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Immune activation of peripheral blood and mucosal CD3$^+$ lymphocyte cytokine profiles in children with autism and gastrointestinal symptoms

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Abstract

Gastrointestinal pathology, characterized by lymphoid nodular hyperplasia and entero-colitis, has been demonstrated in a cohort of children with autistic spectrum disorder (ASD). Systemic and intestinal mucosal immune dysregulation was assessed in ASD children with gastrointestinal (GI) symptoms ($n=18$), and typically developing controls ($n=27$), including non-inflamed controls (NIC) and inflamed GI control children with Crohn's disease (CD), by analysis of intracellular cytokines in CD3$^+$ lymphocytes. In both peripheral blood and mucosa, CD3$^+$ TNFα$^+$ and CD3$^+$ IFNγ$^+$ were increased in ASD children compared with NIC ($p<0.004$) and reached levels similar to CD. In contrast, peripheral and mucosal CD3$^+$ IL-10$^+$ were markedly lower in ASD children with GI symptoms compared with both NIC and CD controls ($p<0.02$). In addition, mucosal CD3$^+$ IL-4$^+$ cells were increased ($p<0.007$) in ASD compared with NIC. There is a unique pattern of peripheral blood and mucosal CD3$^+$ lymphocytes intracellular cytokines, which are consistent with significant immune dysregulation, in this ASD cohort.

Keywords: Inflammation; Mucosa; TNFα; IL-10; Autism

1. Introduction

Autistic spectrum disorders (ASD) are complex, pervasive developmental disorders of childhood characterized by qualitative impairments in social interaction, deficits in verbal and non-verbal communication, and restricted repetitive and stereotyped patterns of behavior and interests (DSMIV criteria). It is likely that within the ASD population distinct phenotypes may be identified according to patterns of clinical presentation; for example, regression versus non-regression, specific behavioral traits, genetics, brain structure and structural imaging, and the profile of biochemical and immunological markers. A subset of ASD children with gastrointestinal (GI) symptoms (ASD^{GI}) and inflammatory mucosal pathology has been described and confirmed in independent studies (Horvath et al., 1999; Wakefield et al., 2000; Furlano et al., 2001). The exact prevalence of GI symptoms in ASD is unknown. Two retrospective studies that analyzed representative populations of children with autism, reported GI symptoms in approximately 20% of young children previously diagnosed with autism (Fombonne et al., 2001; Taylor et al., 2002). This contrasts with prospective reports from pediatric gastroenterology and general autism clinics which have described GI symptoms in 46–84% of patients with ASD (Horvath et al., 1999). However, prevalence estimates from population-based epidemiologic studies are largely lacking.

Associated functional GI abnormalities in ASD^{GI} children include: low activities of disaccharidase enzymes (Horvath et al., 1999), defective sulfation of ingested phenolic amines such as acetaminophen (Alberti et al., 1999), bacterial overgrowth with greater diversity and number of clostridia species (Finegold et al., 2002), increased intestinal permeability (D'Eufemia et al., 1996) and a beneficial effect of

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gluten/casein dietary exclusion on behavior cognition (Knivsberg et al., 1995, 2002). The intestinal pathology described in this cohort of ASD patients, includes chronic ileo-colonic lymphoid nodular hyperplasia (LNH), entero-colitis, gastritis and esophagitis (Wakefield et al., 2000, 2005; Furlano et al., 2001; Torrente et al., 2002). The intestinal pathology differs from established inflammatory bowel diseases such as Crohn's disease (CD) and ulcerative colitis in a number of respects (Ashwood et al., 2003, 2004; Wakefield et al., 2005). Flow cytometric and immunohisto-chemical analyses of mucosal lymphocyte populations in ASD^{GI} children have demonstrated qualitatively consistent abnormalities at different anatomical sites including stom-ach, duodenum, ileum and colon (Furlano et al., 2001; Torrente et al., 2002, 2004; Ashwood et al., 2003, 2004). Mucosal lymphocyte infiltration, histological acute and chronic inflammation, γδT cells, eosinophils, Paneth cells and intraepithelial lymphocytes, are all increased in these ASD^{GI} children compared with typically developing healthy controls. In addition, there are findings of focal deposition of serum IgG from ASD^{GI} children which co-localize with complement C1q on the basolateral enterocyte membrane—changes which are not seen in histologically normal or inflamed mucosa of typically developing children or children with cerebral palsy—is suggestive of an inflammatory process that may perturb the intestinal barrier function in this population (Torrente et al., 2002). Co-localization of immunoglobulin and complement components on the epithelial membrane have been found in both gastric and duodenal specimens and may be indicative of an autoim-mune process directed against self-antigen contained within epithelial cells (Torrente et al., 2002, 2004). Furthermore, compared with pediatric controls, increased basement membrane thickness and abnormal patterns of epithelial glycosaminoglycans have been reported in children with ASD, and may be indicative of inflammatory degradation that could contribute to disruption of the intestinal barrier function (Furlano et al., 2001).

Many ASD children are on gluten and casein exclusion diets and improvements in behavior have been reported (Knivsberg et al., 1995, 2002). The rationale for a modified diet includes the removal of precursors for exorphins with their potential for neurotoxicity. Moreover, there is a possible beneficial effect of these exclusion diets on the associated intestinal lesion, given the potential for immu-nologic reactivity to gluten and casein in the GI mucosa. In ASD, it has been demonstrated that in vitro stimulation of peripheral blood mononuclear cells (PBMC) with dietary protein, resulted in elevated pro-inflammatory cytokine production (most notably TNFα) when compared with typically developing pediatric controls, (Jyonouchi et al., 2001). While immune dysregulation and inflammatory phenomena are increasingly recognized as part of the pathogenesis of autism in some children, there is an apparent divergence of opinion on the predominant polarity of the dysregulated immune response in ASD, with both raised IL-12 and raised IL-4 having been reported (Gupta et al., 1998; Singh, 1996).

This study tested the hypothesis that both systemic and mucosal immune dysregulation, with an increase in pro-inflammatory cytokine producing $CD3^+$ lymphocytes, is present in a subset of children with ASD undergoing investigation for GI symptoms. We sought to characterize lymphocyte intracellular cytokine profiles in ileal biopsies and PBMC and to make comparisons between children with ASD and typically developing pediatric controls, with histologically normal ileal mucosa and those with ileal Crohn's disease. We examined the possibility that, as in Crohn's disease, overlapping mucosal and PBMC cytokine profiles might reflect the presence of systemically activated immune cells in the presence of a primary mucosal pathology.

2. Methods

2.1. Subjects

This prospective study consecutively examined 45 children, referred to the tertiary pediatric gastroenterology unit at the Royal Free Hospital, London for investigation of GI symptoms. All patients required a diagnostic colono-scopy and biopsy based on clinical grounds. All biopsy specimens examined in this study were obtained from the same anatomical site (i.e. terminal ileum mucosal lymphoid tissue). All GI diagnoses were made by experienced pediatric gastroenterologists, based upon clinical, serologi-cal, microbial, endoscopic, and routine histological assess-ment of mucosal biopsies by experienced histopathologists. Children were investigated consecutively, in order of their referral to the clinic and consent to participation, to avoid any selection bias. Inclusion criteria for all cases and controls were GI symptoms sufficient to warrant invasive ileo-colonoscopy investigation including: abdominal pain and bloating, chronic abnormal bowel habit and failure to thrive, and that there was no contraindication to anesthetic for ileo-colonoscopy. The developmental diagnoses of for the ASD^{GI} children were made prior to referral to the unit by a suitably qualified pediatric psychiatrist, developmental pediatrician or psychologist and fit the diagnostic criteria of the Diagnostic and Statistical Manual-IV (DSMIV criteria, 4th edition, 1994) for psychiatric disorders and ICD-10 criteria. Patients with Fragile X were excluded.

Control ileal mucosa was obtained from typically developing pediatric controls undergoing investigation of GI symptoms. All haematoxylin and eosin stained histolog-ical sections were independently reviewed by a routine histopathologist who was blinded to the flow cytometry data. Following flow cytometry analysis, typically develop-ing controls were subdivided into those with Crohn's disease (CD), and histologically non-inflamed controls (NIC), based on the clinical assessment and histopathology review. Clinical data for each child including: gender, age,

GI symptom history, medication and dietary intervention, were recorded. Peripheral blood specimens were obtained in the majority of children at the same time as colonoscopy.

Spontaneous mucosal intracellular cytokine production was assessed in intraepithelial (IEL) and lamina propria (LPL) $CD3^+$ $CD8^+$ and $CD3^+$ $CD8^-$lymphocytes using multi-color flow cytometry. Terminal ileal lymphocytes were obtained from children with ASD^{GI} ($n=18$, median age 8 years, range 4–15, 14 male), age matched typically developing non-inflamed controls (NIC) ($n=15$, median age 10 years, range 2–17, 11 male) and age matched children with Crohn's disease (CD) ($n=12$, median age 11, range 4–17, 9 male). ASD^{GI} were not on any anti-inflammatory or immunomodulatory therapy. Diagnoses included autism (17) and Asperger's syndrome (1) Children had a similar history of achieving normal developmental milestones followed by loss of acquired skills and onset of aberrant behaviors. Of the ASD^{GI} children, 14 had chronic constipation, 4 had diarrhea, and 2 had alternating constipation and diarrhea. These symptoms were accompanied by abdominal pain and bloating in many ASD^{GI} children. Some ASD^{GI} children were on dietary restriction including a gluten-free ($n=3$), casein free ($n=1$), and gluten/casein-free ($n=4$). The remaining 10 ASD^{GI} children were on unrestricted (conventional) diets. Dietary histories were obtained from the parents and cross-checked with in-patient nursing records. Serum anti-endomyseal and anti-glaidin antibody titers to screen for celiac disease and serological pathogen screenings were negative in ASD^{GI} children. No high abnormal values were noted for routine hematological markers. In the Crohn's disease group, 3 children were not currently receiving therapy. Four were on dietary modification in combination with 5-ASA, while one child was taking 5-ASA alone. In the remaining four children, therapy included Azathioprine and Asacol ($n=2$), and Prednisolone and Asacol ($n=2$).

In all cases, written informed consent to participate in the study was obtained from the parents. This study received ethical approval from the relevant local institutional human Research Ethics Committee.

2.2. Cell isolation and flow cytometric assessment

Isolation of mucosal lymphocytes was performed as previously described (Ashwood et al., 2003). In brief, single cell suspensions from multiple mucosal pinch biopsies were prepared in two stages: firstly, the epithelial layer was removed using calcium-free Hanks' balanced salts solution and continuous agitation (Sigma, UK). Second, the remaining lamina propria tissue was digested with collagenase 2 mg/ml (Sigma) for 3 h. Mononuclear cell viability was >90% by 0.1% trypan blue exclusion. In addition, peripheral blood mononuclear cells (PBMC) from ASD ($n=12$), CD ($n=10$) and NIC ($n=14$), were isolated using Lymphoprep™ density gradient centrifugation (Nycomed, Oslo, Norway). PBMC were added to tissue culture medium (consisting of RPMI-1640 and 10% fetal calf serum, Gibco BRL, UK). To optimize intracellular staining and prevent cytokine release, Brefeldin A (GogiPlug) and Monensin (GolgiStop) were added, according to the manufacturer's recommendation (Pharmingen, UK). PBMC were either cultured alone or stimulated with PMA (50 ng/

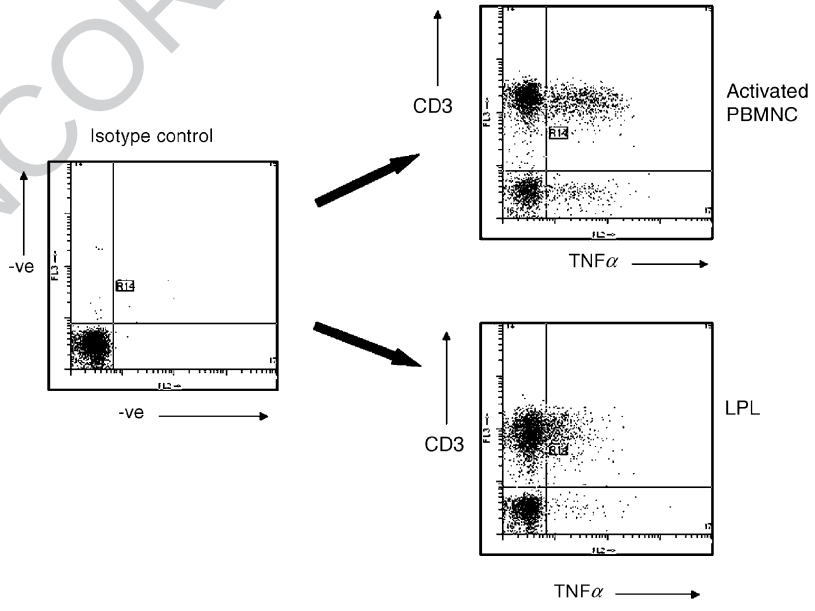

Fig. 1. Representative TNFα staining in activated PBMC and terminal ileal mucosal biopsies $CD3^+$ lymphocytes from an ASD^{GI} child. Staining with concentration and isotype-matched negative controls allows for the elimination of background staining. PMA activated $CD3^+$ and mucosal $CD3^+$ lamina propria (LPL) show increased staining for intracellular TNFα.

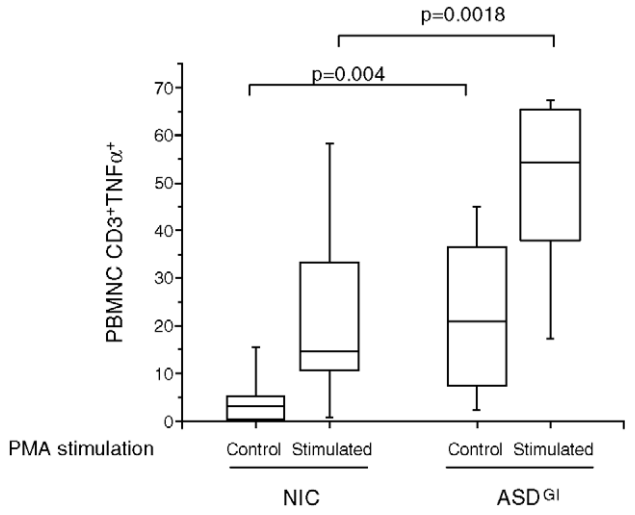

Fig. 2. Peripheral blood-TNFα response in children with ASD and GI symptoms (ASD^{GI}) and non-inflamed controls (NIC) controls before and after PMA stimulation. $CD3^+$ cells from ASD^{GI} ($n=12$) and age matched NIC children ($n=14$), were assessed for intracellular TNFα production following culture with or without stimulation with PMA. Significantly increased $CD3^+$ $TNF\alpha^+$ cells were present in ASD^{GI} children compared with NIC in both the untreated and treated groups.

ml) and Ionomycin (1 μM final concentration) (Sigma) for 4 h at 37 °C in a humid, 5% CO_2 incubator. Mucosal lymphocytes were only sufficient in number for measurement of spontaneous cytokine profiles. Following isolation, cells from each compartment were washed and maintained in PBS containing bovine serum albumin (0.2%, Sigma) and sodium azide (0.02%, Sigma) prior to staining and flow cytometry analysis.

Standard intracellular cytokine analysis was performed using anti-IFN-γ-FITC, IL-12-FITC, TNFα-PE and IL-4-PE (R&D Systems, UK) and anti-IL-10-PE (Pharminogen, UK). Cells were labeled with the cell-surface lineage markers anti-CD3-PeCy5 and CD8-APC (Dako, UK) antibodies for 30 min, washed and fixed with 4% paraformaldehyde for 10 min. Cells were again washed, permeabilized in 1% saponin solution (Sigma) and stained for the respective intracellular cytokines according to the manufacturer's recommendation, for 30 min. Concentration and isotype-matched control antibodies were used to determine non-specific binding and optimum fluorescence quadrant markers (mouse IgG1-FITC, ECD, PC5 and IgG2a-PE from Coulter-Immunotech, UK). Unstained mucosal cells and PBMC were used as further controls. Multi-color flow cytometry was performed on a 4-channel Galaxy 2000 (Dako) equipped with a 488 nm argon laser and 633 nm diode laser.

Data acquired by flow cytometry were analyzed using Winlist Version 4.0. A gate was drawn around the lymphocyte population and back-gated in relation to $CD3^+$. A minimum of 10,000 events within this gate was required for analysis. Quadrants were generated for $CD3^+$, $CD3^+$ $CD8^+$ and $CD3^+$ $CD8^-$ populations and individual cytokines, with the lower limit for distinguishing cytokine-positive cells being determined for each antibody using either the isotype-matched controls or unstained mucosal and peripheral blood lymphocytes (Fig. 1). Staining was confirmed using one-dimensional histogram plots of the gated $CD3^+$, $CD3^+$ $CD8^+$ and $CD3^+$ $CD8^-$ populations and differences in mean fluorescence intensity (MFI) were recorded for each cytokine. The percentage of total $CD3^+$, $CD3^+$ $CD8^+$ and $CD3^+$ $CD8^-$ cells within each compartment, and the percentage of cytokine-producing cells in each lymphocyte population, was determined. All data are expressed as median (interquartile range). Statistical analyses using the Mann–Whitney *U* test (Bonferroni-corrected) were performed using SPSS software, version 10.1 and results were considered significant if $p<0.05$.

3. Results

3.1. Cytokine profiles of peripheral blood lymphocytes

Significantly increased frequencies of $TNF\alpha^+$ peripheral lymphocytes were noted in the unstimulated cell cultures from ASD^{GI} children compared with typically developing NIC ($p<0.004$, Figs. 1 and 2, Table 1) and reached similar levels to those in children with CD. However, the frequency of lymphocytes positive for the regulatory cytokine IL-10 were reduced in children with ASD^{GI} compared with both CD

t1.1 Table 1
t1.2 PBMC responses in children with ASD and GI symptoms (ASD^{GI}) with or without stimulation

		Unstimulated					Stimulated				
t1.4		IL-12	IL-4	TNFα	IFNγ	IL-10	IL-12	IL-4	TNFα	IFNγ	IL-10
t1.5	ASD^{GI}	0.68	3.7	20.9*	4.7‡	0.9‡+	6.2‡	7.8	54.5*	16.7	1*+
t1.6		(0.42–2.03)	(0.7–13.2)	(9.2–36.7)	(0.3–16.2)	(0.3–10.7)	(2.06–6.62)	(1.2–19.2)	(39.5–65.3)	(12.3–31.2)	(0.2–9.6)
t1.7	NIC	0.68	1.6	3.1	0.3	13.9	2.49	3.7	14.7	19.3	14.5
t1.8		(0.51–1.13)	(0.5–2.4)	(0.8–4.9)	(0–0.6)	(7.5–27.5)	(0.23–4.22)	(1.6–6.3)	(11.9–31.6)	(6.8–42.6)	(8.2–26.5)
t1.9	CD	1.44	3.6	25.7#	1	14.2	9.09#	7.4	37#	26.7	18.3
t1.10		(0.74–6.38)	(1.3–6)	(14.5–39.7)	(0.4–3.3)	(6.1–27.2)	(0.42–15.2)	(1.3–19.6)	(20.3–54.1)	(11.4–33.4)	(4–32.4)

t1.11 *$P<0.004$ Children with ASD plus GI symptoms (ASD^{GI}) ($n=12$) compared with non-inflamed controls (NIC) ($n=14$).
t1.12 ‡$P<0.05$ ASD^{GI} children compared with NIC.
t1.13 +$P<0.05$ ASD^{GI} children compared with Crohn's disease controls (CD) ($n=10$).
t1.14 #$P<0.05$ CD compared with NIC.
t1.15 Data are expressed as median (interquartile ranges) of percentage positive cells.

Table 2
Non-stimulated mucosal intracellular lymphocyte $CD3^+$ cytokine profiles from the terminal ileum of children with ASD and GI symptoms (ASD^{GI})

		CD3	IL-12	IL-4	IFNγ
LPL	ASD^{GI}	58.7*	2	3.6*	3.8*
		(51.22–68.39)	(1.4–5)	(0.9–6.5)	(0.8–7.2)
	NIC	40.3	0.9	0.5	0.4
		(32.65–48)	(0.5–1.35)	(0.3–0.9)	(0.3–2.1)
	CD	59.94#	3.5#	1.8	3.9
		(52.67–69.16)	(0.5–9.6)	(0.5–3.7)	(0.2–8.3)
IEL	ASD^{GI}	79.05	2.2	3.5	3.5*
		(56.6–93.68)	(0.7–6)	(1.2–5.9)	(1.6–10.6)
	NIC	74.94	1.4	1.4	0.6
		(54.42–89.12)	(0.7–2.8)	(1.1–2)	(0–1.6)
	CD	80.02	5.3	2.4	3.1#
		(62.67–92.16)	(1.8–14.5)	(0.8–3.7)	(1.2–5.5)

*$P<0.05$ Children with ASD and GI symptoms (ASD^{GI}) ($n=18$) compared with non-inflamed controls (NIC) ($n=15$).
#$P<0.05$ Crohn's disease controls (CD) ($n=12$) compared with non-inflamed controls.
Data are expressed as median (interquartile ranges) of percentage positive cells.

and NIC ($p<0.04$, Table 1). Elevated frequencies of $CD3^+$ $IFN\gamma^+$ lymphocytes were detected in ASD^{GI} children compared with NIC ($p<0.035$, Table 1) and were similar to levels in CD. In addition, there was a trend for increased $CD3^+$ $IL\text{-}4^+$ cells in children with ASD^{GI} compared with NIC although this did not reach statistical significance (Table 1). When $CD3^+$ subsets—$CD3^+$ $CD8^+$ and $CD3^+$ $CD8^-$ (as an indicator of $CD3^+$ $CD4^+$ cells)—were examined independently, there was a similar differential cytokine pattern seen for both subsets in children with ASD^{GI} compared with the corresponding lymphocyte populations in either typically developing control group. Both the frequency of $CD3^+$ $CD8^-$ $TNF\alpha$ and $CD3^+$ $CD8^+$ $TNF\alpha$ subsets were increased in children with ASD^{GI} compared with NIC ($p=0.016$). In addition, there was a decreased frequency of $CD3^+$ $CD8^-$ $IL\text{-}10^+$ cells ($p=0.02$) and a trend to a lower frequency of $CD3^+$ $CD8^+$ $IL10^+$ ($p=0.06$) cells in children with ASD^{GI} compared with either typically developing control group.

Following PMA stimulation, the frequency of $CD3^+$ cells positive for either $TNF\alpha$ or $IFN\gamma$ were increased in all patient groups. The significant excess in $CD3^+$ $TNF\alpha^+$ cells in children with ASD^{GI} compared with NIC, was maintained following stimulation ($p=0.0018$, Fig. 2, Table 1). Within the $CD3^+$ population the frequency of $CD3^+$ $CD8^-$ $TNF\alpha^+$ and $CD3^+$ $CD8^+$ $TNF\alpha^+$ cells in the ASD^{GI} group were again increased compared with the NIC ($p=0.019$). There was no corresponding rise in $CD3^+$ $IL\text{-}10^+$ cells noted in any group following stimulation. Frequency of $CD3^+$ $IL\text{-}10^+$ cells remained significantly lower in ASD^{GI} children compared with CD following stimulation ($p=0.04$).

3.2. Terminal ileum lymphocytes

Macroscopic and endoscopic pathology of the ileum in this cohort of ASD^{GI} children was similar to that described in previous reports (Wakefield et al., 2000). Moderate-to-severe degrees (grades 2–3) of ileal lymphoid nodular hyperplasia were observed macroscopically in 11 of 18 (61%) ASD^{GI} children. Histologically, 8 (44%) ASD^{GI} children had evidence of chronic ileal inflammation alone, and 2 (11%) showed both acute and chronic inflammation. In 3 (17%) children there was eosinophilic inflammation of the mucosa.

In accordance with previous flow cytometric and morphometric data (Ashwood et al., 2003; Torrente et al., 2002) the frequency of $CD3^+$ and $CD8^+$ T lymphocytes were increased in the mucosal lamina propria of ASD^{GI} children compared with NIC (mean ± S.E.M., $CD3^+$: 58.7 ± 6.4% vs. 40.3 ± 7.4% and $CD8^+$: 30.5 ± 4.7% vs. 15 ± 7.1%, $p<0.03$) and reached levels similar to those seen in children with CD. The detection of intracellular cytokines in this study was consistent with previous published data on cytokine levels in intestinal mucosal tissue (O'Mahony et al., 1998; O'Keefe et al., 2001). In biopsies from children with CD, there were increased frequencies of populations of $CD3^+$ cells that were positive for IL-12, IFNγ, TNFα (Table 2, Fig. 3) and (where sufficient sample was available) IL-6, when compared with

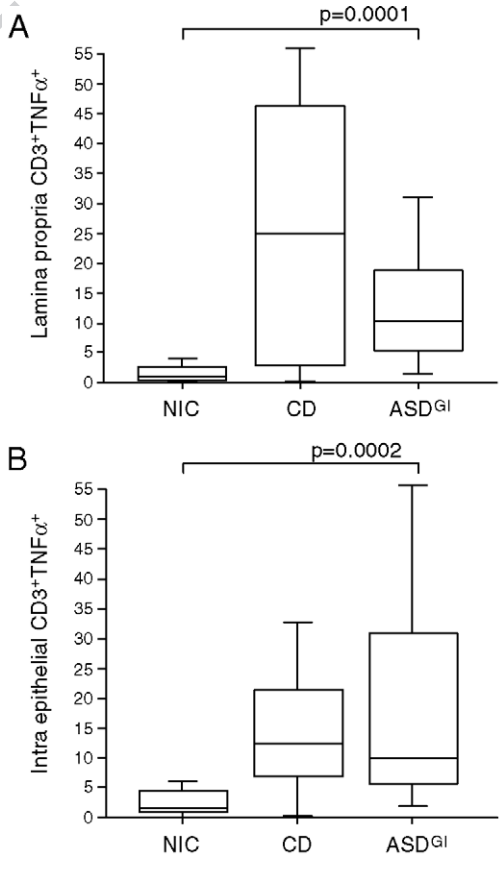

Fig. 3. Mucosal lymphocyte intracellular TNFα production in children with ASD and GI symptoms (ASD^{GI}), Crohn's disease (CD) and non-inflamed controls (NIC), in non-stimulated cells. Increased $CD3^+$ $TNF\alpha^+$ lymphocytes were observed in ASD^{GI} children ($n=18$) compared with NIC ($n=15$) and reached levels similar to those for CD ($n=12$), in both the lamina propria (A) and epithelial (B) mucosal compartments.

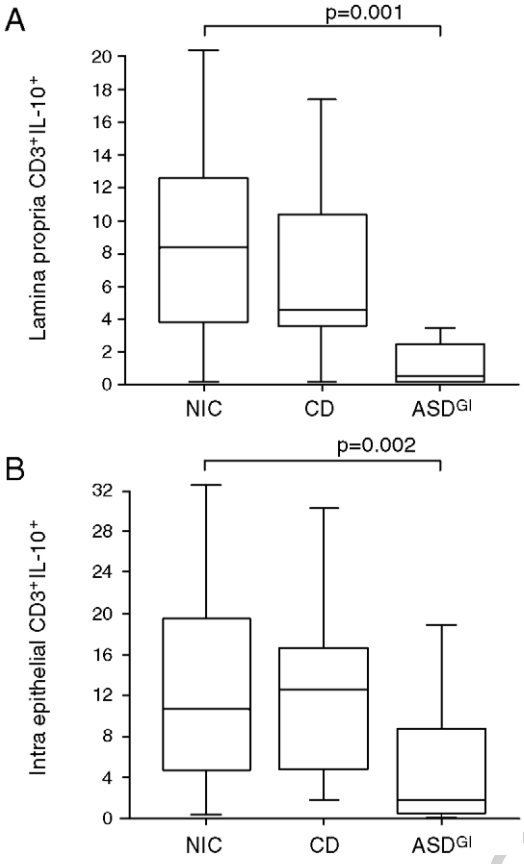

Fig. 4. Mucosal lymphocyte intracellular IL-10 production in children with ASD and GI symptoms (ASD^{GI}), Crohn's disease (CD) and non-inflamed controls (NIC) in non-stimulated cells. Significantly decreased $CD3^+$ $IL\text{-}10^+$ lymphocytes were observed in ASD^{GI} children ($n=18$) compared with NIC ($n=15$) and CD controls ($n=12$) in both the lamina propria (A) and epithelial (B) mucosal compartments.

NIC. This finding is consistent with the previously reported T_H1 profile of patients with CD.

Within the terminal ileal lamina propria, the frequency of $CD3^+$ $TNF\alpha^+$ lymphocytes were increased in children with ASD^{GI} compared with NIC ($p<0.0001$, Figs. 1 and 3A), but not CD controls. In the epithelial layer, there was a similar pattern, with increased frequency of $CD3^+$ $TNF\alpha^+$ lymphocytes detected in children with ASD^{GI} compared with NIC ($p<0.0002$, Fig. 3B). As with the observations in peripheral blood lymphocytes of children with ASD^{GI}, there were no differences in the frequency of $CD3^+$ $CD8^-$ $TNF\alpha^+$ and $CD3^+$ $CD8^+$ $TNF\alpha^+$ subpopulations, which were both significantly increased compared with the NIC ($p=0.03$). There was a decrease in the frequency of $CD3^+$ $IL\text{-}10^+$ population in children with ASD^{GI} compared with NIC, in both the lamina propria ($p<0.001$, Fig. 4A, Table 2) and epithelial layer ($p<0.02$, Fig. 4B, Table 2). Similarly, the frequency of $CD3^+$ $IL\text{-}10^+$ cells were decreased in children with ASD^{GI} compared with CD controls ($p<0.006$, Table 2). Decreased frequency of IL-10 was evident in both $CD3^+$ $CD8^-$ and $CD3^+$ $CD8^+$ subpopulations in ASD^{GI} children. Moreover, when the ratio between IL-12 and IL-10 positive cells within the lamina propria was considered, a vastly increased $CD3^+$ IL-12/IL-10 ratio was observed in children with ASD^{GI} compared with NIC (4.81 (1.86–5.64) vs. 0.08 (0.05–0.30), $p<0.001$ (median (interquartile range)). In addition, there were increased frequencies in lamina propria $CD3^+$ $IFN\gamma^+$ lymphocytes ($p<0.02$, Table 2) and intra-epithelial $CD3^+$ $IFN\gamma^+$ lymphocytes ($p<0.003$, Table 2) compared with NIC, but not CD. If the ratio between IFNγ and IL-10 positive cells within the lamina propria was considered, an increased $CD3^+$ IFNγ/IL-10 ratio was observed in ASD^{GI} children compared with NIC (2.1 (0.91–9.9) vs. (0.05 (0.03–0.4), $p<0.001$). In children with ASD^{GI} the lamina propria $CD3^+$ $IL\text{-}4^+$ lymphocytes were increased compared with NIC ($p<0.009$, Table 2). Where comparisons were possible, the frequency of lamina propria $CD3^+$ $IL\text{-}5^+$ lymphocytes were increased in ASD^{GI} compared with CD controls (4.82 (2.84–12.05) ($n=8$) vs. 0.74 (0.5–1.28) ($n=11$), $p<0.006$). When extended gated regions that included potential blast cells were used, similar significant differences were observed in cytokine profiles between cases and controls.

Eight of the ASD^{GI} children were on exclusion diets, including gluten-free ($n=3$), casein free ($n=1$), and gluten/casein-free ($n=4$). The low numbers of subjects limited the power of any analysis. Comparing lymphocyte cytokines in the lamina propria between children with ASD^{GI} currently using some form of dietary restriction showed a non-statistically significant decrease in the frequency of $CD3^+$ $TNF\alpha^+$ (5.4 (4.4–16.6) compared with those on no dietary restriction (16.1 (9.4–22.5), $p=0.17$). There was a lack of statistical association between the majority of cytokines and the degree of inflammation. However, $CD3^+$ $IFN\gamma^+$ cells in the lamina propria compartment and $CD3^+$ $IL\text{-}12^+$ in the intraepithelial compartment were significantly greater in those ASD^{GI} children who had mucosal inflammation compared with those without inflammation ($p=0.004$).

4. Discussion

In this study, we have made the novel observation that peripheral blood and mucosal $CD3^+$ lymphocytes derived from ileal lymphoid tissue of ASD^{GI} children show significant immune dysregulation with an enhanced pro-inflammatory cytokine profile. This pattern was characterized by increased pro-inflammatory TNFα and INFγ, and in the mucosa, increased IL-4 and IL-5 also, with a marked reduction in the frequency of cells expressing the regulatory cytokine IL-10 in both mucosa and PBMC. This pattern of intracellular cytokines is consistent with that reported for duodenal and colonic biopsies from a symptomatically similar population of children (Ashwood et al., 2004) and concords with observations of increased TNFα protein production in PBMC of ASD^{GI} children, following in vitro stimulation (Jyonouchi et al., 2001). The aggregated findings of a complex and apparently idiosyncratic $CD3^+$ cytokine

profile (Ashwood et al., 2004), pan-enteric inflammation (Ashwood et al., 2003) with morphologic characteristics that distinguish the mucosal lesion from well recognized inflammatory mucosal diseases (Wakefield et al., 2000, 2005, Furlano et al., 2001; Torrente et al., 2002) confirm a novel disease process in this ASD^{GI} population of children.

Cytokine profiles in children with CD, including increased IL-12, IL-6 and IFNγ, were consistent with previous reports of a dominant T_H1 pattern associated with this disease. In comparison in ASD^{GI} children, there was no clear differential pattern of cytokines, either in the peripheral blood or mucosal specimens, that could be defined within the T_H1 and/or T_H2 paradigm. Instead, the data would suggest that there is a distinctive immune activation in this cohort of children which leads to a dysregulated pro-inflammatory cytokine profile. The balance between pro- and anti-inflammatory signals is significantly disrupted with a decrease in IL-10 synthesizing lymphocytes. This imbalance in mucosal pro- and anti-inflammatory signals can be illustrated by considering the relationship between IL-12 and/or IFNγ, known inducers of mucosal inflammation and the regulatory cytokine IL-10. Even though the frequency of mucosal $CD3^+$ $IL\text{-}12^+$ cells were not increased in ASD^{GI} above control levels, the ratio between IL-12 and IL-10 was significantly increased in ASD^{GI} children compared with both NIC and CD typically developing controls. In PBMC, spontaneous IFNγ was higher in ASD^{GI}, compared with NIC, but following in vitro stimulation this difference was not maintained between cases and controls. Interestingly, in autoimmunity, there is a common finding that cells which have high baseline (spontaneous) values for cytokines cannot be further stimulated in vitro to produce additional cytokines, such as the case here for the cytokine IFNγ and to some extent TNFα. This response may represent polyclonal activation leading to elevated baseline levels of cytokines rendering the cells less able to respond beyond their already activated state. (Gershwin et al., 1979; Vervliet et al., 1985).

The apparent deficiency of a $CD3^+$ $IL\text{-}10^+$ response in ASD^{GI} children, at levels significantly below those seen in typically developing NIC and CD controls, may well have functional consequences for peripheral and mucosal immune dysregulation. A number of studies have observed that even in healthy controls the gut is a site of "tightly controlled inflammation" (Boland, 1998; Carol et al., 1998), with inflammatory signals such as from IL-12 kept in check by signals induced by IL-10 and TGFβ1. However, $CD3^+$ lymphocytes are unlikely to be the only source of IL-10 (or IL-12) in mucosal biopsies and a study of the overall cytokine milieu is necessary. Nonetheless, the $CD3^+$ $IL\text{-}10^+$ response may be a potential biomarker for this specific ASD phenotype, particularly when expressed in a ratio that incorporates the profiles of TNFα, IFNγ and IL-12 with regulatory cytokines such as IL-10.

It would be of interest to examine ASD children who do not have GI symptoms in a similar manner, in order to determine to what extent the cytokine patterns segregate phenotypically distinct ASD groups that share a common behavioral diagnosis. However, the ethical constraints of performing invasive procedures on asymptomatic children mean that this comparison is not feasible. Although the ASD^{GI} patients reported in this study appear to be representative of our broader experience of GI symptoms in this population, since they were referred to a tertiary pediatric unit they may well be at the more severe end of the spectrum. The PBMC cytokine response to stimulation in our population accords with that seen by Jyonouchi et al. in similarly affected children referred to a non-GI unit (Jyonouchi et al., 2001), suggesting that these pro-inflammatory immune responses reflect more than just a referral bias. Another potential short-coming of this study is that the expert developmental diagnosis was not specifically re-evaluated in our unit. This has been performed in previous studies (Wakefield et al., 2000) and we have no reason, based upon these prior observations, to doubt the accuracy of the original diagnoses based on DSM/ICD-10. Furthermore, all children studied remain under review by local developmental pediatricians, and we are unaware of any case where the diagnosis has been revised or reverted.

The pathology identified by ileocolonscopy is likely to be an underestimate of the prevalence of mucosal inflammation in these children. Confirmation and extension of the original GI findings has been provided recently by Balzola et al. who included capsule enteroscopy in their assessment of the small intestine (Balzola et al., 2005a,b). The data confirmed frequent jejunal and ileal disease and the potential patchy pan-enteric distribution of the mucosal inflammation in ASD^{GI} patients. These findings and the focal nature of the pathology may explain, at least in part, the lack of a correlation between cytokine profiles and histological inflammation.

Biopsy specimens were taken from the same defined anatomical region (i.e. ileal lymphoid tissue), allowing valid comparisons between groups. A potential technical problem in this study is the efficiency of lymphocyte isolation from intestinal biopsies, due to variable size of the specimens obtained, although this appears not to have been an issue, technically, and should not have led to systematic differences between patient groups. Similarly, random sampling including possible contributions from cells from Peyer's patches, would be the same between groups. Expression of the data as a proportion of 10,000 $CD3^+$ cells in all patient groups allowed for valid comparisons between groups.

Cytokines and products of immune activation have widespread effects on neuronal pathways, and have been suggested to contribute to common features of ASD such as mood and sleep disturbances. Systemic cytokines such as IFN-α, IL-2 and TNF-α administered at therapeutic doses, have side effects including mood depression, sleep disorder, impaired cognitive function, decreased exploratory behavior, and changes in motivation (Larson, 2002; Licinio et al., 1998). Systemic cytokine administration can also cause increases in noradrenergic, dopaminergic and serotonergic

metabolism in the hypothalamus, hippocampus and nucleus accumbens (Merali et al., 1997; Mohankumar et al., 1991; Shintani et al., 1993). Importantly, cytokines can activate and exert trophic effects on glial cells, which in turn can produce cytokines and chemokines following such activation. As the CNS is largely populated by astroglia and microglial cells, these cytokine–cell interactions are important for neuronal cell functioning and development. Alternatively, afferent neurons may be directly responsive to peripheral cytokine stimulation (Dantzer et al., 1998). The relationship between the various immune abnormalities that have been reported in individuals with ASD and GI symptoms and the development of neurologic changes is not yet clear. However, it is evident that successful neurodevelopment is contingent upon a strong interface between the cellular immune system and the neurologic network. There has been speculation that exposure of the developing neuronal system during critical periods to enhanced or aberrant immune activation may result in the brain pathology of ASD and/or in phenotypic differences in the disease dependent upon rates of neuronal and immune development.

Recently, Vargas et al. presented compelling evidence for an active neuroimmune activation in some patients with ASD, with marked activation of microglia and astroglia and altered cytokine profiles responses in the brain and spinal fluid (Vargas et al., 2005). Of particular interest is the observation that cytokine profiles were quantitatively and qualitatively different when comparing brain tissue extracts and CSF; reasons for the relatively high levels of these cytokines in CSF and the presence of a pattern distinct from that in the cerebral parenchyma, are unknown. In this study, the abundance of lymphocyte-derived cytokines in the CSF, and yet the absence of either a CSF lymphocytosis or a lymphocyte infiltrate in brain tissue, argues against a primary intra-cerebral source for these specific cytokines. The tissue reaction in the brain may represent secondary activation of the resident innate immune response following cytokine elaboration elsewhere; the authors suggest possible primary sources as the leptomeninges and choroid plexus. It is plausible that systemic and intestinal immune dysregulation occurs concomitantly due to a central defect that has a global effect on the immune response in this cohort of children with ASD and GI symptoms. However, it is also plausible that these data reflect a primary intestinal immune activation and immunopathology, which leads to heightened systemic immune activation and neuroinflammation as a consequence. Indeed, in a recent study the generation of chronic colitis in an animal model through the rectal administration of trinitrobenzene sulfonic acid (TNBS), not only caused GI inflammation, but also led to activation of brain areas that are abnormal in autism, as measured by c-*Fos* expression (Welch et al., 2005). Encephalopathy following primary intestinal pathology is well known (reviewed by Wakefield et al., 2002). Central, Vagus nerve-independent, autonomic activation, and increased blood brain permeability have also been reported in this same model (Assen et al., 2003; Hathaway et al., 1999; Natah et al., 2005). In celiac disease, it is recognized that primary mucosal immunopathology due to gliadin intolerance can produce secondary neurological disease including: cerebral inflammation, dementia, cerebellar ataxia, epilepsy, and heterotopic cerebral calcification (Hadjivassiliou et al., 1996; Gobbi et al., 1992; Bushara, 2005). Further investigation of gut–brain interactions in this cohort of children with ASD and GI symptoms is necessary to clarify the potential links with the intestinal pathology and the effect on behaviors.

This study adds to the growing literature that a dysregulated or aberrant immune response may be central to the development of autism in some children. The increased synthesis of pro-inflammatory cytokines and reduced level of counter regulatory cytokines leads to an imbalanced immune response in a cohort of children with autism and associated GI immunopathology. This altered immune profile is not restricted to the mucosal compartment but is mirrored in the periphery. The accumulating data suggest that, potentially, a primary mucosal immunopathology may lead to secondary systemic and CNS immunopathology that may impact neurodevelopment in a way that is relevant to some of the clinical features of autism in this cohort.

5. Uncited references

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O'Farrelly, 1998

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