

# Aberrant responses to TLR agonists in pediatric IBD patients; the possible association with increased production of Th1/Th17 cytokines in response to candida, a luminal antigen

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Toll like receptors (TLR) regulate innate immune responses sensing byproducts of intestinal microbiota. We examined responses to TLR agonists in children with inflammatory bowel disease (IBD). Peripheral blood mononuclear cells (PBMC) obtained from children with IBD [Crohn's disease (CD, n = 10), ulcerative colitis (UC, n = 10)], children with non-IgE-mediated food allergy (NFA, n = 20), and controls (n = 15) were tested for their production of proinflammatory and counter-regulatory cytokines with TLR agonists in comparison with their cytokine production against milk protein and candida. IBD patients were all in the inactive state. IBD PBMC produced more IL-6 with all the TLR agonists tested than controls. CD PBMC produced more counter-regulatory cytokines with TLR agonists, while UC PBMC produced more IL-1 $\beta$  and IL-10 with TLR 7/8 agonist than controls. Cytokine production by NFA PBMC did not differ from controls. CD but not UC PBMC produced more IFN- $\gamma$  and IL-17 with candida. Aberrant responses to TLR agonists may be associated with increase in IFN- $\gamma$ /IL-17 production against candida in CD children.

**Harumi Jyonouchi<sup>1</sup>, Lee Geng<sup>1</sup>,  
Agnes Cushing-Ruby<sup>1</sup> and  
Iona M. Monteiro<sup>2</sup>**

<sup>1</sup>Division of Allergy/Immunology and Infectious Diseases, University of Medicine and Dentistry of New Jersey (UMDNJ), New Jersey Medical School (NJMS), Newark, NJ, USA, <sup>2</sup>Division of Pediatric Gastroenterology, Pediatrics, University of Medicine and Dentistry of New Jersey (UMDNJ), New Jersey Medical School (NJMS), Newark, NJ, USA

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Harumi Jyonouchi, Division of Allergy/Immunology and Infectious Diseases, University of Medicine and Dentistry of New Jersey (UMDNJ), New Jersey Medical School (NJMS), Newark, NJ, 07101-1709, USA

Tel.: 973 972 1414

Fax: 973 972 5895

E-mail: jyanouha@umdnj.edu

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Inflammatory bowel disease (IBD) is thought to be mediated by aberrant immune responses against commensal flora. Recent genome-wide linkage analyses and candidate gene-based studies have identified possible multiple IBD susceptibility loci (1–3). Genes identified from such loci include those associated with innate immune defense, indicating that aberrant innate immune responses can be risk factors for IBD.

It is crucial for the gut mucosal immune system to establish immune homeostasis that permits us to (i) cohabit with commensal flora under a mutual beneficial relationship, (ii) digest huge amounts of macronutrients without provoking

immune reactions (oral tolerance), and (iii) maintain effective immune defense against pathogens. This somewhat conflicting task may make the gut mucosal immune system error-prone, requiring active counter-regulatory measures such as development of regulatory T (Treg) cells (4). These measures appear to develop gradually during the first few years of life and this is why infants are prone to be reactive to common dietary proteins (DP) in IgE or non-IgE dependent manner. However, most children outgrow this condition by 2–3 yr of age without developing reactivity to commensal flora (5). This is in contrast to IBD patients; aberrant

immune responses against commensal microbiota are implicated with development of IBD. Although the events that lead to abolishment of mucosal immune homeostasis are not well understood, genetic analysis of IBD patients indicate a role of innate immunity (1–3).

Innate immune responses are mounted by innate immune cells (macrophages, dendritic cells, natural killer cells, etc.) in an antigen (Ag)-independent manner via receptors recognizing common microbial byproducts, pattern recognition receptors (PRR) (6, 7). PRR are genetically predetermined to sense non-human microbial byproducts such as endotoxin, proteoglycans, unmethylated CpG sequences, and double or single stranded RNA (6). Among PRR, toll like receptors (TLR) are studied most extensively. Polymorphisms of certain TLRs are now implicated with altered responses to certain microbes (8–10). Other PRR families include nucleotide-binding oligomerization domain (NOD) like receptors (NLR) (7). Loss of function mutations of NOD2, a member of the NLR family, is associated with increased susceptibility to Crohn's disease (CD) at least in western countries (1, 2). NOD2 is reported to exert synergistic effects on TLR2-mediated pathways which are important for recognizing peptidoglycans produced by gram positive bacteria (11). NOD2 is also reported to modulate TLR4 and TLR3 mediated pathways that sense endotoxin produced by gram negative bacteria and viral-derived double stranded RNA respectively (6, 11).

Innate immune responses profoundly affect subsequent adaptive immunity and T-helper (Th) cell differentiation via activation, migration, and maturation of antigen presenting cells. For example, IL-12 production by innate immune cells promotes differentiation of Th1 cells while increased production of IL-1 $\beta$  and IL-6 promote differentiation of Th17 cells (12, 13). Although innate immune responses are crucial for the first line immune defense, aberrant innate immune responses can be detrimental. For example, IL-6 produced by innate immune cells prevents activation-induced T-cell apoptosis and may prolong inflammation along with activation of acute phase responses (14, 15).

In this study, we hypothesized that TLR-mediated signaling pathways may be altered in IBD patients. Since TLR are genetically predetermined with little post-natal variation, such abnormalities, if present, are associated with genetic variation and can be detected in the peripheral blood (PB) monocytes in IBD patients with inactive disease state. We also hypothesized

that such abnormalities are not found in children who outgrew non-IgE mediated food allergy (NFA) once they establish mucosal immune homeostasis. This study evaluated TLR responses in stable IBD patients followed in our clinic and compared their responses to those of healthy controls and children who have outgrown NFA. The results are evaluated in comparison with their responses to representative luminal Ag.

## Materials and methods

### Study subjects

This study includes 20 IBD children [CD n = 10, UC n = 10], healthy control children (n = 16), and control children with history of non-IgE mediated food allergy (NFA, n = 20). IBD and NFA children were diagnosed and treated in the Pediatric Gastroenterology and Pediatric Allergy/Immunology Clinics, respectively, at our institution. IBD diagnosis was made on the basis of endoscopic and histological findings. Demographics of the study subjects are summarized in Table 1. The treatment and the disease status of IBD patients at the time of sample obtainment are summarized in Table 2 (16, 17). None of the IBD patients were on oral or IV steroids for at least 3 wk prior to the venipuncture and they are considered to be in the inactive disease state (Table 2). Before the venipuncture, all the subjects were given a physical examination and found not to have active infection. NFA children who revealed aberrant immune reactivity to DP served as controls with immune reactivity to benign luminal Ag. These NFA children were already on the restricted diet avoiding offending food and were in stable condition with resolution of GI symptoms.

Table 1. Demographics of the study subjects

Study group	Age median (range)	Sex (M:F)	Ethnicity
Crohn's disease (n = 10)	14.7 (11.4–17.8)	7:3	1 W†, 7 AA†, 2 other
Ulcerative Colitis (n = 10)	13.2 (6.6–17.5)	4:6	1 W, 1 Asian, 7 Hispanic, 1 other
NFA* control (n = 20)	3.6 (1–8.8)	17:3	15 W, 1 AA, 1 Asian, 3 Hispanic
Control children (n = 16)	6.7 (2.5–11.3)	14:2	13 W, 1 AA†, 2 Hispanic

\*NFA, non-IgE mediated food allergy. These patients were diagnosed with NFA mainly to cow's milk protein and treated by restricted diet, avoiding the offending food. Blood samples were obtained after their NFA symptoms were under control.

†AA, African American; W, non-Hispanic Caucasian.

Table 2. Clinical characteristics of CD and UC patients

Characteristics	CD (n = 10)	UC (n = 10)
Location		
Small bowel	8	
Colon	8	10
Years from diagnosis: median (range)	2 (1–10)	0.7 (0.2–7.9)
Disease activity		
CRP*	1.4 (0.3–7.7)	1.3 (0–12.2)
CDAI*‡: median (range)	2.5 (0–50)	
PUCAI*§: median (range)		13 (0–25)
Therapy		
None (n)	0	0
5-ASA* (n)	9	10
Steroids (n)	0	0
Immunosuppressors (n)†	8	3
Infliximab (n)	1	1
Surgery (n)	0	0

\*5-ASA, 5-aminosalicylic acid; CRP, C reactive protein; CDAI, Crohn's Disease Activity Index; PUCAI, Pediatric Ulcerative Colitis Activity Index.

†6- or azathiopurine.

‡CDAI < 150 is considered to be in the inactive disease state.

§PUCAI: <10; no disease, 10–35 mild disease, 35–64, moderate disease, >65 severe disease.

#### Toll like receptor responses

Peripheral blood mononuclear cells were isolated by Ficoll-Hypaque density gradient centrifugation. Innate immune responses were assessed by incubating PBMCs ( $10^6$  cells/ml) overnight with TLR4 agonist (LPS; 0.1  $\mu$ g/ml, GIBCO-BRL, Gaithersburg, MD, USA), TLR2/6 agonist (zymosan; 50  $\mu$ g/ml, Sigma-Aldrich, St. Luis, MO, USA), TLR3 agonist (Poly I:C, 0.1  $\mu$ g/ml, Sigma-Aldrich), and TLR7/8 agonist (CL097, water-soluble derivative of imidazoquinoline, 20  $\mu$ M, InvivoGen, San Diego, CA, USA) in RPMI 1640 with additives as previously described (18). No endotoxin was detectable in TLR2, 3, and 7/8 agonists used in the study (Sigma-Aldrich). Levels of proinflammatory [tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), IL-1 $\beta$ , IL-6, IL-12p40, and IL-23] and counter-regulatory [IL-10, transforming growth factor- $\beta$  (TGF- $\beta$ ) and soluble TNF receptor II (sTNFR<sub>II</sub>)] cytokines in the culture supernatant were then measured. Overnight incubation was adequate to induce the optimal responses in this setting. Type 1 interferon (IFN) production was also measured in response to TLR 7/8 agonists; we found minimal production of type 1 IFN with stimuli of other agonists. We also tested responses to TLR9 agonist (CpG, 5  $\mu$ g/ml; InvivoGen) in the 15 study subjects secondary to limited numbers of cells. In these limited numbers of study subjects, we obtained the similar results seen in responses to TLR3 agonist.

#### Reactivity to luminal antigens

Cellular reactivity to common luminal Ag was assessed by incubating PBMCs ( $10^6$  cells/ml) with the Ags for 4 days and measuring levels of IFN- $\gamma$ , TNF- $\alpha$ , IL-5, IL-10, IL-12p40, IL-17, and TGF- $\beta$  in the culture supernatant (19). The representative luminal Ags used included; (i) crude extract of cow's milk protein (CMP, 100  $\mu$ g/ml, kindly provided by Ross Products Division/Abbott Laboratories, Columbus, OH, USA) and (ii) extract of *Candida albicans* (5  $\mu$ g/ml, Greer, Lenoir, NC, USA). Four days' incubation period resulted in the optimal production of these cytokines in this setting in the initial titration studies. It should be noted that prior to implementation of a dairy-free diet, NFA PBMC revealed significant reactivity to CMP as indicated by the production of higher than control mean (CM) + 1 s.d. of TNF- $\alpha$ /IL-12p40 (19). No endotoxin was detected in these antigen stimuli (Sigma-Aldrich).

#### Cytokine ELISA

Cytokine levels were measured by an enzyme-linked immunosorbent assay (ELISA), using OptEIA™ Reagent Sets (BD Pharmingen, San Diego, CA, USA) for TNF- $\alpha$ , IL-1 $\beta$ , IL-5, IL-6, IL-10, IL-12p40 and ELISA reagent set (R & D, Minneapolis, MN, USA) for sTNFR<sub>II</sub>, IL-17 (IL-17A), and TGF- $\beta$ . IL-23 ELISA kit was purchased from eBioscience (San Diego, CA, USA). Type 1 IFN were measured using IFN- $\alpha$  multi species bioassay ELISA kit (US biological, Swampscott, MA, USA). Intra- and inter-variations of cytokine levels were < 5%.

#### Statistics

For comparison of test values with control values, Wilcoxon signed-rank test was used. For comparison of values of multiple groups, Kruskal–Walls test was used. These tests were performed using R.2.5.1 (20). A value of  $p < 0.05$  was considered to be significant.

## Results

#### Toll like receptor responses

*Toll like receptor 4 responses (Table 3).* Crohn's disease and UC PBMC produced more IL-6 than normal and NFA controls but production of other cytokines did not differ among the study groups.

Table 3. Cytokine production in response to TLR4 agonists

Study group	Proinflammatory cytokines				Counter-regulatory cytokines		
	TNF- $\alpha$	IL-6	IL-1 $\beta$	IL-12	sTNFR $\text{II}$	IL-10	TGF- $\beta$
CD	1109.2* (226.3–3455.3)	37237.5 (16051.8–59199.1) p < 0.005	1788.9 (296.9–3828.9)	246.0 (7.2–1288.0)	1277.0 (764.4–1903.3)	1360.5 (585.8–1661.1)	2152.1 (496.9–2656.7)
UC	1200.6 (19–2461)	35722.8 (18276.2–96856) p < 0.005	1369.1 (1924.–2796.6)	218.5 (<3.9–1225.4)	1266.1 (220.9–2310.6)	1358.7 (882.1–1849.3)	863.5 (721.6–2588.4)
NFA control	786.1 (256.4–2646.3)	19478.9 (2446.3–38871.9)	1457.9 (375.7–3317.4)	302.3 (9.9–951.1)	813.2 (157.9–1625.3)	1137.1 (316.7–1666.2)	920.7 (126.8–2405.1)
Normal control	813.6 (155.3–1681.8)	22457.7 (10399.2–34649.4)	1264.6 (383.6–2864.6)	129.5 (<3.9–664.0)	1016.8 (526.6–1647.0)	1156.2 (655.4–1827.9)	1055.0 (134.6–2218.4)

TNF- $\alpha$ , tumor necrosis factor-alpha; IL, interleukin; sTNFR, soluble TNF receptor; TGF, transforming growth factor; TLR, Toll like receptors; CD, Crohn's disease; UC, ulcerative colitis; NFA, non-IgE-mediated food allergy.

\*The results were expressed as median (range) ng/ml. The results significantly higher than normal controls were high-lighted with p values (Wilcoxon signed ranks test).

*TLR2/6 responses (Table 4).* Crohn's disease and UC PBMC produced higher amounts of IL-6 and IL-10 than both the control groups. CD PBMC also produced more sTNFR $\text{II}$  than both the control groups. Production of other cytokines did not differ among the study groups.

*TLR3 responses (Table 5).* Crohn's disease and UC PBMC produced higher amounts of IL-6 and IL-10 than both the control groups. CD PBMC also produced more TGF- $\beta$  than both the control groups. Production of other cytokines did not differ among the study groups.

*TLR7/8 responses (Table 6).* Crohn's disease and UC PBMC also produced higher amounts of IL-6. In addition, CD PBMC revealed higher production of sTNFR $\text{II}$  and TGF- $\beta$  than both the control groups. In contrast, UC PBMC

revealed higher IL-1 $\beta$  and IL-10 production than normal controls. Production of other cytokines did not differ among the study groups.

*Other responses.* Interleukin-23 and type 1 IFN are also implicated with development of certain autoimmune conditions (12, 21). We thus measured IL-23 production in response to TLR4, 2/6, and 7/8 agonists (Fig. 1). We also measured type 1 IFNs in response to TLR 7/8 agonist (Fig. 2); we found minimal production of type 1 IFNs with other stimuli. CD but not UC PBMC produced higher amounts of IL-23 with TLR4 agonist (Fig. 1) but IL-23 production with other TLR agonists did not differ among the study groups (median of IL-23 production: < 120 pg/ml for TLR 2/6 agonist and < 3.9 pg/ml for TLR 3 and 7/8 agonists). As for type 1 IFN, only NFA PBMC revealed higher amounts of type 1

Table 4. Cytokine production in response to TLR2/6 agonists

Study group	Proinflammatory cytokines				Counter-regulatory cytokines		
	TNF- $\alpha$	IL-6	IL-1 $\beta$	IL-12	sTNFR $\text{II}$	IL-10	TGF- $\beta$
CD	1560.1* (153.5–1916.7)	3026.3 (1741.6–4369.2) p < 0.005	803.2 (362.5–2963.4)	246.0 (<3.7–1282)	969.1 (292.3–1594.6) p < 0.01	357.1 (91.3–1277.2) p < 0.01	1447.3 (452–2257.9)
UC	1216.6 (646.3–1643.5)	2969.0 (2070.9–3881.5) p < 0.005	640.0 (111.9–2308.7)	123.2 (<3.9–1053.5)	765.6 (77.1–2234.6)	274.7 (9.4–1488.5) p < 0.05	822.8 (467.3–1118.6)
NFA control	1298.8 (315.4–2146.8)	1893.6 (792.8–3162.5)	474.1 (84.6–3708.8)	288.5 (<3.7–1881.1)	377.7 (187.3–1197.0)	102.8 (<3.7–1081.7)	588.3 (96.9–2001.9)
Normal control	1266.3 (594.8–1582.7)	2131.4 (1569.4–2715.2)	413.0 (126.0–1223)	206.7 (<3.9–965.8)	428.0 (23.6–792.8)	148.5 (26.6–561.9)	616.0 (69.0–1603.3)

TNF- $\alpha$ , tumor necrosis factor-alpha; IL, interleukin; sTNFR, soluble TNF receptor; TGF, transforming growth factor; TLR, Toll like receptors; CD, Crohn's disease; UC, ulcerative colitis; NFA, non-IgE-mediated food allergy.

\*The results were expressed as median (range) ng/ml. The results significantly higher than normal controls were high-lighted with p values (Wilcoxon signed ranks test).

## Aberrant responses to TLR agonists in pediatric IBD patients

Table 5. Cytokine production in response to TLR3 agonists

Study Group	Proinflammatory cytokines				Counter-regulatory cytokines		
	TNF- $\alpha$	IL-6	IL-1 $\beta$	IL-12	sTNFR $\text{II}$	IL-10	TGF- $\beta$
CD	93.0* (<3.9–1348.8)	1327.2 (40.7– 3129.7) p < 0.01	<3.9 (<3.9–959.8)	<3.9 (<3.9–964.1)	559.9 (25.6–1676) p < 0.005	<3.9 (<3.9–1095.5)	1181.1 (35.8–2590.8) p < 0.05
UC	1372 (<3.9–1699.7)	890.2 (<3.2–3221.8) p < 0.05	<3.9 (<3.9–527.0)	<3.9 (<3.9–196.5)	874.3 (41.3–1365.5) p < 0.05	76.1 (<3.9–501) p < 0.05	1265 (145.2–1884.7)
NFA control	<3.9 (<3.9–107.2)	87.9 (<3.2–1162.8)	<3.9 (<3.9–56.8)	<3.9 (<3.7–141.8)	127.7 (<15.7–422.7)	<3.9 (<3.9–67.7)	890.7 (117.7–2790.1)
Normal control	<3.9 (<3.9–55.8)	43.6 (<3.2–842.8)	<3.9 (<3.9–15.5)	<3.9 (<3.9–38.0)	136.9 (<15.7–477.2)	<3.9 (<3.9–19.8)	1056.4 (163.7–1927.6)

TNF- $\alpha$ , tumor necrosis factor-alpha; IL, interleukin; sTNFR, soluble TNF receptor; TGF, transforming growth factor; TLR, Toll like receptors; CD, Crohn's disease; UC, ulcerative colitis; NFA, non-IgE-mediated food allergy.

\*The results were expressed as median (range) ng/ml. The results significantly higher than normal controls were high-lighted with p values (Wilcoxon signed ranks test).

Table 6. Cytokine production in response to TLR7/8 agonists

Study Group	Proinflammatory cytokines				Counter-regulatory cytokines		
	TNF- $\alpha$	IL-6	IL-1 $\beta$	IL-12	sTNFR $\text{II}$	IL-10	TGF- $\beta$
CD	1393.3* (135.8–1705.7)	3200.4 (1099.8–4361.4) p < 0.02	1240.5 (5.8–5035.9)	1595.6 (28.2–6941.3)	1016.4 (550.1–1814.5) p < 0.01	845.1 (84.7–1352.0)	2361.1 (5.38–2558.4) p < 0.05
UC	1372 (<3.9–1699.7)	3077.2 (708.0–3569.5) p < 0.01	2318.3 (25.5–3668.8) p < 0.05	717.0 (<3.9–1507.9)	1096.7 (170.5–2016.0)	878.1 (137.0–1272.4) p < 0.05	913.6 (620.7–1880.0)
NFA control	894.8 (48.6–1911.4)	2313.3 (1093.9–3114.2)	1443.9 (33.0–3511.0)	378.9 (<3.7–1536.6)	600.7 (99.3–1409.7)	363.3 (83.6–1038.0)	788.1 (115.2–2995.3)
Normal control	535.5 (116.8–1978.5)	2252.4 (1178.3–2756.3)	805.1 (63.7–2717.4)	569.5 (72.7–1808.0)	696.4 (194.9–1124.1)	307.3 (101.9–885.0)	1088.7 (134.1–1975.9)

TNF- $\alpha$ , tumor necrosis factor-alpha; IL, interleukin; sTNFR, soluble TNF receptor; TGF, transforming growth factor; TLR, Toll like receptors; INF- $\gamma$ , interferon-gamma; CD, Crohn's disease; UC, ulcerative colitis; NFA, non-IgE-mediated food allergy.

\*The results were expressed as median (range) ng/ml. The results significantly higher than normal controls were high-lighted with p values (Wilcoxon signed ranks test).

IFNs than normal controls in response to TLR 7/8 agonist (Fig. 2).

Production of proinflammatory and counter-regulatory cytokines with TLR3 agonist was lower as compared with other TLR agonists except for IL-6 in our culture system (data not shown). Nevertheless, CD and UC PBMCs produced more IL-6 than control groups (p < 0.01 for CD and p < 0.05 for UC PBMC).

### Responses to luminal antigens

Crohn's disease but not UC PBMC produced higher amounts of Th1 (IFN- $\gamma$ ) and Th17 (IL-17) cytokines with candida Ag but not with CMP (Table 7). Production of regulatory cytokines (TGF- $\beta$  and IL-10) or Th2 cytokine (IL-5) did not differ among the study groups. It is of note that CMP but not candida Ag induced produc-

tion of fair amounts of IL-10 in all the study groups. We observed similar results when PBMC were tested for their responses to  $\beta$ -lactoglobulin, a major component of CMP (data not shown).

### Discussion

Disrupted immune homeostasis that allows aberrant immune responses against commensal microbiota has been implicated in the development of IBD. This disruption may be triggered by aberrant innate immune responses mediated by the families of PRR including TLR and NLR. In fact, the first identified genetic risk factor for CD is a negative mutation of NOD2, a receptor of the muramyl dipeptide component of peptidoglycan produced by certain microbes and a member of NLR (11, 22). NOD2 is found to modulate TLR signaling pathways (11). TLR per

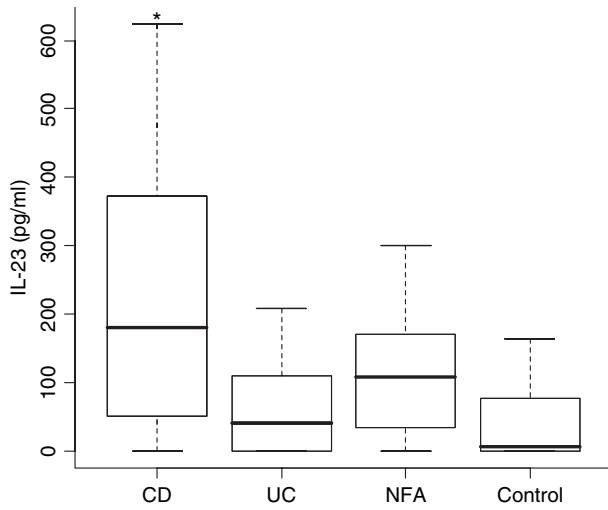


Fig. 1. Production of interleukin (IL)-23 by peripheral blood mononuclear cells (PBMC) obtained from children with Crohn's disease, ulcerative colitis, non-IgE-mediated food allergy or healthy controls in response to Toll like receptors 4 (TLR4) agonist. PBMC ( $10^6$ /ml) were stimulated overnight with TLR4 agonist as described in the method section. The 'I' bar marks the range of IL-23 levels and the thick black line marks the median. The box illustrates where the 'interquartile range' falls. 'Outliers' are marked separately as 'o'. \*, higher than control cells ( $p < 0.02$ , Wilcoxon signed ranks test).

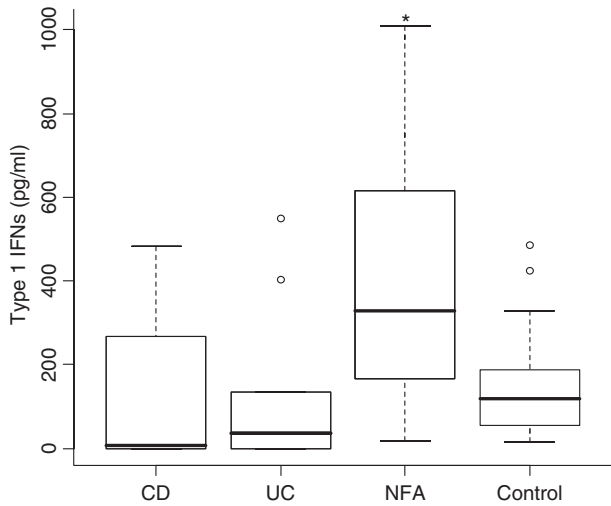


Fig. 2. Production of type 1 interferons (IFN) by peripheral blood mononuclear cells (PBMC) obtained from children with Crohn's disease, ulcerative colitis, non-IgE-mediated food allergy or healthy controls in response to Toll like receptors 7/8 (TLR7/8) agonist. PBMC ( $10^6$ /ml) were stimulated overnight with TLR7/8 agonist as described in the method section. The 'I' bar marks the range of IFN levels and the thick black line marks the median. The box illustrates where the 'interquartile range' falls. \*, higher than other study groups ( $p < 0.02$ , Wilcoxon signed ranks test).

se are also included in the candidate genes for IBD development. Activation of TLR/NOD pathways leads to the production of various

Table 7. Cytokine production in response to luminal antigens (milk protein and candida)

Study Group	Cow's milk protein							
	IFN- $\gamma$	TNF- $\alpha$	IL-5	IL-12	IL-17	L-10	TGF- $\beta$	
CD	10.5 (<3.9-716.6)*	234.7 (<3.9-477.1)	<3.9 (<3.9)	197.7 (<3.6-987.6) $p < 0.02$	42.1 (<3.9-181.4)	680.0 (19.0-1326.2)	1451.0 (513.7-2128.1)	
UC	13.8 (<3.9-480.5)	144.4 (<3.9-588.4)	<3.9 (<3.9)	226.2 (<3.9-1540.8)	<3.9 (<3.9-185.2)	691.2 (118.3-1263.3)	1039.8 (477.2-2040.3)	
NFA control	31.8 (<3.9-234.1)	198.2 (8.1-862.1)	<3.9 (<3.9-81.9)	540.3 (29.2-2267.5)	10.8 (<3.9-101.8)	767.9 (393.7-1404.0)	1159.8 (159.2-3445.5)	
Normal control	23.8 (<3.9-203.3)	139.7 (<3.9-389.5)	<3.9 (<3.9)	276.2 (63.1-627.4)	9.0 (<3.9-117.3)	747.0 (308.9-1428.8)	1085.8 (324.7-2262.2)	
				Candida albicans				
CD	245.9 (5.5-1393.4) $p < 0.05$	27.0 (<3.9-292.9)	IL-5 <3.9 (<3.9-92.0)	IL-12 <3.9 (<3.9-28.0)	IL-17 180.9 (11.4-573.0)	IL-10 18.8 (<3.9-111.8)	TGF- $\beta$ 1128.0 (198.2-1744.8)	
UC	28.8 (<3.9-670.0)	12.4 (<3.9-644.6)	<3.9 (<3.9-22.6)	<3.9 (<3.9-19.9)	21.0 (<3.9-429.3)	13.4 (<3.9-155.3)	1291.5 (326.2-1960.6)	
NFA control	23.0 (<3.9-790.7)	<3.9 (<3.9-615.1)	<3.9 (<3.9-298.7)	<3.9 (<3.9-207.9)	4.1 (<3.7-172.1)	5.25 (<3.9-97.7)	880.1 (102.1-2932.6)	
Normal control	8.2 (<3.9-193.7)	<3.9 (<3.9-116.4)	5.9 (<3.9-91.4)	<3.9 (<3.9-37.9)	21.0 (<3.9-177.7)	15.4 (<3.9-53.2)	1170.1 (326.9-2309.2)	

TNF- $\alpha$ , tumor necrosis factor-alpha; IL, interleukin; sTNFR, soluble TNF receptor; TGF, transforming growth factor; TLR, Toll like receptors; INF- $\gamma$ , interferon-gamma; CD, Crohn's disease; UC, ulcerative colitis; NFA, non-IgE-mediated food allergy.

\*The results were expressed as median (range) ng/ml. The results significantly higher than normal controls were high-lighted with p values (Wilcoxon signed ranks test).

proinflammatory and counter-regulatory cytokines. Among them, TNF- $\alpha$  is known to play a key role in IBD, as evidenced in striking therapeutic effects of TNF- $\alpha$  inhibitors in CD patients (23). In addition, Cantó et al. reported increase in TNF- $\alpha$  production by PBMC in response to zymosan, a TLR 2/6 agonist, in IBD patients with active but not inactive disease state (24).

T-helper (Th) 17 cells, a newly identified T-cell subset, draw significant interest in their role in autoimmunity. That is, although Th17 cells appear important in immune defense against certain pathogens such as candida and mycobacterium (13, 25), Th17 cells can exert detrimental effects in autoimmune conditions including IBD (26). It is now generally agreed that both Th1 and Th17 cells have roles in CD pathogenesis, while Th2 skewed responses are implicated in UC (27, 28). Micro-environmental factors influence differentiation of Th1 and Th17 cells. Recent studies indicate reciprocal development of Treg vs. Th17 cells, depending on cytokine levels in the microenvironment. Namely, TGF- $\beta$  and IL-2 promote differentiation of Treg cells, while IL-1 $\beta$ , IL-6, and IL-23 promote differentiation of Th17 cells in humans (13, 29). It was also shown that concentrations of IL-6 vs. retinoic acid in the gut microenvironment affect Th17 vs. Treg cell differentiation in rodents (30, 31).

Given these findings, we hypothesized that TLR responses are dysregulated in IBD patients, leading to aberrant Treg vs. Th1/Th17 responses against common luminal Ags. TLR mediated signaling pathways are genetically predetermined and TLRs are widely distributed in the body. Thus we also hypothesized that such aberrant TLR responses can be detected in peripheral blood monocytes in IBD subjects with the inactive disease state when such responses are less affected by inflammation associated with IBD. Thus in this study, we examined cytokine production against a panel of TLR agonists and common luminal Ag in CD and UC children with the inactive disease state.

Our results revealed production of significantly higher amounts of IL-6 by IBD PBMC than both the NFA and normal control cells in response to TLR agonists. With these TLR agonists, production of neither TNF- $\alpha$  nor IL-1 $\beta$  was higher in IBD children than in controls except for higher IL-1 $\beta$  production by UC PBMCs with TLR7/8 agonist. This is consistent with the previous report that TNF- $\alpha$  production with zymosan was not elevated in IBD patients in the inactive disease state (24). IL-6 is a pleiotropic cytokine that plays a central role in immune defense but also in inflammatory responses. IL-6 induces

signaling mediated by signal transducer and activator of transcription-3 (STAT3) in mucosal T cells and protects them from apoptosis (15). Accumulation of pathogenic T cells mediated by resistance to apoptosis is implicated in the development of IBD (15, 32). Therefore such an increase in IL-6 production triggered by TLR responses may be associated with pathogenesis of IBD.

When we assessed the production of counter-regulatory cytokines, we also found significant increase in production of IL-10, TGF- $\beta$ , and sTNFR<sub>II</sub> in CD children and less evidently in UC children. In this study, as a TLR 2/6 agonist, we employed zymosan, a well known suppressor of IL-10 in normal individuals (33). However, zymosan suppressed IL-10 production less effectively in IBD patients than control children. Impaired IL-10 production was previously implicated in the pathogenesis of IBD as evident in IL-10 knock out mice, one of the rodent models of IBD (15, 34). Our finding of higher IL-10 production with TLR 2/6 and TLR7/8 agonists in CD children may be contradictory to the findings in such mouse models. The same holds true for the increased production of sTNFR<sub>II</sub> and TGF- $\beta$  in CD children. This difference may reflect the counter-regulatory action of the immune system resulting from chronic GI and systemic inflammation in CD patients. Although counter-regulatory cytokines are indispensable for immune homeostasis, excessive IL-10, TGF- $\beta$ , and sTNFR<sub>II</sub> may hinder the clearance of pathogens and could contribute to the alteration of commensal flora in CD patients.

Nucleotide-binding oligomerization domain 2 mutation is the first described genetic risk factor for IBD and NOD2 is reported to modulate TLR2, 3, and 4 but not TLR 7/8 signaling in rodents (11). In this study, we found altered production of multiple cytokines in response to TLR2/6 and 7/8 agonists in IBD patients. Such changes may indicate an association between polymorphisms of TLR7/8 and/or TLR 2/6 instead of NOD2 in IBD patients. However, polymorphisms of TLR7/8 are not well known; one case control study reported negative results in single nucleotide polymorphisms of TLR7 in IBD (35). Alternatively, our findings may also be the result of abnormalities of signaling pathways shared by these TLRs.

In NFA children, aberrant (undesired) cellular immune responses to macronutrients (DP) are thought to be due to their immature gut immune system, a condition that they will eventually outgrow (5). Thus we reasoned that control NFA children, unlike IBD subjects, reveal normal

TLR responses once their NFA condition is under control. Indeed, their TLR responses did not differ from those of normal controls in this study. The only exception was higher production of type 1 IFNs with TLR 7/8 agonist in NFA children as compared with other study groups. Type 1 IFN are produced by multiple lineage cells but plasmacytoid dendritic cells (pDC) that expresses TLR7 and TLR9 are the major source for systemic production of type 1 IFN (36). We observed higher numbers of pDC in young children (H. J. et al. unpublished observation). Thus the increase in type 1 IFN in NFA children may be a reflection of their young age (Table 1). In summary, our results in IBD patients in comparison with NFA children further support possible abnormalities in TLR signaling pathways in IBD children.

Our findings of increase in production of IL-6 and TGF- $\beta$  in IBD patients are intriguing given the fact that these two cytokines appear critical for differentiation of Th17 vs. Treg cells (13, 27). To further address the possibility of altered Treg vs. Th1/Th17 responses, we assessed a profile of T cell cytokine production against two common luminal Ag, CMP and candida. We employed CMP, since control NFA children had revealed cellular immune reactivity to CMP prior to implementation of the dairy-free diet. Our results revealed higher IL-17 and IFN- $\gamma$  production by CD cells with candida but not with CMP. In contrast, neither UC nor NFA cells revealed higher IFN- $\gamma$  or IL-17 production with candida. This is consistent with previously noted Th1/Th17 skewed responses in CD and Th2 skewed responses in UC (15). As for milk protein, we observed fair amounts of IL-10 production with CMP in all the study groups but this is not the case for candida Ag. Others reported that CMP-specific Treg cells producing IL-10 were found in children who outgrew NFA to CMP (37). For DP which are present at high concentrations in the gut lumen, oral tolerance may be maintained by IL-10 producing Treg cells or other means. For luminal microbial Ags, such regulatory mechanisms may not be induced and aberrant TLR responses may trigger or aggravate Th1/Th17 immune reactivity to commensal microbes.

In summary, our results revealed aberrant TLR responses detectable in PBMC in CD and UC patients. The most intriguing result is altered IL-6 production in IBD patients with all TLR agonists tested. These altered patterns of cytokine production could favor Th1/Th17 cell differentiation in CD patients, resulting in increased IFN- $\gamma$  and IL-17 production against commensal microbiota. However, we did not find significant

association between ethnicity/treatment measures and IL-6 production. This may be attributed to the small number of the study subjects and also to the fact that all the IBD patients were in the inactive disease state. Alternatively, our findings may just reflect predisposing innate immune abnormalities associated with development of IBD. Such questions needs to be addressed in larger scale studies using some of the parameters tested in this study in both active and inactive states of IBD patients.

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