASD PANS patients. Interestingly, ASD subjects with PANS like behavioral symptoms also revealed lower production of IL-6 (**Table 6**). IL-6 is associated with terminal differentiation of B cells and is reported to be lower in patients with antibody deficiency such as common variable immunodeficiency [35]. On the other hand, IL-6 has also be implicated with neuronal development, following neuronal insult during fetal and newborn periods [36, 37]. Reduced IL-6 production may reflect subsequent suppression, following prior IL-6 mediated neuroinflammation. If so, lowering IL-6 production may have evolved into impaired antibody production in some ASD subjects who had suffered from IL-6 mediated inflammation in their early years.

In subjects with autoimmune encephalitis refractory to rituximab, IL-6 blockers such as tocilizumab which is an inhibitor of the IL-6 receptor, are reported to be effective [38, 39]. However, in ASD subjects with lower IL-6 production, the use of IL-6 blockers may not be effective, even if the PANS like behavioral symptoms are attributed to AE. IL-10 production was also lower in ASD patients with SAD, however this is not associated with the presence of PANS like symptoms. Changes in IL-10 production is reported in patients with common variable immunodeficiency (CVID) [40]. Therefore, this finding may be associated with the pathogenesis of antibody deficiency.

When we assessed associations between sleep disorders and changes in monocyte cytokine profiles, we expected to see changes in inflammatory monocyte cytokines, since ASD subjects with PANS like symptoms often suffer from sleep disorders. However, we mainly found lower production of TGF-ß which is considered to be a counter-regulatory cytokine and associated with tissue repair, promoting fibrotic changes [41]. Our results may indicate a decrease in counter-regulatory measures in neuroinflammation in sleep disorders in ASD subjects. The etiology of and the role neuroinflammation plays in sleep disorders in ASD are not well understood. Our finding may indicate that impairment of TGF-mediated pathways may play a role in sleep disorders in ASD.

Our previous studies indicated that IL-1ß/IL-10 ratios can be general markers for dysregulatred innate immune responses in ASD subjects [12]. However, in this study, we did not find strong associations with this parameter to specific co-morbid medical conditions, except for seizure disorder. This parameter may be associated with general inflammation caused by immune mediated inflammation. However, in order to more fully assess treatment options for co-morbid medical conditions in ASD subjects, detailed analysis of monocyte cytokine profiles is likely required.

Our study may be limited by the relatively small sample side of ASD subjects who had specific co-morbid conditions. Findings in this study need to be validated by a study using a larger numbers of study subjects, in association with responses to specific treatment measures targeted to each co-morbid condition.

5. Conclusion

Our study revealed that associations between monocyte cytokine parameters and specific co-morbid medical conditions exist in the ASD subjects studied, independent of other clinical variables. This indicates that there is a possibility that monocyte cytokine profiles may be used for assessing treatment options in ASD subjects with specific co-morbid medical conditions.

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Conflict of interest

The authors have nothing to disclose.

Abbreviations

AA	African American
ABC	aberrant behavior checklist
AC	allergic conjunctivitis
ADHD	attention deficiency hyperactivity disorder
ADI-R	autism diagnostic interview-revisited
ADOS	autism diagnostic observation scale
AR	allergic rhinitis
ASD	autism spectrum disorder
CCL2	C-C chemokine ligand 2
CSHQ	children's sleep habits questionnaires
CNS	central nervous system
CVID	common variable immunodeficiency
FA	food allergy
FPIES	food induced enterocolitis syndrome
GI	gastrointestinal
IBD	inflammatory bowel disease
IIM	innate immune memory
IL	interleukin
LPS	ipopolysaccharide
MIA	maternal immune activation
NFA	non-IgE mediated FA
PANS	pediatric acute-onset neuropsychiatric syndrome
PBMo	peripheral blood monocytes
PST	prick skin testing

SAD	specific antibody deficiency
SD	standard deviation
SPUH	Saint Peter's University Hospital
TLR	toll-like receptor
sTNFR	soluble TNF receptor
TGF	transforming growth factor
TNF	tumor necrosis factor

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References

[1] Fu C, Armstrong D, Marsh E, Lieberman D, Motil K, Witt R, et al. Consensus guidelines on managing Rett syndrome across the lifespan. BMJ Paediatr Open. 2020;4(1):e000717.

[2] Feliciano DM. The Neurodevelopmental Pathogenesis of Tuberous Sclerosis Complex (TSC). Front Neuroanat. 2020;14:39.

[3] Muskens JB, Velders FP, Staal WG. Medical comorbidities in children and adolescents with autism spectrum disorders and attention deficit hyperactivity disorders: a systematic review. Eur Child Adolesc Psychiatry. 2017;26(9):1093-1103.

[4] Holingue C, Newill C, Lee LC, Pasricha PJ, Daniele Fallin M. Gastrointestinal symptoms in autism spectrum disorder: A review of the literature on ascertainment and prevalence. Autism Res. 2018;11(1):24-36.

[5] Martin CR, Osadchiy V, Kalani A, Mayer EA. The Brain-Gut-Microbiome Axis. Cell Mol Gastroenterol Hepatol. 2018;6(2):133-148.

[6] Saurman V, Margolis KG, Luna RA. Autism Spectrum Disorder as a Brain-Gut-Microbiome Axis Disorder. Dig Dis Sci. 2020;65(3):818-828.

[7] Masi A, Glozier N, Dale R, Guastella AJ. The Immune System, Cytokines, and Biomarkers in Autism Spectrum Disorder. Neurosci Bull. 2017;33(2):194-204.

[8] Siniscalco D, Schultz S, Brigida AL, Antonucci N. Inflammation and Neuro-Immune Dysregulations in Autism Spectrum Disorders. Pharmaceuticals (Basel). 2018;11(2).

[9] Fung TC, Olson CA, Hsiao EY. Interactions between the microbiota, immune and nervous systems in health and disease. Nat Neurosci. 2017;20(2):145-155.

[10] Jyonouchi H, Geng L. Associations between Monocyte and T Cell Cytokine Profiles in Autism Spectrum Disorders: Effects of Dysregulated Innate Immune Responses on Adaptive Responses to Recall Antigens in a Subset of ASD Children. Int J Mol Sci. 2019;20(19).

[11] Jyonouchi H, Geng L, Davidow AL. Cytokine profiles by peripheral blood monocytes are associated with changes in behavioral symptoms following immune insults in a subset of ASD subjects: an inflammatory subtype? J Neuroinflammation. 2014;11:187.

[12] Jyonouchi H, Geng L, Rose S, Bennuri SC, Frye RE. Variations in Mitochondrial Respiration Differ in IL-1ss/IL-10 Ratio Based Subgroups in Autism Spectrum Disorders. Front Psychiatry. 2019;10:71.

[13] Bilbo SD, Block CL, Bolton JL, Hanamsagar R, Tran PK. Beyond infection - Maternal immune activation by environmental factors, microglial development, and relevance for autism spectrum disorders. Exp Neurol. 2018;299(Pt A):241-251.

[14] Rose DR, Careaga M, Van de Water J, McAllister K, Bauman MD, Ashwood P. Long-term altered immune responses following fetal priming in a non-human primate model of maternal immune activation. Brain Behav Immun. 2017;63:60-70.

[15] Fok ET, Davignon L, Fanucchi S, Mhlanga MM. The lncRNA Connection Between Cellular Metabolism and Epigenetics in Trained Immunity. Front Immunol. 2018;9:3184.

[16] Netea MG, Schlitzer A, Placek K, Joosten LAB, Schultze JL.

Innate and Adaptive Immune Memory: an Evolutionary Continuum in the Host's Response to Pathogens. Cell Host Microbe. 2019;25(1):13-26.

[17] Wendeln AC, Degenhardt K, Kaurani L, Gertig M, Ulas T, Jain G, et al. Innate immune memory in the brain shapes neurological disease hallmarks. Nature. 2018;556(7701):332-338.

[18] Neher JJ, Cunningham C. Priming Microglia for Innate Immune Memory in the Brain. Trends Immunol. 2019;40(4):358-374.

[19] Aman MG, Singh NN, Stewart AW, Field CJ. The aberrant behavior checklist: a behavior rating scale for the assessment of treatment effects. Am J Ment Defic. 1985;89(5):485-491.

[20] Owens JA, Spirito A, McGuinn M. The Children's Sleep Habits Questionnaire (CSHQ): psychometric properties of a survey instrument for school-aged children. Sleep. 2000;23(8):1043-1051.

[21] Sparrow SB CD, Vineland DV. Adaptive Behavior Scales Survey Form Manual. American Guidance Service: Cirde Pines, MN. 1985.

[22] Boyce JA, Assa'ad A, Burks AW, Jones SM, Sampson HA, Wood RA, et al. Guidelines for the diagnosis and management of food allergy in the United States: report of the NIAID-sponsored expert panel. J Allergy Clin Immunol. 2010;126(6 Suppl):S1-58.

[23] Butrus S, Portela R. Ocular allergy: diagnosis and treatment. Ophthalmol Clin North Am. 2005;18(4):485-492, v.

[24] Nassef M, Shapiro G, Casale TB. Identifying and managing rhinitis and its subtypes: allergic and nonallergic components--a consensus report and materials from the Respiratory and Allergic Disease Foundation. Curr Med Res Opin. 2006;22(12):2541-2548.

[25] Expert Panel Report 3 (EPR-3): Guidelines for the Diagnosis and Management of Asthma-Summary Report 2007. J Allergy Clin Immunol. 2007;120(5 Suppl):S94-138.

[26] Orange JS, Ballow M, Stiehm ER, Ballas ZK, Chinen J, De La Morena M, et al. Use and interpretation of diagnostic vaccination in primary immunodeficiency: a working group report of the Basic and Clinical Immunology Interest Section of the American Academy of Allergy, Asthma & Immunology. J Allergy Clin Immunol. 2012;130(3 Suppl):S1-24.

[27] Gromark C, Harris RA, Wickstrom R, Horne A, Silverberg-Morse M, Serlachius E, et al. Establishing a Pediatric Acute-Onset Neuropsychiatric Syndrome Clinic: Baseline Clinical Features of the Pediatric Acute-Onset Neuropsychiatric Syndrome Cohort at Karolinska Institutet. J Child Adolesc Psychopharmacol. 2019;29(8):625-633.

[28] Jyonouchi H, Geng L, Ruby A, Zimmerman-Bier B. Dysregulated innate immune responses in young children with autism spectrum disorders: their relationship to gastrointestinal symptoms and dietary intervention. Neuropsychobiology. 2005;51(2):77-85.

[29] Jyonouchi H. Innate immunity and neuroinflammation in neuropsychiatric conditions including autism spectrum disorders: a role of innate immune memory. 2019. In: Cytokines [Internet]. InTech-open

[30] Jyonouchi H. Autism spectrum disorders and allergy: observation from a pediatric allergy/immunology clinic. Expert Rev Clin Immunol. 2010;6(3):397-411.

[31] Friedrich M, Pohin M, Powrie F. Cytokine Networks in the Pathophysiology of Inflammatory Bowel Disease. Immunity. 2019;50(4):992-1006.

[32] Feng B, Chen Z. Generation of Febrile Seizures and Subsequent Epileptogenesis. Neurosci Bull. 2016;32(5):481-492.

[33] Vezzani A, Balosso S, Ravizza T. Neuroinflammatory pathways as treatment targets and biomarkers in epilepsy. Nat Rev Neurol. 2019;15(8):459-472.

[34] Jyonouchi H, Geng, L. Intractable Epilepsy (IE) and Responses to Anakinra, a Human Recombinant IL-1 Receptor Agonist (IL-1ra): Case Reports Journal of Clinical & Cellular Immunology 2016;7:5.

[35] Sharifi L, Aghamohammadi A, Rezaei N, Yazdani R, Rezaei F, Bokaie S, et al. Interleukin-1beta and interleukin-6 in Common Variable Immunodeficiency and their association with subtypes of B cells and response to the Pneumovax-23 vaccine. Eur Cytokine Netw. 2019;30(4):123-129.

[36] Gumusoglu SB, Fine RS, Murray SJ, Bittle JL, Stevens HE. The role of IL-6 in neurodevelopment after prenatal stress. Brain Behav Immun. 2017;65:274-283.

[37] Rudolph MD, Graham AM, Feczko E, Miranda-Dominguez O, Rasmussen JM, Nardos R, et al. Maternal IL-6 during pregnancy can be estimated from newborn brain connectivity and predicts future working memory in offspring. Nat Neurosci. 2018;21(5):765-772.

[38] Lee WJ, Lee ST, Moon J, Sunwoo JS, Byun JI, Lim JA, et al. Tocilizumab in Autoimmune Encephalitis Refractory to Rituximab: An Institutional Cohort Study. Neurotherapeutics. 2016;13(4):824-832.

[39] Randell RL, Adams AV, Van Mater H. Tocilizumab in Refractory Autoimmune Encephalitis: A Series of Pediatric Cases. Pediatr Neurol. 2018;86:66-68.

[40] Berron-Ruiz L, Lopez-Herrera G, Vargas-Hernandez A, Santos-Argumedo L, Lopez-Macias C, Isibasi A, et al. Impaired selective cytokine production by CD4(+) T cells in Common Variable Immunodeficiency associated with the absence of memory B cells. Clin Immunol. 2016;166-167:19-26.

[41] Varela-Eirin M, Loureiro J, Fonseca E, Corrochano S, Caeiro JR, Collado M, et al. Cartilage regeneration and ageing: Targeting cellular plasticity in osteoarthritis. Ageing Res Rev. 2018;42:56-71.