BRIEF ARTICLE

Immune phenotype in children with therapy-naïve remitted and relapsed Crohn's disease

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Abstract

AIM: To characterize the prevalence of subpopulations of CD4+ cells along with that of major inhibitor or stimulator cell types in therapy-naïve childhood Crohn's disease (CD) and to test whether abnormalities of immune phenotype are normalized with the improvement of clinical signs and symptoms of disease.

METHODS: We enrolled 26 pediatric patients with CD. 14 therapy-naïve CD children; of those, 10 children remitted on conventional therapy and formed the remission group. We also tested another group of 12 children who relapsed with conventional therapy and were given infliximab; and 15 healthy children who served as controls. The prevalence of Th1 and Th2, naïve and memory, activated and regulatory T cells, along with the members of innate immunity such as natural killer (NK), NK-T, myeloid and plasmocytoid dendritic cells (DCs), monocytes and Toll-like receptor (TLR)-2 and TLR-4 expression were determined in peripheral blood samples.

RESULTS: Children with therapy-naïve CD and those in relapse showed a decrease in Th1 cell prevalence. Simultaneously, an increased prevalence of memory and activated lymphocytes along with that of DCs and monocytes was observed. In addition, the ratio of myeloid /plasmocytoid DCs and the prevalence of TLR-2 or TLR-4 positive DCs and monocytes were also higher in therapy-naïve CD than in controls. The majority of alterations diminished in remitted CD irrespective of whether remission was obtained by conventional or biological therapy.

CONCLUSION: The finding that immune phenotype is normalized in remission suggests a link between immune phenotype and disease activity in childhood CD. Our observations support the involvement of members of the adaptive and innate immune systems in childhood CD.

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INTRODUCTION

Crohn's disease (CD) is a chronic gastrointestinal disease characterized by segmental inflammation of the intestinal mucosa associated with a dysregulated action of the mucosal immune system to the otherwise innocuous luminal antigens in a genetically susceptible host. About 10%-15% of patients with CD are diagnosed before 18 years of age[1]. Certain features are unique to pediatric CD in comparison to adult onset disease such as different disease location, altered response to immunosuppressive therapy and different genetic and immune phenotype[2,3]. Different characteristics may suggest differences in the pathomechanism of CD in children compared to that in adults. Theories regarding CD pathomechanism include malfunctioning of the immune system. Indeed, several studies carried out in adult CD indicate the involvement of either adaptive or innate immunity[4-7].

In adult CD, peripheral Treg prevalence is diminished in therapy-naïve patients and in the active state of disease or relapses and is increased with therapy or in remission[8-10], while intestinal Treg prevalence is increased in active CD[9-11]. Simultaneously, the prevalence of effector T cells[12-14] and activated T cells[15-17] is increased in the periphery in active CD. The shift of T lymphocytes toward Th1 commitment in peripheral blood and biopsy specimens is a widely observed phenomenon in CD[18].

In addition, the prevalence of antigen presenting cells (APCs) including dendritic cells (DCs) decrease in remission, and even more so in relapses at the periphery, but is increased in tissues, simultaneously with an increased expression of Toll-like receptor (TLR)-2 and 4 in therapy-naïve and treated CD adults[19-21]. Monocytes/macrophages, another group of APCs, are comparable at the periphery but increased in biopsies and showed upregulation of TLR-2 and TLR-4 in the active state and in remission of CD[22-25]. Other cell types of innate immunity such as natural killer (NK) and natural killer T (NKT) cells are less prevalent in active CD[17,26-28].

There have been a limited number of studies specifically performed to investigate alterations of the immune system in children with CD. In addition, the majority of available data are on treated CD children. While in untreated CD children some alterations in adaptive immunity have been reported, such as the skewness of the Th1/Th2 ratio to Th2 (this finding is in contrast with that observed in adult CD)[29-32], recent data suggest that the disturbance of innate immune functions is also a major factor contributing to CD in children. Indeed, in untreated children the central macrophage prevalence is increased[33], and TLR-2 and 4 receptor expression is also enhanced[34]. The role of innate immune system in the pathomechanism of CD may be inversely associated with age at disease onset[2].

The immune dysregulation in CD is affected by ongoing therapy. Aminosalicylates and steroids, as well as immunosuppressive drugs used as first-line therapy in CD have strong immunomodulatory effects[35]. In addition, biological therapy including the tumor necrosis factor α inhibitor infliximab (IFX)[36] also has substantial effects on immune cell functions [many of the data are from patients with rheumatoid arthritis (RA)] as it may increase the prevalence of peripheral and central Tregs[8,37], effector and activated[38,39] and Th1 committed T cells[39,40]. IFX also decreases the prevalence of NK cells[38], DCs[41,42] and monocytes[43,44], as well as TLR-2 and TLR-4 expression in peripheral cells[45]. The immunological impact of conventional or IFX therapy on peripheral immune phenotype is, however, known exclusively for adult CD patients and has not been explored fully in CD children.

In this prospective study we aimed to explore the major cell prevalence of the adaptive and innate immune systems in therapy-naïve CD children and its alteration with the improvement of CD obtained by conventional therapy or IFX treatment.

MATERIALS AND METHODS

We enrolled the following patient groups into our study: (1) 14 therapy-naïve CD children. No drug was prescribed for these patients at the time of CD diagnosis. The diagnosis of CD was established by means of "The Porto criteria"[46]; disease activity was determined according to the Pediatric Crohn's disease activity index (PCDAI)[47]; (2) During conventional treatment [steroid, azathioprine (AZT) and 5-aminosalicylate (5-ASA)], 10 children responded forming the remission group. Clinical remission was defined as a PCDAI < 10; (3) IFX therapy (5 mg/kg IFX at weeks 0, 2, and 6) was started in 12 CD children who failed to respond to conventional therapy forming the "relapsed group". Non-responsiveness was defined as moderately increased PCDAI (PCDAI > 30) in patients under conventional therapy; and (4) Fifteen age- and gendermatched children with functional abdominal pain served as controls. All patients and controls were diagnosed, treated and followed up in the Outpatient Clinic of the First Department of Pediatrics, Semmelweis University between September 2007 and August 2009. The Institutional Ethical Committee approved our study; written parental informed consent was obtained.

The patients' clinical characteristics are shown in Table 1. Small and large bowel was involved in 11 of 14 treatment naïve CD patients, according to the literature (L3 localization, Montreal criteria[48]). Therapy-naïve CD patients and CD patients with relapse had lower body mass index than controls. Lower body weight and body mass index is a common presenting sign in pediatric patients with CD[49]. Reduced food intake, postprandial abdominal cramps, systemic release of cytokines and malabsorptive diarrhea were listed as factors responsible for this phenomenon[50].

Table 1 Clinical data and patient characteristics

	Control	Therapy-naïve (before conventional therapy)	First remission (with conventional therapy)	Relapse (before IFX therapy)	IFX therapy (before 2nd infusion)	IFX therapy (before 3rd infusion)
Clinical data						
n (boys/girls)	15 (6/9)	14 (6/8)	10 (4/6)		12 (5/7)	
Age (yr)	12 (8-16)	10 (8.5-13)	11.5 (9.5-15.5)	14 (11-16)	14.5 (11.5-16)	14.5 (12-16)
Body mass index (kg/m^2)	19.5 (16.5-22.3)	13.9 (12.5-15.8)[d]	18.6 (14.4-19.2)[c]	17.4 (14.1-19.5)[a]	18.8 (14.6-21.3)	19.25 (16.1-22.1)
Body weight (percentile)	58 (35-79)	13 (4-23)[d]	24 (19-44)[c]	21 (7-28)[a]	23 (15-63)	35 (10-74)
Disease duration (mo)	-	10 (8.5-13)	10.5 (8.5-15.5)	11.5 (9.25-15)	12 (9.5-15)	12 (10-15)
Activity index (PCDAI)	-	45 (39-58)	0 (0-5)[f]	45 (25-48)[j]	20 (7-28)[g]	13 (2-22)[l]
Localization (*n*, montreal criteria)	-	L1 (1), L2 (2), L3 (11)	L1 (0), L2 (2), L3 (8)		L1 (1), L2 (2), L3 (9)	
Laboratory data						
White blood cell count (g/L)	7.9 (5.7-10.1)	12.4 (9.4-14.2)[b]	11.7 (8.4-14.4)	10 (8.2-11.2)	7.9 (5.5-10.6)	6.7 (4.5-10.4)[e]
Platelet (g/L)	346 (288-375)	657 (451-746)[b]	442 (308-624)	479 (396-750)[b]	392 (293-523)	383 (308-483)[e]
Serum iron (μmol/L)	16 (14-21)	4 (2-12)[a]	8 (4-10)[a]	4 (2-7)[d]	5 (4-7)[d]	8 (6-8)[b,e]
Serum albumin (g/L)	45 (44-49)	37 (34-39)[d]	42 (42-45)[c]	40 (36-41)[d]	41 (38-45)[b]	42 (38-45)[b]
C-reactive protein (mg/L)	0 (0-1)	21 (5-65)[d]	7 (2-11)[b,c]	27 (9-55)[h, d]	9 (3-11)[d]	5 (1-14)[d,e]

[a]$P < 0.05$, [b]$P < 0.01$; [d]$P < 0.01$ *vs* control; [c]$P < 0.05$, [f]$P < 0.01$ *vs* therapy-naïve; [h]$P < 0.01$, [j]$P < 0.01$ *vs* first remission; [e]$P < 0.05$, [g]$P < 0.05$, [l]$P < 0.001$ *vs* relapse. IFX: Infliximab; PCDAI: Pediatric Crohn's disease activity index; L1: Small bowel; L2: Large bowel; L3: Small bowel and large bowel localization according to Montreal criteria[48].

Together with other routine blood sampling 6 mL of lithium-heparin anticoagulated blood was taken from therapy-naïve patients at the time of diagnosis, at the time of first remission in the remission group and at the initiation of IFX therapy, and then 2 and 6 wk later in the relapsed groups. From peripheral blood mononuclear cells (PBMCs), the identification of markers (6B11, CCR4, CD3, CD4, CD8, CD11c, CD14, CD25, CD45RA, CD45RO, CD123; CD161, CXCR3, HLA-DR and Lin-1 BD Biosciences Pharmingen, San Diego, CA, USA; TLR-2 and TLR-4 eBioscience, San Diego, CA, USA) and FoxP3 assay (eBioscience, San Diego, CA, USA) were performed with a BD FACS Aria (BD Biosciences Pharmingen, San Diego, CA, USA)[51]. Briefly, from whole blood, PBMCs were separated with gradient centrifugation using Ficoll-Paque (GE Healthcare Life Sciences, Pittsburgh, PA, USA). The isolated PBMCs were washed twice with Phosphate Buffered Saline pH 7.4 (PBS, Central Pharmacy of Semmelweis University, Budapest, Hungary) and were stained with the appropriate fluorescent antibodies according to the manufacturers' instructions. For intracellular staining, cells were incubated first with Fixation/Permeabilization Buffer (eBioscience, San Diego, CA, USA), then washed twice with Permeabilization Buffer (eBioscience, San Diego, CA, USA) and stained by FoxP3 and isotype control. At the end of staining, cells were washed twice with PBS and with Permeabilization Buffer for the detection of cell surface markers and FoxP3, respectively. Samples were resuspended in PBS and were measured within 1 h recording at least 50 000 events in the lymphocyte gate.

Our data did not follow normal distribution, therefore non-parametric statistical tests and median and interquartile ranges were used. Mann-Whitney and Friedman tests with Dunn's post hoc comparison and Spearman's correlation were used for statistical analysis, the level of significance was 5% ($P < 0.05$). All data are expressed as median (interquartile range). Statistical analysis was performed with Statistica 8 (Statsoft, Tulsa, OK, USA).

RESULTS

Major clinical characteristics and laboratory data are summarized in Table 1. The investigated cell prevalence values and cell ratios of the adaptive and innate immune systems are summarized in Tables 2 and 3, respectively. We also highlighted the most important alterations in Figures 1 and 2.

First, we compared the immune phenotype in therapy-naïve CD patients with that of healthy controls. In CD children, the prevalence of activated T cells (i.e. CD4$^+$CD25$^+$ cells) increased. At the same time, the prevalence of T cells with Th1 commitment (i.e. CD4$^+$CXCR3$^+$ cells) decreased resulting in a skewness of Th1/Th2 to Th2. The prevalence of memory (i.e. CD4$^+$CD45RO$^+$) cells increased and, therefore, a shift in the naïve/memory ratio toward memory cells was observed. The prevalence of regulatory T (i.e. CD4$^+$CD25^{hi}FoxP3$^+$) cells was comparable between the two groups.

Striking differences in cell prevalence values of innate immunity were obtained between therapy-naïve CD and healthy children. The occurrence of NK and NKT cells (marked as CD3$^-$CD161$^+$ and CD3$^+$6b11$^+$, respectively) was lower in CD children. Interestingly, the prevalence of the APCs investigated differed largely between CD and healthy children. DCs (i.e. those with Lin1$^-$HLADR$^+$ expression) were more prevalent and, within DC cells, the myeloid DCs (mDCs, i.e. CD11c$^+$ cells) were more prevalent, while plasmocytoid DCs (pDCs, i.e. CD123$^+$ cells) were less frequent in CD than in healthy children. This leads to skewness of mDCs in the mDC/pDC ratio. The prevalence of peripheral monocytes (i.e. CD14$^+$ cells) also increased in therapy-naïve CD patients. In addition, the prevalence of DC cells and monocytes expressing TLR-2 and TLR-4 receptors was also increased in CD. Of note,

Table 2 Prevalence and ratios of cellular members of adaptive immunity

Cell prevalence in parent population	Control	Therapy-naïve (before conventional therapy)	First remission (with conventional therapy)	Relapse (before IFX therapy)	IFX therapy (before 2nd infusion)	IFX therapy (before 3rd infusion)
CD4+ in PBMC	35.16 (24.81-47.79)	36.52 (18.61-44.06)	37.42 (22.27-44.26)	39.12 (24.15-51.18)	39.77 (27.71-50.30)	35.65 (24.31-48.81)
Activated in CD4+	5.92 (2.39-6.51)	10.61 (3.93-19.04)[a]	5.03 (3.37-7.93)[g]	7.84 (5.30-11.67)[a]	7.24 (5.67-12.25)[a]	9.98 (5.45-16.19)[b]
Naïve in CD4+	64.57 (59.86-73.03)	63.36 (41.19-74.86)	61.31 (39.09-73.05)	66.31 (60.44-71.79)	64.47 (53.90-77.87)	58.11 (53.51-64.96)
Effector in CD4+	21.91 (13.94-33.96)	31.29 (22.80-55.78)[a]	33.97 (28.54-39.44)[a]	29.47 (26.54-36.19)[a]	35.23 (27.74-40.36)[a]	37.62 (35.74-45.24)[b,i]
Naïve/effector ratio	2.73 (1.60-4.97)	1.57 (1.02-2.54)[a]	1.43 (1.14-2.21)[a]	2.34 (1.66-2.68)	1.79 (1.47-2.36)	1.52 (1.19-1.74)[a,e]
Th1 in CD4+	16.19 (9.91-25.26)	6.49 (4.50-7.67)[d]	12.89 (5.12-19.03)[c]	10.9 (4.03-14.38)[a]	12.73 (8.12-15.81)	18.87 (12.51-38.11)[e]
Th2 in CD4+	4.59 (2.78-5.51)	4.7 (1.98-6.03)	5.06 (1.42-8.48)	3.97 (2.27-7.19)	4.61 (2.55-5.65)	4.46 (2.11-8.16)
Th1/Th2 ratio	3.98 (3.06-5.12)	1.60 (0.50-2.11)[d]	1.95 (0.43-3.62)[b]	2.68 (0.85-5.07)	2.68 (1.42-6.14)	3.25 (1.37-4.80)[e]
Treg in CD4+	1.25 (1.10-2.37)	1.36 (1.09-2.48)	1.41 (1.07-3.50)	1.31 (1.16-2.63)	1.33 (1.07-2.99)	1.96 (1.47-3.77)[b,e]

[a]$P < 0.05$, [b]$P < 0.01$, [d]$P < 0.01$ *vs* control; [c]$P < 0.05$, [g]$P < 0.01$ *vs* therapy-naïve; [e]$P < 0.05$, [i]$P < 0.01$ *vs* relapse. Data are expressed as median (interquartile range). IFX: Infliximab; PBMC: Peripheral blood mononuclear cell; Activated (CD25+); Naïve (CD45RA+); Effector (CD45RO+); Th1: T helper 1 committed, CXCR3+; Th2: T helper 2 committed, CCR4+; Treg: Regulatory T lymphocytes, CD25hiFoxP3+.

Table 3 Prevalence and ratios of cellular members of innate immunity

Cell prevalence in parent population	Control	Therapy-naïve (before conventional therapy)	First remission (with conventional therapy)	Relapse (before IFX therapy)	IFX therapy (before 2nd infusion)	IFX therapy (before 3rd infusion)
NKT in PBMC	1.39 (0.81-2.42)	0.7 (0.11-1.33)[a]	0.74 (0.37-1.34)	0.72 (0.45-1.23)	0.73 (0.37-1.20)	0.83 (0.31-2.05)
NK in PBMC	3.17 (1.71-4.76)	1.73 (0.72-3.49)[a]	1.98 (1.65-2.97)	1.95 (0.72-4.81)	2.14 (0.66-4.78)	2.45 (1.26-4.85)
DC in PBMC	0.61 (0.10-2.15)	1.05 (0.54-3.32)[a]	1.16 (0.58-2.98)[a]	1.45 (1.09-3.46)[a]	0.86 (0.30-2.80)	0.82 (0.52-2.31)
mDC in DC	46.04 (37.14-52.30)	61.66 (45.37-72.74)[b]	45.19 (37.83-56.68)[c]	69.01 (40.11-74.79)[a,e]	60.01 (48.03-68.50)[a]	57.39 (37.19-64.14)[a,g]
pDC in DC	27.38 (20.65-33.94)	19.26 (15.08-24.58)[a]	21.61 (14.76-32.61)[a]	18.33 (16.59-26.42)[a]	19.03 (17.26-32.38)	27.39 (19.68-36.70)[g]
mDC/pDC ratio	1.85 (0.44-1.46)	3.05 (2.32-6.11)[d]	2.35 (1.80-9.10)[b,c]	3.5 (1.74-7.79)[d]	3.04 (2.03-4.65)[d]	2.20 (1.66-5.54)[b,g]
TLR-2 in DC	8.8 (4.14-17.35)	55.08 (37.27-57.94)[d]	17.32 (6.32-32.54)[c]	38.7 (19.31-54.59)[b,e]	25.89 (5.69-60.43)	16.70 (2.29-42.61)[g]
TLR-4 in DC	2.24 (0.90-2.78)	14.36 (6.26-19.20)[b]	2.73 (1.35-7.21)[f]	10.11 (2.29-19.55)[a,e]	5.31 (2.81-13.42)	3.32 (0.10-6.05)[g]
Monocyte in PBMC	2.01 (1.38-3.82)	8.57 (3.82-16.72)[b]	5.96 (2.49-21.22)[a,c]	7.3 (2.65-15.44)[a]	5.58 (2.78-17.52)[a]	5.31 (3.54-6.94)[a]
TLR-2 in monocyte	16.44 (10.31-19.69)	29.65 (17.83-39.77)[a]	20.92 (7.87-29.15)[c]	20.63 (7.05-29.01)	19.89 (13.13-29.32)	18.52 (10.29-28.96)
TLR-4 in monocyte	7.17 (0.45-14.73)	14.59 (3.01-35.23)[a]	4.43 (1.82-8.31)[c]	10.39 (2.10-26.54)	8.26 (2.71-17.56)	6.30 (2.48-18.94)

[a]$P < 0.05$, [b]$P < 0.01$, [d]$P < 0.001$ *vs* control; [c]$P < 0.05$, [f]$P < 0.01$ *vs* therapy-naïve; [e]$P < 0.05$ *vs* first remission; [g]$P < 0.05$ *vs* relapse. Data are expressed as median (interquartile range). IFX: Infliximab; PBMC: Peripheral blood mononuclear cell; NK: Natural killer, CD3-CD161+; NKT: Natural killer T, CD3+6b11+; DC: Dendritic cell, Lin1-HLA-DR+; mDC: Myeloid dendritic cell, CD11c+; pDC: Plasmocytoid dendritic cell, CD123+; Monocyte (CD14+); TLR: Toll-like receptor.

4 of the 14 therapy-naïve CD children did not respond to conventional therapy. Their immune phenotype at the therapy-naïve phase did not differ from those children who responded to conventional therapy (data not shown).

In the second phase of our study, we prospectively tested the alteration in cell prevalence values during therapy. At the time of first remission with conventional therapy, the Th1/Th2 ratio shifted to Th1 and normalized along with activated T cell prevalence. Memory T cells remained elevated, while all the other cell types of adaptive immunity were comparable to that measured before therapy. For innate immune cells, NK and NK-T remained lower than normal and total DC prevalence remained higher than the control. However, mDC and pDC ratios, total monocyte prevalence and cells expressing TLR-2/TLR-4 receptor values (including monocytes and DCs) were normal.

In children who relapsed with conventional therapy, immune phenotype again became comparable to that in therapy-naïve CD. Therefore, we measured lower Th1, increased activated T, higher DC and higher macrophage prevalence as well as higher TLR-2 and TLR-4 expression in comparison to controls. In addition, the prevalence of mDCs, simultaneously with TLR-2 and TLR-4 expressing DCs was higher in relapsed than in remitted CD. During IFX therapy, immune cell prevalence was measured at two time points (i.e. 2 and 6 wk after the initiation of therapy). Th1, activated T and Treg prevalence increased significantly by week 6 of therapy. Total DC, mDC, pDC, total monocyte, along with TLR-2 and TLR-4 expressing DC and macrophage prevalence were normal at this time. Of note, although no significant alteration was observed at week 2, some tendencies were already present (Figures 1 and 2).

DISCUSSION

In our study we investigated the major components of adaptive and innate immunity in a simultaneous manner in CD children. While CD4 numbers in therapy-naïve CD children were normal as in early studies with adult patients[52,53], we noticed a shift to the Th2 direction in Th1/Th2 committed T lymphocytes. This finding is in line with other reports on blood[29,30,52] or biopsy specimens of therapy-naïve CD children[31]. Similar to adults, we also found a higher than normal prevalence of activated CD4+ cells and effector memory cells and a decrease in effector cell/naïve CD4 cell ratios[12-17]. We tested the idea that this was
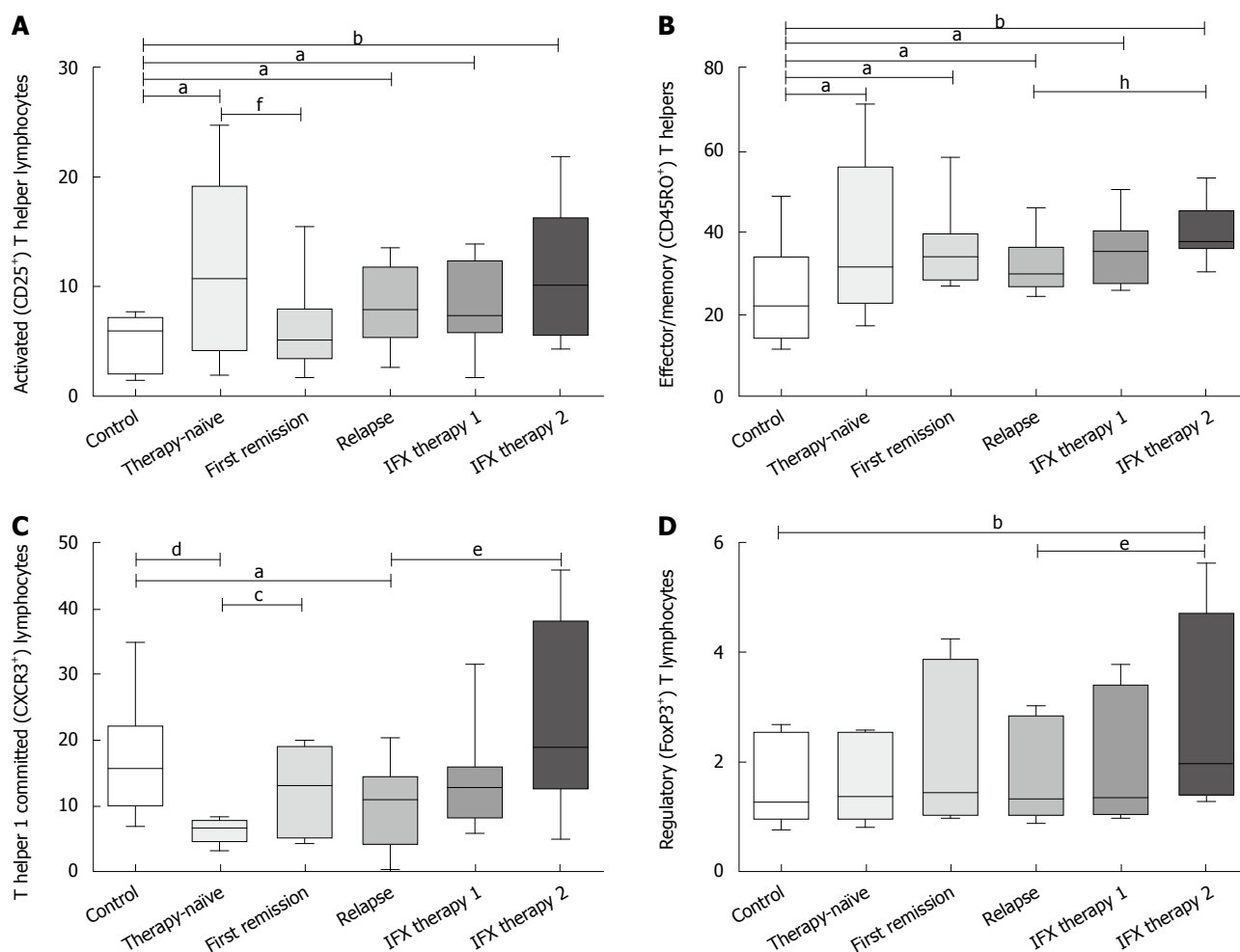
Figure 1 Prevalence of cellular members of adaptive immunity. The prevalence of activated (i.e. CD4+CD25+) (A), effector or memory (i.e. CD4+CXCR3+) (B), Th1 committed (i.e. CD4+CXCR3+) (C) and regulatory T (i.e. CD4+CD25hiFoxP3+) cells (D). [a]P < 0.05, [b]P < 0.01, [d]P < 0.001 *vs* control; [c]P < 0.05, [f]P < 0.01 *vs* therapy-naïve; [e]P < 0.05, [h]P < 0.01 *vs* relapse.

due to low Treg numbers, but our data showing a normal prevalence of FoxP3 expressing CD4 cells do not support this notion. This finding is in contrast with that suggesting a diminution of Treg cells in untreated CD adults. However, in an earlier study Tregs were identified according to CD4+CD25^{high} positivity[8] currently not regarded as a sensitive marker for this cell type.

Our study provides novel information on the possible contribution of the innate immune system to a Th2 shift in therapy-naïve CD children. While some authors suggested that altered NK and NKT function may be a component in adult CD[17,26-28], our data are the first to show lower than normal NK and NKT prevalence in therapy-naïve CD children. In addition, we also observed a marked increase in monocyte and DC prevalence with an increase in the mDC/pDC ratio. These cell populations are major triggers of immune response and may be linked with the increase in memory T cell prevalence. As recent studies emphasized, mDC and pDC have distinct regulatory properties as mDC may shift the immune response not only toward Th1, but also in the Th2 direction[54], while pDC can induce Tregs[55]. Therefore, an increase in the mDC/pDC ratio in our patients may contribute to a lower Th1/

Th2 ratio. Furthermore, we also measured an increased prevalence of TLR-2 and TLR-4 expressing monocytes and DCs that may also play a role in the activation of immune cells. This finding is in accordance with our previous observation of high TLR-2 and TLR-4 expression in the colonic mucosa of therapy-naïve CD children[34].

We tested prospectively the link between immune phenotype and disease activity index in our patients. The majority of immune system alterations in therapy-naïve CD are normalized with the normalization of PCDAI. While Th1 prevalence significantly increased compared to the therapy-naïve state and almost normalized during therapy, Th1/Th2 was still in the normal range at first remission suggesting a difference in CD immune phenotype between adults and children[4-7].

In remitted patients, NK and NKT prevalence increased and the difference between therapy-naïve CD and healthy controls disappeared. This finding does not support previous reports on decreased NK and NKT numbers in treated CD adults[17,26-28]. We also observed that the mDC/pDC ratio, prevalence of monocytes and that of TLR-2 and TLR-4 expressing DCs and monocytes were also normalized. This may suggest that normalization of immune phe-
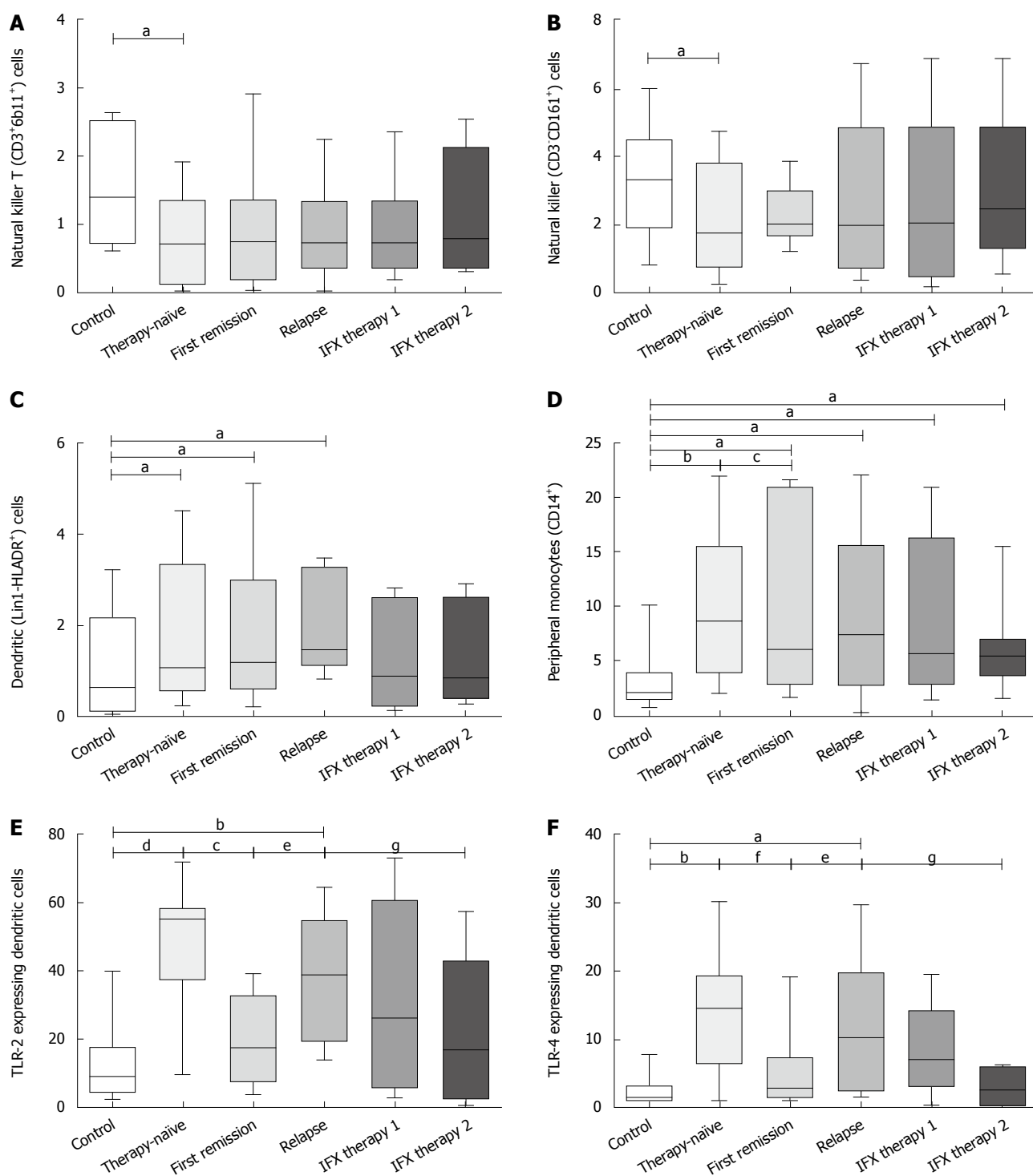
Figure 2 Prevalence of cellular members of innate immunity. The prevalence of natural killer T (i.e. CD3+6b11+) (A), natural killer (i.e. CD3-CD161+) (B), dendritic cell (i.e. Lin1-HLA-DR+) (C), peripheral monocyte (i.e. CD14+) (D), Toll-like receptor 2 expressing dendritic cell (E) and Toll-like receptor 4 expressing dendritic cell (F). [a]$P < 0.05$, [b]$P < 0.01$, [d]$P < 0.001$ *vs* control; [c]$P < 0.05$, [f]$P < 0.01$ *vs* therapy-naïve; [e]$P < 0.05$ *vs* first remission; [g]$P < 0.05$ *vs* relapse.

notype is linked to an improvement in PCDAI. This may be a specific feature in childhood CD as a number of studies reported a higher than normal prevalence of activated and effector T lymphocytes even in treated CD adults[12-17]. Tregs probably do not play a role in this normalization as their prevalence was not altered in remission.

We performed another prospective study in 12 CD children who relapsed with conventional therapy and were treated with IFX. In these CD children, the immune phenotype was similar to that observed in therapy-naïve CD children (with the only exception of normal prevalence of NK and NKT cells). During IFX therapy, however, marked changes occurred in the 6th wk of IFX treatment and the prevalence of Th1 cells and the APCs investigated were normalized. Interestingly, the prevalence of activated T cells, memory cells and Treg cells were elevated further.

Recently, increased numbers of Th1 cells and high blood levels of Th1-type cytokines were found in adult patients with rheumatoid arthritis (RA) after IFX treatment[39,40]. Others also found an increase in effector and activated T cells with IFX[38,39]. While the explanation for this is still unclear, an attractive hypothesis may be that IFX inhibits the homing of Th1 and activated T cells to the inflammation site and transiently increases their peripheral occurrence[38,40].

Theoretically, this immune phenotype may refer to an increased risk for infections. However, our patients did not exhibit major clinical signs and symptoms of infection during IFX therapy. Interestingly, simultaneously with these changes, IFX therapy also increased the prevalence of peripheral Treg cells. This phenomenon - which may be due to a resistance of Tregs to IFX-induced apoptosis - was also demonstrated previously in the peripheral blood of adult CD patients[8], and in colonic samples from CD children[37].

In our patients, the prevalence of monocytes, mDCs, mDC/pDC ratio and TLR-2 and TLR-4 expressing DCs normalized during IFX therapy indicating the possible impact of biological therapy on innate immunity. This is in line with the observation of others investigating RA and CD patients[41,43-45].

Our study has three major limitations. First, although we did correct for multiplicity when performing pairwise comparisons, the study was not powered for the multitude of statistical tests we performed, thus some significances could occur by chance alone. Second, peripheral cell prevalence values do not necessarily reflect the intestinal phenotype. Third, our results may have been affected by ageing of the patients during the follow-up period. The short duration of our prospective study (i.e. about 10 mo until the first remission or 6 wk from the beginning of IFX therapy), however, makes this bias less likely.

CD exhibits several abnormalities in adaptive immunity (such as a decrease in Th1 cell and an increase in memory and activated T cell prevalence) and innate immunity (such as an increase in DC, monocyte and TLR-2 and TLR-4 exhibiting APC prevalence). The majority of the observed alterations of the innate immune system are normalized with the improvement of clinical signs and symptoms of CD, irrespective of whether this is obtained by conventional therapy or add-on IFX therapy. This finding suggests a link between immune phenotype and disease activity in childhood CD.

If these results are reinforced by other groups, the observations may raise the possibility that immune phenotype is a potential biomarker for clinical response in CD children.

COMMENTS

Background
About 10%-15% of patients with Crohn's disease (CD) are diagnosed before 18 years of age. Pediatric CD is a unique subtype of CD due to different location, altered responsiveness to therapy and different susceptibility factors compared with those in adult CD.

Research frontiers
Several studies indicate both the malfunction of adaptive and innate immunity in adulthood CD. Fewer data are available for CD children. The alteration of immune phenotype with treatment is also unclear in this population.

Innovations and breakthroughs
In this prospective study, the authors demonstrated marked alterations in both adaptive and innate immunity in childhood CD. These abnormalities were resolved in infants responding to conventional therapy but not in non-responding children. Immune phenotype depends on disease activity.

Applications
These observations support the involvement of members of the adaptive and innate immune systems in childhood CD. They also identify immune phenotype as a possible biomarker for the follow-up of therapeutic success.

Peer review
In this study, the authors prospectively investigated the major components of adaptive and innate immunity in CD children. The authors showed several abnormalities in adaptive and innate immunity in CD children. They also showed that the majority of observed alterations were normalized in remission stage and suggest a link between immune phenotype and disease activity in childhood CD. Although the number of CD patients enrolled to this study was small, this prospective study demonstrated clinically interesting results in CD children.

REFERENCES

1   **Dubinsky M**. Special issues in pediatric inflammatory bowel disease. *World J Gastroenterol* 2008; **14**: 413-420

2   **Nieuwenhuis EE**, Escher JC. Early onset IBD: what's the difference? *Dig Liver Dis* 2008; **40**: 12-15

3   **Shaoul R**, Karban A, Reif S, Weiss B, Shamir R, Tamir A, Davidovich O, Halevi J, Silver EL, Levine A. Disease behavior in children with Crohn's disease: the effect of disease duration, ethnicity, genotype, and phenotype. *Dig Dis Sci* 2009; **54**: 142-150

4   **Zenewicz LA**, Antov A, Flavell RA. CD4 T-cell differentiation and inflammatory bowel disease. *Trends Mol Med* 2009; **15**: 199-207

5   **Himmel ME**, Hardenberg G, Piccirillo CA, Steiner TS, Levings MK. The role of T-regulatory cells and Toll-like receptors in the pathogenesis of human inflammatory bowel disease. *Immunology* 2008; **125**: 145-153

6   **van Lierop PP**, Samsom JN, Escher JC, Nieuwenhuis EE. Role of the innate immune system in the pathogenesis of inflammatory bowel disease. *J Pediatr Gastroenterol Nutr* 2009; **48**: 142-151

7   **Sartor RB**. Mechanisms of disease: pathogenesis of Crohn's disease and ulcerative colitis. *Nat Clin Pract Gastroenterol Hepatol* 2006; **3**: 390-407

8   **Chamouard P**, Monneaux F, Richert Z, Voegeli AC, Lavaux T, Gaub MP, Baumann R, Oudet P, Muller S. Diminution of Circulating CD4+CD25 high T cells in naïve Crohn's disease. *Dig Dis Sci* 2009; **54**: 2084-2093

9   **Maul J**, Loddenkemper C, Mundt P, Berg E, Giese T, Stallmach A, Zeitz M, Duchmann R. Peripheral and intestinal regulatory CD4+ CD25(high) T cells in inflammatory bowel disease. *Gastroenterology* 2005; **128**: 1868-1878

10 **Saruta M**, Yu QT, Fleshner PR, Mantel PY, Schmidt-Weber CB, Banham AH, Papadakis KA. Characterization of FOXP3+CD4+ regulatory T cells in Crohn's disease. *Clin Immunol* 2007; **125**: 281-290

11 **Makita S**, Kanai T, Oshima S, Uraushihara K, Totsuka T, Sawada T, Nakamura T, Koganei K, Fukushima T, Watanabe M. CD4+CD25bright T cells in human intestinal lamina propria as regulatory cells. *J Immunol* 2004; **173**: 3119-3130

12 **Roman LI**, Manzano L, De La Hera A, Abreu L, Rossi I, Alvarez-Mon M. Expanded CD4+CD45RO+ phenotype and defective proliferative response in T lymphocytes from patients with Crohn's disease. *Gastroenterology* 1996; **110**: 1008-1019

13 **Alkim C**, Balci M, Alkim H, Dağli U, Parlak E, Tezel A, Ulker

A. The importance of peripheral immune cells in inflammatory bowel disease. *Turk J Gastroenterol* 2007; **18**: 82-88

14   **García de Tena J**, Manzano L, Leal JC, San Antonio E, Sualdea V, Alvarez-Mon M. Active Crohn's disease patients show a distinctive expansion of circulating memory CD4+CD45RO+CD28null T cells. *J Clin Immunol* 2004; **24**: 185-196

15   **Rose M**, Hildebrandt M, Fliege H, Seibold S, Mönnikes H, Klapp BF. T-cell immune parameters and depression in patients with Crohn's disease. *J Clin Gastroenterol* 2002; **34**: 40-48

16   **Liu ZX**, Hiwatashi N, Noguchi M, Toyota T. Increased expression of costimulatory molecules on peripheral blood monocytes in patients with Crohn's disease. *Scand J Gastroenterol* 1997; **32**: 1241-1246

17   **Senju M**, Hulstaert F, Lowder J, Jewell DP. Flow cytometric analysis of peripheral blood lymphocytes in ulcerative colitis and Crohn's disease. *Gut* 1991; **32**: 779-783

18   **Zenewicz LA**, Antov A, Flavell RA. CD4 T-cell differentiation and inflammatory bowel disease. *Trends Mol Med* 2009; **15**: 199-207

19   **Baumgart DC**, Metzke D, Schmitz J, Scheffold A, Sturm A, Wiedenmann B, Dignass AU. Patients with active inflammatory bowel disease lack immature peripheral blood plasmacytoid and myeloid dendritic cells. *Gut* 2005; **54**: 228-236

20   **Baumgart DC**, Thomas S, Przesdzing I, Metzke D, Bielecki C, Lehmann SM, Lehnardt S, Dörffel Y, Sturm A, Scheffold A, Schmitz J, Radbruch A. Exaggerated inflammatory response of primary human myeloid dendritic cells to lipopolysaccharide in patients with inflammatory bowel disease. *Clin Exp Immunol* 2009; **157**: 423-436

21   **Hart AL**, Al-Hassi HO, Rigby RJ, Bell SJ, Emmanuel AV, Knight SC, Kamm MA, Stagg AJ. Characteristics of intestinal dendritic cells in inflammatory bowel diseases. *Gastroenterology* 2005; **129**: 50-65

22   **Frolova L**, Drastich P, Rossmann P, Klimesova K, Tlaskalova-Hogenova H. Expression of Toll-like receptor 2 (TLR2), TLR4, and CD14 in biopsy samples of patients with inflammatory bowel diseases: upregulated expression of TLR2 in terminal ileum of patients with ulcerative colitis. *J Histochem Cytochem* 2008; **56**: 267-274

23   **Kamada N**, Hisamatsu T, Honda H, Kobayashi T, Chinen H, Kitazume MT, Takayama T, Okamoto S, Koganei K, Sugita A, Kanai T, Hibi T. Human CD14+ macrophages in intestinal lamina propria exhibit potent antigen-presenting ability. *J Immunol* 2009; **183**: 1724-1731

24   **Cantó E**, Ricart E, Monfort D, González-Juan D, Balanzó J, Rodríguez-Sánchez JL, Vidal S. TNF alpha production to TLR2 ligands in active IBD patients. *Clin Immunol* 2006; **119**: 156-165

25   **Grimm MC**, Pavli P, Van de Pol E, Doe WF. Evidence for a CD14+ population of monocytes in inflammatory bowel disease mucosa--implications for pathogenesis. *Clin Exp Immunol* 1995; **100**: 291-297

26   **van Ierssel GJ**, van der Sluys Veer A, Verspaget HW, Griffioen G, van Hogezand RA, Lamers CB. Contribution of plasma cortisol to corticosteroid-suppressed peripheral blood natural killer cell activity in Crohn's disease. *Immunopharmacology* 1995; **29**: 11-17

27   **Grose RH**, Thompson FM, Baxter AG, Pellicci DG, Cummins AG. Deficiency of invariant NK T cells in Crohn's disease and ulcerative colitis. *Dig Dis Sci* 2007; **52**: 1415-1422

28   **Kontiainen S**, Scheinin T, Halme L. Number of activated T-helper cells and NK cells in peripheral blood is decreased in severe Crohn's disease. *APMIS* 1996; **104**: 355-361

29   **Mack DR**, Beedle S, Warren J, Davis J, Gross T. Peripheral blood intracellular cytokine analysis in children newly diagnosed with inflammatory bowel disease. *Pediatr Res* 2002; **51**: 328-332

30   **Holland N**, Dong J, Garnett E, Shaikh N, Huen K, Harmatz P, Olive A, Winter HS, Gold BD, Cohen SA, Baldassano RN, Kirschner BS, Heyman MB. Reduced intracellular T-helper 1

interferon-gamma in blood of newly diagnosed children with Crohn's disease and age-related changes in Th1/Th2 cytokine profiles. *Pediatr Res* 2008; **63**: 257-262

31   **Desreumaux P**, Brandt E, Gambiez L, Emilie D, Geboes K, Klein O, Ectors N, Cortot A, Capron M, Colombel JF. Distinct cytokine patterns in early and chronic ileal lesions of Crohn's disease. *Gastroenterology* 1997; **113**: 118-126

32   **Jo Y**, Matsumoto T, Yada S, Fujisawa K, Esaki M, Onai N, Matsushima K, Iida M. CCR4 is an up-regulated chemokine receptor of peripheral blood memory CD4+ T cells in Crohn's disease. *Clin Exp Immunol* 2003; **132**: 332-338

33   **Perminow G**, Reikvam DH, Lyckander LG, Brandtzaeg P, Vatn MH, Carlsen HS. Increased number and activation of colonic macrophages in pediatric patients with untreated Crohn's disease. *Inflamm Bowel Dis* 2009; **15**: 1368-1378

34   **Szebeni B**, Veres G, Dezsõfi A, Rusai K, Vannay A, Mraz M, Majorova E, Arató A. Increased expression of Toll-like receptor (TLR) 2 and TLR4 in the colonic mucosa of children with inflammatory bowel disease. *Clin Exp Immunol* 2008; **151**: 34-41

35   **Diefenbach KA**, Breuer CK. Pediatric inflammatory bowel disease. *World J Gastroenterol* 2006; **12**: 3204-3212

36   **Veres G**, Baldassano RN, Mamula P. Infliximab therapy in children and adolescents with inflammatory bowel disease. *Drugs* 2007; **67**: 1703-1723

37   **Ricciardelli I**, Lindley KJ, Londei M, Quaratino S. Anti tumour necrosis-alpha therapy increases the number of FOXP3 regulatory T cells in children affected by Crohn's disease. *Immunology* 2008; **125**: 178-183

38   **Ferkolj I**, Ihan A, Markovic S, Veceric Z, Pohar M. Infliximab reduces the number of activated mucosal lymphocytes in patients with Crohn's disease. *J Gastrointestin Liver Dis* 2006; **15**: 231-235

39   **Aeberli D**, Seitz M, Jüni P, Villiger PM. Increase of peripheral CXCR3 positive T lymphocytes upon treatment of RA patients with TNF-alpha inhibitors. *Rheumatology* (Oxford) 2005; **44**: 172-175

40   **Maurice MM**, van der Graaff WL, Leow A, Breedveld FC, van Lier RA, Verweij CL. Treatment with monoclonal antitumor necrosis factor alpha antibody results in an accumulation of Th1 CD4+ T cells in the peripheral blood of patients with rheumatoid arthritis. *Arthritis Rheum* 1999; **42**: 2166-2173

41   **Richez C**, Schaeverbeke T, Dumoulin C, Dehais J, Moreau JF, Blanco P. Myeloid dendritic cells correlate with clinical response whereas plasmacytoid dendritic cells impact autoantibody development in rheumatoid arthritis patients treated with infliximab. *Arthritis Res Ther* 2009; **11**: R100

42   **Balanescu A**, Radu E, Nat R, Regalia T, Bojinca V, Ionescu R, Balanescu S, Savu C, Predeteanu D. Early and late effect of infliximab on circulating dendritic cells phenotype in rheumatoid arthritis patients. *Int J Clin Pharmacol Res* 2005; **25**: 9-18

43   **Lügering A**, Schmidt M, Lügering N, Pauels HG, Domschke W, Kucharzik T. Infliximab induces apoptosis in monocytes from patients with chronic active Crohn's disease by using a caspase-dependent pathway. *Gastroenterology* 2001; **121**: 1145-1157

44   **Baert FJ**, D'Haens GR, Peeters M, Hiele MI, Schaible TF, Shealy D, Geboes K, Rutgeerts PJ. Tumor necrosis factor alpha antibody (infliximab) therapy profoundly down-regulates the inflammation in Crohn's ileocolitis. *Gastroenterology* 1999; **116**: 22-28

45   **De Rycke L**, Vandooren B, Kruithof E, De Keyser F, Veys EM, Baeten D. Tumor necrosis factor alpha blockade treatment down-modulates the increased systemic and local expression of Toll-like receptor 2 and Toll-like receptor 4 in spondylarthropathy. *Arthritis Rheum* 2005; **52**: 2146-2158

46   Inflammatory bowel disease in children and adolescents: recommendations for diagnosis--the Porto criteria. *J Pediatr Gastroenterol Nutr* 2005; **41**: 1-7

47   **Hyams JS**, Ferry GD, Mandel FS, Gryboski JD, Kibort PM, Kirschner BS, Griffiths AM, Katz AJ, Grand RJ, Boyle JT.

Development and validation of a pediatric Crohn's disease activity index. *J Pediatr Gastroenterol Nutr* 1991; **12**: 439-447

48   **Satsangi J**, Silverberg MS, Vermeire S, Colombel JF. The Montreal classification of inflammatory bowel disease: controversies, consensus, and implications. *Gut* 2006; **55**: 749-753

49   **Mamula P**, Markowitz JE, Baldassano RN. Inflammatory bowel disease in early childhood and adolescence: special considerations. *Gastroenterol Clin North Am* 2003; **32**: 967-995, viii

50   **Diefenbach KA**, Breuer CK. Pediatric inflammatory bowel disease. *World J Gastroenterol* 2006; **12**: 3204-3212

51   **Svec P**, Vásárhelyi B, Pászthy B, Körner A, Kovács L, Tulassay T, Treszl A. Do regulatory T cells contribute to Th1 skew-

ness in obesity? *Exp Clin Endocrinol Diabetes* 2007; **115**: 439-443

52   **Selby WS**, Jewell DP. T lymphocyte subsets in inflammatory bowel disease: peripheral blood. *Gut* 1983; **24**: 99-105

53   **Yuan SZ**, Hanauer SB, Kluskens LF, Kraft SC. Circulating lymphocyte subpopulations in Crohn's disease. *Gastroenterology* 1983; **85**: 1313-1318

54   **Kool M**, Lambrecht BN. Dendritic cells in asthma and COPD: opportunities for drug development. *Curr Opin Immunol* 2007; **19**: 701-710

55   **de Heer HJ**, Hammad H, Kool M, Lambrecht BN. Dendritic cell subsets and immune regulation in the lung. *Semin Immunol* 2005; **17**: 295-303