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Dysregulated Innate Immune Responses in Young Children with Autism Spectrum Disorders: Their Relationship to Gastrointestinal Symptoms and Dietary Intervention

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Key Words

Tumor necrosis factor-α · Interleukin-10 · Lipopolysaccharide · Autism spectrum disorders · Innate immunity

Abstract

Objective: Our previous study indicated an association between cellular immune reactivity to common dietary proteins (DPs) and excessive proinflammatory cytokine production with endotoxin (lipopolysaccharide, LPS), a major stimulant of innate immunity in the gut mucosa, in a subset of autism spectrum disorder (ASD) children. However, it is unclear whether such abnormal LPS responses are intrinsic in these ASD children or the results of chronic gastrointestinal (GI) inflammation secondary to immune reactivity to DPs. This study further explored possible dysregulated production of proinflammatory and counter-regulatory cytokines with LPS in ASD children and its relationship to GI symptoms and the effects of dietary intervention measures. ***Methods:*** This study includes ASD children (median age 4.8 years) on the unrestricted (n = 100) or elimination (n = 77) diet appropriate with their immune reactivity. Controls include children with non-allergic food hypersensitivity (NFH; median age 2.9 years) on the unrestricted (n = 14) or elimination (n = 16) diet, and typically developing children (median age 4.5 years, n = 13). The innate immune responses were assessed by measuring production of proinflammatory (TNF-α, IL-1β, IL-6, and IL-12) and counter-regulatory (IL-1ra, IL-10, and sTNFRII) cytokines by peripheral blood mononuclear cells (PBMCs) with LPS. The results were also compared to T-cell responses with common DPs and control T-cell mitogens assessed by measuring T-cell cytokine production. ***Results:*** ASD and NFH PBMCs produced higher levels of TNF-α with LPS than controls regardless of dietary interventions. However, only in PBMCs from ASD children with positive gastrointestinal (GI(+)) symptoms, did we find a positive association between TNF-α levels produced with LPS and those with cow's milk protein (CMP) and its major components regardless of dietary interventions. In the unrestricted diet group, GI(+) ASD PBMCs produced higher IL-12 than controls and less IL-10 than GI(–) ASD PBMCs with LPS. GI(+) ASD but not GI(–) ASD or NFH PBMCs produced less counter-regulatory cytokines with LPS in the unrestricted diet group than in the elimination diet group. There was no significant difference among the study groups with regard to cytokine production in responses to T-cell mitogens and other recall antigens. ***Conclusion:*** Our results revealed that there are findings limited to GI(+) ASD PBMCs in both the unrestricted and elimination diet groups. Thus our findings indicate intrinsic defects of innate immune responses in GI(+) ASD children but not in NFH or GI(–) ASD children, suggesting a possible link between GI and behavioral symptoms mediated by innate immune abnormalities.

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Introduction

Autism spectrum disorders (ASDs) are complex developmental disorders diagnosed on the basis of behavioral symptoms. However, like other disorders involving multiple organ systems, ASD children often suffer from other medical problems early in life. Gastrointestinal (GI) inflammation confirmed by imaging and endoscopic studies occurs frequently in young ASD children [1–4]. Many parents of these ASD children report favorable responses to the elimination diet (typically, a gluten-free, casein-free diet) with resolution of GI symptoms in addition to improvement in behavioral symptoms. Other medical problems frequently encountered in young ASD children include recurrent ear infection/rhinosinusitis/upper respiratory infection, adverse reactions to multiple medications, and a prolonged course of illness as compared to typically developing siblings. In a subset of ASD children, others also indicate a clustering of autoimmune disorders in their family members [5] and high prevalence of autoantibodies against neuronal cells [6–8]. These findings indicate that substantial numbers of young ASD children suffer from medical problems associated with immunodysregulation and autoimmunity but conventional immune workup is generally unrevealing.

To address possible immune abnormalities in ASD children, we initially assessed innate immune responses that are not routinely screened in a conventional immune workup. Innate immunity mounts the initial first-line, nonspecific immune responses in an antigen (Ag)-independent manner, but proper innate immune responses are essential for development of effective adaptive immunity, especially early in life [9, 10] and are also closely associated with CNS functions [11]. Our previous studies revealed a high prevalence of excessive production of proinflammatory cytokines (most notably tumor necrosis factor-α, TNF-α) with stimulation of endotoxin (lipopolysaccharide, LPS), in a substantial number of ASD children [12]. Endotoxin is one of the major microbial products produced in the GI mucosa and provokes potent innate immune responses through TLR4 and other pattern-recognition receptors [13]. ASD children with excessive TNF-α production with LPS often reveal cellular immune reactivity to common dietary proteins (DPs) as observed in children with non-allergic food hypersensitivity (NFH) [14]. Thus our previous findings suggested a role of innate immune abnormalities in the development of a cell-mediated immune reaction to common DPs in ASD children. Since excessive inflammation induced by innate immunity can be detrimental to the body, the immune system can also exert counter-regulatory measures partly through production of counter-regulatory cytokines [13].

Taken together, we hypothesized that ASD children with positive GI symptoms (GI(+)) and cellular immune reactivity to DPs likely reveal dysregulated production of inflammatory and counter-regulatory cytokines with LPS, and that such innate immune abnormalities likely persist even after dietary intervention measures, given the fact that these ASD children also frequently suffer from other medical problems as described above. This study determined a profile of proinflammatory and counter-regulatory cytokines production by peripheral blood mononuclear cells (PBMCs) in response to LPS, using cells from young ASD children on an unrestricted or an elimination diet and compared the results with their responses to common DPs and T-cell mitogens. NFH children and typically developing children served as controls.

Patients and Methods

This study includes ASD and control children (age 1–10 years) in Turner stage 1 to exclude the effects of hormonal changes associated with puberty. ASD children were recruited from those referred to the Autism Center, NJMS, UMDNJ, Newark, N.J. ASD diagnosis was made or ascertained by DSM-IV and/or ICD-10 criteria, ADI-R (Autism Diagnostic Interview-Revised) and ADOS (Autism Diagnostic Observational Schedules). NFH children and typically developing children served as control groups and were recruited from those seen in the Allergy/Immunology Clinic and General Pediatrics Clinic, NJMS, UMDNJ. ASD children on neuropsychiatric medications were excluded from the study. Children with known immunodeficiency, metabolic disorders, genetic disease, and illnesses involving the major organs were excluded from the study. Blood samples were obtained following obtainment of the IRB-approved signed consent form. At the time of venipuncture, all the study subjects were not febrile and had no evidence of acute microbial illnesses.

The demographics of the study subjects are summarized in table 1. ASD and NFH children are subdivided into 2 groups: children on an unrestricted diet or those on an elimination diet appropriate on the basis of their immune reactivity to common DPs. Children in the unrestricted diet group were on an unrestricted diet or within 2 weeks of starting the dietary intervention at the time of blood sampling. Children in the elimination diet group had been on the elimination diet for >4 months: they were on the casein-free/gluten-free/soy-free or casein-free/soy-free diet. None of the children on the casein-free/soy-free diet had elevated (higher than normal range) immune reactivity to gliadin assessed by cytokine production [14]. ASD children in each diet group were also subdivided into 2 groups on the basis of the presence or absence of GI symptoms: GI(+) or GI(–) ASD children. We defined GI symptoms as vomiting, diarrhea, chronic loose stool with undigested food, colic and GI cramping, constipation (often alternated with explosive diarrhea), and gastroesophageal reflux disease reported by the par-

Table 1. Demographics of the study subjects

Study group	Age median (range) years	Subjects sex (M:F)	Atopy (asthma, AR, and AD[1])	Immunization reaction	Length of dietary intervention median months (range)	Response to dietary intervention
Unrestricted diet[2]						
GI (+) ASD	4.8 (1.8–10.6)	71 (59:12)	20/71 (28.2%)	10/71 (14.1%)		
GI (–) ASD	5.4 (2.1–10.2)	29 (27:2)	10/29 (34.5%)	2/29 (6.9%)		
NFH	2.5 (1–7.8)	14 (9:5)	2/14 (14.3%)	0/14		
Controls	4.5 (1–8.3)	13 (5:8)	unknown	0/13		
Restricted diet[3]						
GI (+) ASD	4.7 (2.2–10.9)	68 (62:6)	17/68 (25.0%)	16/68 (23.5%)	12 (4–48)	66/68 (97.1%)[2]
GI (–) ASD	4.6 (2.3–7.4)	9 (7:2)	4/9 (44.4%)	1/9 (11.1%)	6 (4–12)	0/9 (0%)
NFH	3.2 (1–7.5)	16 (9:7)	2/16 (12.5%)	1/15 (6.7%)	6 (4–24)	16/16 (100%)

[1] In the unrestricted diet group, atopic dermatitis (AD) was diagnosed in 6/71 (8.5%) GI(+) ASD, 3/29 (10.3%) GI(–) ASD, and 1/14 (7.1%) NFH children. In the restricted diet group, AD was reported in 4/68 (5.9%) GI(+) ASD, 0/9 GI(–) ASD, and 1/16 (6.3%) NFH children. The skin symptoms of AD patients were affected by intake of causative food allergens (milk, egg, and peanut).

[2] In GI(+) ASD children, 32, 28, and 11 of them were diagnosed as having autism, pervasive developmental disorder not otherwise specified (PDD-NOS), and ASD, respectively. In GI(–) ASD children, 12, 14, and 3 of them were diagnosed as having autism, PDD-NOS, and ASD, respectively. Pediatric autoimmune neuropsychiatric disorders associated with streptococcal infection (PANDAS)-like clinical features are reported in 5/71 (7.0%) GI(+) ASD children and 2/29 (6.9%) GI(–) ASD children. None of the NFH control children revealed PANDAS-like features.

[3] In GI(+) ASD children, 31, 33, and 4 of them were diagnosed as having autism, PDD-NOS, and ASD, respectively. In GI(–) ASD children, 4 and 5 of them were diagnosed as having autism and PDD-NOS, respectively. Responses to dietary interventions are on the basis of improvement of GI symptoms. Two of 68 subjects reported persistent GI symptoms. Most parents reported better behavioral symptoms along with resolution of GI symptoms. One of 2 non-responders revealed clinical features of PANDAS. One responder had worsening autistic behaviors following resolution of GI symptoms: his clinical features are similar to PANDAS. One of 9 GI(–) ASD children also revealed PANDAS-like clinical features. Parents of GI(–) ASD children reported no behavioral changes following implementation of the restricted diet.

ents/care takers or physicians. All the NFH patients on the unrestricted diet revealed significant cellular immune reactivity to common DPs along with significant GI symptoms and poor weight gain. NFH children all responded well to the elimination diet with resolution of GI symptoms in relatively short periods of time (<4 months) without significant complications.

The presence of atopic disorders and immunization reactions in each study group are also shown in table 1. Diagnoses of atopic asthma, allergic rhinitis, and allergic dermatitis were made by the presence of typical clinical features with positive skin test reactivity and/or the presence of allergen-specific IgE against common aero and/or food allergens. The prevalence of atopic disorders in our study subjects was similar to those reported in the general population [15]. The prevalence of allergic dermatitis was not high in our study subjects (table 1, footnote). Immunization reactions reported by parents included prolonged fever (>24 h), febrile seizure, lethargy (>24 h), systemic urticaria/angioedema, extreme irritability (>24 h), and loss of speech (within 1 week after immunization). Most of the immunization reactions are associated with either DTP/DTaP and/or MMR with concurrent administration of other infant vaccines. However, as revealed in table 1, GI symptoms appeared more prevalent than immunization reactions in ASD children.

Assessment of Immune Response

Innate Immune Responses. PBMCs were cultured overnight with LPS (0.1 μg/ml, GIBCO-BRL, Gaithersburg, Md., USA) [12]. LPS was used as a representative endotoxin: LPS is known to be a major stimulant of innate immunity produced by G(–) bacteria in the gut and exerts a potent stimulating action through TLR4 [16]. This suboptimal concentration of LPS was chosen based on our previous reports [12]. For assessing innate immune responses, we measured the levels of proinflammatory cytokines (TNF-α, IL-1β, IL-6, IL-12p40) and counter-regulatory mediators (sTNFRII, IL-10, and IL-1ra) in the culture supernatant.

Adaptive Immune Responses. We used common recall Ags (tetanus toxoid and dust mite extract) and polyclonal T-cell stimulants (phytohemagglutinin, 10 μg/ml, and concanavalin A (Con A), 1 μg/ml) for assessing T-cell functions [12, 14]. With these stimulants, we measured IFN-γ, TNF-α, IL-5, IL-10, and IL-12p40. At the same time, we also tested the reactivity to common DPs including crude extracts of cow's milk and soy, gliadin (a major wheat protein), and major components of cow's milk protein (CMP). These data are reported elsewhere, but the results of examining the association between cytokine production with β-lactoglobulin and LPS are included in this study (not reported elsewhere).

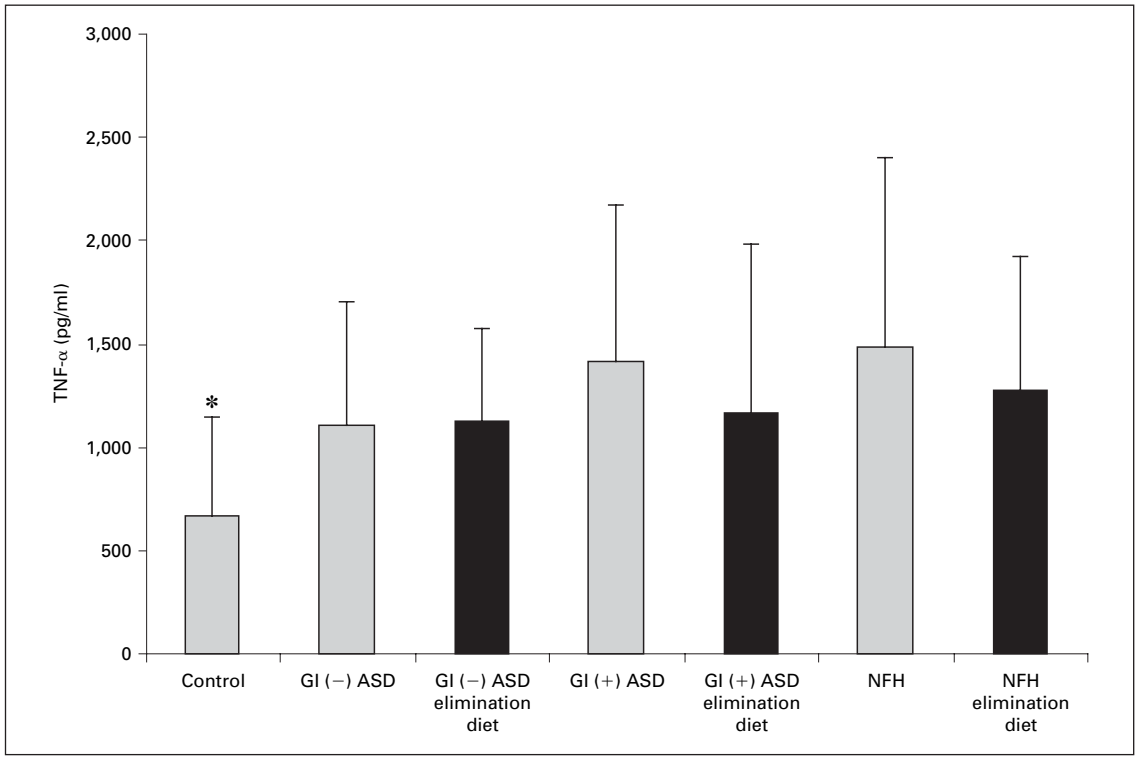

Fig. 1. The levels of TNF-α produced with LPS in each study group. The results are expressed as mean values and standard deviation (SD). The levels of TNF-α produced by control PBMCs were significantly lower compared to all the other study groups.

ELISA

Cytokine levels in the culture supernatants were measured by ELISA, using OptEIA™ Reagent Sets (BD Pharmingen, San Diego, Calif., USA) for IFN-γ, TNF-α, IL-5, IL-10, IL-12, IL-6, and IL-1β. sTNFRII and IL-1ra were measured using the regent sets from R & D (Minneapolis, Minn., USA). Intra- and inter-variations of cytokine levels were less than 5%.

Statistical Analysis

The equality of two sets of data values was evaluated by Mann-Whitney test (two sets of independent samples) or Wilcoxon weighed ranks test (two sets of related samples). Comparison of multiple values was done by Kruskal-Wallis test. Correlation of two parameters was assessed by two-tailed Kendall τ-b test. Differences with $p < 0.05$ were considered significant.

Results

Proinflammatory and Counter-Regulatory Cytokine Production with LPS

The results of proinflammatory (TNF-α, IL-1β, IL-6, and IL-12p40) and counter-regulatory cytokines (sTNFRII, IL-1ra, and IL-10) were summarized in table 2. Consistent to our previous findings [14], GI(+) ASD, GI(–) ASD, and NFH PBMCs produced more TNF-α with LPS than control cells in both the unrestricted and elimination diet groups (table 2; fig. 1). GI(+) ASD PBMCs tended to produce higher amounts of TNF-α than GI(–) ASD PBMCs in the unrestricted diet group: 20/71(28.6%) GI(+) ASD PBMCs produced higher than normal range TNF-α while so did only 3/29 (10.3%) GI(–) ASD PBMCs and 2/14 (14.3%) NFH PBMCs. In the elimination diet group, higher than normal range TNF-α production was observed in 12/69 (17.6%) GI(+) ASD, 1/9 (11.1%) GI(–) ASD, and 2/16 (12.5%) NFH PBMCs, respectively.

Other cytokine levels produced with LPS did not differ from controls in all the study groups, except for higher levels of IL-12 in GI(+) ASD PBMCs in the unrestricted diet group and higher levels of IL-6 in NFH-inhibitory PBMCs in the elimination diet group. In GI(+) ASD PBMCs, production of counter-regulatory cytokines (sTNFRII, IL-1ra, and IL-10) was lower in the unrestricted diet group than in the elimination diet group. In contrast, NFH PBMCs tended to produced higher levels of counter-regulatory cytokines in the unrestricted diet group (table 2; fig. 2).

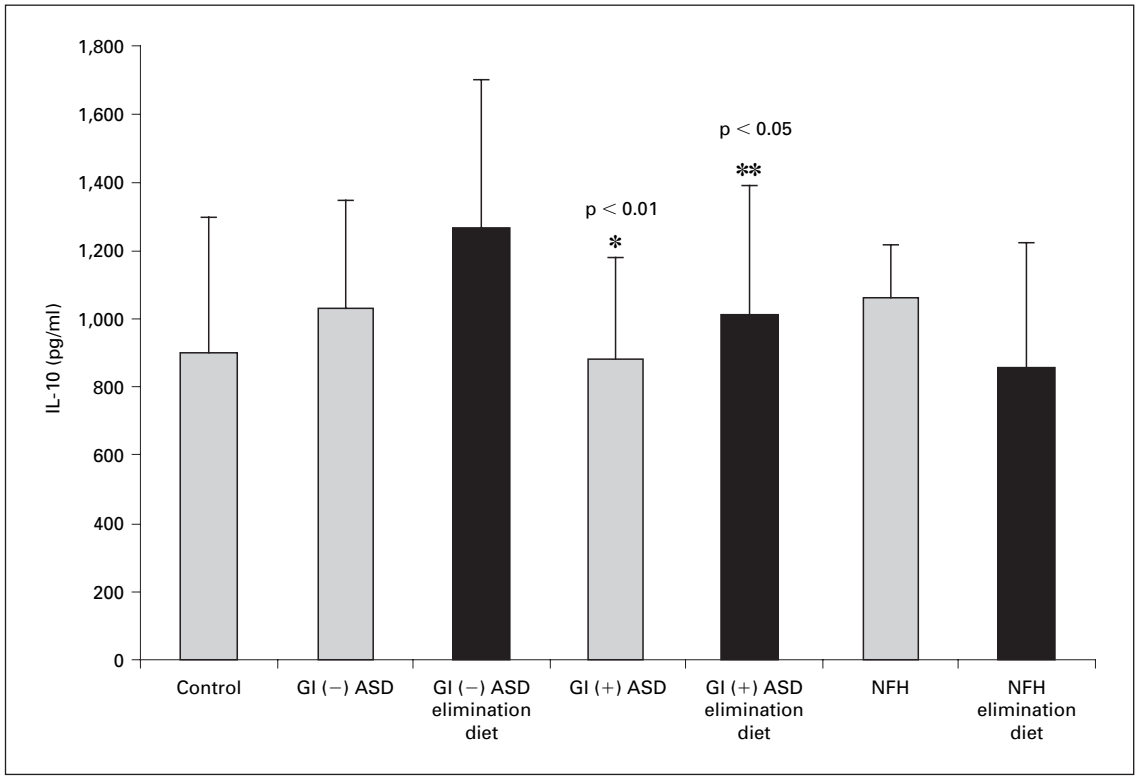

Fig. 2. The levels of IL-10 produced with LPS in each study group. The results are expressed as mean values and standard deviation (SD). GI(+) ASD PBMCs produced less IL-10 than that produced by NFH PBMCs in the unrestricted diet group ($p < 0.01$). GI(+) ASD PBMCs in the unrestricted diet group also produced less IL-10 as compared to that produced in the elimination diet group ($p < 0.05$).

Table 2. Cytokine production with LPS[1] in the unrestricted and restricted diet groups

Cytokines measured	GI(+) ASD median (range), pg/ml	GI(–) ASD median (range), pg/ml	NFH median (range), pg/ml	Controls median (range), pg/ml
Unrestricted diet	n = 71	n = 29	n = 14	n = 13
TNF-α	1,431 (223.5–3,462) ($p < 0.001$)[2]	926.5 (243.0–2,595.5) ($p < 0.05$)	1,116.0 (624.0–3,519.0) ($p < 0.02$)	690.4 (38–1,788.0)
IL-12p40	<15.7 (<15.7–1,294.0) ($p < 0.02$)	<15.7 (<15.7–877.6)	<15.7 (<15.7–483.1)	<15.7 (<15.7–98.0)
IL-6	30,740 (11,975–191,934)	30,430 (12,186–56,601)	32,947 (19,623–62,736)	3,021 (20,426–51,675)
IL-1ß	2,240 (76.7–5,631.0)	2,375.0 (553.5–5,351.7)	1,955.0 (242.4–4,934.0)	249 (497.5–4,767)
sTNFRII	4,222.0 (1,458.0–9,834.0)	5,521.5 (1,516.0–21,582)	5,495.0 (1,908.0–10,177)	519 (2,161–10,356)
IL-1ra	3,157.5 (318.0–12,044)	4,168.5 (28.4–9,835.2)	3,611.0 (988.6–10,919)	410 (2,97.2–12,425)
IL-10	8,74.0 (211.8–1,692.0)	1,054.0 (458.2–1,937.0)	1,043.0 (857.4–1,313.0)	975.6 (314.1–1,421)
Restricted diet	n = 68	n = 9	n = 16	
TNF-α	914.4 (204.8–4,031.0) ($p < 0.01$)[2]	974.0 (707.5–2,068) ($p < 0.05$)	11,37.0 (392.0–2,663.5) ($p < 0.02$)	
IL-12p40	<15.7 (<15.7–375.6)	<15.7 (<15.7–29.8)	91.4 (<15.7–515.6)	
IL-6	30,727 (12,145–54,414)	38,302 (14,470–51,230)	22,430 (1,5912–41,100) ($p < 0.05$)	
IL-1ß	1,795.0 (33.3–5,082.0)	1,500 (941.5–5,340)	1,262.0 (364.9–3,760)	
sTNFRII	5,737.6 (1,371–18,712)[4]	6,906 (1,691–17,632)	3,002.0 (1,675–10,452)[3]	
IL-1ra	4,786.0 (620.8–17,256)[4]	4,537 (1,080–9,316)	2,833.0 (157.6–6,423)[3]	
IL-10	1,053.5 (319.2–2,063.0)[4]	1,159 (756.6–2,142)	781.7 (4,37.4–1,392.5)	

[1] LPS concentration used was 0.1μg/ml.
[2] p values in comparison with control data by Mann-Whitney test.
[3] These values are lower than those for GI(+) ASD children in the restricted diet group ($p < 0.05$ for sTNFRII and $p < 0.01$ for IL-1ra).
[4] These values are higher as compared to GI(+) ASD children on the unrestricted diet ($p < 0.005$ for sTNFRII, $p < 0.02$ for IL-1ra, and $p < 0.05$ for IL-10).

Table 3. Cytokine production with Con A

Cytokines measured[1]	GI(+) ASD median (range), pg/ml	GI(–) ASD median (range), pg/ml	NFH median (range), pg/ml	Controls median (range), pg/ml
Unrestricted diet	n = 71	n = 29	n = 14	n = 13
IFN-γ	834.4 (123.7–9,776)	480.0 (136.5–4,512)	1,174.8 (107.5–3,811.0)	627.3 (137.8–2,280)
IL-5	29.1 (<3.9–303.0)	44.9 (<3.9–272.9)	49.0 (<3.9–661.5)	<3.9 (<3.9–386.1)
TNF-α	440.0 (<3.9–2,858.0) ($p < 0.05$)[1]	364.4 (<3.9–1,348.0)	294.5 (<3.9–4,308.0)	97.7 (<3.9–536.0)
IL-10	433.8 (80.3–1,401.0)	625.8 (195.6–1,025.6)	685.6 (434.4–1,055.0)	257.6 (153.5–715.8)
IL-12	<15.7 (<15.7–4,095.0)	<15.7 (<15.7–1,143.8)	80.0 (<15.7–646.6)	<15.7 (<15.7–323.0)
Restricted diet	n = 68	n = 9	n = 16	
IFN-γ	720.9 (167.0–3,027.0)	596.4 (149.3–2,419)	654.2 (272.8–2,883.0)	
IL-5	13.6 (<3.9–653.0)	56.6 (<3.9–203.3)	61.0 (<3.9–188.9)	
TNF-α	227.1 (<3.9–1,180)	91.3 (<3.9–1,052.0)	671.0 (<3.9–1,477.0)	
IL-10	587.0 (56.7–1,224.0)	607.9 (157.4–1,644.0)	532.0 (50.7–1,235.6)	
IL-12	<15.7 (<15.7–874.5)	<15.7 (<15.7–32.4)	<15.7 (<15.7–1,087)	

[1] p values in comparison with data from control children by Mann-Whitney test. There are no significant statistical differences between the study groups.

Table 4. The ratio of proinflammatory and counter-regulatory cytokines produced with LPS

Ratio	GI(+) ASD median (range)	GI(–) ASD median (range)	NFH median (range)	Controls median (range)
Unrestricted diet	n = 71	n = 29	n = 14	n = 13
TNF-α/IL-10	1.465 (0.068–5.417) ($p < 0.02$)[1,2]	1.018 (0.156–2.562)	0.932 (0.598–2.603)	0.763 (0.030–2.198)
IL-6/IL-10	35.33 (14.26–125.0)	33.49 (10.97–60.87)	27.93 (20.23–40.13) ($p < 0.02$)[3]	47.59 (16.38–90.84)
IL-1ß/IL-1ra	0.740 (0.057–6.931)	0.544 (0.117–2.588)	0.539 (0.023–1.436)	0.381 (0.049–9.341)
TNF-α/sTNFRIIa	1.612 (0.284–5.021) ($p < 0.02$)	1.260 (0.426–4.306)	1.344 (0.367–4.152)	1.225 (0.019–3.428)
Restricted diet	n = 68	n = 9	n = 16	
TNF-α/IL-10	0.950 (0.151–7.506)	0.916 (0.334–1.885)	1.207 (0.451–5.469)	
IL-6/IL-10	32.99 (12.54–453.07)	30.61 (13.44–49.35)	35.4 (14.23–48.05)	
IL-1ß/IL-1ra	0.415 (0.016–3.360)[4]	0.623 (0.161–1.165)	0.529 (0.205–23.15)	
TNF-α/sTNFRIIa	1.4657 (0.247–5.547)	1.130 (0.264–2.325)	2.690 (0.517–5.620) ($p < 0.05$)[5]	

[1] p values were obtained in comparison with control values (Mann-Whitney test).
[2] This value is also higher than those in GI(–) ASD PBMCs from the unrestricted diet.
[3] This value is lower than control children as well as GI(+) ASD children on the unrestricted diet ($p < 0.05$).
[4] This value is also higher than that obtained in GI(–) ASD children on the restricted diet ($p < 0.01$).
[5] This value is lower than GI(+) ASD PBMCs from the unrestricted diet ($p < 0.05$).

Cytokine Production with T-Cell Stimulants

We measured Th1 (IFN-γ and TNF-α), Th2 (IL-5), and regulatory (IL-10 and IL-12p40) cytokines in response to T-cell stimulants (Con A, phytohemagglutinin, and recall Ags). These levels did not differ among the study groups as reported before [14], regardless of the dietary intervention measures (table 3, and unreported data). However, GI(+) ASD PBMCs in the unrestricted diet group produced more TNF-α with Con A than control cells. We also observed that 11/71 (15.8 %) G(+) ASD PBMCs produced elevated levels of IFN-γ (>4,000 pg/ml) with Con A in the unrestricted diet group.

Ratio of Proinflammatory and Counter-Regulatory Cytokines Produced with LPS

In the unrestricted diet group, GI(+) ASD PBMCs showed higher TNFα/IL-10 and TNF-α/sTNFRII ratios than controls with LPS. The TNFα/IL-10 ratio produced

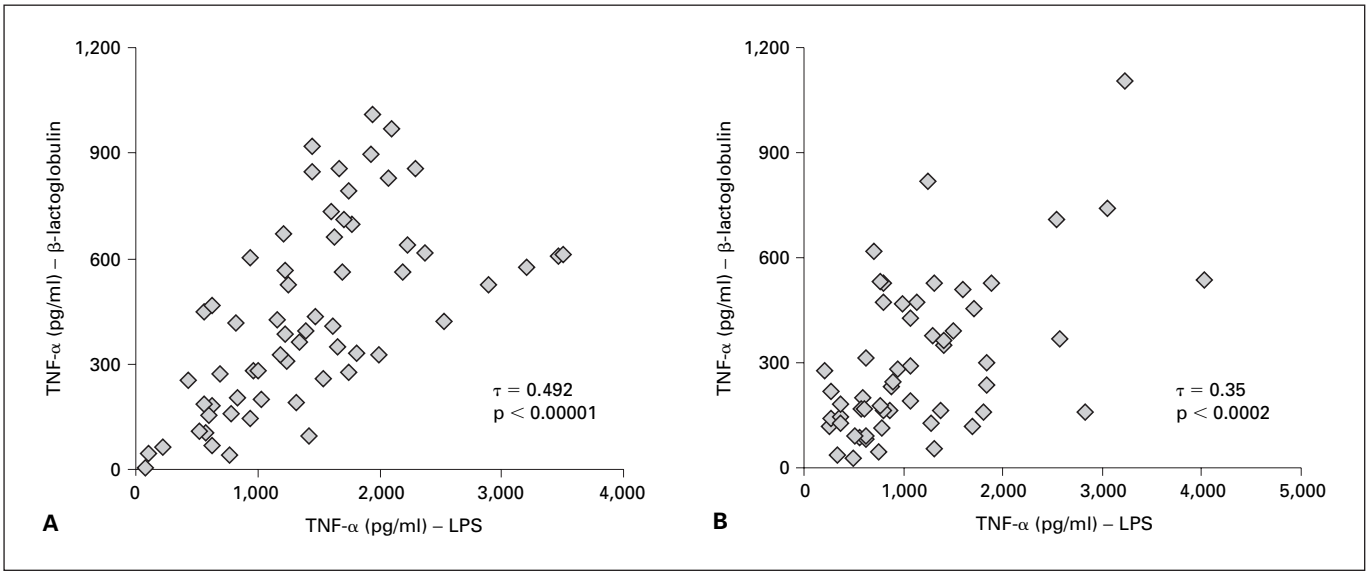

Fig. 3. Correlation of TNF-α levels stimulated with LPS (0.1 μg/ml) and β-lactoglobulin in GI(+) ASD children on the unrestricted diet (**A**) or on the elimination diet (**B**).

by GI(+) ASD PBMCs with LPS was also higher than that by GI(–) ASD PBMCs (table 4). NFH PBMCs revealed a lower IL-6/IL-10 ratio than GI(+) ASD and control PBMCs in the unrestricted diet group (table 4). In the elimination diet group, these ratios did not differ in the study groups except for a higher TNF-α/sTNFRII ratio in NFH PBMCs than other study groups ($p < 0.02$). When these ratios were compared between unrestricted and elimination diet groups, they did not differ among the study groups except for a lower IL-1β/IL-1ra ratio in the restricted diet group than in the unrestricted diet group in GI(+) ASD PBMCs.

Correlation of TNF-α Levels Produced by Common Dietary Proteins and LPS

We compared TNF-α levels produced with LPS with TNF-α produced with DPs in each study group: the cytokine production profile with DPs in each study group are reported elsewhere. In the unrestricted diet group, GI(+) ASD PBMCs revealed a positive correlation between TNF-α production with LPS and that with β-lactoglobulin (fig. 3), α-lactoalbumin ($\tau = 0.354$, $p < 0.0001$), and CMP ($\tau = 0.33$, $p < 0.0002$). In the elimination diet group, such a correlation was also found in GI(+) ASD PBMCs with β-lactoglobulin (fig. 3), α-lactoalbumin ($\tau = 0.253$, $p < 0.01$), and CMP ($\tau = 0.364$, $p < 0.0001$). No such correlation was found in GI(–) ASD or NFH PBMCs ($p > 0.05$).

Discussion

This study revealed elevated TNF-α production with LPS in GI(+) and GI(–) ASD PBMCs as well as NFH PBMCs in both unrestricted and elimination diet groups, but the levels tend to be higher in the unrestricted diet group. We also observed other abnormalities that appear limited to GI(+) ASD PBMCs. These results may indicate the intrinsic natures of dysregulated innate immune responses in GI(+) ASD children, which may predispose them to adverse reactions to benign environmental factors including DPs and aggravation of behavioral symptoms.

Innate immunity provides an initial immune defense by activating innate immune cells (macrophage/monocytes, dendritic cells, natural killer cells, etc.). They mount nonspecific immune responses by recognizing pathogen-associated molecular patterns and tissue-derived danger signals through pattern-recognition and other receptors [9, 17]. Innate immune responses produce proinflammatory cytokines including TNF-α, IL-6, IL-1β, and IL-12. To avoid excessive inflammatory responses, the effects of proinflammatory cytokines are counter-regulated by suppressive cytokines and soluble receptors including IL-10, sTNFRI, sTNFRII, and IL-1ra. Thus, cytokines such as TNF-α, its soluble receptors, and IL-6, present in the periphery and the CNS, comprise a link between peripheral immune stimulation and CNS-mediated behaviors [18,

19]. Dysregulated or excessive innate immune responses could lead to prolonged illnesses, various adverse reactions to benign environmental factors, and even to autoimmunity [13, 20, 21]. Innate immunity is especially crucial early in life, secondary to relatively immature adaptive immunity.

Many physicians taking care of the medical aspects of ASD children, including the authors, have been alarmed by the high prevalence of medical problems present in ASD children in early life. Parents often report recurrent otitis media, chronic rhinosinusitis, and upper respiratory illnesses in young ASD children with a prolonged course of illnesses. Young ASD children also frequently reveal GI symptoms with abnormal findings on GI workup [1–4]. Many of these medical problems appear to improve or become more stabilized with age, although they tend to persist longer in ASD children than in typically normally developing children. Autoimmune phenomena also appear to be prevalent in a subset of ASD children with the frequent presence of autoantibodies against neuronal cells in the serum and cerebrospinal fluid [8]. Some ASD children exhibit waning and waxing of behavioral symptoms (obsessive-compulsive behaviors, anxiety, tics, and ataxic movement) in association with streptococcal infection and possibly with other microbial infections. These ASD children often have multiple family members suffering from autoimmune disorders including lupus, rheumatoid arthritis, inflammatory bowel diseases, and autoimmune thyroiditis [5, 6]. These observations do suggest intrinsic defects of innate immune responses in ASD children with the above-described symptoms. Our previous results indicated that ASD children have a high prevalence of innate immune abnormalities and that such innate immune abnormalities could predispose these ASD children to cellular immune reactivity to DPs and other environmental factors [12, 14].

Secondary to immature gut mucosal immunity, infants often develop immune responses against common DPs, most frequently against milk and soy proteins. These immune responses are mainly associated with cell-mediated, delayed-type immune responses with predominantly elevated TNF-α production with DPs [22–25]. The innate immune abnormalities we initially described include excessive proinflammatory innate immune responses, which could lead to excessive adaptive immune responses and undesired immune reactions to benign environmental factors. Subsequently our results also indicate an association between innate immune abnormalities and the development of cellular immune reactivity to DPs in ASD children [14].

To address the role of innate immune abnormalities and adverse reactions to DPs in ASD children and the effects of the dietary intervention measures, we have been conducting cross-sectional and prospective clinical studies. The data presented here are those obtained from cross-sectional studies in which we evaluated cellular immune reactivity to common DPs by PBMCs in comparison with their responses to LPS, other recall Ags, and polyclonal T-cell stimulants in GI(+) and GI(–) ASD children with or without dietary intervention.

Our study revealed a close association between TNF-α production with CMP and its major protein components (α-lactoalbumin and β-lactoglobulin) and the presence of GI symptoms. Appropriate dietary intervention led to resolution of GI symptoms along with a decrease in TNF-α production with causative DPs in both G(+) ASD and NFH children. However, responses to dietary intervention tend to be slower in GI(+) ASD children than NFH-inhibited children. ASD children also tend to suffer from other medical problems, even GI inflammation associated with cellular immune reactivity to DPs are better taken care of. These results raise the question of whether intrinsic defects exist in the innate immunity of GI(+) ASD children.

As reported before [14], TNF-α-production with LPS was higher in GI(+), GI(–), and NFH PBMCs than in controls regardless of the dietary intervention, although the unrestricted diet group tended to reveal higher TNF-α production. Changes in T-cell functions are unlikely to be associated with elevated TNF-α production with LPS, since T-cell cytokine production did not differ among the study groups. These findings indicate that excessive TNF-α production with LPS could be nonspecific reflecting the on-going inflammatory condition regardless of the GI symptoms. However, we also observed a positive correlation between LPS-induced TNF-α production and that with CMP and its major components in GI(+) ASD PBMCs but not in GI(–) ASD or NFH PBMCs. This finding indicates a role for excessive TNF-α production with LPS in the development of immune reactivity to DPs at least in GI(+) ASD children.

Thus we further explored the differences in LPS-induced responses between GI(+) ASD PBMCs and other study groups. NFH PBMCs revealed a tendency to produce more counter-regulatory cytokines in the unrestricted diet group than in the elimination diet group with LPS. NFH children also tended to reveal higher IL-10 production with DPs in the unrestricted diet group (reported elsewhere). This tendency was less apparent in the elimination diet group in NFH PBMCs, indicating liberation

of counter-regulatory measures following implementation of dietary intervention. GI(–) ASD PBMCs did not reveal significant changes in IL-10 production. In contrast, GI(+) ASD revealed less counter-regulatory cytokine production and resulted in higher TNF-α/IL-10 and TNF-α/sTNFRII ratios with LPS in the unrestricted diet group than in the restricted diet group. It may be postulated that in NFH children on the unrestricted diet, innate immunity exerts more effective suppressive actions by increasing production of regulatory cytokines. In contrast, GI(+) ASD children may not institute effective counter-regulatory measures in response to inflammatory responses. This could lead to subsequent suboptimal counter-regulatory measures in T-cell responses: we did find less IL-10 production with CMP in GI(+) ASD PBMCs than in NFH PBMCs (manuscript submitted). An increase in counter-regulatory cytokine production in GI(+) ASD PBMCs with dietary intervention may also indicate dysregulated regulatory measures of innate immunity in GI(+) ASD children. This could lead to a prolonged course of microbial illnesses and development of adverse reactions to benign environmental factors other than DPs [16, 26].

In summary, our results indicate the complex nature of apparent intrinsic defect(s) of innate immunity in GI(+) ASD children. Better understanding of these abnormalities in relation to CNS functions will be helpful to develop preventive and therapeutic measures to ensure optimal cognitive development and avoid other medical complications in these ASD children. Clinically, the presence of GI symptoms in young ASD children could be a risk factor for the development of other medical complications.

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