Pediatric patients with inflammatory bowel disease exhibit increased serum levels of proinflammatory cytokines and chemokines, but decreased circulating levels of macrophage inhibitory protein-1β, interleukin-2 and interleukin-17

GIULIO KLEINER[1], VALENTINA ZANIN[1], LORENZO MONASTA[1], SERGIO CROVELLA[1,2], LORENZO CARUSO[3], DANIELA MILANI[3] and ANNALISA MARCUZZI[1]

[1]Department of Advanced Diagnostic and Clinical Trials, Institute for Maternal and Child Health - IRCCS 'Burlo Garofolo', Trieste 34137; [2]Department of Medicine, Surgery and Health Sciences, University of Trieste, Trieste 34127; [3]Department of Morphology, Surgery and Experimental Medicine, University of Ferrara, Ferrara 44121, Italy

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Abstract. Inflammatory bowel disease (IBD) is a chronic and progressive inflammatory condition of the gastrointestinal tract. Although the causative events that lead to the onset of IBD are yet to be fully elucidated, deregulation of immune and inflammatory mechanisms are hypothesized to significantly contribute to this disorder. Since the onset of IBD is often during infancy, in the present study, the serum values of a large panel of cytokines and chemokines in pediatric patients (<18 years; n=26) were compared with age-matched controls (n=37). While elevations in the serum level of several proinflammatory and immune regulating cytokines were confirmed, such as interleukin (IL)-1β, IL-5, IL-7, interferon (IFN)-γ-inducible protein-10, IL-16, cutaneous T-cell-attracting chemokine, leukemia inhibitory factor, monokine induced by γ-IFN, IFN-α2 and IFN-γ, notably decreased levels of IL-2, IL-17 and macrophage inhibitory protein-1β were also observed. Therefore, while a number of proinflammatory cytokines exhibit increased levels in IBD patients, pediatric IBD patients may also exhibit certain aspects of a reduced immunological response.

Introduction

Inflammatory bowel disease (IBD) is an inflammatory condition of the gastrointestinal tract that comprises two forms: Ulcerative colitis and Crohn's disease. IBD is characterized by chronic inflammation, remitting and relapsing episodes, and a progressive course at diagnosis (1). Four aspects are known to be involved in the etiopathogenesis of IBD, including the external environment, the genetic signature of the patient, the microbiota and the immune system (2). Specific pathogenic agents or the physiological microbial flora provide antigenic stimulation of cell-mediated immune responses in genetically susceptible individuals, while the presence of dysbiosis or impaired gastrointestinal mucosal barriers, due to genetic factors or environmentally-induced injury, represent risk factors for the development of an inappropriate immune response (3,4).

Innate and adaptive immunity are known to play a crucial role in triggering and maintaining inflammatory events in IBD (5,6). Furthermore, an important underlying mechanism is the production and release of cytokines and chemokines, which are able to drive inflammatory events at a local and systemic level. In a recent study, the normal production of cytokines and chemokines was shown to vary consistently with the individual growth of healthy subjects and the physiological sharpening of the immune system (7). The diagnosis of IBD is particularly challenging in patients of a pediatric age, since presenting symptoms can vary widely and may only consist of subtle extraintestinal manifestations. Often children present IBD-like phenotypes and symptoms; however, they do not suffer from IBD. A long period of unmanaged symptoms can significantly impact on growth; thus, early treatments are essential to preserve the long-term quality of life of patients (8,9).

Although non-invasive tests for IBD already exist, including antibody tests, imaging-based screens and fecal biomarkers (10), a comprehensive comparison of the serum levels of cytokines and chemokines in pediatric IBD patients compared with normal age-matched controls has yet to be performed. Therefore, the aim of the present study was to assess a large panel (n=48) of inflammatory cytokines and chemokines in pediatric IBD patients, and compare the results with the same panel in age-matched healthy controls.

Correspondence to: Dr Giulio Kleiner, Department of Advanced Diagnostic and Clinical Trials, Institute for Maternal and Child Health - IRCCS 'Burlo Garofolo', Via dell'Istria 65/1, Trieste 34137, Italy
E-mail: giulio.kleiner@burlo.trieste.it

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Table I. Profile of the controls and the patients with IBD.

Group	Cases (n)	Age (years)[a]	Gender, M/F (n)	Pathology
Controls	37	11 (1/17)	15/22	-
IBD	26	9 (1/16)	14/12	Crohn's disease (n=15) Ulcerative colitis (n=11)

[a]Results are expressed as the median (minimum/maximum). M, male; F, female; IBD, inflammatory bowel disease.

Subjects and methods

Patients and normal controls. The independent Ethics Review Board of the Institute for Maternal and Child Health - IRCCS 'Burlo Garofolo' (Trieste, Italy) provided approval of the study (n.185/08, 19/08/2008). For a child to be eligible, informed consent was required from the parents or guardians. For ethical reasons, the study population of pediatric patients was restricted to those who were undergoing a medically indicated peripheral venous blood sampling prior to elective surgical interventions or within the scope of elective diagnostic procedures. Furthermore, for the control group, subjects were excluded from the study if they had an acute or chronic infectious disease, any clinically significant disorder, or if they were on any medication with a known effect on immunological factors, such as corticosteroids. Blood samples were collected from the control subjects (CTRL, n=37) and IBD patients (IBD, n=26). The clinical characteristics of the subjects included in the study are shown in Table I. Patient history, with regard to breast-feeding, vaccinations, previous infectious diseases and allergy, was documented but not evaluated as covariates in the study, since subgroup analysis required a larger population size.

Determination of the cytokine and chemokine levels. The investigated panel comprised 48 cytokines or chemokines known to or hypothesized to be involved in inflammatory processes. The panel included interleukin (IL)-receptor antagonist (RA), IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12 (p70), IL-13, IL-15, IL-17, eotaxin, fibroblast growth factor (FGF)-basic, granulocyte-colony stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon (IFN)-γ, IFN-γ-inducible protein (IP)-10, monocyte chemoattractant protein (MCP)-1, macrophage inflammatory protein (MIP)-1α, platelet-derived growth factor (PDGF)-BB, MIP-1β, regulated on activation, normal T cell expressed and secreted (RANTES), tumor necrosis factor (TNF)-α, vascular endothelial growth factor (VEGF), IL-1α, IL-2Rα, IL-3, IL-12 (p40), IL-16, IL-18, cutaneous T-cell-attracting chemokine (CTACK), growth regulated oncogene (GRO)-α, hepatocyte growth factor (HGF), IFN-α2, leukemia inhibitory factor (LIF), MCP-3, macrophage colony-stimulating factor (M-CSF), macrophage migration inhibitory factor (MIF), monokine induced by γ-IFN (MIG), β-nerve growth factor (NGF), stem cell factor (SCF), stem cell growth factor (SCGF)-β, stromal cell-derived factor (SDF)-1α, TNF-β and TNF-related apoptosis-inducing ligand (TRAIL). Analysis of the panel was performed with the collected serum samples, using a magnetic bead-based multiplex immunoassay (Bio-Plex®; Bio-Rad Laboratories, Inc., Milan, Italy), as previously described (11-13). Experiments were performed according to the manufacturer's instructions. Data from the reactions were acquired using a Bio-Plex® 200 reader (Bio-Rad Laboratories, Inc.), while a digital processor (Toshiba Intel® Celeron® CPU B820; Toshiba, Tokyo, Japan) was used to manage the data output. Application of Bio-Plex Manager® software (Bio-Rad Laboratories, Inc.) enabled presentation of the data as the median fluorescence intensity and concentration (pg/ml).

Statistical analysis. For each set of experiments, the cytokine levels are presented as the median values with the interquartile range. To compare the differences between the values in the two distinct groups, a non-parametric Mann-Whitney rank-sum test was applied, where $P \leq 0.05$ was considered to indicate a statistically significant difference. In the case of multiple comparisons, the Bonferroni correction was applied, where 0.05 was divided by the number of multiple comparisons. Analyses were performed using GraphPad Prism 5.0 software (GraphPad Software, Inc., La Jolla, CA, USA) and Stata/IC 11.2 software (StataCorp LP, College Station, TX, USA).

Results

Pediatric IBD patients exhibit a distinct cytokine profile when compared with the normal controls, with 10 cytokines/chemokines exhibiting significantly higher values in IBD patients. All the serum concentrations of cytokines and chemokines, acquired following the magnetic bead-based multiplex immunoassay, in the pediatric IBD patients and controls are reported in Table II. Among the 48 investigated cytokines and chemokines, eight cytokines/chemokines were not detectable in the sera of the controls or IBD pediatric patients, which included IL-15, GM-CSF, IL-1α, IL-3, IL-12 (p40), MCP-3, β-NGF and TNF-β. The majority of cytokines and chemokines (n=27) did not exhibit a statistically significant difference when comparing the values in the IBD and CTRL groups. These cytokines and chemokines were IL-1RA, IL-4, IL-6, IL-8, IL-9, IL-10, IL-12 (p70), IL-13, eotaxin, FGF-basic, G-CSF, MCP-1, MIP-1α, PDGF-BB, RANTES, TNF-α, VEGF, IL-2Rα, IL-18, GRO-α, HGF, M-CSF, MIF, SCF, SCGF-β, SDF-1α and TRAIL. However, 10 cytokines/chemokines exhibited significantly higher concentrations ($P<0.05$) in the IBD patients when compared with the CTRL group. These cytokines and chemokines

Table II. Concentrations of cytokines and chemokines in the patients with IBD and the healthy controls.

Cyto/chemokine	Controls	IBD
IL-1β	nd	3.21 (nd-7.09)[a]
IL-1RA	160 (133.7-193)	239.3 (139-370.9)
IL-2	12.86 (9.93-16)	4.33 (1.31-12.62)[a]
IL-4	8.01 (7.75-8.7)	10.29 (5.54-16.14)
IL-5	nd	3.31 (nd-6.97)[a]
IL-6	12.3 (10.41-15.22)	18.16 (11.2-30.9)
IL-7	11.1 (2.29-14.97)	31.17 (13.15-49.76)[a]
IL-8	32.28 (28.74-38.29)	38.57 (24.67-64.12)
IL-9	23.5 (16.38-29.81)	21.34 (12.93-33.6)
IL-10	9.35 (nd-11.43)	5.29 (nd-16.54)
IL-12 (p70)	40.5 (23.61-50.38)	17.73 (10.38-43.35)
IL-13	9.36 (7.97-11.68)	4.26 (1.52-8.13)
IL-15	nd	nd
IL-17	111 (98.37-132.5)	44.59 (15.46-99.85)[a]
Eotaxin	11.73 (nd-46.26)	42.52 (nd-171.8)
FGF-basic	38.6 (33.23-49.16)	35.07 (19.55-59.91)
G-CSF	43.97 (34.28-53.47)	52.88 (35.79-93.16)
GM-CSF	nd	nd
IFN-γ	163.8 (147.1-177.4)	1105 (238-2412)[a]
IP-10	542.5 (390.2-872.6)	3486 (576.3-6348)[a]
MCP-1	50.78 (26.87-77.01)	23.37 (13.37-45.71)
MIP-1α	7.02 (6-8.02)	5.84 (3.48-8.23)
PDGF-BB	8479 (6714-10519)	5182 (3387-11838)
MIP-1β	99.38 (79.2-131.7)	52.59 (35.96-72.8)[a]
RANTES	4633 (nd-5457)	1592 (nd-17660)
TNF-α	30.44 (23.81-36.31)	38.82 (23-55.57)
VEGF	82.2 (46.72-124.9)	91.71 (54.33-209.2)
IL-1α	nd	nd
IL-2Rα	145.4 (76.49-199.7)	173.6 (68-338.5)
IL-3	nd	nd
IL-12 (p40)	nd	nd
IL-16	144 (83.92-195.8)	473.4 (164.3-1343)[a]
IL-18	196 (102.1-284.4)	120.6 (74.95-180.5)
CTACK	406 (259.3-527.8)	716.2 (548.6-889.3)[a]
GRO-α	11.6 (78.63-195.9)	158.3 (58.62-250.6)
HGF	404.2 (290.3-555.5)	382.1 (279.1-513.5)
IFN-α2	nd	54.05 (30.27-74.11)[a]
LIF	nd	nd (nd-12.09)[a]
MCP-3	nd	nd
M-CSF	8.02 (nd-13.22)	nd (nd-15.05)
MIF	393.5 (73.17-3127)	3731 (572.1-7002)
MIG	393 (228.3-676)	2523 (1141-3821)[a]
β-NGF	nd	nd
SCF	1048 (113-1514)	135.5 (64.75-233.3)
SCGF-β	36615 (22073-73267)	39607 (22791-60520)
SDF-1α	37.46 (17.34-75.02)	47.59 (nd-352.3)
TNF-β	nd	nd
TRAIL	85.28 (49.52-104.9)	71.41 (49.84-118.4)

Values are expressed as the median (interquartile range) in pg/ml. [a]P<0.05, vs. control group (Mann-Whitney test with Bonferroni correction for multiple comparisons). nd, non-detectable; IBD, inflammatory bowel disease; IL, interleukin; IL-1RA, interleukin-1 receptor antagonist; FGF, fibroblast growth factor; G-CSF, granulocyte-colony stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; IFN, interferon; IP, IFN-γ-inducible protein; MCP, monocyte chemoattractant protein; MIP, macrophage inflammatory protein; PDGF, platelet-derived growth factor; RANTES, regulated on activation, normal T cell expressed and secreted; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor; CTACK, cutaneous T-cell-attracting chemokine; GRO, growth regulated oncogene; HGF, hepatocyte growth factor; LIF, leukemia inhibitory factor; M-CSF, macrophage colony-stimulating factor; MIF, macrophage migration inhibitory factor; MIG, monokine induced by γ-IFN; NGF, nerve growth factor; SCF, stem cell factor; SCGF, stem cell growth factor; SDF, stromal cell-derived factor; TRAIL, TNF-related apoptosis-inducing ligand.

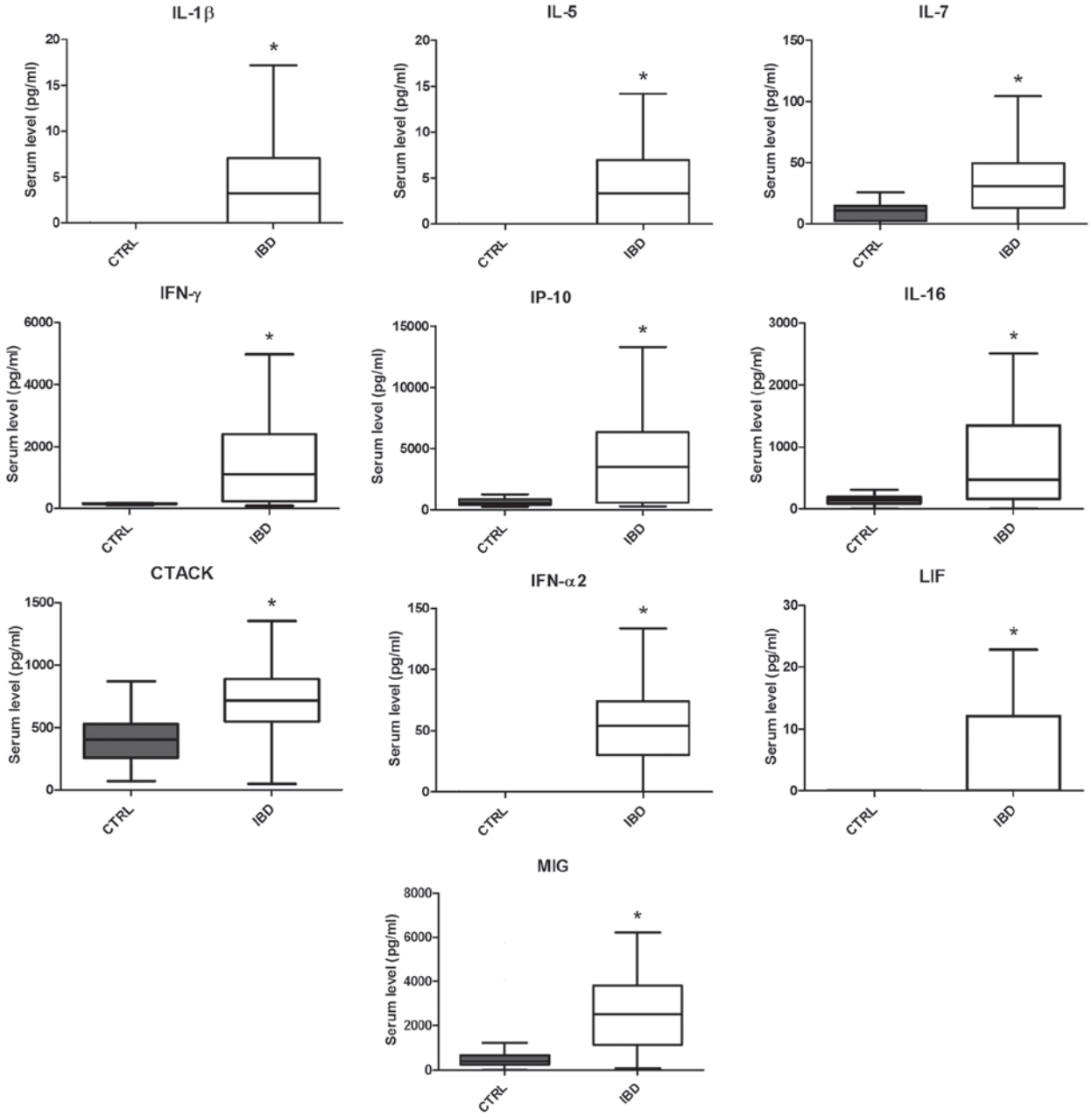

Figure 1. Box plots showing the serum levels (pg/ml) of IL-1β, IL-5, IL-7, IFN-γ, IP-10, IL-16, CTACK, IFN-α2, LIF and MIG in the CTRL (gray) and IBD (white) groups. Whiskers were calculated using the Tukey method and outliers are not shown. *P<0.05, vs. CTRL group (Mann-Whitney test with Bonferroni correction for multiple comparisons). IL, interleukin; IFN, interferon; IP, IFN-γ-inducible protein; CTACK, cutaneous T-cell-attracting chemokine; LIF, leukemia inhibitiory factor; MIG, monokine induced by γ-IFN; CTRL, control; IBD, inflammatory bowel disease.

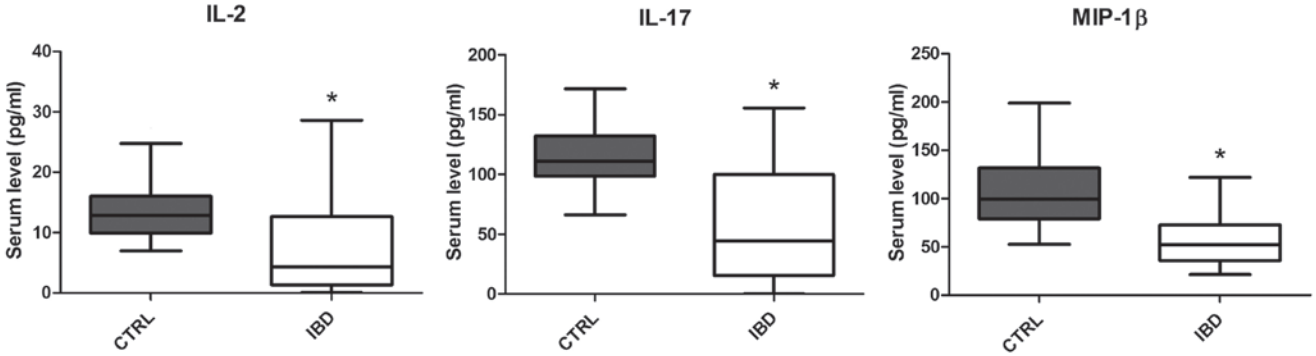

Figure 2. Box plots showing the serum levels (pg/ml) of IL-2, IL-17 and MIP-1β in the CTRL (gray) and IBD (white) groups. Whiskers were calculated using the Tukey method and outliers are not shown. *P<0.05, vs. CTRL (Mann-Whitney test with Bonferroni correction for multiple comparisons). IL, interleukin; MIP, macrophage inflammatory protein; CTRL, control; IBD, inflammatory bowel disease.

included IL-1β, IL-5, IL-7, IFN-α2, IFN-γ, IP-10, CTACK, IL-16, LIF and MIG (Fig. 1).

Levels of certain circulating cytokines are significantly decreased in pediatric IBD patients when compared with the normal controls. The majority of cytokines and chemokines were unaffected or significantly ($P<0.05$) increased in the pediatric IBD patients when compared with the age-matched control subjects. However, it is particularly notable that two cytokines, IL-2 and IL-17, and one chemokine, MIP-1β, exhibited significantly decreased levels in the pediatric IBD patients when compared with the age-matched controls (Fig. 2).

Discussion

The presence of deregulated cytokines and chemokines has been previously reported in cases of IBD, primarily in studies performed at the level of the inflamed mucosa, while studies on the circulating levels of cytokines and chemokines in IBD are less frequent (14-17). Among the panel of cytokines and chemokines investigated in the present study, 10 proteins, including IL-1β, IL-5, IL-7, IFN-α2, IFN-γ, IP-10, CTACK, IL-16, MIG and LIF, were shown to be significantly upregulated in the group of pediatric IBD patients, as compared with the age-matched controls. While a number of these molecules, including IL-1β, IP-10 and IL-16 in particular, are known to have a strong proinflammatory activity (18-24), certain other molecules, such as IFNs, may account for compensatory mechanisms aimed at decreasing the inflammatory status (25).

However, the most notable finding of the present study was that a small number of cytokines and chemokines were significantly downregulated in the serum of the IBD pediatric patients when compared with the age-matched healthy controls. These data are completely novel and unexpected, particularly considering that in IBD patients, concentrations of MIP-1β, IL-2 and IL-17 (26-30) have been previously reported to be upregulated at the mucosal level. The data concerning IL-17 are particularly significant, since IL-17 has been proposed as a potential therapeutic target for IBD (31). In addition, the results may aid the interpretation of certain paradoxical effects resulting from anti-TNF therapy that have been observed in IBD patients, such as the occurrence of psoriasis in a subset of IBD patients (32). Therefore, the results demonstrate, in accordance with the observations of a previous study (33), that the role of IL-17 in the physiopathology of IBD is complex.

In conclusion, the results of the present study indicate that the immunological characteristics of IBD are more complex than originally hypothesized, and may comprise certain aspects of immune-deficiency. To date, the precise balance between proinflammatory and antiinflammatory cytokines/chemokines during the progression of IBD remains unknown. The correct evaluation of cytokines and chemokines involved in the progress of the disease is crucial for identifying potential novel targets for drug treatments.

Further studies are necessary to improve the understanding of IBD etiology and to clarify whether there are differences between Crohn's disease and ulcerative colitis. Other parameters to be considered include genetic background, disease onset, the age of the patients and pharmacological treatment (34,35).

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