Inflamm. Res. (2014) 63:943–950
DOI 10.1007/s00011-014-0768-7

Inflammation Research

ORIGINAL RESEARCH PAPER

Elevated levels of Th17 cells and Th17-related cytokines are associated with disease activity in patients with inflammatory bowel disease

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Received: 11 October 2013 / Revised: 4 August 2014 / Accepted: 6 August 2014 / Published online: 18 August 2014

Abstract
Objective Interleukin-17(IL-17)-producing T helper(Th)17 cells are considered as a new subset of cells critical to the development of inflammatory bowel disease (IBD). We aimed to investigate the distribution of Th17 cells, the expressions of Th17-related cytokines (IL-17, IL-21 and IL-22) and their association with disease activity in IBD patients.
Methods We collected intestinal tissue biopsies from 40 patients with active ulcerative colitis (UC), 20 patients with active Crohn's disease (CD) and 20 healthy controls. The distribution of Th17 cells and expressions of Th17-related cytokines in colonic tissues were evaluated by a standard immunohistochemical procedure. Serum IL-17, IL-21 and IL-22 levels were determined by ELISA. Pearson's and Spearman's correlation analyses were performed to analyze the correlation between the number of Th17 cells, the expressions of Th17-related cytokines and disease activity index, endoscopic and histological grading, and CRP and PLT levels, respectively.
Results Compared with healthy controls, the number of Th17 cells and the expressions of IL-17, IL-21 and IL-22 were significantly increased in active IBD patients ($P < 0.05$). In addition, Pearson's and Spearman's correlation analyses showed that the number of Th17 cells and the expressions of Th17-related cytokines were correlated with disease activity index, endoscopic and histological grading, CRP and PLT levels ($P < 0.05$).
Conclusions Th17 cells and Th17-related cytokines (IL-17, IL-21 and IL-22) were increased in the intestinal mucosa in active IBD patients and may play an important role in disease activity and mucosal damage.

Keywords Inflammatory bowel disease (IBD) · Ulcerative colitis (UC) · Crohn's disease (CD) · Th17 Cells · IL-17 · IL-21 · IL-22

Responsible Editor: Ikuo Morita.

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Introduction

Inflammatory bowel disease (IBD) is characterized by chronic relapsing inflammation of the gastrointestinal tract including two major phenotypes: ulcerative colitis (UC) and Crohn's disease (CD). Although the etiology and pathogenesis of IBD remain incompletely clear, complex interactions of genetic, immunological, microbiological and environmental factors are likely to contribute to IBD [1]. Furthermore, the interaction of genetic and environmental factors is believed to promote an excessive mucosal inflammation response directed against components of the luminal microflora [2]. Several subsets of T helper (Th) cells contribute to immune response at inflammatory sites. UC was believed to be mediated by Th2 cytokines and CD was mediated by Th1 cytokines [3]. Recently, a novel population of effector T lymphocytes was identified, which is separated from Th1 and Th2 and characterized by the secretion of IL-17, so its designation as Th17. It was reported that Th17 cells and Th17-related cytokines, such

as IL-17, IL-21 and IL-22, were increased in IBD patients [4–6]. However, the relationship between these Th17-related cytokines and disease activity and mucosal damage in IBD is still unclear. In this study, we investigated the distribution of Th17 cells and Th17-related cytokine levels in active IBD patients. Moreover, to analyze the association of Th17-related cytokines with disease activity and mucosal damage in IBD, we evaluated the correlation between Th17-related cytokine levels and endoscopic grading, histological grading, C-reactive protein (CRP) and platelet (PLT), respectively.

Materials and methods

Patients and samples

Forty cases of ulcerative colitis, 20 cases of Crohn's disease and 20 healthy controls from the First Affiliated Hospital of Nanjing Medical University between 2009 and 2011 were included. Healthy controls underwent colonoscopy to screen for cancer or polyps. UC and CD patients were diagnosed based on clinical, endoscopic and histological criteria [7]. All patients had not been treated with salicylates, corticosteroids, or immunosuppressive or biological agents. Healthy controls showed normal colonic mucosa without tumor, autoimmune diseases and IBD family history. The main clinical characteristics of the study population are summarized in Table 1. There was no significant difference in age and sex distribution among the UC group, CD group and normal control (NC) group.

This study was approved by the Ethics Committee of the First Affiliated Hospital of Nanjing Medical University.

The disease activity of UC was assessed by Truelove and Witts Index and Southerland Disease Activity Index. CD disease activity was assessed by Harvey–Bradshow Index (Simplified CD Activity Index). The endoscopic activity of UC was evaluated according to the Ulcerative Colitis Endoscopic Index of Severity (UCEIS). The histological grade of UC was described by Truelove and Richards. The endoscopic activity of CD was evaluated according to simplified endoscopic activity score for Crohn's disease (SESCD) [8].

Table 1 The basic characteristics of the IBD patients and healthy individuals

Clinical characteristics	NC ($n = 20$)	UC ($n = 40$)	CD ($n = 20$)
Mean age ± SD (years)	33.2 ± 7.0	31.7 ± 9.3	25.1 ± 10.5
Age range (years)	25–46	21–47	13–47
Sex (male/female)	11/9	24/16	13/7
Disease activity *n* (%)			
Mild		12 (30.0)	6 (30.0)
Moderate		20 (50.0)	8 (40.0)
Severe		8 (20.0)	6 (30.0)

Immunohistochemical detection in tissues

Samples were fixed in 10 % buffered formalin, dehydrated in ethanol and embedded in paraffin. The endogenous peroxidase activities were blocked by 3 % (v/v) H_2O_2 at room temperature for 10 min. The samples were heated in citrate solution (0.01 M; pH 6.0) at 92 °C for 15 min to reactivate the antigens. Expression and distribution of Th17-related cytokines (IL-17, IL-21, and IL-22) were investigated by immunohistochemical analysis. Slides were added with anti-IL-17 antibody (polyclonal, 1:100, Abcam, UK), anti-IL-21 antibody (polyclonal, 1:100, Millipore, USA) and anti-IL-22 antibody (polyclonal, 1:100, Abcam, UK) and incubated at 4 °C overnight, then incubated sequentially with a second antibody at room temperature for 20 min. PBS was used to rinse the slides after each step. Nuclear counterstaining was performed with Mayer's HE for 50 s. Negative controls were treated with PBS instead of primary antibodies. The slides were observed under a light microscope and photomicrographs taken.

For double-labeling experiments involving a combination of anti-IL-17 and anti-CD4 antibodies, tissue sections were incubated with the primary antibodies (anti-IL-17, 1:100) at 4 °C overnight and then washed with PBS. For the secondary antibody, FITC (green) goat anti-rabbit (1:500, Vector) was added for 30 min at RT in the dark and then washed with PBS. Slides were fixed in 4 % paraformaldehyde in PBS for 15 min and then washed with PBS. Subsequently, monoclonal mouse anti-human CD4 (1:100, Novocastra, Dossenheim, Germany) was applied overnight at 4 °C. Rhodamine red-labeled goat anti-mouse IgG (1:500, Rockland, Gilbertsville, Pennsylvania, USA) was applied for 120 min at room temperature. Images were obtained by the digital confocal laser scanning system MRC-600 (Bio Rad, Hercules, California, USA). Normal goat IgG was used as a negative control.

Assessments of Th17-related cytokines (IL-17, IL-21, and IL-22) immunostaining

The immunohistochemical positive reactions were assessed in cytoplasm. The percentage of positive staining cells was determined by counting positively stained cells and total cells in five separate areas of lamina propria under 400× magnifications and the average counts were recorded. Integrated optical density (IOD): expression intensity was quantified as IOD. Magnified at 400×, the integrated

intensity/area (integrated optical density [IOD]) of the staining of each tissue core was evaluated by two observers blinded to tissue status using Image-Pro Plus software (Version 6.0, Media Cybernetics Inc., USA). The IOD represented the sum density of Th17-related cytokines staining in a fixed field with a total pixel area of about 345 × 345. An average of five pictures per tissue core was taken for analysis. The average values were calculated for each image [9, 10].

Enzyme-linked immunosorbent assay (ELISA)

Whole blood samples from 40 UC patients, 20 CD patients and 20 healthy individuals were obtained. IL-17, IL-21 and IL-22 serum levels were measured using ELISA (Molecular Devices, USA). Fifty microliters of standard was added to each standard well. Ten microliters of testing sample was added in the testing sample well. Then, 100 μl of HRP-conjugated reagent was added to each well. The wells were then covered with an adhesive strip and incubated for 60 min at 37 °C. Each well was aspirated and washed. This process was repeated four times. Washes were performed with wash solution (400 μl) using a squirt bottle, manifold dispenser or auto-washer. After the last wash, any remaining wash solution was removed by aspiration or decanting. The plate was inverted and blotted against a clean paper towel. Then 50 μl chromogen solutions A and B were added to each well. These were gently mixed and each well was incubated for 15 min at 37 °C away from light. Optical density (OD) was read at 450 nm using a microtiter plate reader within 15 min of incubation.

Statistical analysis

Continuous data are expressed as mean ± standard deviation (SD) and were analyzed by t test or analysis of variance (ANOVA) test. Correlations between IOD, serum levels and disease activity index, CRP and PLT levels, UC endoscopic grading and CD endoscopic grading were analyzed by Pearson's correlation analysis, respectively. Correlations between IOD, serum levels and UC histopathological grading were analyzed by Spearman's correlation analysis, respectively. SPSS 17.0 (SPSS Inc., USA) statistical software was used for data analysis. $P < 0.05$ was considered to be statistically significant.

Results

Distribution of Th17 cell in intestinal tissue

To observe the distribution of Th17 cells, intestinal tissue sections from active UC or CD patients were double stained with fluorescence-labeled anti-CD4 and anti-human IL-17 antibodies (Fig. 1). The result showed that there were more CD4/IL-17 double staining positive T cells in the inflamed mucosa of UC and CD patients compared with normal control (Fig. 1f, i). The result from Fig. 1 also showed that Th17 cells were mainly increased in the lamina propria of active UC and CD patients.

Distribution and expression of Th17-related cytokines

To evaluate the expression of IL-17, IL-21 and IL-22, intestinal tissue samples were stained with anti-IL-17, anti-IL-21 and anti-IL-22 antibodies, respectively. As shown in Fig. 2a–c, there were almost no positive cells in normal intestinal tissues. In contrast, IL-17-, IL-21- and IL-22 positive staining cells separately increased in active IBD patients (Fig. 2d–i). In active IBD patients, IL-17-, IL-21- and IL-22-positive staining cells were mainly located within the lamina propria.

The percentage of Th17-related cytokines positive cells in intestinal tissues of active IBD patients was higher than in the NC group (Table 2, $P < 0.05$). We measured the integrated optical density (IOD) of IL-17, IL-21 and IL-22 in the mucosa from active IBD patients. As shown in Fig. 3, these cytokines' intensities were significantly higher in active IBD patients compared with the controls ($P < 0.05$). Of these, IL-22 intensity was significantly higher in active CD patients than in UC patients ($P < 0.05$).

Compared with the NC group, the mean serum levels of IL-17, IL-21 and IL-22 were significantly higher in active UC patients ($P < 0.05$) (Table 3).

Relationship of Th17-related cytokines with disease activity in IBD patients

We analyzed the correlation between the expressions of these three cytokines and disease activity index of UC/CD. The Pearson correlation showed that the levels of IL-17, IL-21 and IL-22 in serum and colonic tissues were significantly correlated with Truelove and Witts Index, Southerland Index for the UC group (Table 4). The levels of IL-17, IL-21 and IL-22 were significantly correlated with Harvey–Bradshow Index for the CD group (Table 5).

We also analyzed the correlation between the levels of these three cytokines and endoscopic activity grade and histopathological grading in active IBD patients. The Pearson and Spearman correlations showed that the levels of IL-17, IL-21 and IL-22 both in serum and tissues were significantly correlated with endoscopic disease activity and histopathological grading, respectively, in the UC group ($P < 0.05$) (Table 6), while in the CD group, the

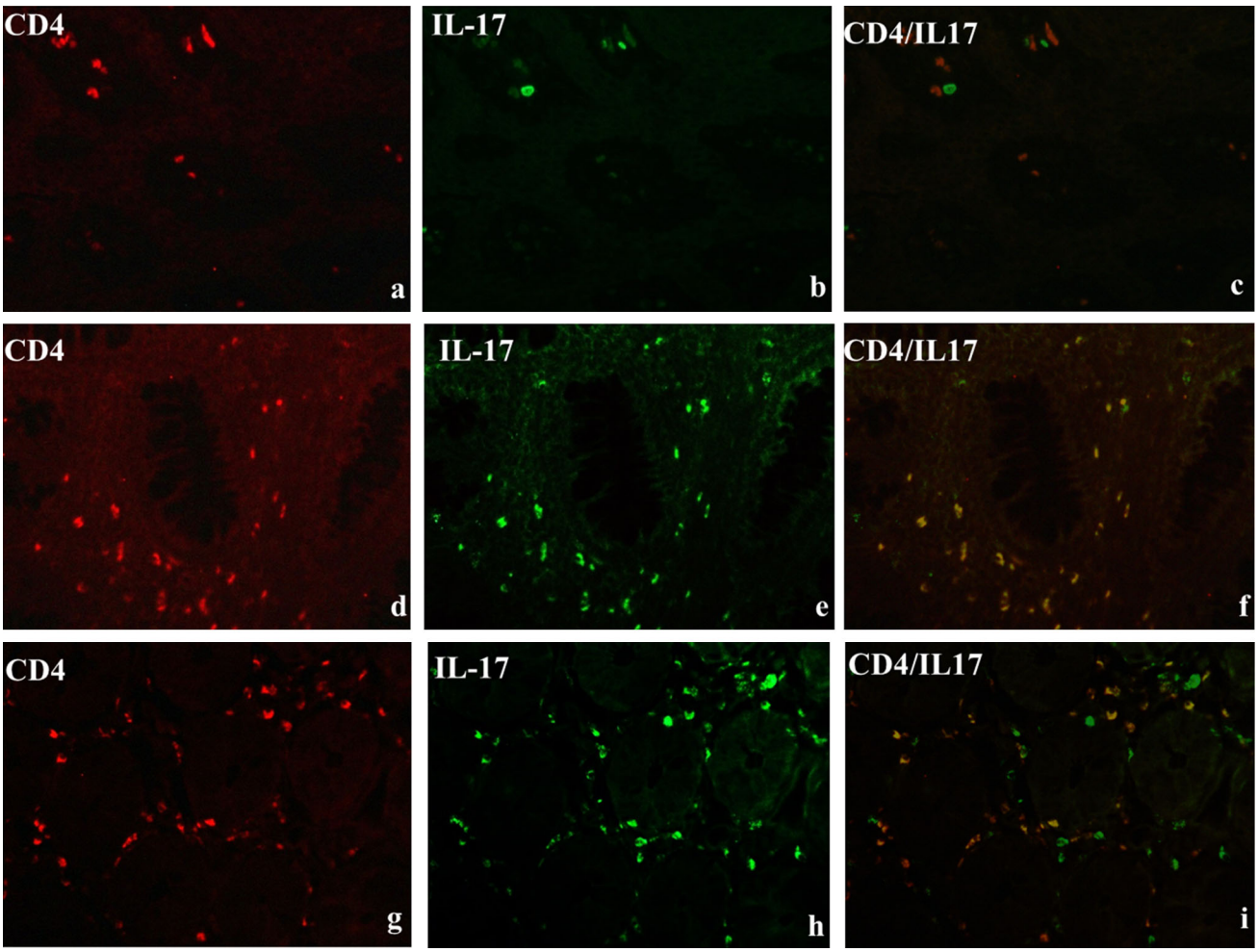

Fig. 1 Distribution of Th17 cell in intestinal tissues in IBD patients. NC group normal control. **a–c** NC group, **d–f** UC group, **g–i** CD group

levels of IL-17, IL-21 and IL-22 were significantly correlated with endoscopic disease activity (Table 7).

Finally, we analyzed the correlation between the IOD of these three cytokines and CRP and PLT levels in active UC/CD patients. The Pearson correlation results showed that the IOD of IL-17, IL-21 and IL-22 expressions in intestinal tissues significantly correlated with CRP and PLT levels ($P < 0.05$) both in UC and CD group (Table 8). In addition, the correlation between the serum levels of these cytokines and CRP and PLT levels was analyzed in active UC patients. As shown in Table 8, the serum level of IL-17, IL-21 and IL-22 significantly correlated with CRP and PLT levels ($P < 0.05$).

Discussion

Th17 cells were identified as a new subset of T helper cells, which play an important role in the activation of the immune system. As known, Th17 cells are associated with host defense against extracellular pathogens and the development of autoimmunity and inflammatory response, such as multiple sclerosis, rheumatoid arthritis and IBD [11–13]. The number of Th17 cells significantly increased in the peripheral blood of patients with active IBD compared with healthy individuals. Fujino [4] found that IL-17^{+} cells were markedly increased in the inflamed region of patients with active IBD compared with inactive IBD patients, while there was no positive expression in the ischemic bowel disease, infectious colitis and controls. These suggested that Th17 cells may be involved in the pathogenesis of IBD [14]. In our study, we also found that Th17 cells were increased in the inflamed intestinal mucosa of active IBD patients, compared with healthy controls. This suggested that Th17 cells may be involved in the immune inflammatory response of the intestinal mucosa of IBD patients.

As known, Th17 cells played an important role in immune inflammatory response through the production of effector cytokines, including IL-17, IL-21, IL-22 and so on.

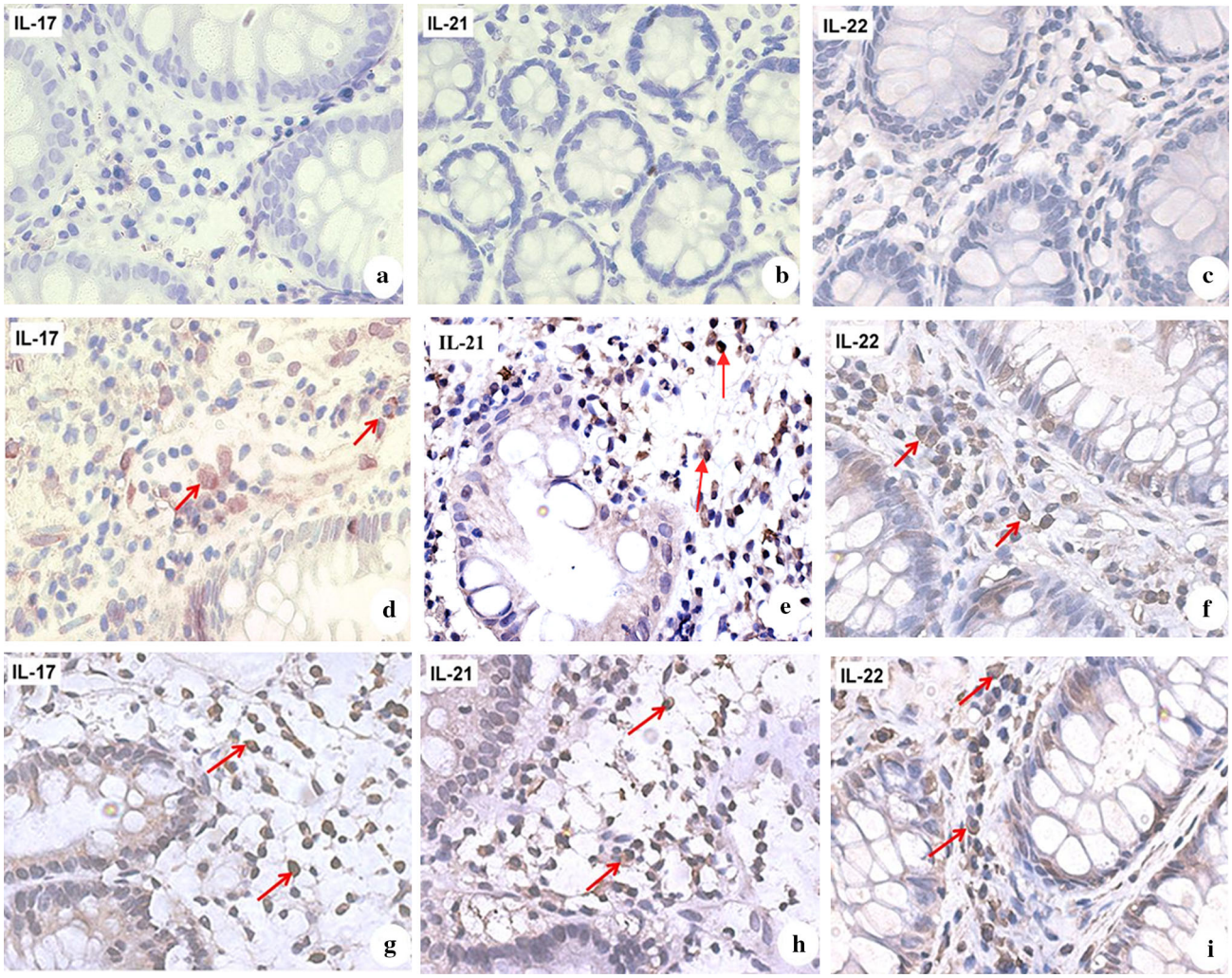

Fig. 2 Distribution of Th17-related cytokines in intestinal tissues in IBD patients. NC group normal control. **a–c** NC group, **d–f** UC group, **g–i** CD group. UC and CD samples from moderately active patients

Table 2 The percentage of positive cells of Th17-related cytokines in intestinal tissues

Group	IL-17 (%)	IL-21 (%)	IL-22 (%)
NC	3.2 ± 0.8	2.8 ± 0.8	3.3 ± 0.7
UC	16.9 ± 5.1*	26.0 ± 2.8*	16.4 ± 3.5*
CD	28.0 ± 9.0*$^{\triangle}$	27.1 ± 6.6*	23.4 ± 6.7*$^{\triangle}$

* versus NC, $P < 0.05$; $^{\triangle}$ versus UC, $P < 0.05$

These cytokines contributed the development of intestinal inflammation and mucosal damage. The study of Hölttä V et al. showed that the activation of IL-17 immunity is a shared feature in pediatric CD and UC. In this paper, they found that colonic interleukin (IL)-17 and IL-22 mRNA were up-regulated and the number of colonic IL-17 cells increased both in pediatric patients with CD and UC [15]. We found that colonic interleukin (IL)-17 and IL-22 protein expressions were up-regulated and the number of colonic Th17 cells increased in adult patients with active CD. IL-17 is a pleiotropic cytokine which induces IL-6 and IL-8 production via mitogen-activated protein kinase (MAPK) pathways, thus contributing to the recruitment of neutrophils at the sites of inflammation [16], triggers T cell proliferation and up-regulates a number of pro-inflammatory molecules, such as inducible nitric oxide synthase and IL-1β [17]. Additionally, IL-17 induces pro-inflammatory cytokine production by macrophages, creating a link between innate and adaptive immunity [18], and plays a key role in host defense against bacteria, particularly at mucosal surfaces [19]. Our results are consistent with the findings of recent studies that showed the levels of IL-17 in serum and inflamed mucosa were significantly increased compared with controls in IBD patients [20].

Yamamoto-Furusho et al. [21] found that IL-21 mRNA expression was significantly increased in rectal mucosa samples of active UC patients, compared with inactive UC

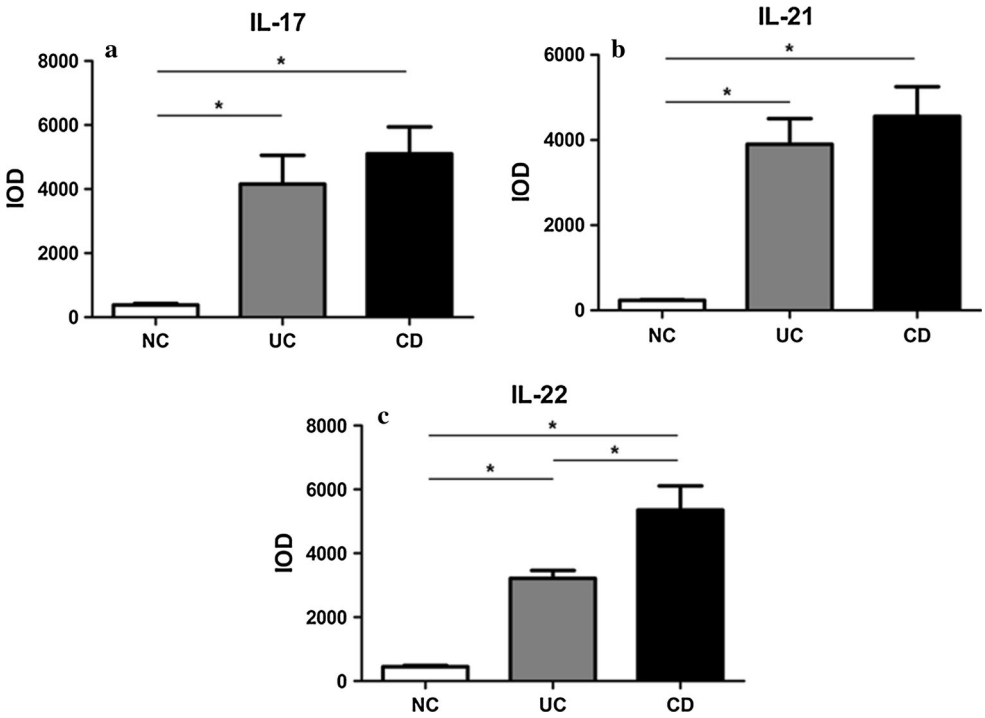

Fig. 3 The expressions of Th17-related cytokines in intestinal tissues in IBD patients. Values expressed as average ± standard error of means of IOD data series for Th17-related cytokines. **a** IL-17 expressions in intestinal tissues in IBD. **b** IL-21 expressions in intestinal tissues in IBD. **c** IL-22 expressions in intestinal tissues in IBD

Table 3 Th17-related cytokines' expression in sera of the UC group (ELISA)

Group	IL-17 (pg/ml)	IL-21 (pg/ml)	IL-22 (pg/ml)
NC	6.0 ± 1.1	136.8 ± 24.0	60.8 ± 11.0
UC	10.6 ± 2.0*	243.1 ± 46.0*	108.5 ± 20.5*

* versus NC, $P < 0.05$

Table 4 Correlation analysis of Th17-related cytokines' expressions and disease activity index in the UC group

	Truelove and Witts Index		Southerland Index	
	Pearson correlation coefficient (r)	P	Pearson correlation coefficient (r)	P
Tissue (IOD)				
IL-17	0.952	0.000	0.879	0.000
IL-21	0.847	0.001	0.901	0.000
IL-22	0.807	0.003	0.822	0.002
Serum (ELISA)				
IL-17	0.879	0.000	0.807	0.003
IL-21	0.871	0.000	0.866	0.001
IL-22	0.895	0.000	0.871	0.000

* $P < 0.05$

Table 5 Correlation analysis of Th17-related cytokine expression and disease activity index in CD group

	Harvey–Bradshow Index Pearson correlation coefficient (r)	P
Tissue (IOD)		
IL-17	0.951	0.000
IL-21	0.913	0.000
IL-22	0.953	0.000

* $P < 0.05$

patients and healthy individuals. In addition, IL-21 mRNA expression was associated with disease activity. However, there is no study on the distribution of IL-21 in the colonic mucosa of active UC patients and the correlation between IL-21 protein expression and disease activity. In our study, we detected the levels of IL-21 in serum and colonic mucosa. The results showed the levels of IL-21, both in serum and colonic mucosa, were increased in active UC. There is positive correlation between IL-21 protein expression and disease activity. IL-21 is also a pleiotropic cytokine which can regulate T cell differentiation and function, especially for the differentiation of Th17 cells [22–24]. In addition, IL-21 is involved in recruiting T cells to the inflamed gut [5]. So, IL-21 is a key component of the inflammatory cascade in the gut. Our finding also supported this opinion.

IL-22 is expressed by both the adaptive arm of the immune system, such as CD4 T cell subsets, and by innate lymphocytes including NK cells and LTi-like cells. As known, IL-22 is expressed in many chronic inflammatory conditions, including rheumatoid arthritis and psoriasis, and its up-regulation often correlates with disease activity

Table 6 Correlation analysis of Th17-related cytokine expression and endoscopic grading and histological grading in the UC group

	Endoscopic grading (UCEIS)		Histological grading	
	Pearson correlation coefficient (r)	P	Spearman correlation coefficient (r)	P
Tissue (IOD)				
IL-17	0.914	0.000	0.906	0.000
IL-21	0.845	0.002	0.812	0.002
IL-22	0.772	0.005	0.807	0.003
Serum (ELISA)				
IL-17	0.916	0.000	0.906	0.000
IL-21	0.839	0.002	0.791	0.004
IL-22	0.862	0.001	0.907	0.000

Table 7 Correlation analysis of Th17-related cytokine expressions and endoscopic grading in the CD group

	Endoscopic grading (SESCD) Pearson correlation coefficient (r)	P
Tissue (IOD)		
IL-17	0.987	0.000
IL-21	0.900	0.000
IL-22	0.964	0.000

* $P < 0.05$

[25]. Andoh [6] found that IL-22 was mainly expressed in the intestinal mucosa of UC patients, while there was no expression in healthy controls. Our results showed that the intensity of IL-22 was significantly higher in active IBD patients than in controls ($P < 0.05$). The IL-22 levels in serum and colonic tissues significantly correlated with disease severity activity; furthermore, our result suggested that IL-22 levels in both serum and tissues were significantly correlated with endoscopic and histopathological grading in UC patients. In CD patients, we found the IL-22 level in the inflamed tissue were significantly correlated with disease severity activity, endoscopic and histopathological grading.

In our study, we found that the levels of IL-17, IL-21 and IL-22 were significantly increased in the intestinal mucosa and serum in active IBD patients. Furthermore, significant positive correlation existed between these changes and disease activity.

The development of IBD is a chronic inflammatory process, but in acute activity stage the behavioral characteristics are similar to acute-phase response (APR). So, CRP and PLT levels, which change in this inflammatory process, correlate with disease activity [26, 27]. In our study, we analyzed the relevance of Th17-related cytokine expressions to the levels of CRP and PLT and found that the expressions of IL-17, IL-21 and IL22 were increased

Table 8 Correlation analysis of Th17-related cytokine expressions and CRP and PLT

	CRP		PLT	
	Pearson correlation coefficient (r)	P	Pearson correlation coefficient (r)	P
UC tissue (IOD)				
IL-17	0.888	0.000	0.833	0.001
IL-21	0.702	0.016	0.756	0.007
IL-22	0.880	0.000	0.791	0.004
UC serum (ELISA)				
IL-17	0.971	0.000	0.831	0.002
IL-21	0.859	0.001	0.846	0.001
IL-22	0.894	0.000	0.817	0.002
CD tissue (IOD)				
IL-17	0.807	0.005	0.895	0.000
IL-21	0.827	0.003	0.960	0.000
IL-22	0.749	0.013	0.867	0.001

* $P < 0.05$

with elevated CRP and PLT levels in patients with active IBD. Thus, Th17-related cytokines is closely related to disease activity.

How about the relationship between Th17 cells, Th17-related cytokines and endoscopic findings and histological features in the mucosa of active IBD patients? In our study, we found that the expressions of IL-17, IL-21 and IL-22 in tissues and serum were gradually increased with the endoscopic disease activity grading and histological grading. These findings suggested that Th17-related cytokines were associated with disease activity and mucosal damage.

In summary, Th17-related cytokines are highly expressed in the intestinal mucosa and serum of patients with active IBD. Moreover, these cytokines were positively correlated with disease activity index, CRP and PLT levels, endoscopic disease activity grading and histological activity grading. Th17 cells and Th17-related cytokines play important roles in the development of IBD. However, future studies are warranted to investigate their molecular mechanisms.

Acknowledgments This work was supported by grants from the National Natural Science Foundation of China (No. 81270469) and the Key Medical Personnel of Jiangsu Province (No. RC2011063).

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