

Innate Immunity Associated with Inflammatory Responses and Cytokine Production against Common Dietary Proteins in Patients with Autism Spectrum Disorder

Harumi Jyonouchi Sining Sun Nanae Itokazu

Department of Pediatrics, University of Minnesota, Minneapolis, Minn., USA

Key Words

Innate immunity · Dietary protein intolerance (DPI) · Endotoxin · TNF- α · Autism spectrum disorder (ASD)

Abstract

Objectives: Children with autism spectrum disorder (ASD) frequently reveal various gastrointestinal (GI) symptoms that may resolve with an elimination diet along with apparent improvement of some of the behavioral symptoms. Evidence suggests that ASD may be accompanied by aberrant (inflammatory) innate immune responses. This may predispose ASD children to sensitization to common dietary proteins (DP), leading to GI inflammation and aggravation of some behavioral symptoms. **Methods:** We measured IFN- γ , IL-5, and TNF- α production against representative DPs [gliadin, cow's milk protein (CMP), and soy] by peripheral blood mononuclear cells (PBMCs) from ASD and control children [those with DP intolerance (DPI), ASD siblings, and healthy unrelated children]. We evaluated the results in association with proinflammatory and counter-regulatory cytokine production with endotoxin (LPS), a microbial product of intestinal flora and a surrogate stimulant for innate immune responses. **Results:** ASD PBMCs pro-

duced elevated IFN- γ and TNF- α , but not IL-5 with common DPs at high frequency as observed in DPI PBMCs. ASD PBMCs revealed increased proinflammatory cytokine responses with LPS at high frequency with positive correlation between proinflammatory cytokine production with LPS and IFN- γ and TNF- α production against DPs. Such correlation was less evident in DPI PBMCs. **Conclusion:** Immune reactivity to DPs may be associated with apparent DPI and GI inflammation in ASD children that may be partly associated with aberrant innate immune response against endotoxin, a product of the gut bacteria.

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Introduction

Autism spectrum disorder (ASD) is a complex developmental disorder diagnosed on the basis of behavioral findings (DSM-IV/ICD-10 criteria). Parents of ASD children frequently report GI symptoms including diarrhea, constipation, colic, gastroesophageal reflux (GER), and GI discomfort. Many parents of ASD children have tried a casein-free/gluten-free (cf/gf) diet, hoping to relieve GI symptoms of their children. Following implementation of

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Harumi Jyonouchi, MD
Division of Allergy/Immunology/Pulmonology/ID
Department of Pediatrics, University of Medicine and Dentistry of New Jersey
185 South Orange Ave., University Heights, Newark, NJ 07103-2714 (USA)
Tel. +1 973 972 5066, Fax +1 973 972 6443

a cf/gf diet, parents often report improvement in speech and communication skills, less hyperactivity, better focusing, and better night sleep with concurrent resolution of GI symptoms. However, it is poorly understood how the cf/gf diet may affect ASD children and there are no objective measures to identify individuals who are likely responders to dietary intervention.

The clinical relevance of gastrointestinal (GI) immune reactions to dietary proteins (DPs) has been a controversial topic partly due to limited epidemiological data and poor understanding of underlying mechanisms. Recent progress in mucosal immunity indicates that IgE-mediated food allergy accounts for only a small portion of adverse reaction to DPs. Instead, cell-mediated immunity is thought to play a pivotal role and TNF- α produced by T cells and other lineage cells may be closely associated with the pathogenesis of cow's milk protein (CMP) intolerance [1–3]. In most ASD children, GI symptoms develop within the first 1 to 2 years of their lives, yet these children seldom reveal positive IgE antibodies against common DP antigen (Ag). GI workups in ASD children reveal ileocolonic lymphoid nodular hyperplasia and mucosal inflammation at high frequency [4, 5] similar to the gut pathology observed in DPI patients [2]. These findings may indicate that cellular immune response to DPs have a role in various GI symptoms revealed by ASD children.

To generate significant immune responses against DPs, activated Ag-presenting cells (APC, dendritic cells, macrophage/monocytes, etc.) must properly present DPs to T cells. Innate immunity plays a crucial role for activation of APC and subsequent AG sensitization [6]. Innate immunity is triggered by recognition of pathogen-associated molecular patterns (PAMPs) shared by groups of microbial pathogens [6–8]. Aberrant responses to PAMPs may lead to intolerance to benign environmental Ags as observed in DPI. One of the major PAMPs released by the intestinal microflora is lipopolysaccharide (LPS), one of the major endotoxins. We previously reported that ASD children produced excessive proinflammatory cytokines with a suboptimal dose of LPS at high frequency [9].

This study tested our hypothesis that aberrant innate (inflammatory) responses to LPS in the gut mucosal tissue may make ASD children more prone to sensitization to common dietary proteins. We assessed immune reactivity to DPs by peripheral blood mononuclear cells (PBMCs) from ASD children by measuring production of IFN- γ , IL-5, and TNF- α against common DPs. Both IFN- γ , a type 1 T cell (T1) cytokine, and TNF- α are implicated with CMP intolerance [1, 10–12]. IL-5 was measured as a

representative type 2 T cell (T2) cytokine [13]. For controls, we used PBMCs from normally developing unrelated children, healthy siblings, and children with DPI. The results were evaluated in comparison with proinflammatory (IL-1 β , IL-6, and TNF- α) and counter-regulatory (sTNFR1, sTNFR2, and IL-1ra) cytokines with a stimulus of LPS.

Patients and Method

Study Subjects

The study included ASD children (n = 72), children with DPI (n = 24), healthy unrelated children (n = 15), and developmentally normal siblings (n = 26) (table 1). ASD children include autism (n = 62), PDD-NOS (n = 9) and Asperger's syndrome (n = 1). Parents, teachers, and therapists reported improvement of behavior, speech and/or GI symptoms following implementation of a cf/gf/soy-free, (cf/sf/gf) diet in 56/60 ASD patients (table 1). Elevated IgE levels (>100 IU/ml), presence of allergen-specific IgE antibody (Ab), and/or positive reactivity to prick skin test (PST) was reported in 13/72 (19.1%) ASD children; the frequency of atopy is equivalent to that observed in healthy siblings (6/26, 23%) and in the general population [14]. Blood samples were obtained when study subjects were afebrile, not on antibiotics or steroids, and did not have symptoms indicating viral syndrome or other common childhood infections. In most DPI patients, blood samples were obtained prior or within 12 weeks following implementation of an elimination diet. All the ASD patients studied had either never been on steroid or were off steroid at least 4 weeks. Any ASD patient who had tried IV secretin was off secretin for >6 weeks when the blood samples were obtained. The study protocol was approved by the IRB committee, University of Minnesota, and a signed consent form was obtained prior to sample obtainment.

Assessment of Immune Response

Innate Immune Responses. PBMCs were obtained by Ficoll-Hypaque density gradient and were cultured overnight with lipopolysaccharide (LPS; 0.1 μ g/ml, GIBCO-BRL, Gaithersburg, Md., USA) [9]. We elected to use LPS, since LPS produced by gram(-) bacteria is likely the major microbial product recognized by innate immunity in the GI mucosa [7, 8]. For assessing innate immune responses, we measured the levels of proinflammatory cytokines (TNF- α , IL-1 β , IL-6) and counter-regulatory mediators (sTNFR1, sTNFR2, and IL-1ra) in the supernatant. LPS concentration was chosen on the basis of our previous study [9].

Adaptive Immune Responses against Dietary Proteins. We assessed immune responses against DPs by measuring production of IFN- γ , TNF- α , and IL-5 by PBMCs. IFN- γ and IL-5 were selected as representative T1 and T2 cytokines, respectively. Macrophage and monocyte lineage cells are generally regarded as a major source of TNF- α and they do not respond to soluble protein Ag such as DPs. However, TNF- α can be produced by other lineage cells including T cells [15, 16] and activated T cells can in turn induce TNF- α production by macrophages and monocytes. In fact, TNF- α production by PBMCs has been implicated with pathogenesis of CMP intolerance and dietary protein enterocolitis [1, 10, 17]. Thus we measured TNF- α production in this setting.

Table 1. Demographics of the study subjects

Study group	Age years	Sex M/F	Clinical features and laboratory results
ASD patients n = 72	1–17 median: 5	59/13	Diagnosis: Asperger's syndrome: 1; PDD-NOS: 9; Autism: 62 Response to an elimination diet: 56/61 (91.8%) ² IgE (>100 IU/ml) and/or positive allergen specific IgE/PST: 13/72 (18.1%) ³
DPI children n = 24	0.5–13 median: 4	17/7	Response to an elimination diet: 24/24 IgE (>100 IU/ml) and/or positive allergen specific IgE/PST: 1/14 (7.1%)
Control children n = 15	1–16 median: 9	12/3	
Siblings n = 26	0.5–20 median: 5.5	18/8	IgE (>100 IU/ml) and/or positive allergen specific IgE/PST: 6/26 (23%) ³

ASD was diagnosed on the basis of DSM-IV and/or ICD-10 criteria, ADI-R (Autistic Diagnostic Interview – Revised) and ADOS (Autistic Diagnostic Observational Schedules). Three patients with autism have seizure disorders confirmed with EEG and have been treated with valproic acid. DPI was diagnosed with onset of various GI symptoms (colic, diarrhea, GI cramping, vomiting, etc.) several hours after ingestion of proteins, poor weight gain, lack of IgE antibody against dietary proteins, marked improvement with an elimination diet and use of free amino acid formulas, and recurrence of symptoms with reintroduction of DPs to which the child reacts.

¹ The reference values obtained from control adults (n = 17, age 20–45 years) were equivalent to those obtained from control children.

² Clinical improvement observed by therapists/teachers/parents following implementation of a casein-free, gluten-free, and/or soy-free (cf/gf/sf) diet includes improvement of GI symptoms/speech/autistic behavior, less hyperactivity and better focusing/night time sleep.

³ The frequencies of elevated IgE and positive allergen-specific IgE/PST (prick skin test) reactivity are equivalent to those observed in general population (around 20%).

Table 2. T cell cytokine production against dietary protein antigens

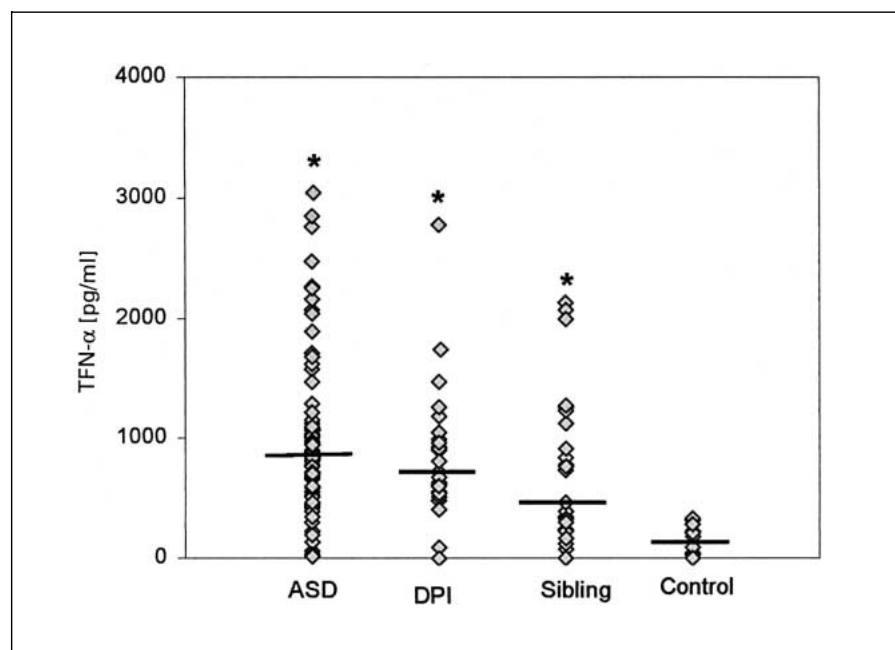
Cytokines measured pg/ml	ASD children (n = 72)				Children with DPI (n = 24)				Siblings (n = 26)				Control children (n = 15)	
	median	range	p	>2SD ¹ , %	median	range	p	>2SD, %	median	range	p	>2SD, %	median	range
<i>IFN-γ</i>														
Medium	<3.9	<3.9–701.7		19.4	6.8	<3.9–802.3		16.7	<3.9	2.8–245.3		7.7	<3.9	<3.9–111.9
Milk	208.6	0.6–1,850	<0.005 ²	41.6	210.4	<3.9–946.9	<0.005	37.5	97.9	<3.9–874.8	<0.05	26.9	39.4	<3.9–146.9
Gliadin	72.8	0.2–854.1	<0.02	19.4	144.2	<3.9–1,154	<0.005	48.5	195.7	<3.9–524		19.2	43.5	1.4–232.4
Soy	114.3	0.5–1,029	<0.05	27.8	206.2	2.8–996.6	<0.02	41.7	45.5	3.9–330.6		11.5	38.7	<3.9–227.1
<i>TNF-α</i>														
Medium	5.6	0.4–1,177		31.9	7.5	3.3–715		41.7	<3.9	<3.9–387.8		19.2	4.5	1.1–33.1
Milk	891.4	28–3,045	<0.001	84.7	762.2	<3.9–2,774	<0.001	91.7	494.8	<3.9–2,069	<0.02	53.8	214.5	<3.9–325.3
Gliadin	356.6	2.1–3,232	<0.001	61.1	493.2	50.6–1,088	<0.001	87.5	139.3	<3.9–1,489		38.5	92.9	5.53–216.7
Soy	89.9	2.6–1,087		31.9	317.8	<3.9–4,043 ³	<0.005	62.5	78.9	<3.9–585.1		11.5	41	1.9–281.9
<i>IL-5</i>														
Medium	4.3	2.1–130	<0.02	19.4	<3.9	<3.9–142.6		20.8	8.2	0.3–57.5		11.5	<3.9	<3.9–62.4
Milk	<3.9	2.0–168.5		27.8	<3.9	<3.9–484.1		16.7	<3.9	0.3–102.1		15.4	<3.9	<3.9–41.5
Gliadin	<3.9	0.5–248.4		25.0	<3.9	3.7–142.6		16.7	<3.9	<3.9–53.9		19.3	<3.9	<3.9–11.9
Soy	34.8	2.5–328.2		11.1	19.4	3.2–310.5		4.2	41.3	<3.9–327.2		3.8	40.1	<3.9–173.7

¹ The percentage of patients whose PBMCs produced >2SD above the CM values.

² p values as compared to controls (Mann-Whitney test)

³ Higher than those produced by ASD PBMCs (p < 0.05).

Fig. 1. TNF- α production by PBMCs from ASD and DPI children, ASD siblings, and control normal children in response to cow's milk protein. * Significantly higher than control children (Mann Whitney test).



As representative DPs, we used crude cow's milk and soy protein extracts (kindly provided by Ross Products Division/Abbott Laboratories, Columbus, Ohio, USA) and gliadin, bovine casein, α -lactalbumin, and β -lactoglobulin (all obtained from Sigma, St. Louis, Mo., USA). We detected <1 ng/ml endotoxin in these DPs (1 mg/ml solution) (Endotoxin kit, Sigma). We used recall Ag other than dietary proteins (tetanus toxoid and dust mite extract), T cell mitogens [phytohemagglutinin, PHA (10 μ g/ml) and concanavalin A, Con A (1 μ g/ml)], and IFN- γ inducing cytokines [IL-12p70 (0.2 ng/ml) and IL-18 (1 ng/ml)] as positive control stimuli as reported before [9, 18].

We also measured IL-4, and IL-2 in a few study subjects and control adults. However, we did not observe significant production of IL-2 or IL-4 with DPs in this assay system.

ELISA

Cytokine levels in the culture supernatants were measured by enzyme-linked immunosorbent assay (ELISA). For measuring IFN- γ , TNF- α , IL-1 β , IL-4, IL-5, and IL-6, we used OptEIA™ Reagent Sets (BD Pharmingen, San Diego, Calif., USA). For measuring sTNFRI, and sTNFRII, we used matched antibody pairs and standards from R & D systems, Minneapolis, Minn., USA. Intra- and inter-variations of cytokine levels were less than 5%. The results were reproducible in 14 ASD patients and 8 DPI patients when two sets of samples were obtained at 3–4 months apart.

Statistical Analysis

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 to be significant.

Results

Responses to Dietary Proteins

The results are summarized in table 2. Specifically, 60/72 ASD PBMCs and 22/26 DPI PBMCs produced >2 standard deviations (SD) + the control mean (CM) value of TNF- α in response to CMP (fig. 1). Also, 30/72 ASD and 9/26 DPI PBMCs produced >2 SD + CM value of IFN- γ with CMP. DPI PBMCs also produced >2 SD + CM values of IFN- γ and TNF- α at high frequency (table 2). We also found higher TNF- α and IFN- γ production by sibling PBMCs with CMP. However, TNF- α production by ASD PBMCs with CMP was still higher than by sibling PBMCs ($p < 0.05$). In ASD siblings, 14/26 and 3/26 sibling PBMCs produced >2 SD + CM values of TNF- α and IFN- γ , respectively; these siblings did have symptoms of CMP intolerance.

With a stimulus of gliadin, 44/71 ASD and 21/25 DPI PBMCs produced >2 SD + CM values of TNF- α and 14/71 ASD and 10/26 DPI PBMCs produced >2 SD + CM values of IFN- γ . With soy protein, 20/72 ASD and 11/26 DPI PBMCs produced >2 SD + CM values of IFN- γ .

IL-5 production by PBMCs did not differ among the study groups except for higher spontaneous IL-5 production by ASD PBMCs as compared to control PBMCs. IFN- γ and TNF- α production against control recall Ags (tetanus toxoid and dust mite) did not differ among the

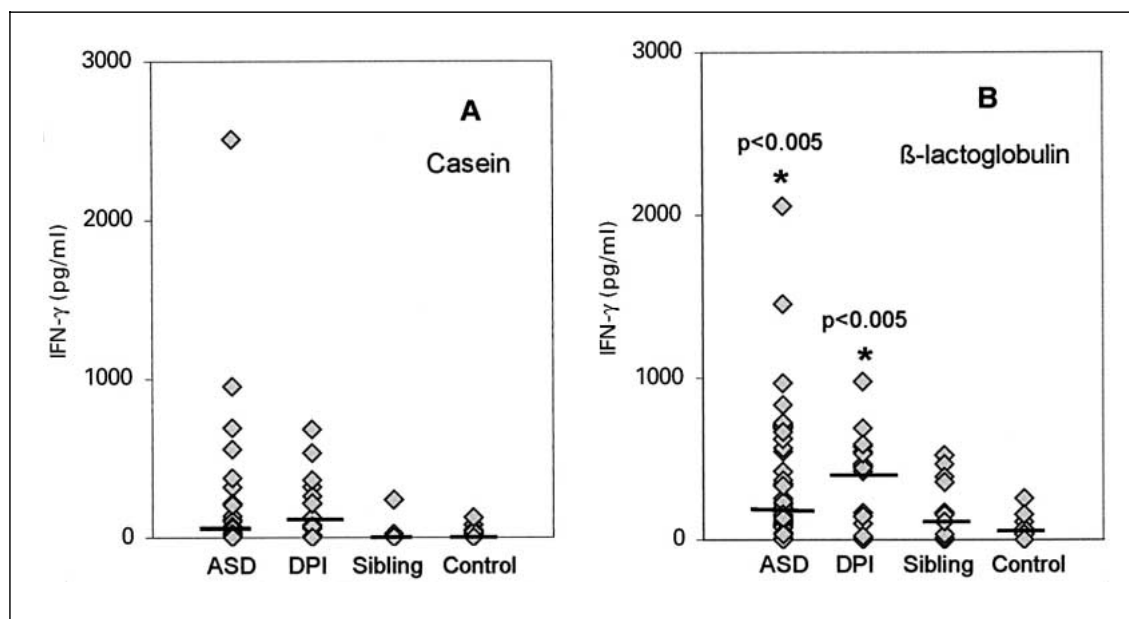


Fig. 2. IFN- γ production with stimuli of casein (A) and β -lactoglobulin (B) in all the study groups. * Significantly higher than control children (Mann Whitney test).

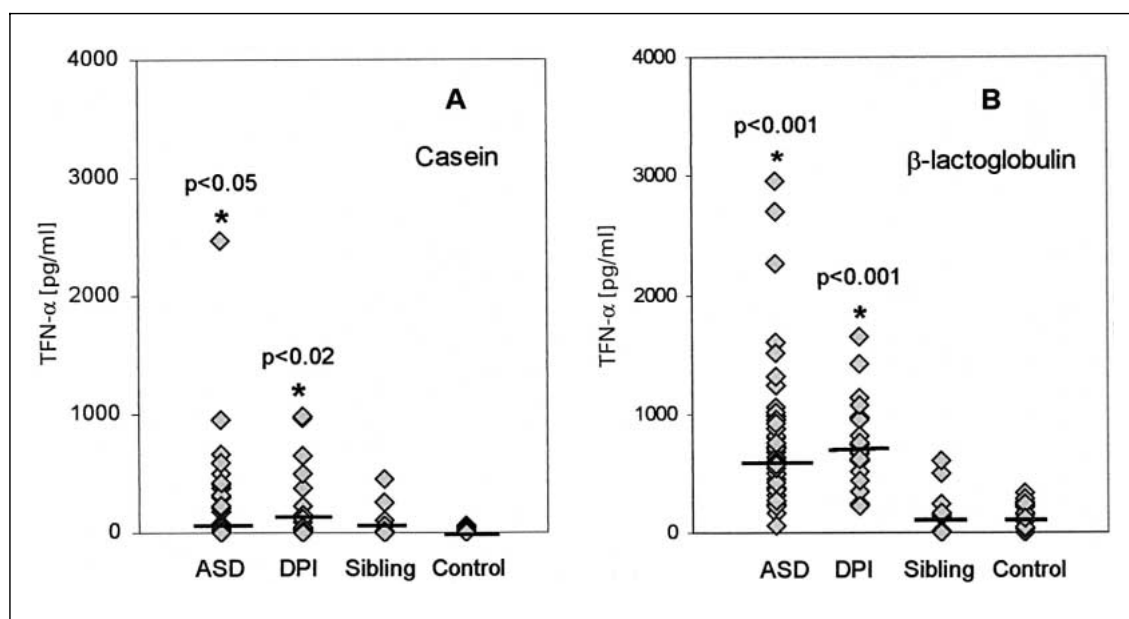


Fig. 3. TNF- α production with stimuli of casein (A) and β -lactoglobulin (B) in all the study groups. * Significantly higher than control children (Mann Whitney test).

study groups (data not shown). IFN- γ and TNF- α production by ASD PBMCs positively correlated with one another against each DP tested ($p < 0.001$), while in DPI PBMCs, only IFN- γ and TNF- α production against soy protein correlated significantly ($p < 0.005$). This is due to the fact that 13 and 10 out of 24 DPI PBMCs produced $>2SD + CM$ values of TNF- α with CMP and gliadin, respectively, but little IFN- γ (<100 pg/ml).

It is of note that parents/teachers/therapists reported improvement of GI symptoms along with some autistic behaviors in 61 of 63 ASD children who were tried on a cf/gf/sf diet. Among responders to the cf/gf/sf diet, 54/61 ASD PBMCs produced $>2SD + MC$ values of TNF- α and INF- γ against DPs, respectively.

Responses to Each Milk Protein Component

Both ASD and DPI PBMCs but not sibling PBMCs produced higher levels of IFN- γ and TNF- α in response to β -lactoglobulin and α -lactoalbumin (fig. 2, 3, and data not shown). IFN- γ and TNF- α production against casein were less remarkable in both ASD and DPI PBMCs (fig. 2, 3). IFN- γ produced by ASD, DPI, and sibling PBMCs with CMP positively correlated with IFN- γ produced with each milk component (casein, β -lactoglobulin, and α -lactoalbumin) ($p < 0.01$). In contrast, only ASD PBMCs revealed positive correlation between TNF- α production with CMP and that with each milk component ($p < 0.01$).

Relationship to Innate Immune Responses

As we reported before [9], ASD PBMCs produced $>2SD + CM$ values or proinflammatory cytokines (TNF- α , IL-6, and IL-1 β) with or without LPS (0.1 μ g/ml) at high frequency (table 3). DPI and sibling PBMCs tended to produce $>2SD + CM$ values of TNF- α , IL-6, and IL-1 less frequently than ASD PBMCs. Higher sTNFRII production was observed only in ASD PBMCs ($p < 0.05$ with medium only, and $p < 0.001$ with LPS) but not in DPI or sibling PBMCs. sTNFRI or IL-1ra levels produced did not differ among all the study groups.

TNF- α with CMP and gliadin in ASD PBMCs positively correlated with TNF- α and IL-6 production with LPS as summarized in table 4. TNF- α production with CMP by ASD PBMCs also positively correlated with IL-1 β production with LPS. In contrast, TNF- α production with DPs by DPI PBMCs revealed positive correlation only in 2 instances: TNF- α production with CMP and LPS and IL-1 β production with LPS and TNF- α production with gliadin. TNF- α production with CMP by ASD sibling PBMCs revealed positive correlation with TNF- α

Table 3. Percentage of patients with excessive proinflammatory cytokine production with LPS in the study groups.

Cytokines measured	ADC children (n = 72) >2SD + CM ¹	Children with DPI (n = 24) >2SD + CM	Siblings (n = 26) >2SD + CM
TNF- α			
Medium	40.3%	25.0%	30.7%
LPS	45.8%	25.0%	23.1%
sTNFRII			
Medium	22.2%	8.3%	3.8%
LPS	11.1%	8.3%	11.5%
IL-6			
Medium	36.1%	25.0%	11.5%
LPS	15.3%	8.3%	3.8%
IL-1 β			
Medium	37.5%	25.0%	20.7%
LPS	36.1%	33.3%	19.2%

¹ The percentage of patients whose PBMCs produced $>2SD$ above the control mean (CM) values.

and IL-1 β production with LPS. IFN- γ production with CMP by ASD PBMCs also correlated positively with TNF- α and IL-1 β production by LPS. Sibling PBMCs also revealed similar responses. Little correlation was observed between IFN- γ production against DPs and proinflammatory cytokine production with LPS in DPI PBMCs.

Discussion

This study evaluated production of IFN- γ , IL-5 and TNF- α against major DPs associated with DPI in ASD children in comparison with proinflammatory and counter-regulatory cytokine production with LPS, a representative endotoxin. We found elevated IFN- γ and TNF- α production ($<2SD + CM$) by PBMCs against these DPs in ASD children as frequently as observed in DPI children. However, proinflammatory cytokine production by LPS is better correlated with IFN- γ /TNF- α production against DPs in ASD PBMCs than in DPI or sibling PBMCs. Thus our results indicate that aberrant innate immune responses against LPS may predispose ASD children to increase in immune reactivity to common DPs.

ASD children frequently exhibit GI symptoms. Others have reported colitis with ileal lymphoid nodular hyper-

Table 4. Correlation between proinflammatory cytokine production with LPS and TNF- α /IFN- γ production against common DPs

	TNF- α production with LPS			IL-6 production with LPS			IL-1 β production with LPS		
	ASD (n = 72)	DPI (n = 24)	Sibling (n = 26)	ASD (n = 72)	DPI (n = 24)	Sibling (n = 26)	ASD (n = 72)	DPI (n = 24)	Sibling (n = 26)
<i>TNF-α production against DPs</i>									
Cow's milk protein	0.577 ¹ p < 0.0001	0.404 p < 0.02	0.389 p < 0.01	0.257 p < 0.005	-0.216	0.234	0.268 p < 0.002	0.116	0.372 p < 0.01
Gliadin	0.299 p < 0.01	0.310	0.222	0.176 p < 0.05	0.018	0.095	0.136	0.456 p < 0.005	0.127
Soy protein	0.07	0.310	0.124	-0.055	-0.123	-0.233	0.069	0.034	-0.116
<i>IFN-γ production against DPs</i>									
Cow's milk protein	0.317 p < 0.001	0.141	0.455 p < 0.001	0.05	-0.223	0.148	0.234 p < 0.01	0.290	0.274 p = 0.052
Gliadin	0.189 p < 0.05	0.152	0.266	-0.027	-0.375	0.106	0.163	0.173	0.02
Soy protein	0.015	0.275	0.326 p < 0.05	-0.085	-0.275	-0.097	0.083	0.005	0.079

There was no correlation with IL-6 or IL-1 β production with LPS and cytokine production against DPs in normal control children. However, TNF- α production with CMP and gliadin in normal children correlated positively with TNF- α production with LPS (p < 0.05) although the amount of cytokines produced was low as shown in table 2.

¹ Correlation coefficient by two-tailed Kendall τ -b test.

plasia in ASD children; the gut epithelium appears particularly affected [4, 5]. Other abnormal GI findings including increased gut permeability [19], disaccharide malabsorption [20], and increase in calprotectin (a marker for gut inflammation) in the stool [19] also support the presence of chronic GI inflammation in ASD children. Parents of ASD children often report improvement of GI symptoms and even some autism behavioral symptoms with a restricted diet (a cf/gf/sf diet). Such observation may indicate that immune reactivity to DP in the gut mucosa is associated with GI inflammation in some ASD children.

Most ASD children exhibit no immediate GI/systemic symptoms with intake of DPs, but develop GI symptoms several hours after ingestion of DPs. In non IgE-mediated immune responses to DPs, production of inflammatory cytokines, especially TNF- α in the gut mucosa, appears to play a vital role in its pathogenesis [7, 11, 21, 22]. In DP-sensitive enteropathy, local immune reactions to DPs in the gut augment production of inflammatory cytokines, leading to chronic gut inflammation and increase in the gut permeability. This may further lead to sensitization to other DPs, resulting in multiple DPI. In patients with CMP intolerance, others reported the presence of circulat-

ing PBMCs secreting TNF- α /IFN- γ against CMP with TNF- α production against CMP being in good correlation with clinical symptoms [1, 10, 11, 17]. Thus we also determined IFN- γ and TNF- α production by PBMCs with DPs in ASD children as a possible objective marker for DPI.

Our results revealed that PBMCs from a large number of ASD children produced excessive amounts of TNF- α and IFN- γ (>2SD + CM) but not IL-5 against CMP and gliadin. These results were comparable to those observed in DPI PBMCs. Excessive TNF- α production against CMP and gliadin was more remarkable than IFN- γ production in both ASD and DPI PBMCs. TNF- α and IFN- γ against soy protein were less remarkable in ASD PBMCs than in DPI PBMCs. This may be partly due to lack of exposure to soy products in some ASD children; most children with DPI were tried on soy formula before switching to hypoallergenic formula. In summary, our study revealed the frequent presence of PBMCs secreting IFN- γ and TNF- α with CMP/gliadin in ASD children as observed in DPI children, indicating that immune reactivity to DPs has a role in GI inflammation observed in ASD children.

Although the elimination diet and rechallenge of suspected DPs are still the gold standards for DPI, it is not

easy to conduct such procedures in many ASD children. It will be helpful to have a simple objective measure for assessing possible DPI in ASD children. Interestingly, among ASD children who clinically responded to a cf/gf/sf diet by parental reports, 88.5% of their PBMCs produced $>2SD + CM$ values of TNF- α with CMP and/or gliadin while only 49.2% of their PBMCs produced $>2SD + CM$ values IFN- γ . These findings indicate that TNF- α production by PBMCs with DPs can be a simple objective marker for assessing the presence of DPI in ASD children.

Both IFN- γ and TNF- α production against DPs is most remarkable against CMP in ASD children. Major protein components of CMP include casein, α -lactalbumin, and β -lactoglobulin. ASD, DPI, and sibling PBMCs produced more TNF- α with β -lactoglobulin and α -lactalbumin than with casein. In contrast, IFN- γ production with CMP correlated to all three major milk proteins in ASD, DPI, and sibling PBMCs. These results indicate that different components of milk proteins may stimulate PBMCs in a different manner.

We observed that TNF- α production against CMP/gliadin tends to be more elevated in both ASD and DPI PBMCs than IFN- γ production with these DPs. T cells are a major cellular source of IFN- γ with Ag stimuli including DPs, while macrophage and monocyte lineage cells are regarded as a major source of TNF- α . However, other lineage cells including T cells and epithelial cells can produce TNF- α [15, 16, 23, 24]. Activated T cells produce various cytokines that in turn activate macrophages/monocytes to produce TNF- α . This may be one possible explanation of generally higher TNF- α production than IFN- γ production with DPs in ASD and DPI PBMCs.

Upon activation, T cells differentiate into the effector-stage T cell subsets with distinguished patterns of cytokine production [13, 25]. T1 cells produce T1 cytokines (IFN- γ , IL-2, and TNF- β) and facilitate phagocytic cell-mediated immune responses. Type 2 (T2) T cells produce T2 cytokines (IL-4, IL-5, and IL-13) and induce eosinophil dominant immune responses [13, 25]. To test the role of T1 vs. T2 responses against DPs, we also measured production of IL-5, a representative T2 cytokine. We did not detect marked increase in IL-5 production by PBMCs against DPs in any of the study groups. As stated above, T1 cytokines generally activate monocyte/macrophage lineage cells to produce TNF- α and we found marked increase in TNF- α production with DPs in a large number of ASD children (table 2, fig. 1). These results indicate that DP-specific T cells in the peripheral blood may be predominantly T1 cells in ASD children. However, others

report that T2 cells may be dominant in the gut mucosa on the basis of histochemical analysis [4] and an increase in Th2 cells in ASD children [26]. These results may be due to difference in methodologies and cellular sources used for the assay; they did not investigate DP-specific T cells.

To generate immune reactivity to any Ag, the activation of Ag-independent innate immunity is crucial. Innate immunity generally produces rapid inflammatory responses at the site of pathogen invasion and activates Ag-presenting cells (APC). Proinflammatory cytokines (IL-1 β , IL-6, and TNF- α) produced by innate immunity also affects HPA axis inducing stress responses [27–30]. Innate immunity plays a crucial role for activation of APC and subsequent Ag sensitization [6]. Aberrant innate immune responses may lead to undesirable immune responses to benign environmental Ags such as DPs. Innate immunity is commonly triggered by PAMPs, conserved microbial molecular patterns. One of the major PAMPs released by the intestinal microbes in LPS, an endotoxin. ASD children produce excessive proinflammatory cytokines with a suboptimal dose of LPS at high frequency as reported before [9]. Therefore, we tested our hypothesis that aberrant innate immunity with LPS is associated with increased cellular, Th1 cell dominant, immune reactivity against DPs in ASD children.

Our results revealed that a significant correlation between TNF- α and IL-6 production and TNF- α and IFN- γ production against CMP/gliadin in ASD PBMCs. In contrast, DPI PBMCs produced higher levels of TNF- α but not other cytokines with LPS than control PBMCs and positive correlation was much less evident between proinflammatory cytokine production with LPS and IFN- γ /TNF- α production with DPs in DPI PBMCs. These results indicate that in some ASD children but not in DPI children, aberrant innate immune responses with LPS may be partly associated with cellular immune reactions against DPs. That is to say, excessive or dysregulated innate inflammatory responses to LPS stimuli predispose these children to sensitization to DPs. The resultant chronic GI inflammation may further augment some behavioral symptoms of autism in these children secondary to GI discomfort and possibly by increased local production of inflammatory cytokines including TNF- α .

In summary, our results revealed the presence of DP responsive PBMCs in ASD children at high frequency. This finding indicates an association between immune reactivity to DP and GI symptoms observed in ASD children. More importantly, our findings also indicate the possibility that sensitization to DPs may be associated

with aberrant innate immune response with LPS, an endotoxin. Further studies of innate immune responses in the gut mucosa may shed light on the pathogenesis of GI symptoms associated with ASD and the effects of dietary intervention on some behavioral symptoms of autism.

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