

# Spontaneous Mucosal Lymphocyte Cytokine Profiles in Children with Autism and Gastrointestinal Symptoms: Mucosal Immune Activation and Reduced Counter Regulatory Interleukin-10

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A lymphocytic enterocolitis has been reported in a cohort of children with autistic spectrum disorder (ASD) and gastrointestinal (GI) symptoms. This study tested the hypothesis that dysregulated intestinal mucosal immunity with enhanced pro-inflammatory cytokine production is present in these ASD children. Comparison was made with developmentally normal children with, and without, mucosal inflammation. Duodenal and colonic biopsies were obtained from 21 ASD children, and 65 developmentally normal pediatric controls, of which 38 had signs of histological inflammation. Detection of CD3<sup>+</sup> lymphocyte staining for spontaneous intracellular TNF $\alpha$ , IL-2, IL-4, IFN $\gamma$ , and IL-10, was performed by multicolor flow cytometry. Duodenal and colonic mucosal CD3<sup>+</sup> lymphocyte counts were elevated in ASD children compared with noninflamed controls ( $p < 0.03$ ). In the duodenum, the proportion of lamina propria (LP) and epithelial CD3<sup>+</sup>TNF $\alpha$ <sup>+</sup> cells in ASD children was significantly greater compared with noninflamed controls ( $p < 0.002$ ) but not coeliac disease controls. In addition, LP and epithelial CD3<sup>+</sup>IL-2<sup>+</sup> and CD3<sup>+</sup>IFN $\gamma$ <sup>+</sup>, and epithelial CD3<sup>+</sup>IL-4<sup>+</sup> cells were more numerous in ASD children than in noninflamed controls ( $p < 0.04$ ). In contrast, CD3<sup>+</sup>IL-10<sup>+</sup> cells were fewer in ASD children than in noninflamed controls ( $p < 0.05$ ). In the colon, LP CD3<sup>+</sup>TNF $\alpha$ <sup>+</sup> and CD3<sup>+</sup>IFN $\gamma$ <sup>+</sup> were more frequent in ASD children than in noninflamed controls ( $p < 0.01$ ). In contrast with Crohn's disease and non-Crohn's colitis, LP and epithelial CD3<sup>+</sup>IL-10<sup>+</sup> cells were fewer in ASD children than in

nondisease controls ( $p < 0.01$ ). There was a significantly greater proportion of CD3<sup>+</sup>TNF $\alpha$ <sup>+</sup> cells in colonic mucosa in those ASD children who had no dietary exclusion compared with those on a gluten and/or casein diet ( $p < 0.05$ ). There is a consistent profile of CD3<sup>+</sup> lymphocyte cytokines in the small and large intestinal mucosa of these ASD children, involving increased pro-inflammatory and decreased regulatory activities. The data provide further evidence of a diffuse mucosal immunopathology in some ASD children and the potential for benefit of dietary and immunomodulatory therapies.

**KEY WORDS:** Inflammation; mucosa; TNF $\alpha$ ; IL-10.

## INTRODUCTION

Autistic spectrum disorder (ASD) is a complex developmental disorder of childhood characterized by qualitative impairments in social interaction, deficits in verbal and nonverbal communication, and restricted repetitive and stereotyped patterns of behavior and interests, developing within the first 3 years of life (1). The prevalence of autism has increased substantially over the last decade in developed countries (2–4). There is a growing awareness of GI and immunological co-morbidity in some ASD children (5–10) that, for those with GI symptoms, may be associated with later onset of behavioral deterioration (11).

Previous studies have described a characteristic GI immunopathology in a subset of ASD children (5–10) in which chronic ileocolonic lymphoid nodular hyperplasia (LNH) and enterocolitis are key endoscopic and histological features. The mucosal lesion consists of a pan-enteric lymphocytic infiltrate, with a variable degree of acute inflammation and eosinophil infiltration. Focal deposition of serum IgG from ASD children which colocalised with complement C1q on the basolateral enterocyte membrane—changes not seen in histologically

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normal and inflamed mucosa of developmentally normal children and children with cerebral palsy—is suggestive that an autoimmune mechanism is involved in the pathology (9). Flow cytometric and immunohistochemical analyses of mucosal lymphocyte populations in ASD children have demonstrated qualitatively consistent abnormalities at different anatomical sites including duodenum, ileum, and colon (10).

Systemic immune aberrations consistent with a dysregulated immune response have been reported in ASD children. This includes raised pro-inflammatory cytokine production, in particular TNF $\alpha$  (12). However, there is an apparent divergence of opinion on the predominant polarity of the dysregulated immune response, with both raised IL-12 and raised IL-4 having been reported (13, 14).

Many ASD children are on gluten and casein exclusion diets and behavioral improvements have been reported (15, 16). The rationale for diet includes the removal of precursors for exorphins with their potential for neurotoxicity. A possible effect of these diets on the associated intestinal lesion also merits consideration, given the potential for immunologic reactivity to gluten and casein in the GI mucosa.

This study tested the hypothesis that mucosal immune dysregulation exists in ASD children undergoing investigation for GI symptoms, with an increase in pro-inflammatory cytokine producing CD3<sup>+</sup> lymphocytes. We sought to characterize spontaneous lymphocyte intracellular cytokine profiles, and to make comparisons between different anatomical sites (duodenum and colon), between different levels within the mucosa (epithelium and lamina propria (LP)), between ASD children on and not on an exclusion diet, and between ASD children and histologically inflamed and noninflamed, developmentally normal paediatric controls.

#### PATIENTS AND METHODS

In a prospective study we examined 86 children referred to the tertiary Paediatric Gastroenterology unit at the Royal Free Hospital, for investigation of GI symptoms. All patients required diagnostic small and/or large intestinal endoscopy and biopsy on clinical grounds. All GI diagnoses were made by experienced paediatric gastroenterologists, on the basis of clinical, serological, endoscopic, and histological criteria. Children were investigated consecutively in order of referral, in order to avoid selection bias. Inclusion criteria for ASD children were: GI symptoms sufficient to warrant invasive investigation, diagnosis of a pervasive developmental disorder according to the Diagnostic and Statistical Manual for Psychiatric

Disorders (DSM-III-R/DSM-IV) and International Classification of Diseases (ICD-10) criteria, where no other cause for their developmental disorder was identified, e.g. fragile X, and there was no contraindication to anaesthetic. Written informed parental consent was obtained in all cases prior to the procedure. Exclusion criteria were: insufficient biopsy material for analysis, and lack of flow cytometry facilities on the day of endoscopy. ASD children were not on any anti-inflammatory or immunomodulatory therapy. Of the ASD children ( $n = 21$ , mean age 7.7, range 2–16 with 15 males) 15 had chronic constipation, 4 had diarrhoea, and 2 had alternating constipation and diarrhoea. Abdominal pain was the referred symptom in many ASD children but was difficult to assess in the presence of an impaired ability to communicate. Some children were on dietary restriction including a gluten-free ( $n = 4$ ), casein free ( $n = 3$ ), and gluten/casein-free ( $n = 4$ ). The remaining 10 ASD children were on unrestricted diets. Dietary histories were obtained from the parents and cross-checked with inpatient nursing records. Biopsies ( $n = 3$ ) were obtained from the fourth part of the duodenum (D4) and from the transverse colon ( $n = 3$ ).

Developmental diagnoses in ASD children included autism ( $n = 20$ ) and Asperger's syndrome ( $n = 1$ ) and were made prior to referral to our unit by a suitably qualified paediatric psychiatrist, developmental paediatrician or psychologist. In previous studies examining similarly affected children, diagnoses have been supported by internal review by a paediatric psychiatrist (5, 7). ASD children shared a similar history of achieving normal developmental milestones, followed by developmental arrest, loss of acquired skills, and onset of aberrant behaviors.

Paediatric controls are described in Table I. The non-Crohn's colitis group (colitis) consisted of 2 children with ulcerative colitis and 11 with indeterminate colitis. Inclusion criteria for controls were: GI symptoms sufficient to merit ileocolonoscopy, a history of normal development, suitability of anaesthesia, and written parental consent as above. In the Crohn's disease group five children were not currently receiving therapy. Twelve were on dietary modification, including polymeric diet in five. Diet was used alone in two children and in combination with 5-ASA in five, Azathioprine in three, and Prednisolone in four. Two children were taking 5-ASA alone. In the children with colitis, therapy included Prednisolone and Azathioprine ( $n = 4$ ), topical Prednisolone ( $n = 1$ ), dietary intervention ( $n = 2$ ), and etanercept ( $n = 1$ ).

#### Histopathology

All mucosal biopsies underwent routine histological assessment. Haematoxylin and eosin-stained histological

**Table I.** Details of Control Groups

Patient category	No.	Mean Age (Range)	Sex (M)	No. of biopsies examined	
				Duodenum	Colon
Normal nondisease controls	18	9.3 (1–13)	10	12	10
Food intolerance	11	3.6 (1–12)	6	9	2
Coeliac disease	5	6.0 (2–13)	2	5	0
Non-Crohn's colitis (Colitis)	13	10.2 (3–15)	10	7	11
Crohn's disease	18	11.8 (2–17)	14	5	16
Histologically noninflamed	27	10.5 (2–15)	15	26	13

sections were then independently reviewed by a histopathologist, blinded to the cytokine data. Systematic, prospective scoring of tissue sections was performed using a standard *pro forma*, as described and validated previously with a high degree of interobserver reproducibility (8).

#### Flow Cytometric Assessment

Spontaneous mucosal intracellular cytokine profiles were assessed in intraepithelial lymphocyte (IEL) and LP lymphocyte (LPL) CD3<sup>+</sup>, CD3<sup>+</sup>CD8<sup>+</sup>, and CD3<sup>+</sup>CD8<sup>-</sup> populations. Single cell suspensions were prepared in two stages: first, the epithelial layer was removed using calcium-free Hanks' balanced salts solution (Sigma, Poole, Dorset, UK). The remaining LP tissue was digested with collagenase (2mg/mL; Sigma) for 3 h. Lymphocytes viability was >90%. Standard intracellular cytokine analysis was performed (17, 18), using anti-IFN- $\gamma$ -FITC, -IL-2-FITC, -TNF $\alpha$ -PE, and -IL-4-PE (R & D Systems, Oxford, UK) and anti-IL-10-PE (Pharminogen, Oxford, UK). Cells were labeled for 30 min with anti-CD3-PeCy5 and anti-CD8-APC (Dako, Ely, UK) antibodies prior to fixation with 4% paraformaldehyde for 10 min. Cells were washed, permeabilised in 1% saponin (Sigma), and stained for the respective intracellular cytokines for 30 min. Concentration and isotype-matched control antibodies (mouse IgG1-FITC, PC5 and IgG2a-PE, Coulter-Immunotech, Oxford, UK) were used to determine nonspecific binding. Unstained mucosal cells and peripheral blood mononuclear cells (PBMC) were used as further controls. Blocking assays with recombinant TNF $\alpha$  (R & D) were performed as described previously (17). In accordance with previous (17, 18) blocking studies, over 85% of the specific signal could be inhibited. In addition, nonpermeabilised cells were negative for TNF $\alpha$ , confirming intracellular staining. Multicolor flow cytometry was performed on a 4-channel flow cytometer (Dako) equipped with a 488 nm argon laser and 633 nm diode laser. Data were analyzed using Winlist Version 4.0. A tight scatter region was drawn around the lymphocyte population and back-gated

in relation to CD3<sup>+</sup> (10, 17, 18) and the percentage of cytokine producing cells calculated.

#### Statistical Comparisons

Data were analyzed in two different ways. First, data from disease specific groups, i.e. ASD, Crohn's disease, coeliac disease, and colitis were compared with nondisease controls and with each other. Second, in order to take into account heterogeneity in the degrees of inflammation in disease controls because of factors such as treatment, biopsies from disease controls were grouped together as inflamed and noninflamed according to histology of adjacent tissue. When comparing ASD children on the basis of dietary status, those on gluten and/or casein exclusion were considered as one group. All data are expressed as a percentage of positive cells per 10,000 recorded events, and shown as median and interquartile ranges. Statistical analysis was performed using the nonparametric Kruksal Wallis one way ANOVA test for multiple value comparisons and for group comparisons using the two-tailed Mann-Whitney *U* test, by SPSS software, version 10.1. Results were considered significant if  $p < 0.05$ .

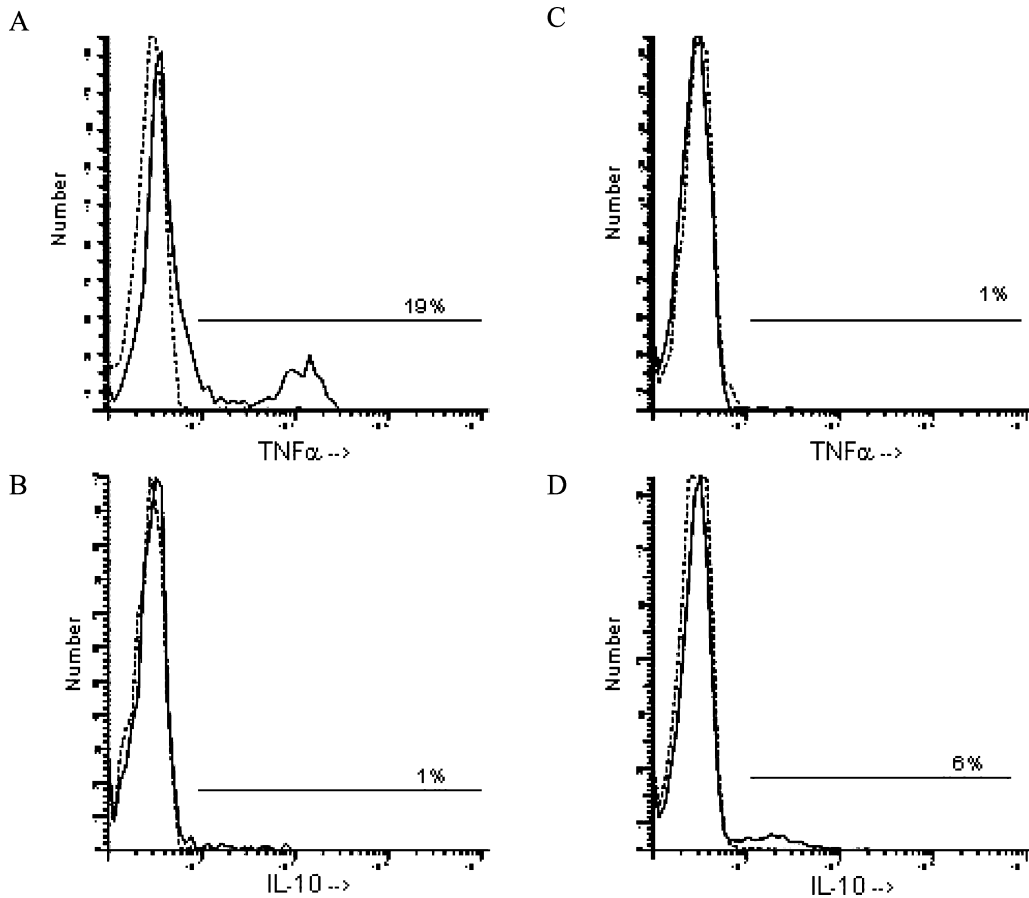
#### Ethical Approval

These studies were approved by the Ethical Practices Committee of the Royal Free Hampstead NHS Trust. Approval was obtained for taking research biopsies for this study. Written parental consent was obtained in each case.

## RESULTS

#### Cytokine Profiles of Duodenal Lymphocytes

*Lamina Propria.* Mucosal CD3<sup>+</sup> cell counts, expressed as a percentage of events within the lymphocytes gated region, were significantly elevated in the duodenal LP in ASD children ( $58.8 \pm 4.4$ ) compared with nondisease, noninflamed controls ( $42.3 \pm 4.9$ ,  $p < 0.04$ ) and reached levels similar to those for inflamed controls ( $58.5 \pm 6.3$ ). The most striking finding was the



**Fig. 1.** A representative histogram of multicolor flow cytometry analysis of  $TNF\alpha^+CD3^+$  and  $IL-10^+CD3^+$  duodenal lymphocytes in an autistic child with gastrointestinal symptoms (A and B, respectively) and a typically developing child with no evidence of histological inflammation (C and D, respectively). The dotted line histogram represents nonspecific staining using concentration and isotype matched antibodies. Similar staining was observed for colonic lymphocytes.

relative proportion of  $CD3^+TNF\alpha^+$  cells in the duodenal LP, which was substantially greater in ASD children than in nondisease controls ( $p < 0.001$ ), histologically inflamed controls (Fig. 1), food intolerance (FI) controls, and IBD controls ( $p < 0.02$ ; Table II). There was no difference compared with coeliac disease ( $p > 0.05$ ). In ASD children,  $TNF\alpha$  staining was elevated equally, in  $CD3^+CD8^+$  and  $CD3^+CD8^-$  populations. Within the duodenal LP, the proportion of  $CD3^+IL-2^+$  cells was significantly greater in ASD children than in nondisease controls ( $p < 0.02$ ; Table II), noninflamed controls ( $p < 0.001$ ; Fig. 1), FI, IBD controls ( $p = 0.02$ ; Table II), and inflamed controls ( $p = 0.01$ ; Fig. 1), but not coeliac disease controls. Similarly, the proportion of  $CD3^+IFN-\gamma^+$  cells was significantly greater in ASD children than in nondisease controls ( $p < 0.05$ ) and inflamed, FI, and IBD controls ( $p < 0.03$ ; Fig. 1, Table II) but was lower than in coeliac disease controls ( $p < 0.03$ ). In ASD children,

increased  $IFN-\gamma$  and  $IL-2$  staining was confined to the  $CD3^+CD8^+$  population (data not shown).  $CD3^+$  lymphocyte staining for the  $T_H2$  cytokine  $IL-4$ , although greater in ASD children than in all control groups, is only significantly elevated compared with IBD controls ( $p = 0.03$ ). Conversely, there was a significantly lower proportion of regulatory  $CD3^+IL-10^+$  T cells in ASD children compared with noninflamed controls ( $p < 0.045$ ; Fig. 2).

*Epithelium.* In the epithelium, the proportions of  $CD3^+TNF\alpha^+$ ,  $CD3^+IL-2^+$ ,  $CD3^+IFN-\gamma^+$ , and  $CD3^+IL-4^+$   $CD3^+$  cells were significantly greater, and  $CD3^+IL-10^+$  significantly lower in ASD children than in noninflamed controls ( $p < 0.03$ ; Figs. 1 and 2). The proportion of  $CD3^+IL-4^+$  cells in ASD children is significantly greater than in coeliac disease and IBD controls ( $p < 0.03$ ; Table II). The proportion of  $CD3^+IL-10^+$  cells in ASD children was significantly lower than in FI and IBD controls ( $p < 0.04$ ).

**Table II.** Lamina Propria Lymphocytes (LPL) and Intraepithelial Lymphocyte (IEL) Cytokine Profiles in Duodenal Mucosa

		Nondisease	Coeliac	FI	IBD	ASD
LPL	TNF $\alpha$	1.49 0.63–4.2	9.56* 3.55–36.26	5.79* 2.7–7.49	0.70 <sup>†</sup> 0.32–4.18	21.14* <sup>§¶</sup> 13.04–25.4
	IL-2	0.39 0.10–0.85	11.70 0.40–35.77	0.61 0.43–0.85	0.45 0.30–1.41	1.99* <sup>§¶</sup> 0.70–11.98
	IL-4	1.02 0.62–3.13	0.80 0.31–1.86	2.01 1.27–20.26	0.80 0.30–2.92	3.56 <sup>¶</sup> 0.79–8.10
	IFN $\gamma$	0.50 0.33–2.35	21.53* 2.54–26.40	0.66 <sup>†</sup> 0.10–1.70	0.57 <sup>†</sup> 0.25–2.17	1.52* <sup>§¶</sup> 0.90–8.22
	IL-10	3.60 0.60–8.90	0.64 0.51–1.55	3.53 0.19–17.41	2.54 0.57–6.67	1.20 0.16–4.20
	IEL	TNF $\alpha$	1.53 0.70–4.97	7.52* 3.16–21.69	6.07 2.82–14.66	1.54 0.90–18.52
	IL-2	0.72 0.20–2.27	5.79 1.17–8.32	2.33* 1.38–3.51	1.73 0.96–3.99	3.49* 0.88–5.90
	IL-4	0.89 0.00–10.68	0.40 0.04–1.07	2.87* <sup>†</sup> 1.05–14.94	1.08 0.65–2.00	3.81* <sup>†¶</sup> 1.07–9.03
	IFN $\gamma$	0.30 0.00–1.77	6.28* 2.06–13.75	1.95 1.10–3.24	0.73 <sup>†</sup> 0.00–3.91	3.46* <sup>¶</sup> 0.88–10.48
	IL-10	4.00 1.14–9.84	0.20 0.00–1.17	5.89 2.09–18.33	4.19 1.05–10.88	0.44* <sup>§¶</sup> 0.00–4.11

Note. Median and interquartile ranges are shown for intracellular CD3<sup>+</sup> cytokine profiles in nondisease control children and those with coeliac disease, food intolerance (FI), inflammatory bowel disease (IBD), and ASD.

\* $p < 0.05$  compared nondisease controls.

<sup>†</sup> $p < 0.05$  compared with coeliac disease.

<sup>§</sup> $p < 0.05$  compared with FI.

<sup>¶</sup> $p < 0.05$  compared with IBD.

Overall, there was no correlation between age or sex and cytokine profiles in any group. Neither dietary restriction nor degree of inflammation significantly was associated with altered cytokine staining in ASD children (data not shown).

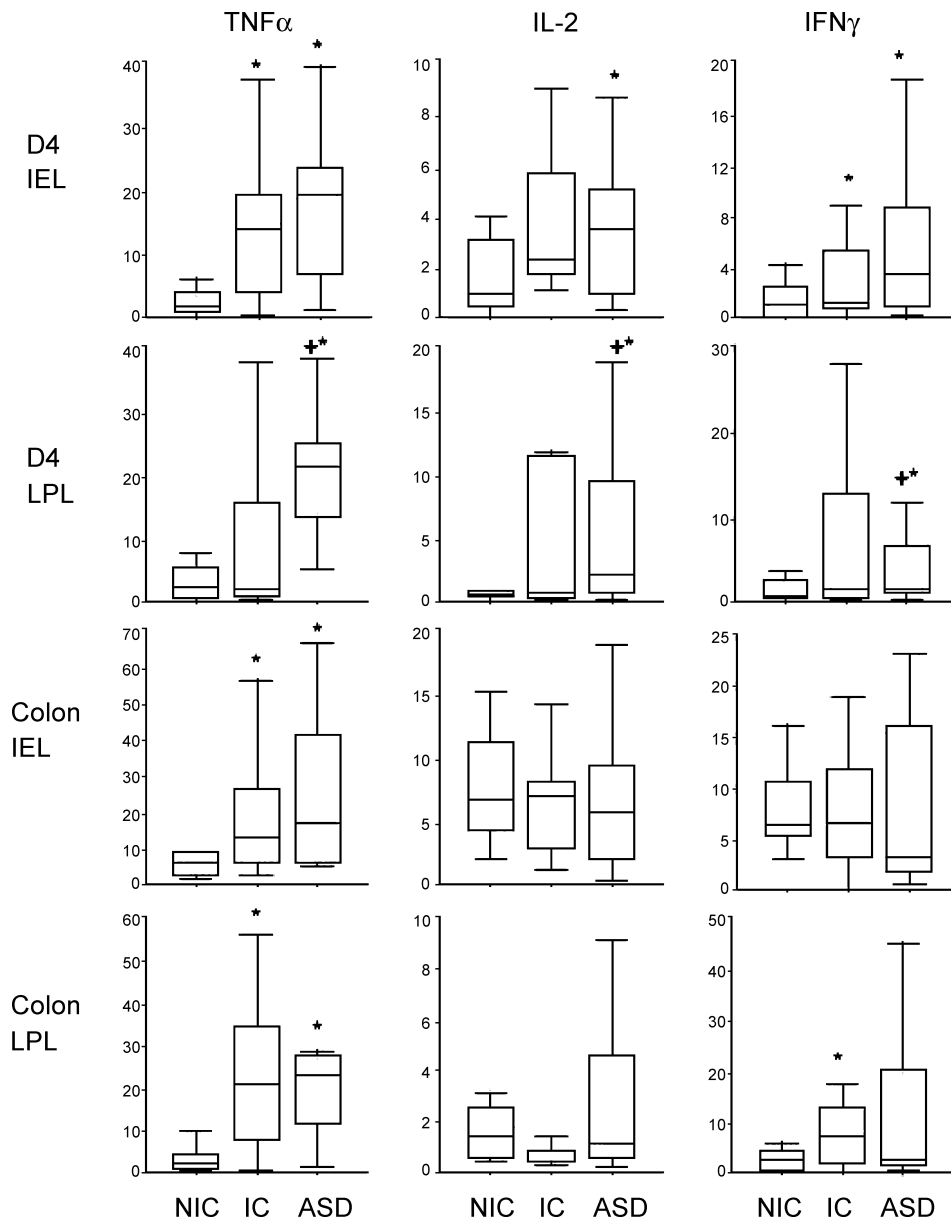
**Upper GI Pathology.** Upper GI biopsies were available from 17 ASD children. Eight of 17 (47%) showed acute and/or chronic inflammation in the oesophagus (2 of 17; 12%), stomach (5 of 17; 30%), and duodenum (1 of 17; 6%). An excess of intraepithelial lymphocytes was observed in the duodenal mucosa in 3 of 17 (18%) biopsies; an excess mucosal eosinophil infiltrate was documented in 3 of 17 (18%). There was no LN<sub>H</sub> in the upper GI biopsies and no villous atrophy was detected in duodenal mucosa. Systematic histological review of all non-inflamed nondisease controls, using the same proforma, confirmed the absence of acute and chronic inflammation, with no detectable increase in either eosinophils or IEL in the mucosa. Upper GI biopsies from four patients with FI and three with inflammatory bowel disease (IBD) showed mild chronic inflammation. *H. pylori* was not detected in any biopsy and coeliac disease was not present in any of the ASD children. On serology, one child had raised antigliadin IgG antibodies but all were negative for antigliadin IgA antibodies and antiendomysial antibodies. Eight ASD children had raised platelet counts above the

upper paediatric reference range (>400); however, the mean counts for the group were within the normal range.

#### Cytokine Profiles of Colonic Lymphocytes

**Lamina Propria.** In ASD children, colonic LP mucosal CD3<sup>+</sup> cell counts, were significantly greater compared with noninflamed controls ( $41.43 \pm 7.7\%$  and  $55.0 \pm 4.7\%$ ,  $p = 0.04$ ) and reached levels similar to those in inflamed controls ( $53.7 \pm 3.0\%$ ). As in the duodenum, the proportion of CD3<sup>+</sup>TNF $\alpha$ <sup>+</sup> cells in ASD children was significantly greater than in noninflamed controls ( $p < 0.002$ ), and reached a level similar to those seen in the inflamed control group (Fig. 1, Table III), and colitis and Crohn's disease controls, analyzed separately. No difference in mean fluorescence intensity for spontaneous intracellular TNF $\alpha$  staining was noted between Crohn's disease and ASD children. The proportions of CD3<sup>+</sup>IL-2<sup>+</sup>, CD3<sup>+</sup>IL-4<sup>+</sup>, and CD3<sup>+</sup>IFN- $\gamma$ <sup>+</sup> cells were similar in ASD children and noninflamed controls. The proportions of CD3<sup>+</sup>IFN- $\gamma$ <sup>+</sup> and CD3<sup>+</sup>TNF $\alpha$ <sup>+</sup> cells in inflamed controls were significantly greater than noninflamed controls ( $p < 0.01$ , Table III). The proportion of CD3<sup>+</sup>IL-10<sup>+</sup> cells was significantly lower in ASD children compared with noninflamed controls ( $p < 0.001$ ) but not inflamed controls ( $p > 0.5$ ; Fig. 2).





**Fig. 2.** Mucosal CD3<sup>+</sup> cytokine profiles in ASD children compared with histologically noninflamed controls (NIC) and histologically inflamed controls (IC). Percentage of TNFα<sup>+</sup>, IL-2<sup>+</sup>, and IFNγ<sup>+</sup> cells are shown at different sites (duodenum and colon) and different levels (epithelial and lamina propria compartments). \**p* < 0.05 compared with NIC, +*p* < 0.05 compared with IC.

*Epithelium.* In colonic epithelium, the proportion of CD3<sup>+</sup>TNFα<sup>+</sup> cells in ASD children was significantly greater than noninflamed controls (*p* < 0.005) and reached levels similar to inflamed controls (Fig. 1). The proportions of CD3<sup>+</sup>IL-2<sup>+</sup> and CD3<sup>+</sup>IFN-γ<sup>+</sup> cells were similar in all groups. No difference was seen in IL-4 staining between ASD and noninflamed groups. However, the proportion of CD<sup>+</sup>IL-4<sup>+</sup> was significantly greater in inflamed controls than ASD children and noninflamed con-

trols (*p* < 0.045). The proportion of CD3<sup>+</sup>IL-10<sup>+</sup> cells in ASD children was significantly lower than in noninflamed controls (*p* < 0.025), Crohn's disease controls (*p* < 0.007), and colitis controls (*p* < 0.031). In ASD children IL-10 producing cells were predominantly of CD3<sup>+</sup>CD8<sup>-</sup> phenotype.

For ASD children there was no correlation between age, sex, or degree of inflammation and the proportion of cytokine-positive CD3<sup>+</sup> cells. However, with respect to

**Table III.** Lamina Propria Lymphocytes (LPL) and Intraepithelial Lymphocyte (IEL) Cytokine Profiles in Colonic Mucosa

		Nondisease	Non-Crohn's colitis	CD	ASD
LPL	TNF $\alpha$	1.53	16.66*	20.43*	23*
		0.75–6.88	5.4–25.04	1.6–42.31	8.72–27.8
	IL-2	1.7	0.84	0.37 <sup>†</sup>	1.12 <sup>§</sup>
		0.6–4.63	0.4–5.33	0.32–0.4	0.54–4.62
	IL-4	1.9	8.53	1.93	3.47
		0.92–2.45	0.75–12.61	1.15–5.94	1.02–13.95
IFN $\gamma$	2	4.79*	3.03	2.4	
IL-10		0.5–7.83	3.79–12.65	0.56–11.92	1.21–21.67
	10.31	1.01	1.34	0.6*	
	1.75–13.81	0.4–7.41	1–8.53	0.09–3.6	
IEL	TNF $\alpha$	7	10.35	12.8	16.81*
		3.9–15.8	4.1–23.17	2.76–30.25	5.9–42.82
	IL-2	6.9	7.9	2.32 <sup>†</sup>	5.7
		3.1–24.88	6.1–12.53	1.28–4.46	1.61–9.52
	IL-4	5.2	7.5	7.15	3.53 <sup>†</sup>
		3.89–11.7	5.89–9.65	2.38–10.79	2.1–8.38
IFN $\gamma$	5.5	7.6	7.24	3.17	
IL-10		4.62–9.71	3.55–11.72	2.72–13.6	1.53–16.58
	4.85	3.36	9.42	1.5* <sup>†§</sup>	
		2.25–13.89	1.7–8.54	1.75–13.25	0.35–2.55

Note. Median and interquartile ranges are shown for intracellular CD3<sup>+</sup> cytokine profiles in nondisease control children or those with non-Crohn's colitis, Crohn's disease, and ASD.

\*  $p < 0.05$  compared nondisease controls.

<sup>†</sup>  $p < 0.05$  compared with non-Crohn's colitis disease.

<sup>§</sup>  $p < 0.05$  compared with Crohn's disease.

dietary history, both colonic IEL and LPL CD3<sup>+</sup>TNF $\alpha$ <sup>+</sup> lymphocytes are significantly lower in those ASD children on an exclusion diet than in those on no dietary exclusion (median  $\pm$  SEM; IEL: 36.7  $\pm$  7.1 versus 8.0  $\pm$  5.1,  $p < 0.018$ , LPL: 25.7  $\pm$  5.3 versus 8.7  $\pm$  3.6,  $p < 0.021$ ).

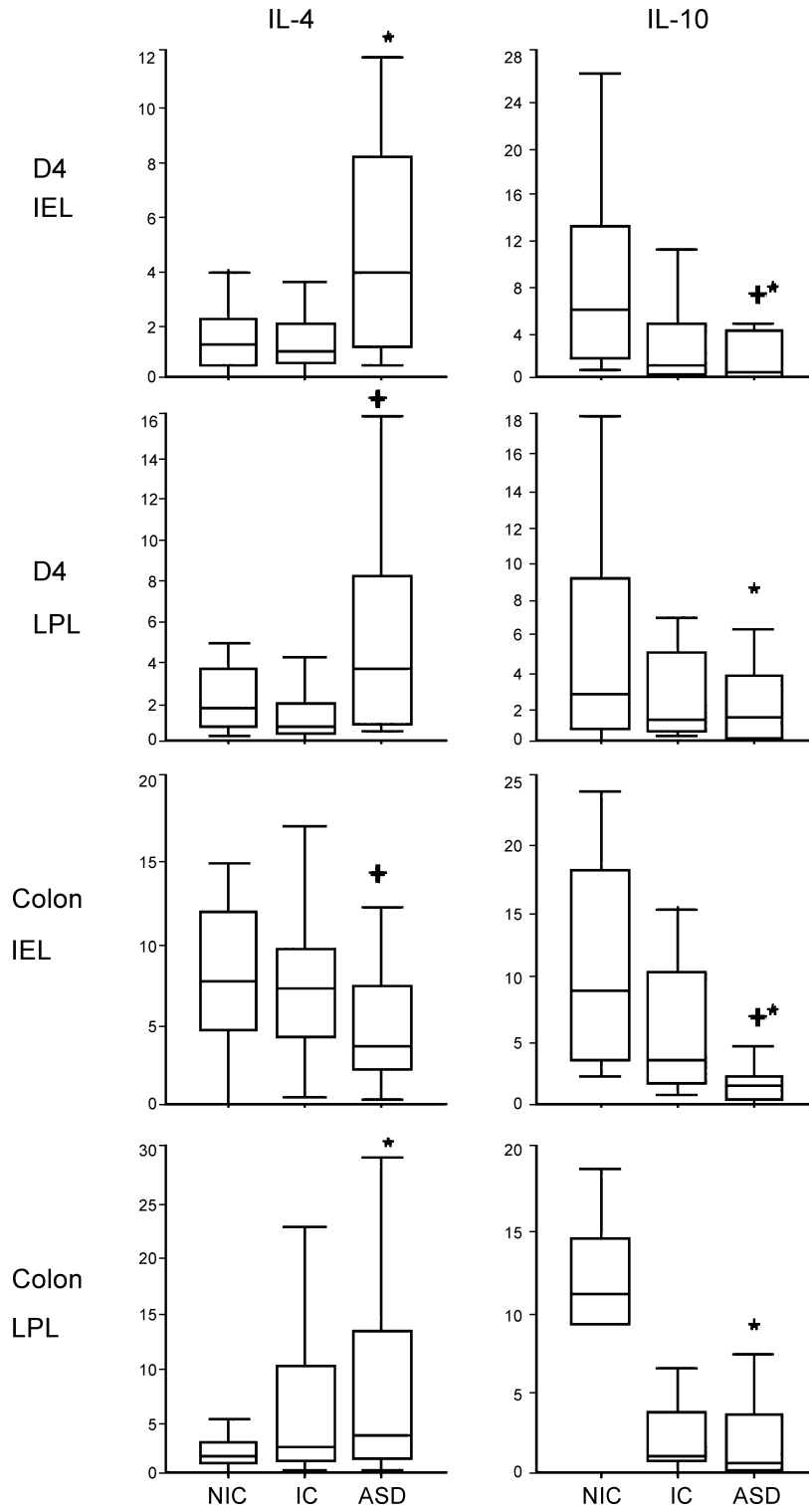
*Lower GI Pathology.* Moderate to severe degrees (grades 2–3) (8) of ileal LNH were observed macroscopically in 15 of 19 (79%) ASD children. Histologically 3 (16%) ASD children had acute and/or chronic ileal inflammation, and 4 (21%) had eosinophilic infiltration of the mucosa. In the colon, 15 of 17 (88%) ASD children showed evidence of acute and/or chronic inflammation. Acute colitis was observed in 7 (37%) and chronic inflammation in 15 (79%) ASD children, with evidence of both acute and chronic inflammation in 37%. Within the LP, an eosinophil infiltrate was reported in 7 (37%) and increased IELs in 3 (16%).

## DISCUSSION

This study provides a functional perspective on the mucosal lymphocyte activity in a novel intestinal immunopathology in a subset of children with ASD and GI symptoms. Our findings support the hypothesis of mucosal immune dysregulation with a pro-inflammatory CD3<sup>+</sup> lymphocyte cytokine profile, particularly for TNF $\alpha$ , in

these children. This pattern is consistent at different anatomical sites (duodenum and colon) and at different levels (LP and epithelium). Of particular interest is the inverse relationship between raised pro-inflammatory and decreased regulatory CD3<sup>+</sup>IL-10<sup>+</sup> activities, which was consistent between sites and mucosal compartments, for ASD children only. The cytokine data and the emerging pathological profile consisting of ileocolonic LNH and a lymphocytic enterocolitis, characterized by a predominant CD8<sup>+</sup> lymphocyte infiltrate, indicate considerable similarities with HIV enteropathy (19) while apparently distinct from other common paediatric mucosal pathologies.

Identification of cytokine-producing cells, using flow cytometry, has been reported previously (17, 18). In combination with phenotypic cell surface markers it has the advantage of defining the cellular source of the respective cytokines. As the enterocolitis in ASD children is predominantly lymphocytic in nature, we sought to characterize the functional status of mucosal lymphocyte populations rather than investigate the overall cytokine milieu of the mucosa. Clearly, other mucosal cell types are capable of synthesizing cytokines, in particular pro-inflammatory IL-1, IL-6, and TNF $\alpha$  from activated macrophages. In order to provide further data on mucosal cytokine status, expression profiling using mRNA microarray and real-time PCR is currently being undertaken. Preliminary studies



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**Fig. 3.** Mucosal CD3<sup>+</sup> cytokine profiles in ASD affected children compared with histologically non-inflamed controls (NIC) and histologically inflamed controls (IC). Percentage of IL-4<sup>+</sup> and, IL-10<sup>+</sup> are shown at different sites duodenum and colon, and different levels the epithelial and lamina propria compartments. \**p* < 0.05 compared with NIC, +*p* < 0.05 compared with IC.



comparing ASD mucosa with inflamed and noninflamed mucosa from developmentally normal children, has confirmed a high degree of concordance between genomic and proteomic cytokine profiles, particularly in the elaboration of pro-inflammatory cytokines in ASD mucosa (20). In light of these data, particularly in relation to elevated IL-4, IL-2, and  $\text{INF}\gamma$  in the duodenum and IL-4 in the colon of ASD children, classification of the disease according to a  $\text{T}_\text{H}1/\text{T}_\text{H}2$  dichotomy does not adequately describe the dysregulated immune status.

It would be of interest to examine ASD children who do not have GI symptoms in a similar manner. However, the ethical constraints of performing invasive procedures on asymptomatic children, means that this comparison is not feasible. A potential shortcoming of this study is that the expert developmental diagnosis was not reevaluated in our unit. This has been performed in previous studies (5, 7) and we have no reason, on the basis of these prior observations, to doubt the accuracy of the original diagnoses. All children studied remain under review by local developmental paediatricians.

The majority of affected children were boys, whereas the controls were relatively evenly distributed between the sexes. However, there were no gender-related differences in mucosal lymphocyte cytokine activity in nondisease controls, helping to rule out a confounding effect of gender. No statistical differences in age were seen between the groups, although children with established inflammatory conditions tended to be older. There is little data about mucosal cell-specific cytokine production with age, especially in the paediatric populations, but it would appear that cytokine production increases with age (21, 22), therefore, tending to bias against the hypothesis.

No correlation was observed between degree of histological abnormality and cytokine activity, specifically for  $\text{TNF}\alpha$ . This may indicate that intermediary steps are necessary between inflammatory cytokine generation and tissue damage, e.g. metalloproteinase induction, which are not fully active or need accessory signals to be induced in ASD children; mRNA expression profiling may help to answer this question. Alternatively, this situation may reflect the patchy nature of the mucosal lesion, such that adjacent biopsies taken for histopathology and cytokine analysis might not be mutually representative. There is potential for variation in efficiency of lymphocyte isolation from intestinal biopsies because of relative size of the specimens obtained. However, this variability should not lead to a systematic bias between groups and is unlikely to account for the observed differences. In addition, detailed morphometric studies of lymphocyte populations in many of the same children included in this study, and reported previously (7, 9, 10), show significant elevation

in relevant lymphocyte subsets, consistent with the data presented here.

Of interest is the apparent effect of diet, and the discordance in  $\text{CD}3^+\text{TNF}\alpha^+$  populations between duodenum and colon, in subjects on and not on exclusion diets. Previous studies have shown that dietary intervention may produce cognitive benefit and have been based upon the premise of removing substrate for certain neuroactive opioid peptides from the food (15, 16). In addition, food antigens may provide a pro-inflammatory stimulus in some ASD children, as suggested by the data of Jyonouchi *et al.* (23). We have previously observed that gluten and/or casein-free (GF-CF) diets do not influence the frequency or degree of ileal LNH, or the nature or extent of mucosal lymphocyte infiltrate (10). However, the statistical association between reduced  $\text{CD}3^+\text{TNF}\alpha^+$  proportions in those on and not on dietary restriction in colonic mucosa appears interesting. Given the small numbers, the conclusions that can be drawn are limited, but clearly the potential influence of these foodstuffs merits further study.

Finally, cytokines play an important role in the cross-talk between the immune and central nervous systems. Cytokines and products of the immune system have widespread effects on neuronal pathways, and may potentially play a role in many common ASD features such as mood and sleep disturbances. Therapeutic modification of cytokine activity may provide some insight into their role in the neurodevelopment features of autism with the theoretical potential for cognitive benefit.

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