Differential immune responses and microbiota profiles in children with autism spectrum disorders and co-morbid gastrointestinal symptoms

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Abstract

Objectives—Many studies have reported the increased presence of gastrointestinal (GI) symptoms in children with autism spectrum disorders (ASD). Altered microbiome profiles, pro-inflammatory responses and impaired intestinal permeability have been observed in children with ASD and co-morbid GI symptoms, yet few studies have compared these findings to ASD children without GI issues or similarly aged typical developing children. The aim of this study was to determine whether there are biological signatures in terms of immune dysfunction and microbiota composition in children with ASD with GI symptoms.

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Methods—Children were enrolled in one of four groups: ASD and GI symptoms of irregular bowel habits (ASD^{GI}), children with ASD but without current or previous GI symptoms (ASD^{NoGI}), typically developing children with GI symptoms (TD^{GI}) and typically developing children without current or previous GI symptoms (TD^{NoGI}). Peripheral blood mononuclear cells (PBMC) were isolated from the blood, stimulated and assessed for cytokine production, while stool samples were analyzed for microbial composition.

Results—Following Toll-Like receptor (TLR)-4 stimulation, the ASD^{GI} group produced increased levels of mucosa-relevant cytokines including IL-5, IL-15 and IL-17 compared to ASD^{NoGI}. The production of the regulatory cytokine TGFβ1 was decreased in the ASD^{GI} group compared with both the ASD^{NoGI} and TD^{NoGI} groups. Analysis of the microbiome at the family level revealed differences in microbiome composition between ASD and TD children with GI symptoms; furthermore, a predictive metagenome functional content analysis revealed that pathways were differentially represented between ASD and TD subjects, independently of the presence of GI symptoms. The ASD^{GI} also showed an over-representation of the gene encoding zonulin, a molecule regulating gut permeability, compared to the other groups.

Conclusions—Overall our findings suggest that children with ASD who experience GI symptoms have an imbalance in their immune response, possibly influenced by or influencing metagenomic changes, and may have a propensity to impaired gut barrier function which may contribute to their symptoms and clinical outcome.

Keywords

ASD; Autism; Cytokines; Gastrointestinal; Immune; Mucosal Immunity; Microbiota; zonulin

1. Introduction

Autism spectrum disorders (ASD) are neurodevelopmental disorders defined by social impairments and the presence of repetitive or stereotyped behaviors. ASD currently affects 1 out of every 68 children in the U.S.(Wingate 2014). The etiology(ies) for the majority of ASD cases are unknown; however, it is generally accepted that both genetic and environmental factors contribute to ASD risk (Abrahams and Geschwind 2008, Geschwind 2008, Landrigan 2010, Hallmayer et al. 2011). Although ASD is primarily considered a condition that affects the brain, co-morbid features in other organs have been described including those within the gastrointestinal (GI) tract (Gillberg and and Billstedt 2000, Bauman 2010, Ballard et al. 2016, Hirata et al. 2016, Mannion and Leader 2016).

Historically, in his original case series, Kanner described vomiting and feeding issues in 6 out of 11 children with autism, a seventh case having diarrhea after small pox vaccination, while another was "obsessed with his feces" (Kanner 1943). Epidemiological studies investigating rates of GI symptoms in children with ASD have varied widely, with the frequency reported at anywhere from 20 to 70% (Horvath and Perman 2002, Molloy and Manning-Courtney 2003, Parracho et al. 2005, Niehus and Lord 2006, Valicenti-McDermott et al. 2006, Xue et al. 2008, Buie et al. 2010, Coury et al. 2012, Chaidez et al. 2014). The differences in reported rates are most likely due to study type (retrospective vs. prospective), how symptoms were reported (parental report vs. medical records), time-span for

experiencing the symptoms, age of participants in the study, lack of standardization regarding the description and definitions of GI symptomology, and differences between population-based case control studies and studies set in specialist pediatric gastroenterology clinics that may introduce certain referral biases (Mannion et al. 2013, Rose and Ashwood 2015). In a recent large population based case-control study conducted within a defined geographic region, data from nearly 1,000 children indicated that those with ASD are 6 to 8 times more likely to have frequent GI symptoms in the past 6 months including gaseousness/bloating, diarrhea, constipation and sensitivity to foods when compared to age-, sex-, region-matched typically developed children (Chaidez et al. 2014). Moreover, the prevalence of clinically diagnosed inflammatory bowel disease (IBD) was shown to be increased in ASD based on data from 3 large study populations (23).

Further reports have indicated that children with ASD and GI symptoms are more likely to experience sleep disturbance, sudden irritability, unexplained crying, aggressive behavior and score worse on behavioral assessments than children with ASD without GI symptoms (Horvath and Perman 2002, Chaidez et al. 2014). Analogous findings of behavioral and neurological problems are seen in other diseases characterized by GI dysfunction (Bushara 2005, Genuis and Bouchard 2010) and support a link between GI dysfunction and behavioral changes. Hans Asperger reported that children with celiac disease also often exhibit psychological problems (Asperger 1961). Anxiety, depression, epilepsy, severe progressive neuropathy, gait ataxia, and limb ataxia are also seen in adult patients with celiac disease (Hallert and Derefeldt 1982, Hadjivassiliou et al. 2002, Bushara 2005, Fasano and Catassi 2005, Genuis and Bouchard 2010). In individuals with diseases/syndromes such as irritable bowel syndrome (IBS), ulcerative colitis and Crohn's disease, psychiatric symptoms have also been reported (Ringel and Drossman 2001, Ringel and Drossman 2002).

Studies focused on assessing the underlying pathology of GI dysfunction in children with ASD have demonstrated low α-1-antitrypsin concentrations suggestive of intestinal protein loss (Walker-Smith and Andrews 1972), increased intestinal permeability as measured by a lactulose:mannitol test (D'Eufemia et al. 1996, de Magistris et al. 2010) and the presence of a diffuse inflammation of the intestinal tract found after endoscopy or colonoscopy as part of clinical work up for GI symptoms (reviewed in (Buie et al. 2010)). The precise nature of this inflammation has been controversial and much debated. However, controversy notwithstanding, evidence from histological, immunohistochemical and flow cytometry studies have suggested a subtle, pan-enteric infiltration of activated lymphocytes, monocytes, natural killer (NK) cells and eosinophils into the walls of the GI tract in children with ASD and GI symptoms, compared with typically developing children with similar GI symptoms (Furlano et al. 2001, Torrente et al. 2002, Ashwood et al. 2003, Ashwood et al. 2004, Torrente et al. 2004).

There are also indications that individuals with ASD and GI symptoms have altered intestinal microbiota, including reports of higher Clostridium counts in the feces compared to controls (Finegold et al. 2002, Finegold et al. 2010), differences in the Clostridium groups C. bolteae and C. cluster I and XI (Song et al. 2004), a higher incidence of C. histolyticum (Parracho et al. 2005), decreased unclassified Veillonellaceae, Prevotella, Coprococcus and unclassified Prevotellaceae species (Kang et al. 2013), altered Bacteroides/Firmicutes ratios

and increased desulfovibrio (Finegold et al. 2010, Tomova et al. 2015), and increased Sutterella and Ruminococcus torques (Wang et al. 2013) compared with controls. Interestingly, one study reported dysbiosis and decreased levels of transcripts for disaccharidases in the mucoepithelium layer in children with ASD and GI symptoms and postulated that decreases in host enzymes could contribute to the dysbiosis (Williams et al. 2011). Taken as a whole, these data suggest that dysbiosis may be a feature of children with ASD who have GI symptoms, but the exact nature and specificity of the microbiota changes or whether these changes are also found in children with ASD without GI symptoms are still far from clear.

Currently, it is not well understood whether children with ASD and GI symptoms are a unique phenotype within ASD or if this is a co-morbidity unrelated to neurodevelopment. Herein we sought to further understand whether the GI microbiota and peripheral immune response are altered in children with ASD and GI symptoms, and to establish whether there are biological signatures in terms of immune dysfunction and microbiota composition compared to children with ASD without GI symptoms. We evaluated differences in the microbiota, the gene encoding zonulin, a molecule regulating gut permeability and peripheral immune response in a well-characterized cohort of children with ASD, both with and without irregular bowel movements, as compared to age equivalent typically developing children.

Methods

2.1. Subjects

Participants for this study were recruited through UC Davis MIND Institute and had all been previously enrolled in the Childhood Autism Risk from Genetics and Environment (CHARGE) study (Hertz-Picciotto et al. 2006). Participants were recruited from 4 groups, 1) children with ASD and GI symptoms of irregular bowel movements (ASD^{GI}); 2) children with ASD and no GI symptoms (ASD^{NoGI}); 3) typically developing children with GI symptoms of irregular bowel movements (TD^{GI}), and; 4) typically developing children without GI symptoms (TD^{NoGI}) (Table 1). A total of 103 participants were included in this study ranging in age from 3 years old to 12 years old, with 88 participants providing both stool and blood samples, 12 participants had only blood analyzed and 3 participants had only stool samples analyzed. After exclusions (see below) only 87 of the 100 blood samples had adequate cell numbers to run the cellular assays. A diagnosis of autism spectrum disorder was confirmed at the UC Davis MIND Institute by trained staff using the Autism Diagnostic Interview-Revised (ADI-R), and the Autism Diagnostic Observation Schedule (ADOS) at the time of enrollment into the original CHARGE study. All subjects were diagnosed prior to 2013 and based on DSM IV. To exclude behavioral and developmental characteristics of ASD in the typically developing group, the Social Communication Questionnaire (SCQ) was used as a screen. Participants in the TD groups had to score within the typical range, i.e. below 15, on the SCQ and above 70 on the Mullen Scales of Early Learning (MSEL) and Vineland Adaptive Behavior Score (VABS). Participants were randomly recruited from the CHARGE database based on inclusion/exclusion criteria (see below) and typically developing children were frequency matched to ASD cases based on age, sex and birth

location. Previous data taken during enrollment in CHARGE were used to initially screen for inclusion criteria, families were contacted only if they had given consent to be contacted about further studies. A telephone interview, along with GI and health questionnaires were used to assess participant eligibility into the current study (outlined below). All participants were also assessed using the Aberrant Behavior Checklist (ABC) to assess impairments within the domains of irritability, lethargy, social withdrawal, stereotypic behavior, hyperactivity and inappropriate speech at the time of enrollment into the current study. Medications and/or behavioral therapies used at the time of enrollment or within the previous year were collected and recorded. Participants were excluded if they had a known diagnosis of other GI pathology (celiac disease or IBD), use of antibiotics or antifungal medications within the prior month, medications affecting GI transit (stool softeners), and/or recent evidence of a GI infection based on stool laboratory tests performed by the child's physician. In addition, participants were excluded if there was evidence of a seizure disorder, genetic disorders (i.e. Fragile X syndrome, Tuberous Sclerosis Complex), liver or pancreatic disease, cystic fibrosis, or chronic infection. Children receiving nutritional monitoring and prescribed dietary interventions under the guidance of trained nutritionists/clinicians, medications, or complementary alternative treatments such as supplements other than a standard daily multivitamin/mineral tablet were also excluded. However, for children who were receiving nutritional modifications that were not overseen by a trained nutritionist, the dietary changes were documented but the participants were not excluded from the study. Similarly, children whose parents reported suspected food sensitivities/intolerances that had not been diagnosed through clinical assessment were also not excluded, but were documented.

2.2. GI symptom evaluation

At the time of recruitment into the current study, the parents or legal guardians of the study participants were asked to complete the CHARGE GI history (GIH) survey and GI symptom survey, based upon Rome III Diagnostic Questionnaire for the Pediatric Functional GI Disorders (Walker LS 2006). The GIH has been reported elsewhere (Chaidez et al. 2014) and rates the frequency of the following symptoms; abdominal pain, gaseousness/bloating, diarrhea, constipation, pain on stooling, vomiting, sensitivity to foods, difficulty swallowing, blood in stool and blood in vomit on a Likert scale [*(0) = never, (1) = rarely, (2) = sometimes, (3) = frequently and (4) = always)*]. Parents/guardians were asked if the participants experienced any allergies to foods, if their diet was restricted, by whom (child, parent or doctor) and for what reason; if any foods caused or worsened symptoms, if the child had any strong food dislikes and what they were, and finally, if any clinical GI diagnosis had ever been given. Parents/guardians answered each of the questions for both current (within the past three months) and previous experiences. The GI symptom survey consisted of 7 sections, each section had 1 to 6 questions to determine if the participant met the criteria for constipation, diarrhea or irritable bowel syndrome (IBS). All questions were multiple choice with the options of answering yes, no or don't know. Sections 1 and 2 determined eligibility criteria for constipation. Sections 3 and 4 determined eligibility criteria for diarrhea. Sections 5 and 6 determined IBS like symptoms if present. Lastly, section 7 asked if stooling had been consistent for the last 6 months (see Supplemental Figure 1 for more detail).

Participants who met the criteria for constipation, diarrhea or IBS on the GI history and symptom surveys were placed in their corresponding GI group (ASD[GI] or TD[GI]), participants who did not meet criteria for constipation, diarrhea or IBS, and had consistent stooling patterns for the past 6 months were placed in their corresponding no GI group (ASD[NoGI] or TD[NoGI]). Participants who did not meet the criteria for constipation, diarrhea or IBS, but had inconsistent stooling patterns during the last 6 months were excluded from this study.

This study was approved by institutional review boards for the State of California and the University of California, Davis. Informed consent is obtained from a legal guardian for all study participants prior to data collection in accordance with the UC Davis IRB protocol.

1.3. Blood collection and cellular assays

Peripheral blood was collected from each subject in acid-citrate dextrose Vacutainers (BD Biosciences; San Jose, Ca). Blood was processed to isolate peripheral blood mononuclear cells (PBMC). Blood was centrifuged at 2100 rpm for 10 minutes, plasma was harvested and the remaining blood cells were layered on lymphocyte separation medium (Corning; Manassas, VA), and centrifuged at 1700 rpm for 30 minutes. PBMC were collected from the buffy layer and washed with Hanks balanced salt solution (Corning; Manassas, VA). PBMC were plated in a 96 well plate at a concentration of 300,000 cells/well in complete media (RPMI 1640 (Invitrogen; Carlsbad, CA) with 10% Fetal Bovine Serum (Corning; Manassas, VA), 100 IU/ml penicillin (Invitrogen; Carlsbad, CA) and 100 IU/ml streptomycin (Invitrogen; Carlsbad, CA)). PBMC were cultured for 24 hours with either 1 μg/mL LPS (Sigma-Aldrich; St. Louis, MO) (a Toll-like receptor (TLR)-4 agonist), 3 μg/mL peptidoglycan (PGN) (Sigma-Aldrich; St. Louis, MO) (a Nucleotide-binding oligomerization domain-containing protein (NOD) ligand), 10 μg/mL phytohemagglutinin (PHA) (Sigma-Aldrich; St. Louis, MO) (a T-cell activator), or in media alone.

1.4. Cytokine Analysis

Following cell culture, supernatants were collected and stored at −80 °C until analysis of the cytokines. To assess pro-inflammatory responses following stimulation, the cytokines IL-1α, IL-1β, IL-6, IL-12 (p40 & p70) and tumor necrosis factor (TNF)α were measured. To assess T cell responses IFNγ (T$_H$1), IL-4 and IL-13 (T$_H$2) were analyzed, and; to assess immune regulation IL-10 and transforming growth factor (TGF)β1 were measured. In addition, IL-5, IL-15 and IL-17 cytokine levels were assessed because they have been implicated in mucosal homeostasis, inflammation and disease. Cytokine analysis was performed using a multiplexing bead immunoassay (Millipore, Billerica, MA). Samples were run according to manufacturer's protocol. Briefly, 25 μL of sample were incubated with antibody coupled fluorescent beads, washed and incubated with biotinylated detection antibodies followed by streptavidin– phycoerythrin. The beads were then `analyzed using flow-based Luminex™ 100 suspension array system (Bio-Plex 200; Bio-Rad Laboratories, Inc.). Standard curves were generated by Bio-plex Manager software to determine unknown sample concentration, reference cytokines were provided by the manufacturer in the kit. TGFβ1 concentration was determined by a separate kit from the other cytokines due to additional processing steps recommended by the manufacturer. Samples underwent an acidification step prior to being

assessed for TGFβ1. Acid-activation is needed to cleave the latent form of TGFβ in order to detect active TGFβ1 by the antibodies used in the Millipore kit (Hale-Donze et al. 2001). 20 μL of sample was incubated with 10 μL of 1N HCl (Fisher Scientific; Pittsburg, PA) for 30 minutes followed by the addition of 10 μL of 1.2N NaOH (Fisher Scientific; Pittsburg, PA) with 0.5M HEPES (Sigma-Aldrich; St. Louis, MO) to return samples to a neutral pH. The minimum detectable amount for the cytokines were as follows: IFNγ 0.8 pg/mL, IL-10 1.1 pg/mL, IL-12p40 7.4 pg/mL, IL-12p70 0.6 pg/mL, IL-13 1.3 pg/mL, IL-15 1.2 pg/mL, IL-17 0.7 pg/mL, IL-1α 9.4 pg/mL, IL-1β 0.8 pg/mL, IL-4 4.5 pg/mL, IL-5 0.5 pg/mL, IL-6 0.9 pg/mL, TGFβ1 9.3 pg/mL and TNFα 0.7 pg/mL. Sample concentrations that were below the limit of detection were given a proxy value as half the limit of detection for statistical comparisons.

Due to the low frequency of GI symptoms in TD children (Chaidez et al. 2014), we were only successful in recruiting 6 TD[GI] children for the immune assays; in addition, many of the TD[GI] participants were significantly younger than the other groups, perhaps reflecting the transient GI symptoms in this population (Table 1). Preliminary comparisons between TD and TD[GI] for induced cytokine levels after stimulation revealed very little differences between the two groups (Supplemental Figure 2). Due to concerns of age and sample size we choose to focus cytokine comparisons of the ASD groups to the TD[NoGI] group and did not include the TD[GI] group in further analysis.

1.5. Haptoglobin (Zonulin) Genotyping

Haptoglobin (Hp) genotype was determined by immunoblot. Immuno blot analysis was performed as routinely described; briefly, plasma samples were electrophoresed through a 4–20% gradient SDS polyacrylamide gel and transferred onto polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). Membranes were blocked in blocking buffer (Tris-buffered saline, 0.1% Tween 20, 5% BSA). The blots were incubated with polyclonal anti-human haptoglobin (Sigma-Aldrich; St. Louis, MO) diluted in blocking buffer (1:5000). After washing, membranes were incubated with anti-rabbit Alexa Fluor 488 (Life Technologies; Carlsbad, CA) diluted in blocking buffer (1:5000). Western blot signal was visualized using an infrared scan LI-COR Odyssey (LI-COR, Lincon, Nebraska, USA).

2.6. Microbiome Analysis

Parents were given collection containers to collect stool samples at home including tubes containing RNA later. Once stool was collected, samples were immediately placed in the freezer and brought back frozen to the clinic within 24 hrs. Total DNA was extracted from 0.25 grams of stool samples that had been preserved and transported in RNA later with PowerSoil DNA isolation kit (Mobio).

Amplification of the V3-V4 regions of the 16S rRNA gene was performed according to Fadrosh et al.(Fadrosh et al. 2014) using a dual-barcode system with fusion primers 338F and 806R. Amplicons were subsequently sequenced on Illumina MiSeq using the 300 bp paired-end protocol at the Genomic Resource Center at the University of Maryland School of Medicine Institute for Genome Sciences. Raw reads were preprocessed to remove the first 3 and last 3 bases if their phred score was lower than 3, read end was trimmed if the average

phred quality score of 4 consecutive bases are below 15. Paired reads were retained if their length was at least 75% of their original length after trimming, which were then assembled using FLASH (Magoc and Salzberg 2011) with overlap by ~90bp on average. Assembled reads were de-multiplexed by binning sequences with the same barcode and quality trimmed in QIIME (version 1.8.0) (Caporaso et al. 2010) (for details, please refer to Fadrosh et al. (Fadrosh et al. under review)). Similar sequences with less than 3% dissimilarity were clustered together using USEARCH (v5.2.32) (Edgar 2010) and *de novo* chimera detection was conducted in UCHIME v5.1 (Edgar et al. 2011). The taxonomic ranks were assigned to each sequence using Ribosomal Database Project (RDP) Naïve Bayes Classifier v.2.2 (Wang et al. 2007), using 0.8 confidence values as the cutoff to a pre-built greengenes database of 16S rRNA sequences (Aug, 2013 vers.) (McDonald et al. 2012).

The heatmap, bar plot were generated using statistical package R (v3.2.1) and Phyloseq R packages (McMurdie and Holmes 2013). The predicted metagenome functional content from 16S rRNA marker gene was performed using PICRUSt (Langille et al. 2013). A Cohen-Friendly association plot (Cohen 1980, Friendly 1992) was produced in R to indicate deviations from independence of KEGG functional categories that are mostly differentially presented among different categories.

2.7. Statistical analysis

Analysis of the data using a Shapiro-Wilk test indicated that distribution of the cytokine data was not normally distributed. Neither log transformation nor square-root transformation normalized all cytokine distributions. Therefore, the Kruskal-Wallis rank sum test was used to compare cytokine levels between groups. Data are expressed as median values (interquartile ranges). A Mann-Whitney non-parametric U-test (with a Holm step down procedure to correct for multiple comparisons) was used in post-hoc analyses to compare cytokine levels between groups and adjusted P values < 0.05 were considered statistically significant. Outliers were removed using ROUT. The behavioral data (ABC subscales) were also analyzed using a Mann-Whitney non parametric U-test, outliers were removed using ROUT. The microbiota data were subjected to two-way ANOVA. These tests are made at the 0.05 significance level using ranked lase discovery rate (FDR) correction for multiple comparisons. The haptoglobin genotype data was analyzed using a two tailed chi-square test.

3. Results

3.1. Immune stimulation assays

3.1.1 Basal stimulation (media alone)—In cell culture supernatants from PBMC cultured with media alone, reduced TGFβ1 production was observed in the ASD[GI] group compared with both ASD[NoGI] and TD[NoGI] children ($p < 0.05$) (Figure 1). We also noted a trend for increased IL-6 in ASD[GI] compared to TD[NoGI] but did not reach statistical significance ($p = 0.0932$) (Table 2).

3.1.2. TLR 4 Stimulation—Following stimulation with TLR-4 ligand, (Table 3), supernatants from PBMC cultures from the ASD[NoGI] group produced approximately 1.5 fold more IL-1α ($p = 0.0003$) and TNFα ($p = 0.0254$), as well as significantly increased

IL-1β (p = 0.0027) compared to TD[NoGI] (Figure 2a-c). Production of IL-6 also trended towards an elevation in the ASD[NoGI] group compared to the TD[NoGI] group but did not reach significance. Decreased levels of mucosa-associated cytokines were observed in ASD[NoGI] compared with TD[NoGI], including IL-15, IL-17 ($p < 0.02$; Figure 2d-e) and IL-12p70 ($p < 0.02$; Table 3).

When comparing the ASD[GI] group with the ASD[NoGI] group we found significant increases in the production of a number of cytokines, including IL-5 (p = 0.0006; Figure 2f), IL-17(p = 0.0057; Figure 2e) and a non-significant trend for IFNγ (p = 0.0531) and IL-15 (p = 0.0829; Figure 2d). Levels of IL-1α were decreased in ASD[GI] compared to ASD[NoGI] (p = 0.0150; Figure 2a). Compared to TD[NoGI] cytokine production in ASD[GI] was increased for IL-5 (p = 0.0021; Figure 2f) and there was a non-significant trend towards increased production of IFNγ (p = 0.0820) and TNFα (p =0.0546); however, IL-17 was decreased (p = 0.0186; Figures 2e).

Of note, TGFβ1 was decreased in ASD[GI] compared with both ASD[NoGI] and TD[NoGI] ($p <$ 0.007; Figure 1).

3.1.3. NOD 1&2 Stimulation—In general, following NOD stimulation, cytokine production in supernatants from PBMC cultures of the ASD[NoGI] group was lower when compared with either ASD[GI] or TD[NoGI] (Table 4). ASD[NoGI] produced less than a third of the amount of IL-1α and IL-1β ($p < 0.001$), was decreased for IL-6, IL-12p40, TNFα, IFNγ ($p < 0.03$), and there was a non-significant trend for decreased IL-5, IL-10 and IL-13 ($p <$ 0.08) when compared with TD[NoGI] (Figure 3a-f). In ASD[GI] there was a 3-fold increase in production of IL-1β, more than double the amount of IFNγ ($p < 0.02$), and a non-significant trend for increased TNFα (p = 0.0647) compared to the ASD[NoGI] group (Figure 3b,e &f). Regulatory cytokines IL-10 and TGFβ were decreased in the ASD[GI] group compared to TD[NoGI]; however, differences were not significant after statistical correction for multiple comparisons.

3.1.4. PHA stimulation—Overall, there were few significant differences between groups following PHA stimulation (Table 5). We found increased IL-15 in the ASD[NoGI] group compared to the TD[NoGI] group (p = 0.0033). Increased production of the T_H1 cytokine IFNγ and decreased regulatory cytokine production of TGFβ1 was observed in the ASD[GI] group compared with the TD[NoGI] group ($p < 0.01$). We also found similar findings of, decreased TGFβ1 and increased IFNγ in ASD[GI] compared to ASD[NoGI] ($p < 0.04$).

3.2. Behavioral Scores

After comparing results of the ABC assessment between the two ASD groups, we found that ASD[GI] had more atypical behavioral scores compared to ASD[NoGI] (Figure 4). Children in the ASD[GI] group had increased ABC subscale scores for irritability and agitation, social withdrawal and lethargy and hyperactivity and noncompliance ($p < 0.02$; Figure 4a-c) compared with ASD[NoGI].

3.3. Haptoglobin analysis

Analysis of Hp genotypes showed a trend of over-representation of the Hp2 gene in ASD subjects over TD groups independent of GI status that did not reach statistical significance (p = 0.077; Supplemental Table 1). However, there was a significant different distribution between ASD^{GI} compared to TD^{GI} (p < 0.01) with an over representation of the Hp2-2 genotype and under-representation of the Hp1-1 genotype in children with ASD^{GI}. No difference was observed between ASD^{NoGI} and TD^{NoGI} or ASD^{GI} and ASD^{NoGI}.

3.4. Microbiome analysis

Gap statistic analysis revealed the presence of three clusters in our samples (Supplemental Figure 3) that correlate with three previously described microbial enterotypes (Arumugam et al. 2011). The first cluster was Bacteroides enriched, while the second cluster lacked any defined dominant bacterial genera. Finally, the third cluster appeared to be dominated by Prevotella. Interestingly, we observed two subgroups within the second cluster that were represented exclusively by children with an ASD diagnosis, irrespective of GI symptoms (i.e. combined group of ASD^{GI} plus ASD^{NoGI}) (Figure 5a). In the third cluster a predominance of subjects without GI symptoms was observed, irrespective of their neurodevelopmental status (i.e. ASD^{NoGI} plus TD^{NoGI}) (Figure 5b). When subjects were segregated based on the presence or absence of GI symptoms, relative abundance analysis showed differences in the microbiome composition at the family level between ASD^{GI} and TD^{GI} children (Figure 6). Specifically, Bacteriodaceae, Lachnospiraceae, Prevotellaceae and Ruminococcaceae appeared more abundant in the ASD^{GI} group compared to the TD^{GI}. Interestingly, the same differences were not detected between ASD^{NoGI} and TD^{NoGI} groups. The Cohen-Friendly association plot was used to predict metagenome functional content in our data set (Supplemental Figure 4). These analyses showed strong differences in some pathway representations between ASD^{GI} and TD^{GI} subjects, and between ASD^{NoGI} and TD^{NoGI}. Specifically, the transporters pathways appeared under-represented while the amino sugars and nucleotide sugars pathways were over-represented in ASD^{GI} compared to ASD^{NoGI}. The starch and sucrose metabolism pathways were also under-represented in ASD^{NoGI}, but slightly up-regulated in ASD^{GI}. Finally, the pathways correlated to the two components system were strongly up-regulated in TD^{GI} and under-represented in ASD^{GI}.

4. Discussion

We report herein that children with ASD^{GI} produced elevated levels of mucosa relevant cytokines, including IL-5, IL15 and IL-17, after exposure to the TLR-4 agonist LPS when compared to children with ASD^{NoGI}. Moreover, production of TGFβ1 was consistently decreased in children with ASD^{GI} under the majority of conditions examined when compared to both ASD^{NoGI} and TD^{NoGI}. In addition to our immune findings, we found differences in microbiome composition in ASD^{GI} compared to TD^{GI} and differences in several transporters and metabolomic pathways in ASD^{GI} patients compared to ASD^{NoGI}. The ASD^{GI} group also scored higher for irritability and agitation, social withdrawal and lethargy, and hyperactivity and noncompliance on the ABC subscales compared to children in the ASD^{NoGI} group. These preliminary data suggest a possible relationship between the microbiome, immune system and behavioral outcome in ASD.

There is a two-way connection between the microbiota and mucosal immune function. In addition to their symbiotic role during development, this complex relationship continues to ensure homeostasis throughout life. For instance, the immune response can shape the composition of the gut microbiota through several different mechanisms including, production of antimicrobial molecules such as REGIIIα, α-defensins and β-defensins, IgA, and T cells that are specific for commensal bacteria (Hooper et al. 2012, Ostaff et al. 2013). In addition, an inflammatory response and the production of reactive oxygen species along with other immune molecules can lead to preferential growth of certain bacterial species, thus contributing to the composition of the microflora. For example, host-derived nitrate has been found to favor E. Coli growth in mice (Spees et al. 2013, Winter et al. 2013), whereas mice with a mutation in the Nos2 gene and are deficient in inducible nitric oxide synthase, do not support E. coli growth (Winter et al. 2013). Furthermore, alterations in NOD2, NLRP6 and TLR immune pathways can lead to dysbiosis and have been linked to several GI conditions (Netea et al. 2004, Eckmann and Karin 2005, Vijay-Kumar et al. 2010, Elinav et al. 2011, Jiang et al. 2013).

Conversely, the microbiota is crucial to ensure a fully developed immune system (Lee and Mazmanian 2010). Germ free (GF) mice show many immune abnormalities such as altered development of gut associated lymphoid tissue (GALT), with smaller and fewer Peyer's patches, smaller mesenteric lymph nodes, the absence of isolated lymphoid follicles, as well as deficiencies in secretory IgA, reduced intraepithelial CD8+ T cells and reduced lamina propria immune cells, including CD4+ T cells (Lee and Mazmanian 2010, Hooper et al. 2012). Reduced CD4+ T cell and smaller germinal centers are seen in the spleens of GF mice demonstrating that the microflora is responsible for influencing the systemic/peripheral immune system in addition to GALT (Lee and Mazmanian 2010). GF mice also exhibit behavioral abnormalities including impairments in social behaviors, specifically in social avoidance and decreased preference for social novelty (Desbonnet et al. 2014). Interestingly, deficits in social avoidance were reversed after bacterial colonization and establishment of the microbiota in GF mice.

The increased production of IL-5, IL-15 and IL-17 in ASD[GI] compared to ASD[NoGI] has a number of implications for mucosal immune function. IL-5 is an important cytokine for antibody class switching and promotes class switching to IgA, the major antibody class in mucosal tissues (Harriman et al. 1988). IgA has been demonstrated to play a role in intestinal protection from pathogenic bacteria, viruses and toxins as well as helping to maintain intestinal homeostasis (Mantis et al. 2011). IL-15 is produced by innate immune cells such as macrophages, dendritic cells (DC) and epithelial cells; and promotes T cell proliferation, survival and cytokine production. It is also involved in the upregulation of NK cell cytokine production, cytotoxicity and survival, as well as B cell survival and isotype switching (Stevceva et al. 2006). In the gut, IL-15 can induce proliferation of intestinal epithelial cells and can influence the expression of the mucosal adhesion integrin, αEβ7, on intraepithelial T cells (Stevceva et al. 2006). IL-15 has also been found to be overexpressed in the gut mucosa of patients with celiac disease, and is thought to contribute to epithelial damage (Mention et al. 2003). Furthermore, a recent study demonstrated that IL-15 blocks induced regulatory T cell (Tregs) generation in the gut by inducing IL-12p70 and IL-23 production in DC (DePaolo et al. 2011). Normally, DC located in the mesenteric lymph

nodes induce the generation of Tregs after being primed by TGFβ1 and retinoic acid (RA) (Coombes and Powrie 2008). However, in the presence of IL-15, RA increases pro-inflammatory cytokine production leading to T_H1 skewing, or with the additional presence of IL-6 leads to T_H17 differentiation (DePaolo et al. 2011). Lastly, increased IL-17 was observed in ASD^{GI} compared to ASD^{NoGI} and is an important inflammatory mucosal cytokine and has been implicated in GI disorders such as Crohn's disease, celiac disease and IBD (Iwakura and Ishigame 2006, O'Connor et al. 2010, Lahdenpera et al. 2012, Huppler et al. 2014). IL-17 is important for priming T cells and recruitment of neutrophils, and also acts on endothelial cells, epithelial cells, neutrophils and macrophages to produce inflammatory cytokines such as IL-1, IL-6 and TNFα (Iwakura and Ishigame 2006, O'Connor et al. 2010). Recent research in a mouse model evaluating the role immune responses have on fetal development, discovered direct injection of IL-17 into the developing brain resulted in early brain overgrowth and ASD-relevant behaviors (Choi et al. 2016). The effects of increased IL-17 in ASD^{GI}, therefore, may have behavioral as well as mucosal consequences.

A major difference between the ASD groups (ASD^{GI} and ASD^{NoGI}) was the decreased production of TGFβ1 that occurred in many of the stimulatory conditions tested, and strongly suggests an imbalance in immune regulation. We, and others, have previously reported finding decreased TGFβ1 levels in plasma of adults and children with ASD (Okada et al. 2007, Ashwood et al. 2008) and the relationship between low plasma levels of TGFβ1 and worse behavior scores (Ashwood et al. 2008). Furthermore, recent studies investigating microRNAs (miRNAs) in individuals with ASD have found differentially expressed miRNAs in individuals with ASD in various tissue samples, which implied alterations in the control of TGFβ1 pathways (Mundalil Vasu et al. 2014, Ander et al. 2015, Huang et al. 2015). TGFβ signaling plays an important role in neurodevelopment as well as immune homeostasis. The TGFβ family participates in regulating neural outgrowth, synapse formation, initiate axon formation, neural migration and may also play a part in synaptogenesis and hippocampal synaptic plasticity (Krieglstein et al. 2011). Reduced TGFβ1 could potentially impact many of these processes, as well as disrupt control of inflammatory immune responses that can also elicit changes in neuronal function.

On a cellular level, the increased IL-17 and decreased TGFβ1 may represent a shift in T cell subsets, moving away from Tregs and towards a more inflammatory T_H17 cell. One recent study reported finding decreased expression of the transcription factor necessary for Tregs, while finding increases in transcription factors important for T_H1, T_H2 and T_H17 lineages (Ahmad et al. 2016). Maintaining homeostasis in the gut is a crucial balancing act providing tolerance to important beneficial microbes and resisting potential pathogenic microbes, and one that rest on the ability of the immune system to make this distinction and respond appropriately. Traditionally, the transcription factor RORγt is expressed and involved in the differentiation of T_H17 cells while Foxp3 is involved in regulatory T cell differentiation (Korn et al. 2009, Harrison and Powrie 2013). However, recent studies have also found populations of T cells in the gut that express both Rorγt and Foxp3, produce the regulatory cytokine IL-10 instead of IL-17 and are functionally equivalent to Tregs (Lochner et al. 2008). The microbiota play an important role in the induction of Rorγ+ Tregs demonstrated by the decreased proportion of colonic Rorγ+ T cells seen in GF mice, and subsequent increases after colonization by various bacterial species (Sefik et al. 2015), again, reiterating

the importance of the immune-microbiota relationship. Studies investigating the stability of Foxp3 have also shown that a subset of cells that once expressed Foxp3 and express signature Tregs surface molecules such as folate receptor 4, CD103 and cytotoxic T lymphocyte-associated antigen could lose Foxp3 expression and take on an activated-memory T cell phenotype under inflammatory conditions, but may also retain the ability to upregulate Foxp3 and regain a Tregs phenotype (Komatsu et al. 2009, Zhou et al. 2009). Better understanding of the plasticity of Tregs in ASD and what factors contribute to shifts in these cell populations in ASD should be investigated further.

While this study was mainly focused on characterizing differences in the ASD[GI] subgroup, we also found children in the ASD[NoGI] group exhibit increased levels of IL-1α, IL-1β and TNFα after TLR-4 stimulation with LPS. Other studies that have looked at induced responses of peripheral immune cells also reported findings of increased innate cytokine production such as TNFα, IL-1β, and IL-6 (Jyonouchi et al. 2001, Jyonouchi et al. 2002, Enstrom et al. 2010, Jyonouchi et al. 2014). In a previous study utilizing a younger cohort of children, 2 to 5 years of age, we found elevated cytokine production, notably IL-1β, after TLR-4 stimulation of isolated blood monocytes from children with ASD (Enstrom et al. 2010). This current study also points to increased innate responses in children with ASD[NoGI] and may also lend support to the idea that increased immune responses persists over time.

Loss of function mutations in NOD genes have been associated with increased susceptibility for Crohn's disease, a GI disorder characterized by severe GI inflammation (Eckmann and Karin 2005). We used PGN to stimulate an innate immune response via signaling through intracellular NOD receptors, NOD1 and NOD2 similar to work by others (McDonald et al. 2005, Sorbara and Philpott 2011). Compared with TD[NoGI] children, decreased cytokine responses were noted in the ASD[NoGI] group. NOD2 activation in specialized epithelial cells, including paneth cells, results in increased production of antimicrobial molecules such as defensins, that can shape the microbiota (Eckmann and Karin 2005). Such activation, also leads to cytokine production, including the anti-inflammatory cytokine IL-10, a decrease in which may lead to inflammation (Netea et al. 2004). Interestingly, a study looking at PBMC in ASD children found that those who have marked behavioral fluctuations and GI symptoms have downregulated immune responses to certain immune stimuli making them more vulnerable to infection (Jyonouchi et al. 2011). Our study highlights context dependent stimulation in ASD that may relate to specific signaling pathways as mentioned above. Further research on the reasons for such differential responses to a range of immune stimuli that are observed in different ASD subsets is warranted.

From the analysis of the microbiome, we were able to identify three different clusters in our set of data; one cluster showing a predominance of samples with GI symptoms and another cluster containing two subgroups comprised exclusively by ASD subjects. Similarly to what other studies have found (Wang et al. 2014, Rosenfeld 2015, Son et al. 2015), our data showed differences in the microbiome composition of children with ASD when compared to TD groups, irrespective of GI symptoms. In the ASD[GI] group compared to the TD[GI], relative abundance analyses were different for several bacterial families (namely increased Bacteriodaceae, Lachnospiraceae, Ruminococcaceae and Prevocellaceae). However, these

data should be approached cautiously due to the limited sample size and younger age of the TD[GI] group. Interestingly, we did not detect the same differences between ASD[NoGI] and TD[NoGI] individuals. In addition, Cohen Friendly analysis showed that some microbiome related biological pathways were differentially represented between groups. Differences in pathway analysis occurred both in trait conditions (i.e. ASD or TD) and in state conditions (i.e. with or without GI symptoms), suggesting that the alterations in the pathways processes are also related to the presence of ASD and not simply to dysbiosis in the GI tract. These disparate pathways may also interact with the reported alterations of several other signaling pathways associated with ASD such as mTOR (Takei and Nawa 2014), Erk1/2 (Pinto et al. 2014), and growth factors important to neurodevelopment (Sadakata and Furuichi 2009).

Based on the lactulose:mannitol test, increased GI permeability has been demonstrated in ASD and may be another mechanism whereby microbiota and the immune system interact in some children. Zonulin, a molecule that regulates intestinal permeability, is the precursor of haptoglobin 2 (pre-Hp2) (Tripathi et al. 2009). Two co-dominant allele variants, termed *HP1* and *HP2*, the latter unique to the human species, are variously distributed in the general population, resulting in three genotypes, HP1-1 (~20%), HP2-1 (~50%), and HP2-2 (~30%) (Melamed-Frank et al. 2001). The structural arrangements of the ensuing α1/α2 variants with a non-polymorphic β chain vary considerably across the three phenotypes, carrying important functional and biologic implications (Papp et al. 2008, Polticelli et al. 2008). Several studies have suggested that the presence of the *HP2* allele correlates with higher risk to develop immune-mediated diseases (Blum et al. 2008, Papp et al. 2008) and *HP2* homozygosis is associated with poor prognosis (Delanghe et al. 1998, Kasvosve et al. 2000) and decreased longevity (Napolioni et al. 2011). We observed a non-significant trend for over-representation of the *HP2* gene product in all ASD patients and a significant difference between ASD[GI] and TD[GI]. With the identification of zonulin as pre-Hp2 it can be postulated that the presence of the *HP2* gene (either in heterozygosis or homozygosis) may lead to predisposition of increase intestinal permeability. Loss of gut barrier function could facilitate the pathogenic bacteria and/or their components (including endotoxins), as well as food-derived peptides into the intestinal mucosal and eventually blood stream leading to subsequent increased interactions with immune cells. Increased blood levels of LPS, a byproduct of the cell walls of gram negative bacteria, has been found in some ASD children; a finding that was associated with increased IL-6 production (Emanuele et al. 2010). Additionally, the *HP* gene is under IL-6 regulation (Oliviero and Cortese 1989) and therefore the increased IL-6 in ASD[GI] children may lead to the overexpression of the zonulin (*HP2*) gene in patients who have at least one copy. A local immune response in the gut can contribute to the degradation of intestinal barrier which may lead to increased transit of intestinal bacterial products in ASD. Cytokines such as IL-6 are known to modify neuronal function and have been associated with increased behavioral impairment in ASD (Onore et al. 2012). Thus, intestinal bacteria may communicate indirectly with the CNS by way of the mucosal and peripheral immune system that could stimulate vagal sensory nerve fibers in the intestine (Goehler et al. 2000).

Commensal and pathogenic microbes can impact the gut through the release of Short-chain fatty acids (SCFA) (MacFabe, 2015). SCFA influence the gut and other organs by affecting metabolism and mitochondria function, acting as signaling molecules, reducing GI motility

and modulating immune cells (Vinolo et al. 2011, den Besten et al. 2013, MacFabe 2015). The most common SCFA produced in the gut are propionic acid (PPA), butyric acid and acetic acid (MacFabe 2015). GI symptoms and metabolic disorders often co-occur within ASD (Frye et al. 2013, Frye et al. 2015, Rose et al. 2017). While SCFA have been shown to have beneficial influences/impact on health, concentration and host factors play a role. For example, one study found PPA exposure on lymphoblastic cell lines increased ATP-linked respiration, reserve capacity and maximal respiration; factors which are important to production of energy and a cells ability to adapt to metabolic stressors. However, increased concentrations of PPA also increased proton leaking and increased reactive oxygen species (ROS) which negatively impact mitochondria. Therefore, at certain concentrations PPA can be beneficial to the cell, but at high concentrations there may be a negative effect (Frye et al. 2016). Host factors also contribute, as children with ASD showed a more robust response to PPA with increases in ATP-linked respiration, reserve capacity and maximal respiration, however, under oxidative conditions the beneficial effects from PPA were lost, as proton leak respiration increased leading to a reduction in reserve capacity (Frye et al. 2016). A follow up study from the same group found that PPA exposure to lymphoblastic cells from children with ASD who demonstrated atypical mitochondrial responses, resulted in upregulation of genes associated with immune activation, including genes responsible for immunoglobulin production and regulating T cells (Frye et al. 2017). The interplay between GI, immune and metabolic function warrants further analyses in large cohort studies. While we are excited about our study findings we feel it's important to note a few limitations. Our study is limited by small sample sizes that impacted how we could stratify our study population. As in other studies it was difficult to recruit typically developing children who experience GI issues due to their low frequency in the general population. Due to limited numbers we were unable to further break down groups to compare differences between specific symptoms clusters (e.g. constipation vs. diarrhea vs. IBS), or microbial composition, etc. This study also primarily consists of males and while the ratios are consistent with the gender ratio of ASD, we did not have sufficient statistical power to analyze sex differences due to the low number of females per group. Finally, our study included a wide age group, ranging from 3 years of age to 12 years of age, with larger sample sizes it may have been possible to compare data across age. Despite these limitations we feel that this study provides valuable insights for children with ASD and GI symptoms.

5. Conclusions

We found several differences when comparing children with ASD who exhibit GI symptoms vs. those that did not. The most notable of these was the reduced regulatory TGFβ1 response of the ASD[GI] groups following stimulation. We also noted an increase in the production of cytokines linked to mucosal inflammation after TLR-4 stimulation in children with ASD[GI] symptoms relative to children with ASD[NoGI]. Our analysis of the microbiome underscore the relationship between the presence of GI symptoms and the host microflora, and suggest a possible role of dysbiosis in the co-morbidity of GI issues in ASD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- Children with ASD and GI symptoms have lower levels of regulatory immune cytokines compared to ASD without GI or healthy typically developing children

- Increased mucosa-related inflammatory cytokines were also found in children with ASD and GI co-morbidities compared to ASD without GI.

- ASD^{GI} had over-representation of the zonulin encoding *HP2* gene and a trend toward over-expression of IL-6, making them at higher risk to increased gut permeability

- The microbiota composition of ASD^{GI} differed from the other three groups and was mechanistically linked to altered pathways possibly involved in ASD pathogenesis and chronic gut inflammation.
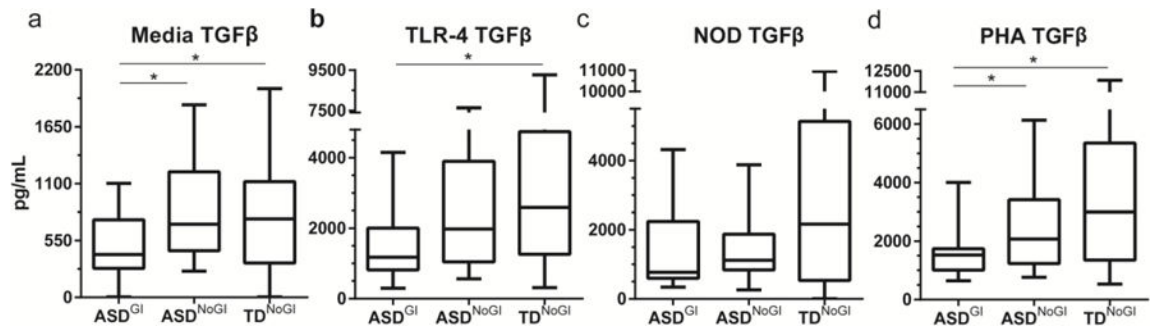
Figure 1.

TGFβ1 production after 24 hours of stimulation with media alone; TLR-4 agonist, LPS; NOD agonist, PGN; or T cell mitogen, PHA. Comparing medians and interquartile range between ASD^{NOGI}, ASD^{GI} and TD^{NOGI}. Concentration of TGFβ1 shown in pg/mL. * denotes p-value < 0.05.
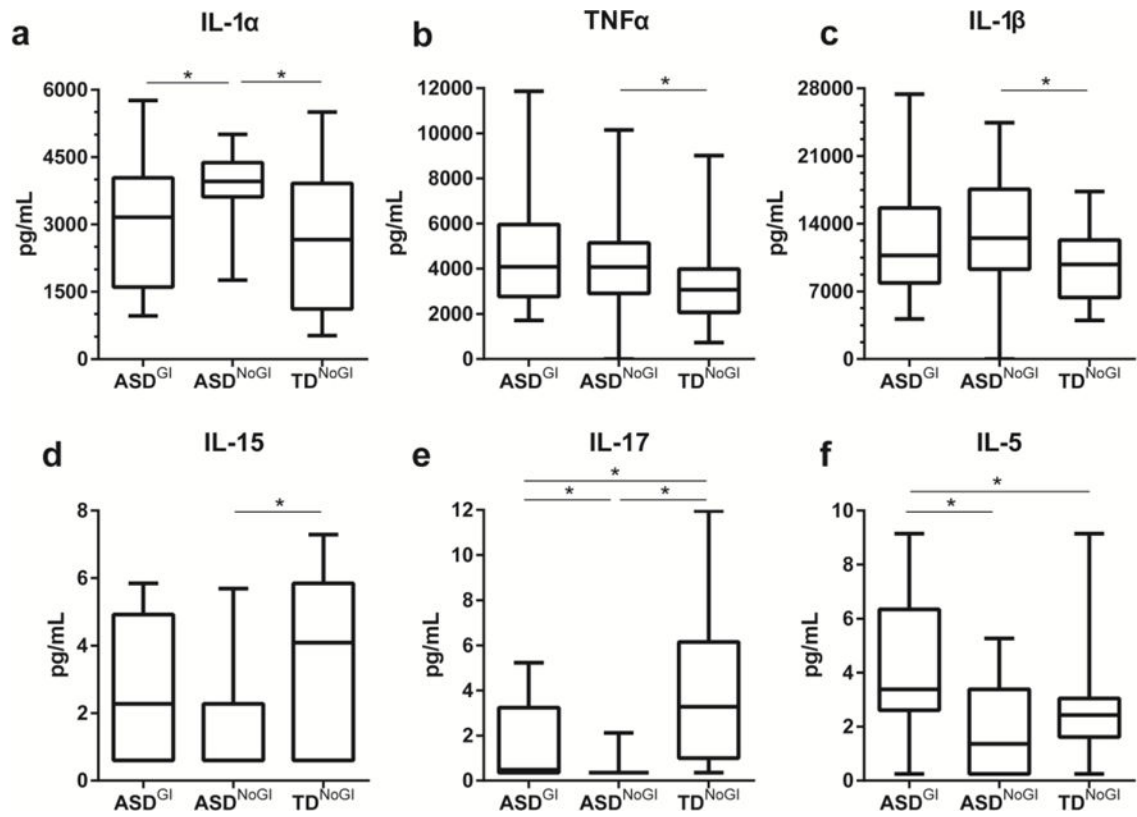
Figure 2.
Induced cytokine responses after stimulation with TLR-4 agonist LPS. Showing responses from IL-1α (a), TNFα (b), IL-1β (c), IL-15 (d), IL-17 (e) and IL-5 (f). Concentrations shown in pg/mL. Data depicted as box and whisker graphs. *denotes p-value < 0.05.
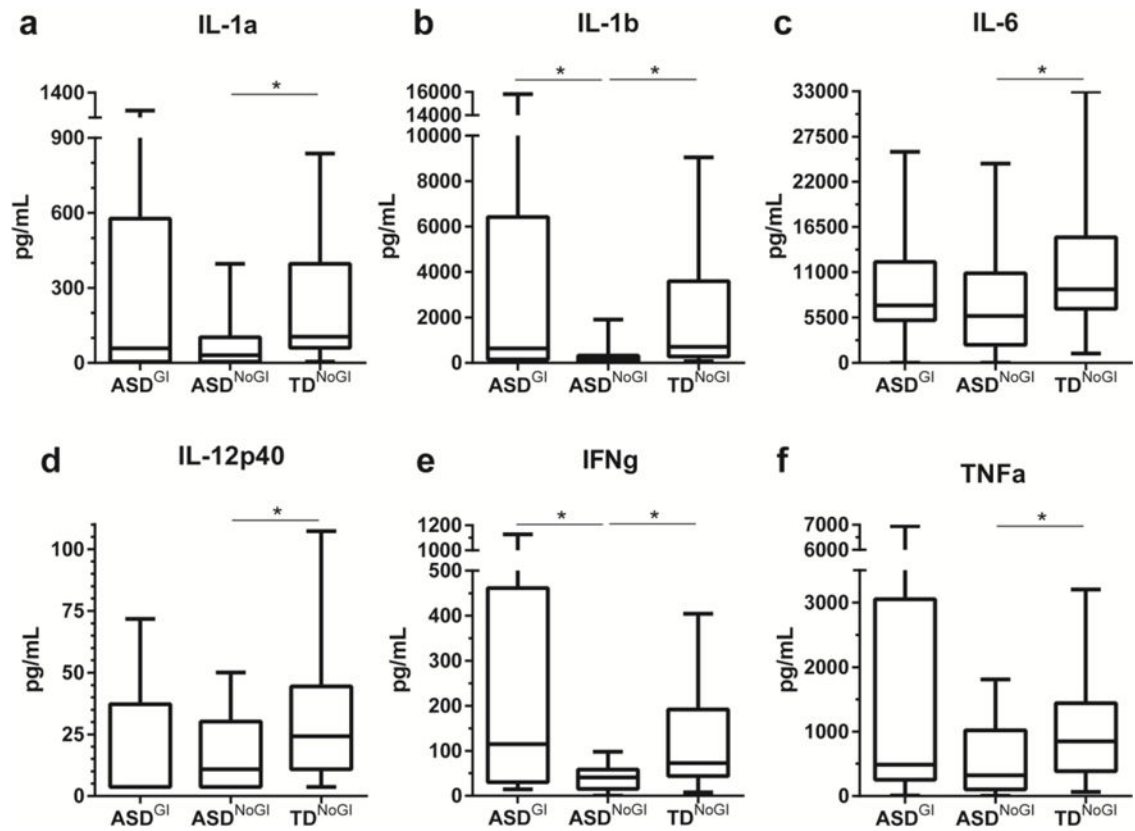
Figure 3.
Induced cytokine responses after stimulation with NOD1 & 2 agonist PGN. Showing responses from IL-1α (a), IL-1β (b), IL-6 (c), IL-12p40 (d), IFNγ (e) and TNFα (f). Concentrations shown in pg/mL. Data depicted as box and whisker graphs. *denotes p-value < 0.05.
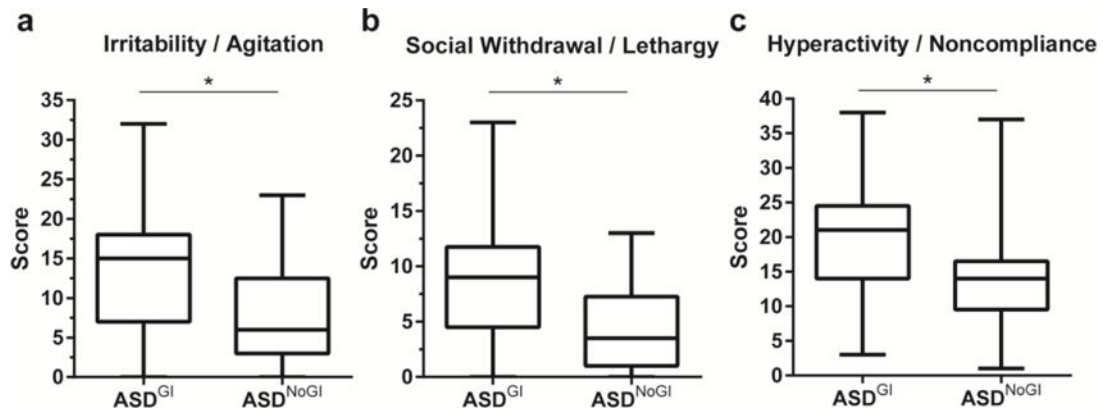
Figure 4.
ABC subscale comparisons between ASD^{NOGI} and ASD^{GI}. Showing irritability/agitation scores (a); social withdrawal/lethargy scores (b); and hyperactivity/noncompliance sores (c). Data depicted as box and whisker graphs. *denotes p-value < 0.05.
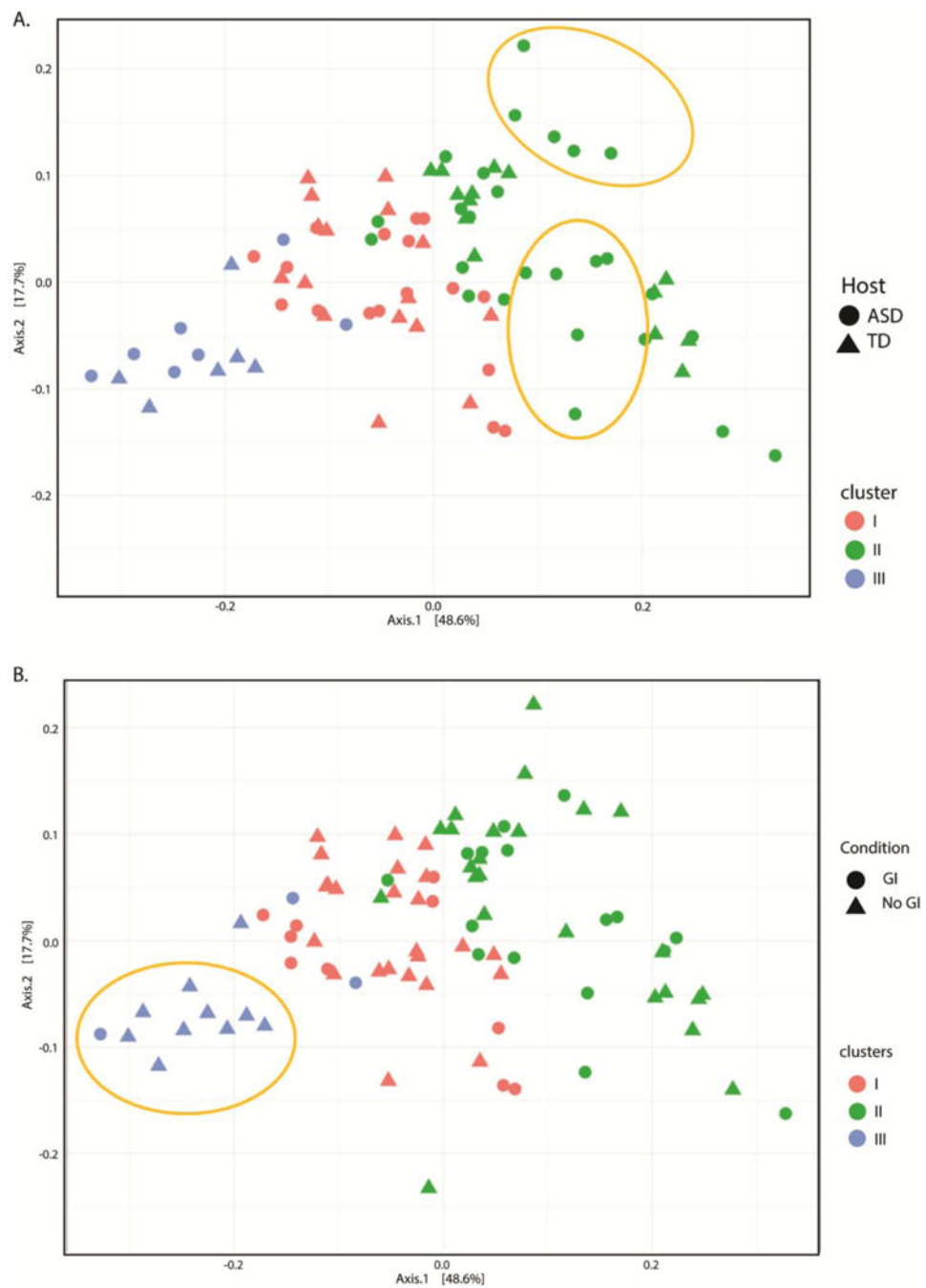
Figure 5.
Principal Coordinates analysis of Unifrac distances between samples. (a) Samples colored by cluster's association and shaped by neurodevelopmental condition. (b) Samples colored by cluster's association and shaped by presence of GI symptoms. In gold potential subgroups of autistic patients included in cluster two and predominance of subjects without GI symptoms in cluster three,

Figure 6.
Bar plot showing the relative abundance at family level in the gastrointestinal microbiota of samples collected from different categories of subjects

Table 1

Demographics of the study participants.

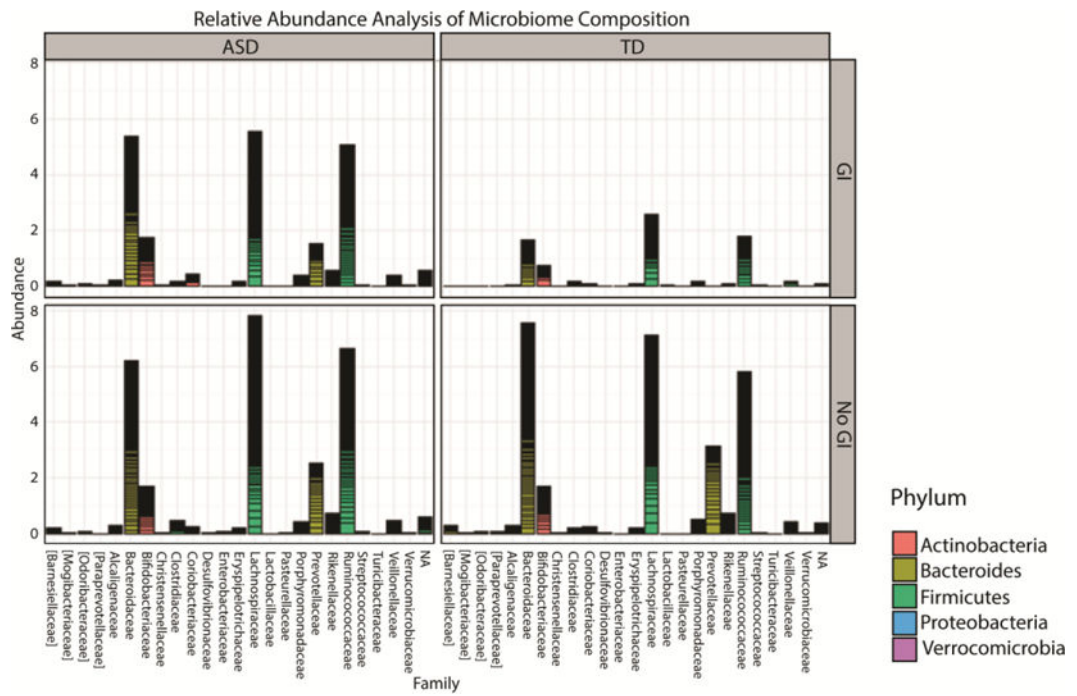|	Demographics							
	Blood				**Stool**			
	ASD[NOGI]	**ASD**[GI]	**TD**[NOGI]	**TD**[GI]	**ASD**[NOGI]	**ASD**[GI]	**TD**[NOGI]	**TD**[GI]
# of subjects	26	20	35	6	29	21	34	7
Age (median)	7.83	5.71	6.75	$5.17^{*\#}$	7.75	6.58	7.09	$5.08^{*\#}$
IQR	(5.46 - 11.23)	(4.77 - 9.06)	(5.50 - 8.33)	(4.10 - 6.00)	(5.25 - 10.54)	(4.79 - 8.86)	(5.56 - 8.50)	(4.33 - 5.83)
# males	19	15	24	5	25	17	32	6
Constipation	NA	8	NA	3	NA	7	NA	4
Diarrhea	NA	4	NA	1	NA	5	NA	1
Both (D+C)	NA	0	NA	0	NA	1	NA	0
IBS like	NA	8	NA	2	NA	8	NA	2

*
significantly different from ASD (p < 0.05)

#
significantly different from TD (p < 0.05)

Table 2

Comparison of PBMC cytokine production in media after 24 hours in vitro between ASD without GI symptoms (ASD^{NOGI}), ASD with GI symptoms (ASD^{GI}) and typically developing children without GI (TD^{NOGI}). Data presented as median and interquartile ranges.

	Media		
	ASD^{NOGI} Median (IQR)	ASD^{GI} Median (IQR)	TD^{NOGI} Median (IQR)
IL-1α	4.70 (4.70-4.70)	4.70 (4.70-4.70)	4.70 (4.70-4.70)
IL-1β	0.40 (0.40-2.0)	0.40 (0.40-4.33)	0.40 (0.40-1.21)
IL-6	3.73 (1.52-15.73)	9.65 (4.05-17.18)	5.25 (2.62-9.44)
IL-12p40	3.70 (3.70-3.70)	3.70 (3.70-3.70)	3.70 (3.70-3.70)
IL-12p70	0.30 (0.30-0.39)	0.30 (0.30-0.73)	0.30 (0.30-0.63)
TNFα	4.47 (2.91-10.04)	8.03 (3.66-17.58)	6.53 (3.74-9.65)
IFNγ	1.93 (0.40-5.96)	1.36 (0.62-2.19)	1.26 (0.4-2.72)
IL-4	2.25 (2.25-2.25)	2.25 (2.25-2.25)	2.25 (2.25-2.25)
IL-13	0.65 (0.65-0.92)	0.65[#] (0.65-0.65)	0.65 (0.65-1.57)
IL-10	4.84 (3.89-7.34)	5.93 (4.08-6.97)	6.32 (4.11-9.81)
TGFβ	2,070[*] (1,235-3,409)	1,523[#] (1,009-1,738)	2996 (1,347-5,351)
IL-5	0.25 (0.25-0.25)	0.25 (0.25-0.25)	0.25 (0.25-0.25)
IL-15	0.60 (0.60-0.60)	0.60 (0.60-0.60)	0.60 (0.60-0.60)
IL-17	0.35 (0.35-0.35)	0.35 (0.35-0.35)	0.35 (0.35-0.35)

[*] p value < 0.05 when comparing ASD GI with ASD no GI

[#] p value < 0.05 when comparing ASD GI with TD

[†] p value < 0.05 when comparing ASD no GI with TD

Table 3

Comparison of PBMC cytokine production after 24 hours of stimulation with TLR-4 agonist between ASD[NOGI], ASD[GI] and TD[NOGI]. Data presented as median and interquartile ranges.

	TLR4 (LPS)		
	ASD[NOGI] Median (IQR)	ASD[GI] Median (IQR)	TD[NOGI] Median (IQR)
IL-1α	3,959[‡*] (3,615-4,376)	3,160 (1,602-4,036)	2,660 (1,115-3,913)
IL-1β	12,506[‡] (9,304-17,562)	10,727 (7,888-15,632)	9,782 (6,370-12,298)
IL-6	35,049 (26,923-46,236)	33,143 (20,277-41,781)	31,468 (21,858-47,848)
IL-12p40	43.69 (19.39-75.11)	39.28 (19.39-96.25)	57.64 (36.21-75.11)
IL-12p70	5.35[‡] (3.34-7.46)	7.46 (3.588-9.65)	7.63 (5.35-9.65)
TNFα	4,072[‡] (2,904-5,139)	4,084 (2,765-5,959)	3,071 (2,074-3,989)
IFNγ	159.90 (53.63-293.20)	253.50 (85.24-878)	168.90 (75.51-288.20)
IL-4	2.25 (2.25-4.16)	2.25 (2.25-11.13)	2.25 (2.25-11.13)
IL-13	19.50 (13.48-28.99)	20.80 (15.14-30.88)	19.50 (14.16-24.65)
IL-10	9,253 (4,421-10,885)	6,151 (3,007-10,047)	6,048 (3,904-8,017)
TGFβ	1,981 (1,043-3,899)	1,179[#] (820.70-2,010)	2,590 (1,261-4,745)
IL-5	1.36* (0.25-3.39)	3.39[#] (2.61-6.35)	2.43 (1.62-3.05)
IL-15	19.50[‡] (13.48-28.99)	20.80 (15.14-30.88)	19.50 (14.16-24.65)
IL-17	0.35[‡*] (0.35-0.35)	0.48[#] (0.35-3.24)	3.28 (1.00-6.145)

*p value < 0.05 when comparing ASD GI with ASD no GI

#p value < 0.05 when comparing ASD GI with TD

‡p value < 0.05 when comparing ASD no GI with TD

Table 4

Comparison of PBMC cytokine production after 24 hours of stimulation with NOD agonist between ASD, ASD[GI] and TD. Data presented as median and interquartile ranges.

	NOD 1&2 (PGN)		
	ASD[NOGI] Median (IQR)	ASD[GI] Median (IQR)	TD[NOGI] Median (IQR)
IL-1α	31.09[‡] (4.70-103.40)	58.80 (4.70-576.40)	104.30 (60.65-395.80)
IL-1β	202.6[‡*] (31.81-327.70)	638.90 (156.40-6415)	713.10 (286.70-3596)
IL-6	5,702 (2,187-10,883)	6,974 (5,174-12,261)	8,919 (6,570-15,244)
IL- 12p40	10.82[‡] (3.70-36.47)	24.18 (3.70-38.21)	24.29 (10.82-51.06)
IL- 12p70	0.30[‡*] (0.30-0.30)	0.30 (0.30-4.00)	0.30 (0.30-5.80)
TNFα	324.10[‡] (102.7-1,022)	488.60 (255-3,053)	850.40 (385.50-1,442)
IFNγ	40.82[‡*] (15.06-58.19)	114.70 (30.37-461.60)	72.63 (43.72-192)
IL-4	2.25 (2.25-2.25)	2.25 (2.25-2.25)	2.25 (2.25-2.25)
IL-13	20.76 (8.29-28.01)	16.17 (8.29-57.57)	29.32 (9.66-58.75)
IL-10	434.60 (163.60-1,080)	473.10 (273.60-1,046)	719.40 (466.20-1,271)
TGFβ	1,118 (837.20-1,877)	771.10 (595.80-2,243)	2,169 (535.40-5,136)
IL-5	2.22 (1.22-3.21)	2.43 (1.72-4.67)	2.72 (2.02-4.99)
IL-15	0.60 (0.60-2.58)	0.60 (0.60-3.51)	2.38 (0.60-3.51)
IL-17	23.79 (8.13-57.22)	15.66 (7.21-30.28)	16.72 (5.59-29.54)

[*] p value < 0.05 when comparing ASD GI with ASD no GI

[#] p value < 0.05 when comparing ASD GI with TD

[‡] p value < 0.05 when comparing ASD no GI with TD

Table 5

Comparison of PBMC cytokine production after 24 hours of stimulation with T-cell mitogen between ASD^{NOGI}, ASD^{GI} and TD^{NOGI}. Data presented as median and interquartile ranges.

	PHA		
	ASD^{NOGI} Median (IQR)	**ASD^{GI} Median (IQR)**	**TD^{NOGI} Median (IQR)**
IL-1α	64.17 (4.70-97.35)	40.11 (4.70-85.84)	52.50 (4.70-77.40)
IL-1β	419.50 (205.00-732.80)	578.90 (329.30-771.40)	405.00 (236.70-709.30)
IL-6	6,851 (5,085-8,863)	6,338 (5,814-11,464)	6,434 (5,060-9,977)
IL-12p40	3.70‡ (3.70-3.70)	3.70$^{#}$ (3.70-3.70)	3.70 (3.70-20.97)
IL-12p70	21.65 (17.05-28.68)	21.65 (12.56-28.94)	17.05 (14.23-27.01)
TNFα	1,438 (900.00-2,205)	1,706 (985.20-2,851)	1,477 (581.20-2,215)
IFNγ	810.30* (431.20-1,208)	1283$^{#}$ (822.80-1,747)	591.80 (395-1,284)
IL-4	558.50 (228.00-893.90)	462.30 (392.20-682.90)	488.60 (292.50-899.60)
IL-13	1,590 (870.80-2,472)	1,452 (1,116-2,743)	1,621 (1,223-2,518)
IL-10	2,020 (1,501-3,739)	2,585 (1,694-3,452)	2,452 (2,035-3,323)
TGFβ	2,070* (1,235-3,409)	1,523$^{#}$ (1,009-1,738)	2,996 (1,347-5,351)
IL-5	205.10 (129.60-331.70)	224.60 (120.80-429.30)	237.20 (180-496.80)
IL-15	12.31‡ (6.40-16.20)	6.88 (1.195-15.23)	6.10 (2.98-8.43)
IL-17	408.50 (265.60-647.50)	412.10 (277.90-583.30)	408.50 (279.20-731.60)

* p value < 0.05 when comparing ASD GI with ASD no GI

$^{#}$ p value < 0.05 when comparing ASD GI with TD

‡ p value < 0.05 when comparing ASD no GI with TD