Elevated systemic levels of free interleukin-18 (IL-18) in patients with Crohn's disease

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ABSTRACT. ***Objectives.*** **Interleukin 18 (IL-18) is a proinflammatory cytokine and a member of the IL-1 family. Animal models and investigations in humans point to an important role for this cytokine in inflammatory bowel diseases (IBD). IL-18 binding protein (IL-18BP) is a naturally occurring antagonist of IL-18.** ***Methods.*** **In this study, we measured IL-18 and IL-18BP plasma concentrations and spontaneous release in cultures of colonic explants from healthy subjects (n = 41), patients with Crohn's disease (CD, n = 135), and patients with ulcerative colitis (UC, n = 93).** ***Results.*** **Both CD and UC patients had higher IL-18BP plasma levels than controls. Plasma levels of free, unbound IL-18 were significantly elevated in CD patients compared to healthy controls, but not in UC patients. Colonic explant cultures from inflamed areas in IBD patients released significantly higher levels of IL-18 than non-inflamed areas and controls. IL-18BP levels from the same cultures were below the detection limit over a culture period of 24 h.** ***Conclusions.*** **Our results confirm the importance of IL-18 in the pathogenesis of IBD and suggest that especially in CD, IL-18BP might be produced in insufficient quantities to counteract the effects of endogenous IL-18.**

Keywords: cytokines, IBD, IL-18, IL-18BP, colonic explant cultures

IL-18 was initially described as an interferon γ-inducing factor [1, 2]. The cytokine plays an important role in the induction of Th1 responses [3]. IL-18 is related to the IL-1 family in terms of its structure, receptor family and signal transduction pathways [4]. Mainly produced by macrophages, monocytes [5, 6], intestinal epithelial cells [7], dendritic cells [6], adrenal cortex cells [8], microglial cells [9] and synovial fibroblasts [10-12], IL-18 acts in synergy with IL-12 for Th1 differentiation [3, 13, 14]. It also exerts pro-inflammatory properties by inducing the production of IL-1β, TNFα, IL-6, IFNγ [3, 13, 15], chemokines [16], nitric oxide and prostaglandins [17]. The pleiotropic activities of IL-18 suggest an important role for this cytokine in triggering and polarization of the immune response [18]. IL-18 has been shown to play an important role in inflammatory bowel disease (IBD). In human Crohn's disease (CD), but not in ulcerative colitis (UC), upregulation of IL-18 in active bowel inflammation has been demonstrated [7, 19, 20]. Also in this disease IL-18 is predominantly expressed by intestinal epithelial cells, macrophages and dendritic cells. Elevated plasma levels of IL-18 in CD patients have been reported [21, 22]. Intestinal mucosal lymphocytes from CD patients express functional IL-18 receptor, and freshly isolated mucosal lymphocytes from these patients also show a significant proliferative response to recombinant IL-18 compared to lymphocytes from healthy controls [21]. In a Crohn's disease-like animal model, blockade of IL-18 activity resulted in reduced inflammatory activity [23-25].

IL-18 binding protein (IL-18BP) is a readily secreted antagonist of IL-18. IL-18BP was purified from human urine, sequenced, cloned and expressed [26]. The genomic sequence of IL-18BP does not contain any exon coding for a transmembrane domain, suggesting that this soluble protein is not derived from cleavage of a membrane-bound receptor. IL-18BP mRNA is constitutively expressed in peripheral blood lymphocytes (PBL), spleen, thymus, colon, small intestine and prostate [26]. *In vitro* experiments demonstrated that this protein impairs the Th 1 response, abolishes the induction of IL-18-induced IFNγ and IL-8 production and activation of NFκB [26]. These results suggest that IL-18BP acts as a soluble decoy receptor for IL-18 and inhibits its biological activity [26]. A modulating role for IL-18BP in human IBD has been shown by Corbaz *et al.* [27]. The importance of IL-18 in IBD was first established by Siegmund in a model of DSS colitis [28] and was later corroborated by studies performed by Ten Hove *et al.* In these latter studies, TNBS-induced colitis in mice could be ameliorated by blockade of IL-18

by IL-18BP [25]. These results also point to a potential therapeutic role for IL-18BP in IBD.
Investigation of the relationship between IL-18 and IL-18BP in human IBD was the goal of the present study. Furthermore, we sought to reveal differences in the IL-18 pathway between CD and UC by measuring both IL-18 and IL-18BP in plasma and in the intestine by analyzing supernatants from colonic explant cultures.

PATIENTS AND METHODS

Patients

IL-18 and IL-18BP levels were measured in EDTA-plasma samples from 93 patients with UC, 135 patients with CD, and 41 healthy subjects. Colonic biopsies for whole tissue cultures were taken from 7 healthy subjects (cancer screening program), 18 UC patients and 21 CD patients. Diagnosis was established by radiological, endoscopic and histological examinations. Patient characteristics are reported in *table 1* and *table 2*. Healthy subjects had a median age of 33 years (range: 24-59 years; 21 females, 20 males). Informed consent was obtained from each patient.

Sample preparation

For each individual enrolled in the study, a 9 mL sample of venous blood was collected on EDTA, and 1 mL aliquots of plasma were stored at -70 °C until assayed. A consecutive code number was assigned to each sample so that assays could be performed in a blinded manner.

Colonic explant cultures

Colonic biopsies were immediately placed in PBS at room temperature, transported to the laboratory, weighed and

Table 1
Clinical features of IBD patients (plasma samples)

	CD	UC	Controls
Female/male	75/60 (56/44%)	35/58 (38/62%)	21/20 (51/49%)
Age (years)	34 (range: 17-76)	48 (range: 18-64)	33 (24-59)
Disease duration (months)	32 (range: 1-372)	36 (range: 0-216)	
Therapy			
No specific therapy	27 (20%)	16 (17%)	41
Aminosalicylates	49 (36%)	47 (51%)	
Aminosalicylates+steroids	20 (15%)	14 (15%)	
Steroids	16 (12%)	5 (5%)	
Azathioprin	23 (17%)	11 (12%)	
Site of inflammation			
Small bowel	41 (30%)		
Colon	42 (31%)		
Small bowel + colon	46 (34%)		
Small bowel + colon + stomach	6 (5%)		
Proctitis		25 (27%)	
Left sided colon		42 (45%)	
Pancolitis		26 (28%)	

Table 2
Clinical features of IBD patients (biopsy samples)

	CD	UC	Controls
Female/male	13/8 (62/38%)	8/10 (44/56%)	4/3 (57/43%)
Age (years)	36 (range: 16-69)	36 (range: 16-57)	53 (49-56)
Disease duration (months)	13 (range: 1-146)	35 (range: 0-216)	
Therapy			
No specific therapy	8 (38%)	3 (17%)	7
Aminosalicylates	3 (14%)	6 (33%)	
Aminosalicylates+steroids	4 (19%)	3 (17%)	
Steroids	0 (0%)	2 (11%)	
Azathioprin	6 (29%)	4 (22%)	
Site of inflammation			
Small bowel	5 (24%)		
Colon	10 (47%)		
Small bowel + colon	6 (29%)		
Small bowel + colon+ stomach	0		
Proctitis		3 (17%)	
Left sided colon		9 (51%)	
Pancolitis		6 (32%)	

washed 3 times in PBS at room temperature. Biopsies were then placed in 48-well flat bottom culture plates containing 1 mL RPMI 1640 supplemented with 10% FCS, 100 U/mL penicillin and 100 µg/mL streptomycin. Cultures were maintained for 24 h at 37 °C in a humidified atmosphere containing 5% CO_2. Culture supernatants were then harvested, centrifuged and the supernatant was stored immediately at -70 °C. Biopsies were taken both from areas which showed endoscopic signs of high inflammatory activity and from endoscopically normal-appearing, non-inflamed mucosa.

ELISA

Plasma levels of IL-18 and IL-18BP were measured by specific enzyme-linked immunosorbent assay (ELISA). IL-18 plasma levels were measured by a commercially available ELISA (MBL Medical & Biological Laboratories CO., LTD, Nagoya, Japan). ELISA was performed according to the manufacturer's recommendations. Briefly, samples were diluted 1:5 with assay diluent provided in the kit. Diluted samples and standards were added to the microwells coated with monoclonal anti-human IL-18 antibody. Incubation for 60 minutes at room temperature was followed by 4 washes with buffer (provided in the kit). The plates were then incubated for 60 minutes with anti-human IL-18 monoclonal antibody conjugated with peroxidase. After the incubation period, the plates were washed and the substrate reagent (TMB/H_2O_2) was added. After 15 minutes the reaction was stopped by addition of 2N H_2SO_4. The optical density of each well was measured at 450 nm in an ELISA reader. The lower detection limit of the assay was 12.5 pg/mL.
The ELISA for IL-18BP has been described [29]. Briefly, 96-well microtiter ELISA plates were coated with a monoclonal anti-human IL-18BP overnight at 4 °C. The plates were washed with PBS/0.05% Tween 20 and blocked with bovine serum albumin. Plasma samples were diluted 1:5 and 100 µL aliquots were added to the wells. rIL-18BP was used to generate a standard curve. The plates were incubated for 2 hrs at 37 °C and washed 3 times. Rabbit anti-rIL-18BP serum was then added and the plates were incubated for a further 2 hrs at 37 °C. The plates were then washed 3 times, a conjugate of goat-anti-rabbit horseradish peroxidase was added and the plates were incubated for 1 h at 37 °C. The plates were then washed 3 times and developed by the addition of peroxidase substrate for 30 min at room temperature. The reaction was stopped by 3N HCl and the absorbance at 492 nm was determined by an ELISA reader.

Calculation of free IL-18

Antibodies used for the IL-18 ELISA detect both free IL-18 and IL-18 bound to IL-18BP but are not able to distinguish between the free and the bound form of IL-18. Therefore, the levels of free IL-18 were calculated, based on methods of previous studies [29]. IL-18BP exhibits a high affinity for its ligand (K*d* = 0.4 nM). This high affinity is mainly due to a very low dissociation rate of the stoichiometric complex. The presence of relatively high levels of IL-18BP in the serum suggests that IL-18 is present in the circulation at least in part as an inactive complex with IL-18BP. Therefore, it was necessary to measure the level of serum IL-18BP in order to calculate the actual level of free IL-18 in the circulation. Thus, levels of free IL-18 were calculated, based on the mass action law [29]. In brief, the calculation was based on a 1:1 stoichiometry in the complex of IL-18 and IL-18BP and a dissociation constant (K*d*) of 0.4 nM [29].

Statistical analysis

Results are expressed as mean ± standard error of mean (SEM). Variables were compared using an ANOVA test followed by *post hoc* Bonferroni testing. Correlations were assessed using Pearson´s correlation coefficient. Results from whole tissue cultures were analyzed by Kruskal-Wallis testing, because in these series the samples were not normally distributed. These results are expressed as median, 25^{th} and 75^{th} percentiles are provided. P values less than 0.05 were considered significant.

RESULTS

IL-18 plasma levels

In CD patients, IL-18 plasma levels were significantly higher (CD: 546 ± 32 pg/mL, n = 135, $P < 0.0001$) than in healthy controls (299 ± 22 pg/mL, n = 41) and UC patients (393 ± 32 pg/mL, n = 93, P for CD *versus* UC: $P < 0.01$). The difference between UC patients and healthy controls was not statistically significant *(figure 1A)*.
When calculated as free, unbound IL-18, the same results were obtained. Healthy controls (232 ± 16 pg/mL) and UC patients (253 ± 24 pg/mL) did not differ significantly. In CD patients, levels of free IL-18 were significantly higher (332 ± 17 pg/mL) than in controls or UC patients ($P < 0.01$ for controls *versus* CD, $P < 0.05$ for UC *versus* CD) *(figure 1B)*. Correlation of IL-18/free IL-18 plasma levels with markers of inflammation, CD activity index (CDAI) and routine laboratory markers are shown in *table 3* for CD and in *table 4* for UC.

IL-18BP plasma levels

Both UC (4.69 ± 0.37 ng/mL, n = 93, $P < 0.001$) and CD (5.01 ± 0.34 ng/mL, n = 135, $P<0.0001$) patients had significantly higher IL-18BP plasma levels than healthy controls (2.10 ± 0.16 ng/mL, n = 41). The difference between UC and CD was not significant *(figure 1C)*. Correlations of IL-18BP plasma levels with markers of inflammation and routine laboratory markers are shown in *table 3* for CD and in *table 4* for UC.

Spontaneous secretion of IL-18 by cultured colonic explants

After 24 h incubation in culture medium, supernatants of colonic biopsy cultures contained measurable levels of

IL-18. Cultured explants from healthy controls (n = 7) secreted 3.91 pg/mL/mg biopsy weight (median, 25th percentile: 3.48 pg/mL/mg, 75th percentile: 4.26 pg/mL/mg). Spontaneous IL-18 release from biopsies of IBD patients taken from macroscopically non-inflamed areas did not differ significantly from tissues from healthy controls (non-inflamed UC: 4.05 pg/mL/mg (3.26-5.75 pg/mL/mg), n = 8, P = 0.82; non-inflamed CD: 5.75 pg/mL/mg (3.76-6.97 pg/mL/mg), n = 9, P < 0.13). Cultured tissues from macroscopically inflamed areas produced significantly higher levels of IL-18 than tissues from non-inflamed areas (inflamed UC: 8.94 pg/mL/mg (6.24-13.05 pg/mL/mg), n = 10, P < 0.001, P < 0.001 *versus* controls; inflamed CD: 9.83 pg/mL/mg (5.44-21.05 pg/mL/mg), n = 12, P < 0.05, P < 0.001 *versus* controls) (*figure 2*).

IL-18BP secretion by colonic explant cultures

Levels of IL-18BP in the supernatants of cultured explants from the different groups were below the detection limit (data not shown).

DISCUSSION

Numerous studies using animal models suggest an important role for IL-18 in the pathogenesis of experimental inflammatory bowel disease [23-25, 28, 30, 31]. Investigations on human inflammatory bowel disease are consistent with these results. Overexpression of IL-18 has been demonstrated in human CD [7, 19, 20, 27]. Recently, an endogenous antagonist of IL-18, IL-18BP, has been characterized, and elevated production has been demonstrated in human CD [27]. In mouse models of inflammatory bowel disease, administration of IL-18BP ameliorated IL-18-mediated inflammation [25]. Thus, these findings suggest a potential therapeutic role for IL-18BP in CD. Nevertheless there is, to our knowledge, no data available about IL-18BP expression in UC.

Therefore, our study aimed to investigate whether there are differences in IL-18/IL-18BP biology in CD and UC. For this purpose, we measured IL-18 and IL-18BP levels both in the circulation and in cultured explants of colonic tissues. We calculated levels of free IL-18, assuming that free IL-18 is the biologically active, not neutralized fraction of secreted IL-18. In addition, we established correlations between IL-18/IL-18BP and markers of inflammatory activity, such as C-reactive protein (CRP), alpha-1-glycoprotein (α-1GP), and Crohn's disease activity index (CDAI).

As already described previously [7, 19, 27], our data gained from colonic explant cultures showed an elevated secretion of IL-18 by inflamed intestinal mucosa in CD, whereas secretion by non-involved areas did not differ from healthy controls. The same pattern of IL-18 expression was found in UC. Plasma levels of IL-18/IL-18BP and especially free IL-18 however, pointed to differences in the biology of IL-18 in CD, compared to UC. According to our data, CD is characterized by a significant increase in IL-18 plasma levels, which results in significantly elevated levels of free, unbound IL-18 despite overexpression of IL-18BP, compared to healthy controls. In contrast, marked elevation of IL-18BP in UC patients efficiently counteracts IL-18 production, resulting in plasma levels of free IL-18,

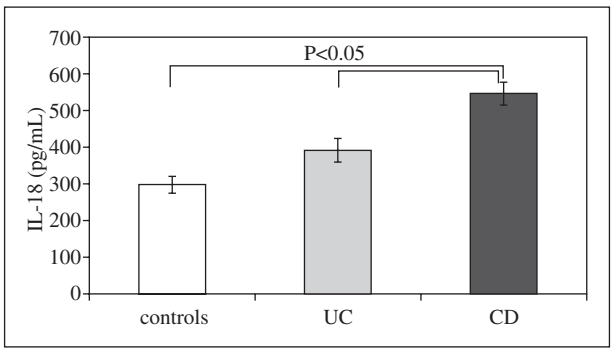

Figure 1A
Plasma levels of IL-18 in ulcerative colitis and Crohn's disease. 228, consecutive unselected patients with IBD were enrolled in the study, 93 with ulcerative colitis (UC) and 135 with Crohn's disease (CD). Forty one healthy subjects served as controls. Results are expressed as mean + SEM. Statistically significant differences between two groups are shown in brackets (P < 0.0001 for controls *versus* CD, P < 0.01 for CD *versus* UC).

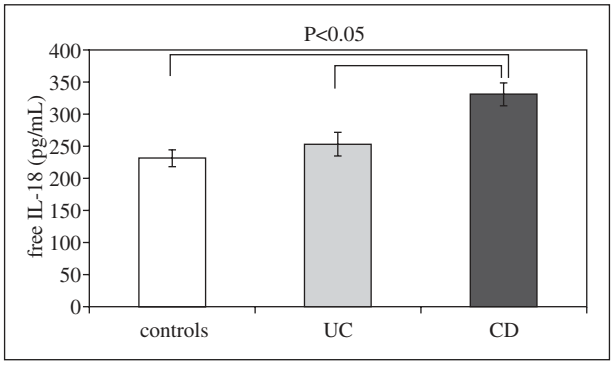

Figure 1B
Plasma levels of free, unbound IL-18 in ulcerative colitis and Crohn's disease. Levels of free IL-18 were calculated, based on the mass action law, after measuring IL-18 and IL-18BP. Results are expressed as mean + SEM. Statistically significant differences between the groups are shown in brackets (P < 0.01 for controls *versus* CD, P < 0.05 for UC *versus* CD).

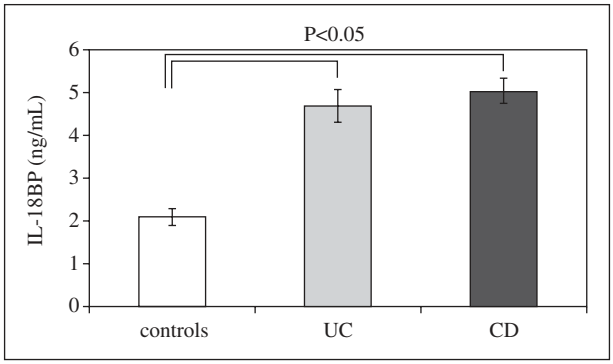

Figure 1C
Plasma levels of IL-18BP in ulcerative colitis and Crohn's disease. 228 unselected, consecutive patients with IBD were enrolled in the study, 93 with ulcerative colitis (UC) and 135 with Crohn's disease (CD). IL-18BP levels were measured using specific ELISA. Results are expressed as mean + SEM. Statistically significant differences between two groups are shown in brackets (P < 0.001 for controls *versus* UC, P < 0.0001 for controls *versus* CD).

Table 3
Correlation of IL-18/IL-18BP plasma levels with laboratory parameters in CD

	IL-18	Free IL-18	IL-18BP
CRP	R=0.345, P<0.01	ns	R=0.487, P<0.001
α-1GP	ns	ns	R=0.386, P<0.01
Leukocyte count	ns	ns	ns
CDAI	ns	ns	ns
Hemoglobin	R=-0.430, P<0.001	R=-0.275, P<0.05	R=-0.365, P<0.01
Ferritin	ns	ns	R=0.465, P=0.001

ns: no significant correlation, CRP: C-reactive protein, α1-GP: alpha-1-glycoprotein.

Table 4
Correlation of IL-18/IL-18BP plasma levels with laboratory parameters in UC

	IL-18	Free IL-18	IL-18BP
CRP	R=0.635, P<0.001	R=0.648, P<0.001	ns
α-1GP	R=0.444, P<0.05	R=0.436, P<0.05	ns
Leukocyte count	ns	ns	ns
Hemoglobin	ns	ns	ns
Ferritin	ns	ns	ns
Rachmilewitz score	ns	ns	ns

ns: no significant correlation, CRP: C-reactive protein, α-1GP: alpha-1-glycoprotein.

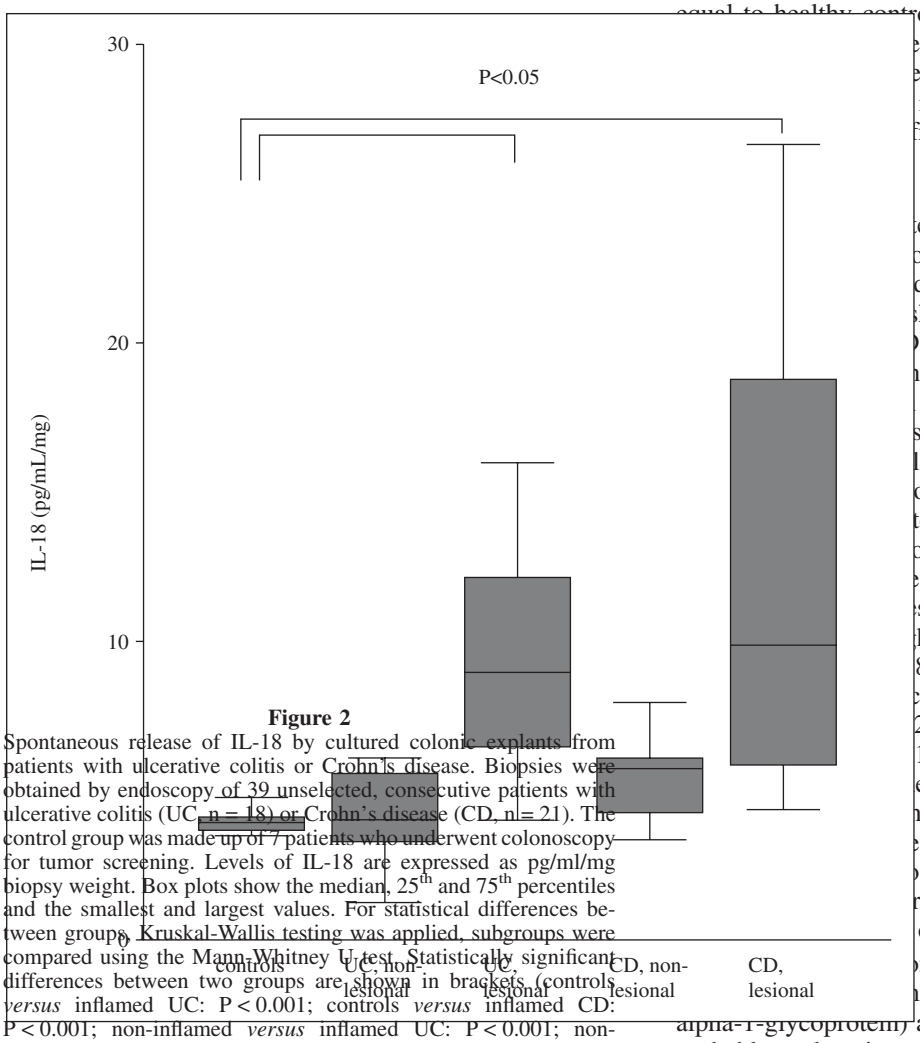

Figure 2
Spontaneous release of IL-18 by cultured colonic explants from patients with ulcerative colitis or Crohn's disease. Biopsies were obtained by endoscopy of 39 unselected, consecutive patients with ulcerative colitis (UC, n = 18) or Crohn's disease (CD, n = 21). The control group was made up of 7 patients who underwent colonoscopy for tumor screening. Levels of IL-18 are expressed as pg/ml/mg biopsy weight. Box plots show the median, 25th and 75th percentiles and the smallest and largest values. For statistical differences between groups, Kruskal-Wallis testing was applied, subgroups were compared using the Mann-Whitney U test. Statistically significant differences between two groups are shown in brackets (controls *versus* inflamed UC: P < 0.001; controls *versus* inflamed CD: P < 0.001; non-inflamed *versus* inflamed UC: P < 0.001; non-inflamed *versus* inflamed CD: P < 0.05).

equal to healthy controls. This observation is consistent
ed data from centenarians with no
ease who have been compared to
ischemic syndromes. In centenar-
free IL-18 were reduced to normal
overexpression despite high levels

ed, free IL-18 in plasma despite
on correlates with data which have
d by Corbaz *et al.* [27], demonstrat-
ship between total IL-18, IL-18BP
in surgically resected specimens
this, plasma levels seem to reflect
in terms of IL-18 biology in CD.
s different. Here, free IL-18 is not
lation, although IL-18 is overex-
olonic mucosa. This might be ex-
temic inflammatory reaction in CD
ocal disease expression in UC. Al-
ession has previously been demon-
sected colonic specimens in active
h we found significantly elevated
8BP in IBD, we could not detect
colonic explant cultures from CD or
24 hour biopsy culture. This may be
18BP is mainly secreted by cells in
e bowel wall (endothelial cells and
hich are not sufficiently assessed by
s obtained by endoscopic biopsies,
produced by intestinal epithelial
ria mononuclear cells [7]. Alterna-
culture may be required.

on of the CDAI with IL-18/IL-18BP
markers of inflammation (i.e. CRP,
alpha-1-glycoprotein) are correlated with IL-18 levels. A probable explanation might be that CDAI is influenced by

different parameters, such as body weight and extraintestinal symptoms. Therefore, CDAI reacts much slower to changes in intestinal inflammation than acute phase reactants such as CRP or alpha-1-GP. In addition, factors other than IL-18 are more likely to influence extraintestinal manifestations of IBD. These factors may be responsible for the lack of correlation with CDAI. Faster reacting markers of inflammation (CRP, alpha-1-GP) are indeed correlated to IL-18 levels.

Although there are clear, significant differences in plasma levels of free IL-18 between UC and CD patients, measuring free IL-18 does not allow us to distinguish between UC and CD, when diagnosis is unclear. Standard deviations are too big in relation to the mean difference, so that there is too much overlap between the two groups.

In mouse models [25], administration of IL-18BP reduced colitis severity. Our data might suggest a potential therapeutic role for IL-18BP in human CD, too. However, in UC too, which is predominantly seen as a Th2-mediated disease [33], blocking IL-18 expression could be beneficial in treating this disease. Our data, which show IL-18-overexpression in active lesions in colonic biopsies in UC, suggest a pathogenic role for IL-18 in UC, too. This is in accordance with recently published data, which demonstrated that IL-18 is also able to enhance Th2 responses [34-38]. In addition, data published by Novick *et al.* [29] showed that the titration curve of IL-18 by IL-18BP is very asymptotic. Hence, even if there is a large excess of endogenous IL-18BP over IL-18 in UC, it may still be beneficial to treat UC patients with exogenous IL-18BP, which will further reduce serum IL-18. However, to confirm these speculations, further research is needed.

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