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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

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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.

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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).

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Table 2
Demographics of the adult study subjects (mothers of the study subjects).

	ASD/Inf	ASD/no-Inf	Normal controls
Subject number	N = 29	N = 28	N = 10
Age (year) : median (range)	40 (32–52)	38 (32–49)	37 (24–48)
Ethnicity	2 AA[a], 3 Asian, 24 W	4 AA, 3 Asian, 21 W	10 W
Co-morbidities			
Autoimmune disease	2 (MS, Lyme disease)	1 (celiac disease)	0

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

agonist (CL097, water-soluble derivative of imidazoquinoline, 20 μM, InvivoGen), and TLR 9 agonist (CpG, 5 μg/ml, InvivoGen) in RPMI 1640 with additives as previously described (Jyonouchi et al., 2005b). We also assessed the responses to dectin 1 agonist [heat killed Candida albicans as a source of β-lactam (10^9 cells/ml)—10 μl/ml, InvivoGen] Overnight incubation was adequate to induce the optimal responses in this setting. Levels of proinflammatory [tumor necrosis factor-α (TNF-α), IL-1β, IL-6, IL-12p40, and IL-23] and counter-regulatory [IL-10, transforming growth factor-β (TGF-β) and soluble TNF-receptor II (sTNFRII)] cytokines in the culture supernatant were then measured by ELISA.

2.3. Reactivity to T cell stimulants

Cellular reactivity to T cell stimulants was assessed by incubating PBMCs (10^6 cells/ml) with T cell mitogens [Con A (2 μg/ml) and PHA (5 μg/ml), Sigma-Aldrich], recall Ags [tetanus toxoid (1:1000, Aventis Pasteur, Swiftwater, PA), candida Ag (5 μg/ml, Greer, Lenoir, NC), β-lactoglobulin (β-LG, Sigma-Aldrich), and α-lactoalbumin (α-LA, Sigma-Aldrich)], and IFN-γ inducing cytokines [IL-12p70 (0.2 ng/ml, BD-Pharminge, San Diego, CA), IL-18 (1 ng/ml, BD-Pharmingen) for 4 days and measuring levels of IFN-γ, TNF-α, IL-5, IL-10, IL-12p40, and IL-17 in the culture supernatant (Jyonouchi et al., 2010). Four days' incubation period resulted in the optimal production of these cytokines in this setting in the initial titration studies.

2.4. Cytokine ELISA

Cytokine levels were measured by ELISA, using OptEIA™ Reagent Sets (BD Biosciences) for IFN-γ, IL-1β, IL-5, IL-6, IL-10, IL-12p40, and TNF-α, and ELISA reagent set (R & D, Minneapolis, MN) for sTNFRII, IL-17 (IL-17A), and TGF-β. IL-23 ELISA kit were purchased from eBiosciences, San Diego, CA. Intra- and inter-variations of cytokine levels were less than 5%.

2.5. Transcription profiling

Peripheral blood (PB) monocytes were purified using an immunoaffinity column following the company's instructions (MACS monocytes isolation kit, Miltenyi Biotec, Auburn, CA). Total RNA were extracted by the RNA easy kit (Quiagen, Valencia, CA). RNA labelling and hybridizations on Agilent Human 4 × 44K arrays (Agilent, Lexington, MA) were done using the Agilent One-Color Microarray-Based Gene Expression Analysis Ver 5.5 protocol (Agilent). All slides ware scanned by Agilent Scanner and normalized numerical data will be obtained by Agilent Feature extraction software 9.

2.6. Statistics

For comparison of test values with control values, a Wilcoxon signed rank test was used and for comparison of multiple groups, a Kruskall–Wallis test was used. These non-parametric statistical measures were used because many of the data sets in this study were not normally distributed. Differences of frequency were tested with a Chi square (χ^2) test. Correlation was tested by using a linear regression analysis. These tests were performed using R.2.10.1 (R-Development Core Team 2009). A p value of <0.05 was considered to be statistically significant. For the analysis of microarrays experiments, Gene Spring GX v11 software (Agilent) was used. After filtering for "present" calls in at least 20% of samples, fold change analysis were performed for group for comparisons on 26992 probes. Genes with at least a two-fold changes as compared to controls are determined as up-regulated or down-regulated. Using a specific module of GeneSpring software (Agilent), pathway enrichment analysis was performed on those genes to see if there is a statistically significant enrichment ($p<0.05$) for specific BioPax pathways.

3. Results

3.1. Clinical features

Demographics of the study subjects are summarized in Tables 1 and 2. As summarized in Table 3 and consistent with our previous results (Jyonouchi et al., 2008), there was no significant difference between the ASD/Inf and control groups regarding frequency of asthma, AR + AC, or FA (both IgE and non-IgE mediated). However, a high frequency of chronic GI condition (19/30, 63.3%) was noted in the ASD/Inf children ($p<0.00001$ by χ^2 test). These children were previously diagnosed with non-IgE mediated FA or FPIES. However, their GI symptoms persisted despite implementation of an appropriate restricted diet (avoidance of offending food). Their GI symptoms were often responsive to antibiotics and anti-fungal agents, albeit transiently (Jyonouchi et al., 2007). Extensive workups of these subjects ruled out inflammatory bowel disease, Celiac disease, eosinophilic gastroenteritis, or other well defined GI conditions. The ASD/Inf children with severe GI symptoms are referred to as ASD/Inf + GI children in this study. The ASD/Inf children without GI symptoms are designated as ASD/Inf-no GI children.

It is also of note that 7/30 ASD/Inf subjects were diagnosed with antibody (Ab) deficiency syndrome; 1 common variable immunodeficiency (CVID) and 6 specific polysaccharide antibody antibody deficiency (SPAD) (Table 3) ($p<0.01$ by χ^2 test). Among these 7 ASD/Inf children, 4 revealed chronic GI conditions, indicating that persistent GI conditions are not solely attributed to Ab deficiency. Recurrent OM and CRS were also common in the ASD/Inf children but

Table 3
Clinical features of the ASD/Inf group subjects.

Co-morbidities	ASD/Inf		ASD/no-Inf (N = 28)	Normal controls (N = 26)
	+ GI (N = 19) no	GI (N = 11)		
Asthma				
IgE mediated	1/19	1/11	0	2/26
Non-IgE mediated	4/19	2/11	2/28	5/26
AR + AC[a]	2/19	2/11	5/28	5/26
Food allergy				
IgE mediated	2/19	1/11	2/28	1/26
Non-IgE mediated	18/19	4/11	22/28	0[b]
Seizure disorders	1 /19	1/11	1/28	0
Chronic GI symptoms	19 /19	0	2/28	0
Immunodeficiency[c]	4/19	3/11	0	0
Recurrent infection				
ROM	11/19	6/11	4/28	1/26
CRS	9/19	5/11	1/28	1/26

[a] Abbreviations used: AR + AC, allergic rhinoconjunctivitis, CRS, chronic rhinosinusitis, ROM, recurrent otitis media.

[b] 2 control subjects had a history of FPIES but had outgrown this condition; no GI symptoms on at the time of blood sampling.

[c] Among 7 children diagnosed with immunodeficiency, 6 subjects were diagnosed with specific polysaccharide antibody deficiency and 1 subject was diagnosed with common variable immunodeficiency (CVID) and all of them are now treated with supplemental intravenous immunoglobulin (IVIG).

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equally distributed in ASD/Inf + GI and ASD/Inf-no GI children (Table 3). It is important to note that loss of cognitive skills was observed repeatedly and over a wide range of ages, and was not limited to infancy or early childhood.

3.2. Innate immune responses

The results for ASD/Inf + GI (N = 19), ASD/Inf-no GI (N = 11), and ASD/no-Inf (N = 28) children were evaluated in comparison with ASD and normal controls. Changes in innate immune responses were more evident in the ASD/Inf + GI children than in the ASD/Inf-no GI children (Figs. 1–4). Namely, ASD/Inf + GI PBMCs produced less IL-6 and IL-1β with TLF7/8 agonist (Fig. 1), less IL-6 with TLR2/6 and TLR9 agonists and in the absence of stimuli (Figs. 1–2). In addition, ASD/Inf + GI PBMCs produced less IL-1β (medium only), IL-23 (TLR4 agonist), TNF-α (TLR9 agonist), and IL-12 (TLR 9 agonist) (Fig. 3). In contrast, ASD/Inf-no GI PBMCs did not reveal significant changes except for lower IL-23 production as compared to control cells (Figs. 1–3). As for counter-regulatory cytokines, ASD/Inf + GI PBMCs produced less IL-10 with TLR 2/6, 7/8, and 9 agonists and in the absence of medium (Fig. 4). ASD/Inf-no GI PBMCs showed no such changes.

3.3. Responses to T cell stimulants

T cell responses did not differ among the study groups in response to T cell mitogens (PHA and Con A) or tetanus toxoid in terms of production of IFN-γ (Fig. 5) and other cytokines (IL-5, IL-10, IL-12p40, TNF-α, and IL-17). However, when cells were stimulated with luminal Ags [candida and milk protein components (αLA and βLG)], ASD/Inf + GI PBMCs produced less IFN-γ (with αLA) and less TNF-α (with αLA, βLG, and candida Ag) as compared to ASD/no-Inf and normal controls (Fig. 6). Such changes were less evident in ASD/Inf-noGI cells.

3.4. Transcription profiling

In ASD/Inf + GI monocytes, large numbers of genes were up- or down-regulated (>2 fold) as compared to ASDno-Inf and normal control cells (Table 4). Such changes were less evident in ASD/Inf-no GI monocytes (Table 4). A total of 292 genes were overlapped in altered expression (>2 fold) in ASD/Inf + GI monocytes, as compared to both ASD/no-Inf and normal controls. In contrast, the numbers of altered transcripts in ASD/Inf-no GI monocytes were substantially less than in ASD/Inf + GI cells, overlapping only in 6 genes (Table 4). Notably, CCL2 and CCL7, key chemokines associated with migration of monocytes to inflamed peripheral tissues (Parihar et al., 2010), are up-regulated 4–5 fold in ASD/Inf + GI monocytes. CCL2 were also up-regulated in ASD/Inf-no GI monocytes (2-fold). The list of overlapping genes in ASD/Inf + GI monocytes is shown in supplemental Table 1 and includes genes associated with risk factors of schizophrenia/autism/depression (TGM2, BZRAP1, KREMEN1, EGR3, and ASMT) (Cai et al., 2008; Bucan et al., 2009; Aleksic et al., 2010; Bradford et al., 2011; Jin et al., 2010; Jonsson et al., 2010; Kim et al., 2010; Kyogoku et al., 2011).

We also studied transcript profiles of PB monocytes in mothers of the study groups. This is secondary to the facts that mothers of ASD children not only share genetic predisposition but also have a potential to affect these children during pregnancy by activation of the immune system (Baharnoori et al., 2010; De Miranda et al., 2010; Hsiao and Patterson, 2010). Transcription profiling of PB monocytes obtained from mothers of the study groups revealed similar findings. Namely, the differences in transcription profiles in PB monocytes was more evident in ASD/Inf + GI mothers than in ASD/Inf-no GI mothers as compared to cells from mothers of the control groups (Table 4). No difference was found in enrichment of transcripts of genes associated with specific pathways in ASD/Inf + GI, as well as ASD/Inf-no GI children. However, a difference was observed between the ASD/Inf + GI mothers and the ASD/no-Inf control mothers; enriched expression of genes associated with

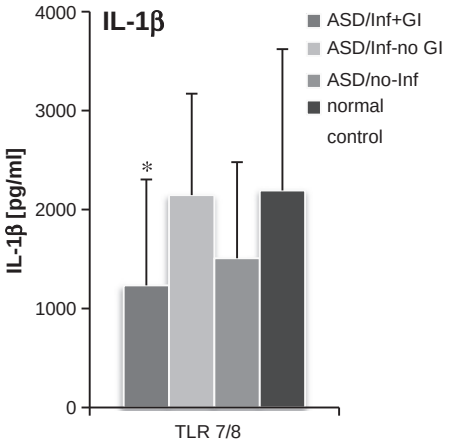

Fig. 2. IL-1β production by PBMCs from ASD-IS, ASD-NS, ASD control, and normal control children when stimulated by a TLR 7/8 agonist overnight. *; p<0.05, **; p<0.01, ***; p<0.005 by Wilcoxon signed rank test as compared to normal controls.

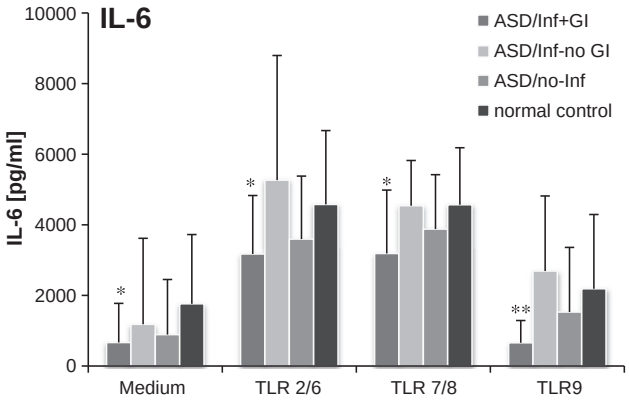

Fig. 1. IL-6 production by PBMCs from ASD-IS, ASD-NS, ASD control, and normal control children when stimulated with TLR agonists over night. Medium: cells were cultured in the medium only without additional stimulants. *; p<0.05, **; p<0.01, ***; p<0.005 by Wilcoxon signed rank test as compared to normal controls.

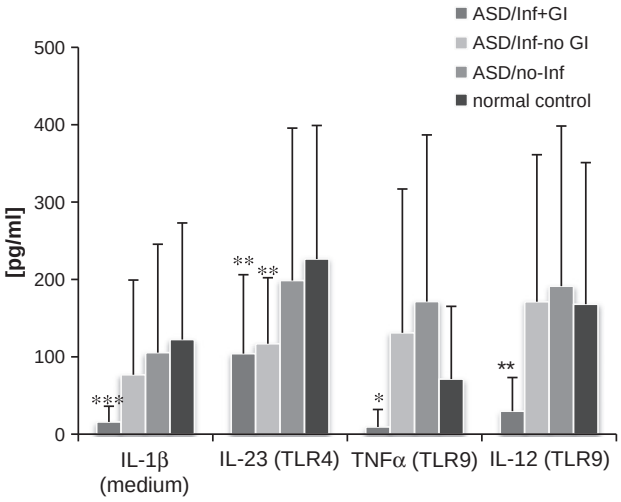

Fig. 3. Cytokine production by PBMCs from ASD-IS, ASD-NS, ASD control, and normal control children when stimulated by TLR agonists as indicated in the figure. Medium: cells were cultured in the medium only without additional stimulants. *; p<0.05, **; p<0.01, ***; p<0.005 by Wilcoxon signed rank test as compared to normal controls.

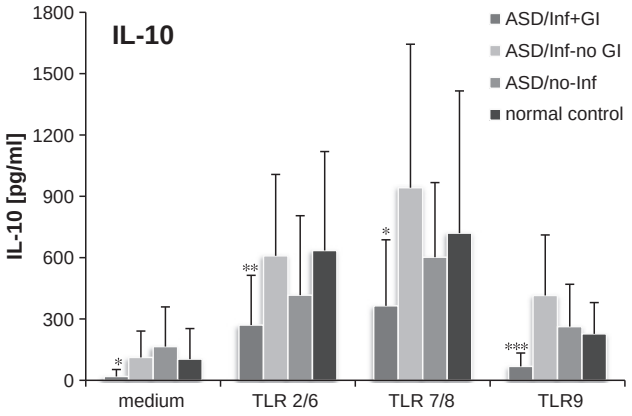

Fig. 4. IL-10 production by PBMCs from ASD-IS, ASD-NS, ASD control, and normal control children when stimulated by TLR agonists as indicated in X axis of the figure. Medium: cells were cultured in the medium only without additional stimulants. *; $p<0.05$, **; $p<0.01$, ***; $p<0.005$ by Wilcoxon signed rank test as compared to normal controls.

Hedgehog and inhibitors of differentiation pathways was found in ASD/Inf + GI mothers.

3.5. CCL2 production

The most notable finding in transcription profiling of ASD/Inf + GI PB monocytes was the up-regulated mRNA expression of CCL2. Concentrations of CCL2 were measured in the culture supernatants obtained after overnight incubation with TLR agonists. CCL2 was found to be high in the setting of medium alone (no stimulant) and tended to decrease after stimuli of TLR agonists in ASD/no-Inf and normal control PBMCs, since monocytes produce CCL2 spontaneously. However, ASD/Inf + GI PBMCs did not reveal a decline of CCL2 production (Fig. 7). ASD/Inf-no GI PBMCs also revealed a tendency of less decline of CCL2 production, albeit this is not as evident as ASD/Inf + GI cells (Fig. 6). These findings are consistent with transcription profiling results.

4. Discussion

Complex gene–gene and gene–environmental factors can affect the intricate networks associated with brain development and functions. GWAS and studies of structural variations such as CNV have provided

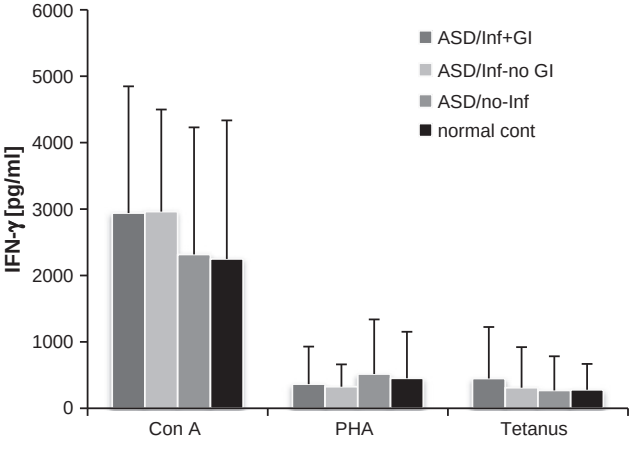

Fig. 5. Cytokine production by PBMCs from ASD-IS, ASD-NS, ASD control, and normal control children when stimulated by recall antigens for 4 days as indicated in the figure. *; $p<0.05$, **; $p<0.01$, ***; $p<0.005$ by Wilcoxon signed rank test as compared to normal controls.

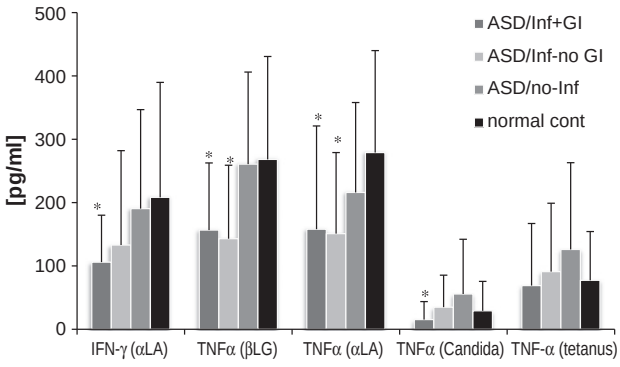

Fig. 6. Cytokine production by PBMCs from ASD-IS, ASD-NS, ASD control, and normal control children when stimulated by polyclonal T cell mitogens and tetanus toxid.

genetic evidence of a substantial polygenetic components in ASD (Cook and Scherer, 2008; El-Fishawy and State, 2010; Rudan, 2010). As a result, it is now known that some gene mutations are non-specifically involved in various psychiatric disorders including ASD, affecting common pathway (s) (Rudan, 2010). In such multi-valent conditions, clinical phenotypes are expected to vary considerably. Modification of treatment measures based on genetic and environmental factors in each patient is likely required, in order to have optimal outcomes.

Given such a heterogeneous ASD population, focusing on well defined gene mutations or genetic defects associated with ASD has been rewarding, as discussed in the introduction (Gonzales and LaSalle, 2010; Wang et al., 2010). However, this strategy is not feasible for the identification of novel factors or the effects of unappreciated pathways. GWAS and CNV analysis have shed a light on numerous genetic mutations that can affect the development of ASD, but these approaches are not sensitive enough to assess the contribution of rare genetic variations that have yet to be identified or effects of unappreciated pathways (Rudan, 2010; Toro et al., 2010).

In addition to behavioral symptoms and impaired cognitive skills, many ASD children suffer from various co-morbidities, with GI symptoms being the most common. Worsening GI symptoms often aggravate the behavioral symptoms in these children (Buie et al., 2010; Jyonouchi, 2010). Our previous studies revealed that certain GI

Table 4
Summary of transcripts profiles in ASD/Inf + GI and ASD/Inf-No GI children.

	Vs. normal controls[a]	Vs. ASD/no-Inf controls	The number of genes overlapping in the 2 control groups
ASD test group			
All ASD/Inf subjects			
Up-regulated	165	99	7
Down-regulated	166	42	3
ASD/Inf + GI			
Up-regulated	1620	322	178
Down-regulated	954	168	114
ASD/Inf-no GI			
Up-regulated	28	378	3
Down-regulated	20	373	3
ASD/Inf group mothers			
All mothers in the ASD/Inf group			
Up-regulated	252	29	0
Down-regulated	105	57	1
ASD/Inf + GI mothers			
Up-regulated	856	72	40
Down-regulated	563	71	41
ASD/Inf-no GI mothers			
Up-regulated	230	546	43
Down-regulated	40	799	12

[a] The numbers of genes up- or down-regulated over 2-fold as compared to normal controls as well as ASD/no-Inf controls were shown in the table.

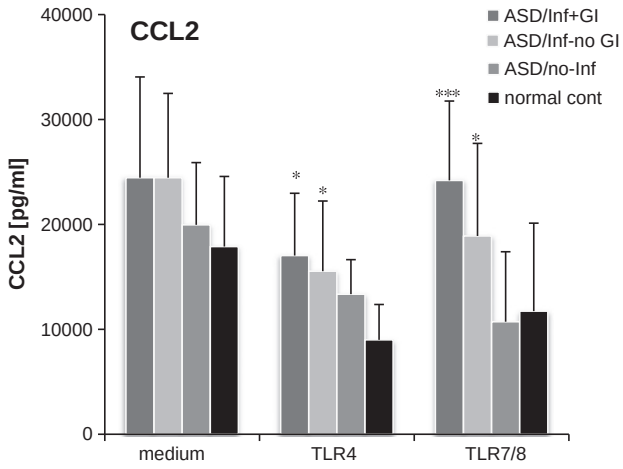

Fig. 7. CCL2 production by PBMCs from ASD-IS, ASD-NS, ASD control, and normal control children when stimulated with TLR 4 or TLR7/8 agonists or without a stimulus overnight. Medium: cells were cultured in the medium only without additional stimulants. *; $p<0.05$, **; $p<0.01$, ***; $p<0.005$ by Wilcoxon signed rank test as compared to normal controls.

symptoms observed in young ASD children can be attributed to delayed type FA, with favorable responses to avoidance of offending food (Jyonouchi et al., 2005a; Jyonouchi, 2009). However, in some ASD children, we also observed persistent GI symptoms that were helped, but not solely controlled, by the dietary intervention (Jyonouchi et al., 2007). These particular ASD children often exhibit markedly fluctuating behavioral symptoms and repeated loss of cognitive skills following immune insults (Jyonouchi et al., 2008).

Various subtle immune abnormalities as well as abnormal findings of gut mucosa or array analysis of whole blood have been reported elsewhere (Ashwood and Wakefield, 2006; Gregg et al., 2008; Jyonouchi, 2009; Singh, 2009; Ashwood et al., 2011a; Enstrom et al., 2010; Ashwood et al., 2011b). However, in general, findings of such abnormalities are subtle and not always reproducible. In most of the studies that have addressed immune abnormalities or GI symptoms in ASD, characterization of ASD subjects were limited to a neuropsychiatric evaluation, without detailed immunological characterization. Studying such a heterogeneous ASD population without defining subgroups may have led to the inconclusive findings in these previous immune studies.

In our prior research, we characterized a subset of ASD children who exhibited fluctuating behavioral symptoms and cognitive skills following immune insults (ASD/inf children). In this ASD subset, we observed innate immune abnormalities as compared to ASD/no-Inf and normal controls (Jyonouchi et al., 2008). Innate immunity senses when the body is under a stressful condition, such as pathogen invasion, by sensing microbial byproducts and damaged tissues products via receptors expressed on innate immune cells. Among these receptor families, TLRs are the most thoroughly studied and are thought to play crucial roles in stress responses. It is known that acute stress responses elicited by innate immune responses are partly mediated by inflammatory cytokines as well as microbial byproducts such as endotoxin, a TLR4 agonist. These mediators are sensed by innate immune cells in the CNS via TLRs or other innate immune receptors (Olson and Miller, 2004; Crack and Bray, 2007). In addition, peripherally activated innate immune cells are recruited to the CNS under certain conditions. For example, PB monocytes can migrate to the CNS, evolving into BMDM cells (Davoust et al., 2008). Thus aberrant innate immune responses have the ability to alter key stress responses, which can then lead to an impairment of the neuroimmune network, hindering brain function. Therefore, our previous results indicate that in this subset of ASD children, the aberrant innate immune responses may be associated with their fluctuating behavioral symptoms and cognitive skills. However, in the initial study, it was unclear how GI symptoms were associated with their clinical features. This was partly due to the low numbers of study subjects and limited measures conducted for assessing innate immunity.

In this study, we were able to reveal that the ASD/Inf children exhibiting chronic GI symptoms (ASD/Inf + GI) revealed distinct innate immune abnormalities as opposed to the ASD/Inf-no GI children , despite the presence of similar fluctuating behavioral symptoms/cognitive activity in both subsets. That is, ASD/Inf + GI PBMCs had decreased production of both proinflammatory (IL-6, IL-1β, IL-12, and IL-23) and counter-regulatory cytokines (IL-10 and sTNFRII) in response to TLR agonists when compared to ASD/no-Inf and normal controls. This was most notable with TLR2/6 and 7/8 agonists. TLR7/8 are intracellular receptors for ssRNA, a byproduct often derived from RNA viruses (Yuan et al., 2010). Thus our findings indicate that impaired TLR7/8 mediated signaling may be associated with recurrent infection. TLR2/6 recognize pathogen associated molecular patterns derived from bacteria and fungi (Yuan et al., 2010). Therefore, suboptimal responses to TLR2/6 agonists in the ASD/Inf + GI children may lead to impaired defense against fungi and other microbes, rendering them more vulnerable to common microbial infection, as well as dysbiosis. Our assumption is indirectly supported by the clinical features displayed by the ASD/Inf + GI children; they manifest frequent infection, persistent GI symptoms, and transient responses to anti-microbial agents (Table 3). Such clinical features indicate that ASD/Inf + GI children may have aberrant mucosal immune responses to commensal flora as is observed in IBD patients (Yamamoto-Furusho and Podolsky, 2007). However, the patterns of TLR responses in ASD/Inf + GI children differed from those observed in IBD patients who were studied using the same methodologies (Jyonouchi et al., 2010).

Both the ASD/Inf + GI and ASD/Inf-no GI children included subjects diagnosed with Ab deficiency syndrome, with the number of subjects with this diagnosis, being 4 and 3, respectively. It should be noted that non-ASD children with Ab syndrome did not reveal the innate immune abnormalities that were observed in the ASD/Inf + GI children (manuscript submitted for publication). Therefore, it is unlikely that their persistent GI symptoms are attributed to aberrant immune responses to commensal flora or Ab deficiency syndrome.

Since the ASD/Inf + GI children revealed lower responses to TLR agonists and TLR responses affect adaptive responses, altered adaptive immune responses may be occurring in these ASD/Inf + GI children. Differences were not observed when PBMCs were stimulated with T cell mitogens or vaccine derived recall Ags among the study groups (Figs. 5–6). However, we did observe decreased production of Th1 cytokines (IFN-γ and TNF-α) in response to milk proteins (αLA and βLG) and candida Ag which may be associated with decreased IL-12 production (Fig. 6). Despite decreased production of IL-1β, IL-6 and TGF-β which are key differentiation factors for IL-17 producing Th17 cells (Kimura and Kishimoto, 2011), we did not observe impaired production of IL-17 in the ASD-IS children. This may be associated with impaired production of Th1 cytokines, that in turn counter-act differentiation of Th17 cells (Kimura and Kishimoto, 2011).

It may be argued that the lower innate immune responses observed in the ASD/Inf + GI children reflect chronic GI inflammation thereby resulting in systemic immune suppression. However, this may not be the case. Namely, chronic inflammation typically causes up-regulated counter-regulatory cytokines while our results showed that production of these cytokines were suppressed in ASD/Inf + GI children. In addition, when the same methodologies were applied for non-ASD children with chronic inflammation (IBD, chronic rhinosinusitis and asthma), their TLR responses differed from those observed in the ASD/Inf + GI children. (Jyonouchi et al., 2008; Jyonouchi et al., 2010) (Unpublished observation). It should be noted that non-ASD children with chronic inflammatory conditions may have different patterns of TLR responses associated with their conditions. Therefore, at this point,

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we cannot definitively rule out the possibility of chronic inflammation playing a role in our findings.

To further address whether innate immune abnormalities are specific to the ASD/Inf + GI children, we conducted transcription profiling of PB monocytes in all the study groups, as well as in the mothers of the study subjects. We elected to study mothers considering the possibility that maternal innate immune abnormalities may have affected these ASD children during pregnancy. The mothers tested in our study were not suffering from overt inflammation/infection/GI symptoms at the time of sample obtainment. Interestingly, our results revealed significant changes in the transcript profiles of monocytes in ASD/Inf + GI children, as well as their mothers (Table 4). These findings make it even more unlikely that the changes in innate immune responses observed in the ASD/Inf + GI children are attributed to chronic GI inflammation. This finding is in contrast to the results of the transcript profiles of the ASD/Inf-no GI monocytes, which revealed similar transcript profiles to normal controls (Table 4). These results indicate that similar transcript profiles of PB monocytes between mothers and children of ASD/Inf + GI children could be attributed to genetic predisposition of innate immunity in ASD/Inf + GI children. It may also be possible that aberrant innate immune responses in the mothers of ASD/Inf + GI children may have also affected these children during pregnancy as reported in rodent models (Baharnoori et al., 2010; De Miranda et al., 2010; Hsiao and Patterson, 2010). It is also of note that genes altered in expression in PB monocytes in ASD/Inf + GI children include genes associated with oxidative stress responses, inflammation, chemotaxis, cell proliferation and differentiation (supplemental Table 1), indicating the altered state of PB monocytes and possible their role of the clinical features in these children. However, further studies are required for verifying these results.

Transcript profiling also revealed a significant up-regulation of CCL2 and CCL7 genes in the ASD-IS children; mRNA expression of these chemokines were more than 4-fold up-regulated as compared to both ASD and normal controls. PB monocytes are divided into two major subsets based on expression of CD14 (LPS co-receptor) and CD16 (FcγIII receptor) (Serbina et al., 2008). These monocytes subsets are physiologically different and changes in their numbers were observed in various disease conditions. $CD14^{++}CD16^{-}$ monocytes express high levels of CCR1, CCR2, CXCR2 and low levels of CX3CR and respond to CCL2 and CCL7 (Serbina et al., 2008). In stroke patients, it has been reported that the number of circulating monocyte subsets is associated with prognosis: $CD14^{++}CD16^{-}$ monocytes numbers are positively associated with poor outcome and higher mortality (Urra et al., 2009). These results indicate that in the presence of CNS inflammation, PB monocytes numbers and functions may be associated with the degree of disease severity. This may be partly attributed to the fact that classical monocytes migrate to the inflamed CNS and evolve into BMDM cells (Djukic et al., 2006; Rodriguez et al., 2007; Davoust et al., 2008; Ransohoff and Cardona, 2010). In ASD/Inf + GI children, clinical features of fluctuating behavioral symptoms and cognitive skills following immune insults also indicate the presence of inflammation mediated by immune mechanisms. In a study of brain tissue obtained from ASD children, up-regulated mRNA expression of CCL2 was reported (Vargas et al., 2005). Interestingly, despite somewhat similar transcript profiles of PB monocytes in the mothers of the ASD-IS children, up-regulation of CCL2 or CCL7 transcripts were not observed. These results indicate that up-regulation of these chemokines in PB monocytes can serve as markers of CNS inflammation in ASD/Inf + GI children.

When we tested the concentration of CCL2 in culture supernatants, we found that CCL2 levels decline with stimuli of TLR agonists. However, this declining trend was less evident in the ASD/Inf + GI children, resulting in higher levels of CCL2 in the ASD/Inf + GI children. ASD/Inf-no GI children revealed a similar tendency, but this was less evident than in the ASD/Inf + GI children (Fig. 6). Our results again indicates that CCL2 can serve as a candidate biomarker for ASD/Inf + GI children, but also indicate the need for further research on chemokine pathways in ASD/Inf + GI children and perhaps in ASD/Inf-no GI children.

Lastly, transcript profiling revealed substantial overlapping of gene expression as compared to both control groups; 292 genes were overlapped, including genes that have been previously implicated with risk for other neuropsychiatric disorders, such as schizophrenia (TGM2, BZRAP1, KREMEN1, EGR3, and ASMT) (Cai et al., 2008; Bucan et al., 2009; Aleksic et al., 2010; Bradford et al., 2011; Jin et al., 2010; Jonsson et al., 2010; Kim et al., 2010; Kyogoku et al., 2011). These particular genes are associated with inflammatory pathways. Therefore, up-regulation of these genes in ASD/Inf + GI children may indicate involvement of common inflammatory pathways associated with other neuropsychiatric conditions including schizophrenia in development of their clinical features such as fluctuating behavioral symptoms in the ASD/Inf + GI children. Further studies of these components may also be beneficial for exploring treatment options by controlling such inflammatory pathways in ASD/Inf + GI children as well as patients with other neuropsychiatric conditions.

In summary, our results revealed a distinct ASD subset, ASD/Inf + GI, with characteristic innate immune abnormalities demonstrated in functional assays, as well as in transcript profiles of PB monocytes. Our findings indicate that classical monocytes (or M1 monocytes) may play a role in disease pathogenesis in ASD/Inf + GI children, although it may not be disease-specific. Nevertheless, our findings may pave the way for finding biomarkers that will identify ASD/Inf + GI children. In addition, our results may also contribute to development of new treatment options for ASD/IS + GI children, by applying already available immune-modulating agents that affect chemokine pathways.

Supplementary materials related to this article can be found online at doi:10.1016/j.jneuroim.2011.07.001.

Conflict of interest

None of the authors have any competing financial interest to declare in relation to the work described.

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