Immune system alterations in patients with inflammatory bowel disease during remission

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Key words: *Th1 and Th2 cytokines; inflammatory bowel disease; Crohn's disease, ulcerative colitis.*

Summary. *Objective. Perturbed immune homeostasis elicited by misbalanced production of proinflammatory and anti-inflammatory cytokines is characteristic of inflammatory bowel disease. The aim of this study was to evaluate cytokine profile in patients with different forms of inflammatory bowel disease – ulcerative colitis and Crohn's disease – during clinical remission phase.*

Material and methods. Production of proinflammatory Th1 cytokines (tumor necrosis factor-alpha (TNF-α), interferon-gamma (IFN-γ)) and anti-inflammatory Th2 cytokines (interleukin-10 (IL-10) and interleukin-13 (IL-13)) was analyzed in peripheral blood mononuclear cells of patients with inflammatory bowel disease (9 with ulcerative colitis and 9 with Crohn's disease) and control subjects (n=11) by enzyme-linked immunosorbent assay (two-site ELISA).

Results. The results of the study revealed that the level of TNF-α after stimulation with phytohemagglutinin in patients with Crohn's disease was significantly higher in comparison to both patients with ulcerative colitis and controls ($P<0.001$ and $P<0.01$, respectively). The secretion of IFN-γ both in patients with Crohn's disease and ulcerative colitis was lower than that in controls ($P=0.05$ and $P<0.01$, respectively), but it normalized after stimulation with phytohemagglutinin. The levels of IL-10 and IL-13 were significantly ($P<0.01$) higher in patients with Crohn's disease than in patients with ulcerative colitis and control group before and after stimulation with phytohemagglutinin.

Conclusions. The results of our study provide evidence that in patients with inflammatory bowel disease, the imbalance between production of proinflammatory Th1 and anti-inflammatory Th2 cytokines persists even during remission of the disease, and disturbances of immune homeostasis are significantly more expressed in patients with Crohn's disease than in patients with ulcerative colitis.

Introduction

Ulcerative colitis (UC, OMIM 191390) and Crohn's disease (CD, OMIM 266600), collectively referred to the inflammatory bowel disease (IBD), represent a group of multifactorial autoimmune disorders of the gastrointestinal tract sharing many clinical and pathological characteristics, however, differing in histological features and cytokine profiles (1–3). The excessive production of either Th1 or Th2 cytokines due to perturbed regulation of immune system activation results in chronic inflammatory processes, and loss of immune homeostasis may be implicated in the genesis of IBD (4–6). Although the etiology of IBD is still unknown, the animal model and human case-control studies have revealed that in the intestinal tissue and peripheral blood during active CD phase, the levels of tumor necrosis factor-alpha (TNF-α), interferon-gamma (IFN-γ), interleukin (IL)-12 and IL-1 are increased, whereas in UC, the secretion of IL-5, IL-4, IL-10, and IL-13 is upregulated, elucidating that immune mechanisms playing a major role in the initiation and perpetuation of both diseases are distinct. Thus, CD is characterized by the typical Th1 cell response and UC by an atypical Th2 cell response (7–11), whereas cytokine profile in IBD patients during the remission phase has not been investigated thoroughly yet.

Therefore, the aim of our study was to explore pro-

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and anti-inflammatory cytokine profile in patients with IBD during the remission of the disease. We used cells of the immune system that are easily accessible in the peripheral blood and can be studied *ex vivo* and investigated spontaneous and stimulated production of TNF-α, IFN-γ, IL-10, and IL-13 in patients with two different IBD forms and in healthy control subjects.

Materials and methods

Patients

Blood samples were collected from 18 IBD patients in remission phase (9 CD patients (men/women, 4/5; mean age, 37.08 years; range, 24–65 years), 9 UC (men/women, 4/5; mean age, 37.00 years; range, 21–55 years)) and 11 unrelated healthy, age- and sex-matched controls. The recruitment of study individuals was performed at the Department of Gastroenterology, Kaunas University of Medicine Hospital. The diagnosis of either CD or UC was based on standard clinical, endoscopic, radiological, and histological criteria (12). For the assessment of disease activity, colitis activity index (CAI) according to Gomes *et al.* (13) and CD activity index (CDAI) (14) were used. Only patients with inactive disease (CAI<4 and CDAI<150) and using 5-aminosalicylate (5-ASA) preparations as maintenance therapy were included into the study. The patients receiving immunosuppressive therapy or treated surgically were excluded from the study. Written informed consent was obtained from all study participants. The study had been approved by the Kaunas Regional Biomedical Research Ethics Committee (Protocol No. BE-2-49).

Cell collection

Peripheral blood mononuclear cells (PBMNC) were separated from EDTA-treated peripheral blood (10 mL) by a standard procedure using Ficoll density gradient centrifugation. After three washes in serum-free RPMI-1640 medium (Biochrom, Germany), the mononuclear cells were resuspended in complete growth medium (RPMI supplemented with 2 mM L-glutamine, antibiotics (100 U/mL penicillin and 100 μL streptomycin) and 10% heat-inactivated fetal bovine serum (FBS, Biochrom, Germany)) and processed cytokine assay as described below.

Cytokine assay

For cytokine assay, cells were adjusted to a concentration of 1×10^6 cells per mL and cultured in complete growth medium in 24-well plates at a 5% CO_2 and 37°C humidified atmosphere (Incubator IR AUTOFLOW Water Jacketed, USA). Cell culturing was

Table. **Mean concentrations of TNF-α, IFN-γ, IL-10, IL-13 (pg/mL) in peripheral blood mononuclear cells (cultivated without and with phytohemagglutinin) in patients with inflammatory bowel disease and control group**

Cytokine		Patients with UC (n=9)		Patients with CD (n=9)		Control group (n=11)		P value		
		Mean±SE	95% CI	Mean±SE	95% CI	Mean±SE	95% CI	UC *vs.* control	CD *vs.* control	UC *vs.* CD
TNF-α (pg/mL)	Spont.	29.73±5.76	25.30–34.16	38.30±28.38	32.01–44.59	35.53±18.13	16.47–54.60	0.56	0.78	**0.02**
	+PHA	604.45±58.00	414.50–794.40	1198.86±101.00	1043.58–1354.14	811.81±123.00	599.16–1024.47	0.13	**<0.01**	**<0.001**
IFN-γ (pg/mL)	Spont.	34.12±0.57	32.80–35.45	40.57±3.13	33.34–47.79	59.45±7.74	42.21–76.70	**<0.01**	**0.05**	0.12
	+PHA	1062.09±17.55	1021.62–1102.55	1047.55±27.72	983.63–1111.46	946.27±58.05	816.93–1075.61	0.1	0.16	0.57
IL-10 (pg/mL)	Spont.	44.46±3.63	36.09–52.84	87.76±9.27	66.39–109.12	64.26±2.84	57.93–70.59	**<0.001**	**0.02**	**<0.001**
	+PHA	127.02±5.27	114.87–139.17	213.47±14.43	180.20–246.75	129.39±4.99	118.27–140.51	0.75	**<0.001**	**<0.001**
IL-13 (pg/mL)	Spont.	23.54±1.31	20.52–26.57	38.09±1.61	34.36–41.81	22.82±0.56	21.56–24.08	0.6	**<0.001**	**<0.001**
	+PHA	186.80±14.99	152.23–221.37	314.70±31.75	241.48–387.93	89.14±7.77	71.83–106.45	**<0.001**	**<0.001**	**<0.01**

CD – Crohn's disease; UC – ulcerative colitis; SE – standard error; CI – confidence interval; spont. – spontaneous cytokine secretion in peripheral blood mononuclear cells; +PHA – cytokine secretion in peripheral blood mononuclear cells stimulated with phytohemagglutinin.
Results achieving statistical significance for a distinction between cases and controls are shown in bold.

performed without and with addition of phytohemagglutinin (PHA; 5 μg/mL) (Biochrom, Germany). After 24 hours, supernatants were collected and stored at –20°C until assay. Concentrations of cytokines TNF-α, IFN-γ, IL-10, and IL-13 in supernatants were determined with commercially available two-site ELISA kits (Amersham Biosciences, UK). The lowest limit of sensitivity of test systems for TNF-α was 4 pg/mL; for IFN-γ, <2 pg/mL; for IL-10, <3 pg/mL, and for IL-13, <7 pg/mL. The optical densities at 450 nm and at a correction wavelength of 620 nm were measured on a microplate reader (DigiScan 400, Asys Hitech).

Statistical analysis

Statistical analyses were done using the SPSS statistical package (version 12.0; Chicago, IL). The data in text, table, and figures are presented as means±SE. The difference between two independent samples was compared using Mann–Whitney U test. An analysis of variance (ANOVA one-way) was used to compare data on immunological analysis between different study groups. A P value of <0.05 was accepted as statistically significant.

Results

Proinflammatory cytokines

The spontaneous secretion of proinflammatory TNF-α cytokine was highest in CD and lowest in UC patient groups (Table, Fig. 1). The difference between two IBD groups was statistically significant (P=0.02). After stimulation with PHA, the production of TNF-α increased from 20- to 30-fold in all study groups and retained the same production patterns as in cells cultivated without PHA; however, the cytokine level in CD patients became significantly higher not only in comparison to UC patients, but also to controls. The concentrations of IFN-γ (Table, Fig. 2) in both IBD patient groups were lower than in controls (P<0.01 for UC; P=0.05 for CD). However, the difference disappeared after the stimulation with PHA.

Anti-inflammatory cytokines

The level of the production of anti-inflammatory cytokine IL-10 was highest in CD patients (Table, Fig. 3), and the difference was significant in comparison to UC and control subjects (P<0.001 and P=0.02, respectively). The difference became even more prominent after the stimulation with PHA. In UC patients, IL-10 level before stimulation was lower than in controls (P<0.001); however, the difference disappeared after cultivation of cells with PHA.

In CD patients, the concentration of IL-13 before stimulation was significantly higher when comparing with UC patients and controls (P<0.001), and this pattern was retained after the stimulation of cells with PHA (Table, Fig. 4). In UC subjects, the level of spontaneous IL-13 production was similar to the levels observed in the control group; however, after the

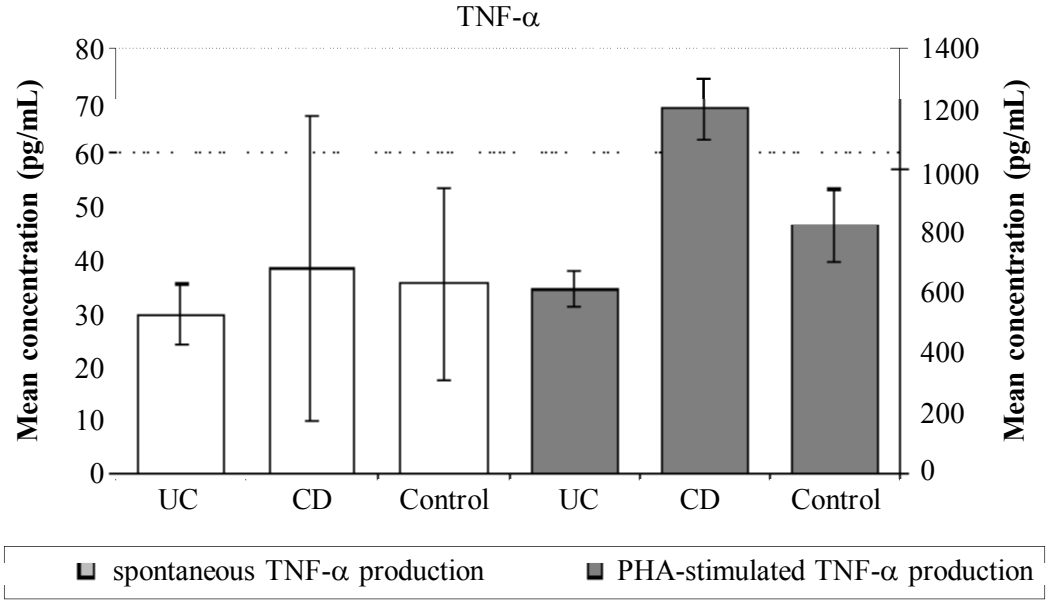

Fig. 1. **Level of TNF-α (pg/mL) in peripheral blood mononuclear cells cultivated without (white columns) and with phytohemagglutinin (PHA) (dark columns) in patients with Crohn's disease (CD) and ulcerative colitis (UC) and control group**

Data are presented as mean±SE.

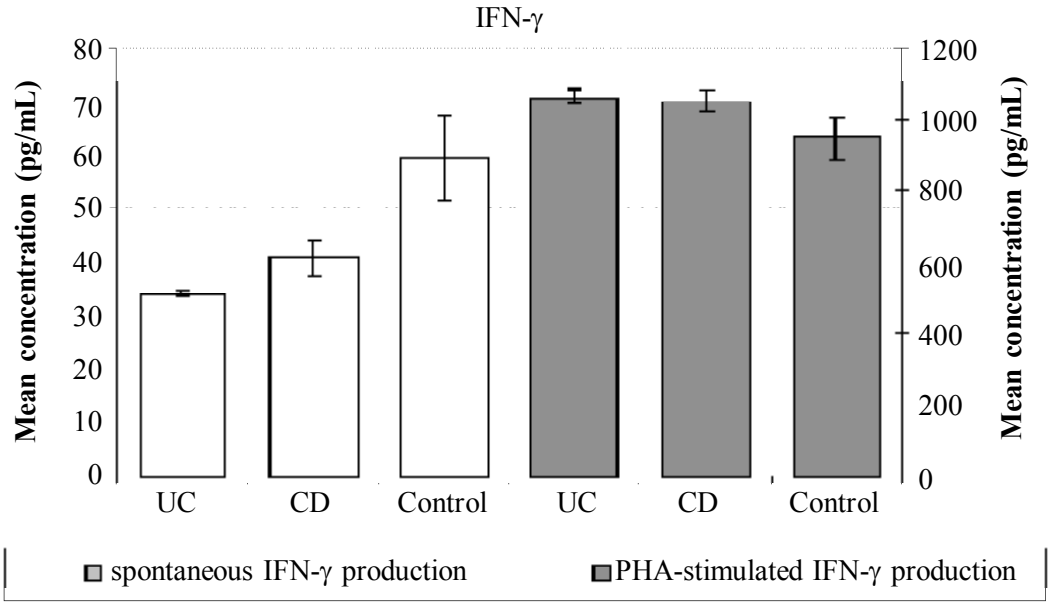

Fig. 2. **Level of IFN-γ (pg/mL) in peripheral blood mononuclear cells cultivated without (white columns) and with phytohemagglutinin (PHA) (dark columns) in patients with Crohn's disease (CD) and ulcerative colitis (UC) and control group**

Data are presented as mean±SE.

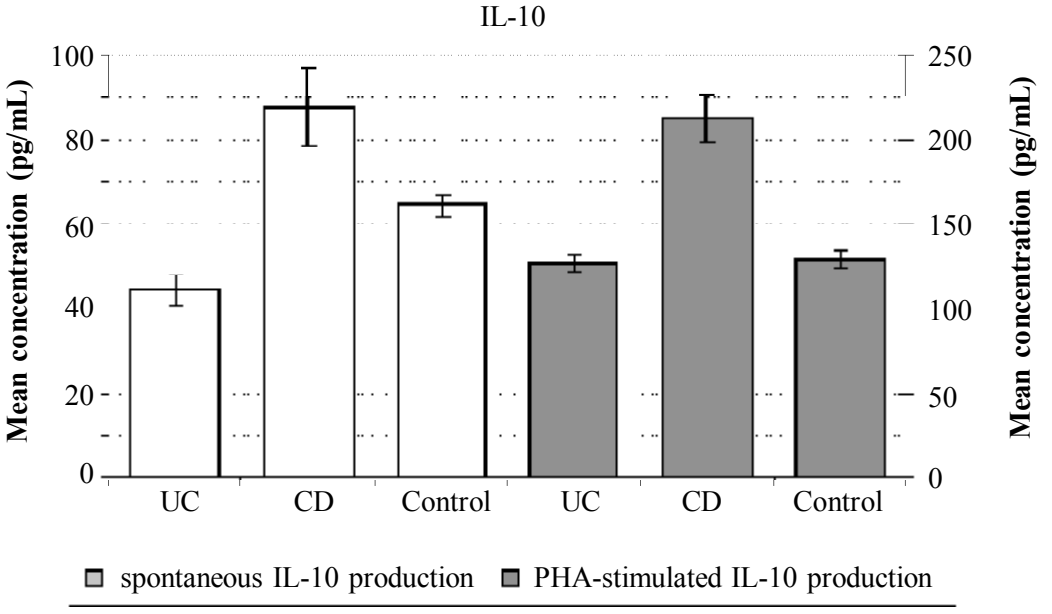

Fig. 3. **Level of IL-10 (pg/mL) in peripheral blood mononuclear cells cultivated without (white columns) and with phytohemagglutinin (PHA) (dark columns) in patients with Crohn's disease (CD) and ulcerative colitis (UC) and control group**

Data are presented as mean±SE.

stimulation, the cytokine concentration became significantly higher than in controls.

Discussion

It is generally accepted that the excessive production of either pro- and anti-inflammatory cytokines plays a predominant role in immune and inflammatory reactions and loss of immune homeostasis in patients with IBD (7–11).

The increased production of Th1 cytokines has been established to be a feature of active CD but not of UC (7–11, 15), while the number of studies analyz-

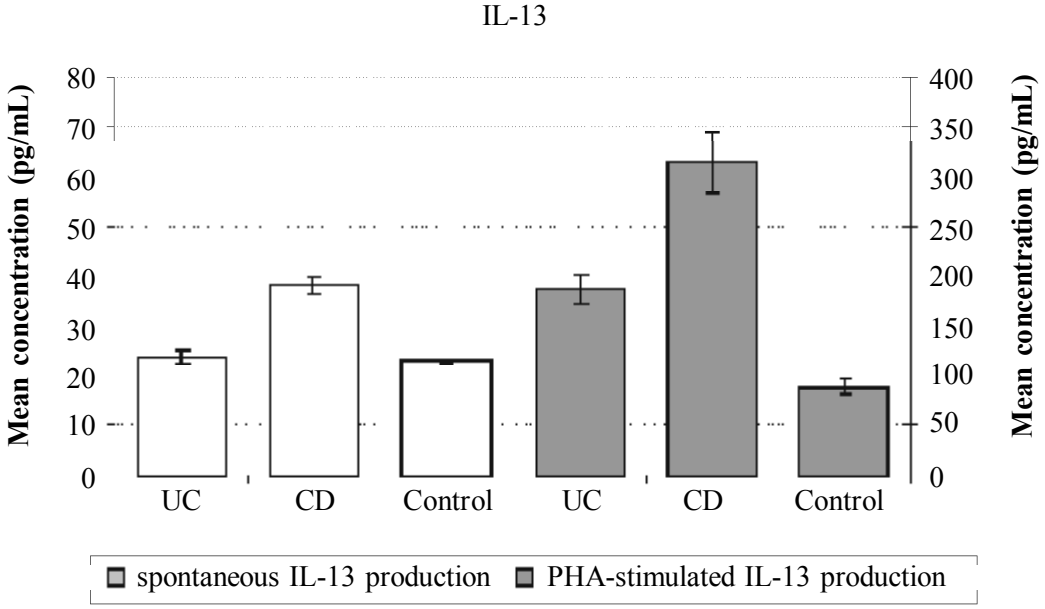

Fig. 4. **Level of IL-13 (pg/mL) in peripheral blood mononuclear cells cultivated without (white columns) and with phytohemagglutinin (PHA) (dark columns) in patients with Crohn's disease (CD) and ulcerative colitis (UC) and control group**

Data are presented as mean±SE.

ing the generation of the proinflammatory subset of cytokines (especially in PBMNC) during IBD remission is limited (16–19). The results of our study revealed that the concentration of TNF-α in both CD and UC patients has not differed from controls; however, the stimulation with PHA significantly increased the production of TNF-α in CD patients. The secretion of IFN-γ in both IBD groups was even lower than that in controls, and the difference disappeared after the stimulation. Normal (TNF-α) or even hyposecretion (IFN-γ) of proinflammatory Th1 cytokines in CD patients with inactive disease is consistent with findings reported by other authors (16, 17). However, our data revealed that the stimulation with PHA caused a significant increase in TNF-α production, typical for CD, even in case of inactive disease. Hyposecretion of IFN-γ in IBD patients may be related to the refractoriness of T cells that have been continuously activated *in vivo* as proposed by Wilde *et al.* (20) or to the down-regulation of the proinflammatory cytokines as the result of therapy with 5-ASA preparations, which also have the suppressive effect on the immune system (21, 22).

The existence of the Th2-biased pattern production in UC but not CD during active state of disease has been confirmed by some studies (7–11), whereas there are only scarce data on cytokine profile in case of noninflammatory or inactive disease (22, 23). In our study, we found the increased production of IL-10, which is critical for temporizing the proinflammatory processes via strong down-regulation of Th1 cytokines (15, 16) during remission phase in CD patients compared with UC patients and control group. These results oppose to recently published publication by Mitsuyama *et al.* (23), indicating the increased secretion of IL-10 during disease recovery in patients with UC, but not CD. Our study is the first report analyzing IL-13 levels in IBD patients during remission phase. We found the increased production of this cytokine in CD group as compared to UC and control groups. Anti-inflammatory cytokines have been reported to have inhibitory effects on activated macrophages producing different cytokines and effector functions on monocytes and T cells (24–26). CD during relapse phase has been reported to have a proinflammatory ("Th1-like") cytokine profile (7–11); therefore, the up-regulation of the anti-inflammatory cytokines seems to be due to response to proinflammatory cytokine activity. The data of our study suggest that IL-10 and IL-13 act as natural dampers of immune proliferation and inflammatory responses. However, it has already been ascertained that an excessive production of either proinflammatory or anti-inflammatory cytokines may also result in the inappropriate suppression of the immune response and may provoke a compensatory reaction (11).

Although the data in our study were obtained from isolated peripheral blood monocytes, relevance to the

gut may be inferred since peripheral blood monocytes are precursors of tissue macrophages. Monocytes thus provide an opportunity to study the mononuclear phagocyte cell line before the monocytes migrate from the blood into the bowel mucosa where they became activated. These immunological responses seen in the peripheral blood are events that mirror systemic activation in the gut (27).

Conclusions

The results of our study provide evidence that in patients with inflammatory bowel disease, the dissonance between production of proinflammatory Th1 and anti-inflammatory Th2 cytokines persists even during the remission of the disease, and disturbances of immune homeostasis are significantly more expressed in patients with Crohn's disease than in patients with ulcerative colitis.

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The authors have to declare no conflicts of interest.

Sergančiųjų uždegiminėmis žarnų ligomis imuninės sistemos pokyčiai ligos remisijos metu

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Raktažodžiai: Th1 ir Th2 citokinai, uždegiminė žarnų liga, Krono liga, opinis kolitas.

Santrauka. *Darbo tikslas.* Imuninės sistemos homeostazės sutrikimas, pasireiškiantis nesuderinta uždegiminių ir priešuždegiminių citokinų gamyba, būdingas uždegiminėms žarnų ligoms. Tyrimo tikslas. Ištirti uždegiminių citokinų profilį: opiniu kolitu ir Krono liga sergantiems ligoniams ligos remisijos metu.

Tyrimo medžiaga ir metodai. Uždegiminių citokinų: naviko nekrozės faktoriaus alfa (TNF-α), interferono gama (IFN-γ) ir priešuždegiminių citokinų: interleukino-10 (IL-10) ir interleukino-13 (IL-13) koncentracija buvo tiriama uždegiminėmis žarnų ligomis sergančių ligonių (opinis kolitas – 9; Krono liga – 9) ir kontrolinės grupės asmenų (n=11) kraujo vienbranduolinėse ląstelėse naudojant imunofermentinės analizės metodą (angl. *two-site ELISA*).

Rezultatai. Tyrimų duomenys rodo, kad TNF-α išskyrimas po stimuliacijos fitohemagliutininu (FHA) buvo padidėjęs sergančiųjų Krono liga grupėje palyginus su opiniu kolitu sergančiųjų grupėje bei kontroline grupe ($p<0,001$ ir $p<0,01$). IFN-γ išskyrimas buvo mažesnis abiejose grupėse palyginus su kontroline grupe ($p=0,05$ ir $p<0,01$), tačiau po stimuliacijos FHA tapo normalus. IL-10, IL-13 išskyrimas buvo reikšmingai ($p<0,01$) didesnis Krono liga sergančiųjų grupėje palyginus su opiniu kolitu sergančiųjų grupės ir kontrolinės grupės asmenimis tiek prieš, tiek ir po stimuliacijos FHA.

Išvados. Sergant uždegiminėmis žarnų ligomis, sutrikęs balansas tarp uždegiminių ir priešuždegiminių citokinų gamybos išlieka ir ligos remisijos metu, be to, yra būdingesnis sergant Krono liga palyginus su opiniu kolitu.

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References

1. Brown SJ, Mayer L. The immune response in inflammatory bowel disease. Am J Gastroenterol 2007;102(9):2058-69.
2. Žvirblienėe A, Kiudelis G, Kupčinskas L. Retrospective analysis of case histories of patients with ulcerative colitis and Crohn's disease. Medicina (Kaunas) 2003;39(8):745-50.
3. Žvirblienė A, Kiudelis G, Žalinkevičius R, Kupčinskas L. Dietary characteristics of patients with inflammatory bowel diseases. Medicina (Kaunas) 2006;42(11):895-9.
4. Nagler-Anderson C. Man the barrier! Strategic defences in the intestinal mucosa. Nat Rev Immunol 2001;1(1):59-67.
5. O'Shea JJ, Ma A, Lipsky P. Cytokines and autoimmunity. Nat Rev Immunol 2002;2(1):37-45.
6. Dignass AU, Baumgart DC, Sturm A. Review article: the aetiopathogenesis of inflammatory bowel disease – immunology and repair mechanisms. Aliment Pharmacol Ther

2004;20(4):9-17.
7. Jurjus AR, Khoury NN, Reimund JM. Animal models of inflammatory bowel disease. J Pharmacol Toxicol Methods 2004;50(2):81-92.
8. Strober W, Fuss IJ, Blumberg RS. The immunology of mucosal models of inflammation. Annu Rev Immunol 2002;20:495-549.
9. Elson CO, Cong Y, McCracken VJ, Dimmitt RA, Lorenz RG, Weaver CT. Experimental models of inflammatory bowel disease reveal innate, adaptive, and regulatory mechanisms of host dialogue with the microbiota. Immunol Rev 2005;206: 260-76.
10. Martinez-Montiel MP, Munoz-Yague MT. Biologic therapies for chronic inflammatory bowel disease. Rev Esp Enferm Dig 2006;98(4):265-91.
11. Bouma G, Strober W. The immunological and genetic basis of inflammatory bowel disease. Nat Rev Immunol 2003;3(7): 521-33.
12. Podolsky DK. Inflammatory bowel disease (1). N Engl J Med 1991;325(13):928-37.
13. Gomes P, Du Boulay C, Smith CL, Holdstock G. Relationship between disease activity indices and colonoscopic findings in patients with colonic inflammatory bowel disease. Gut 1986;27:92-5.
14. Best WR, Becktel JM, Singleton JW, Kern F. Development of a Crohn's disease activity index. National Cooperative Crohn's Disease Study. Gastroenterology 1976;70:439-44.
15. Bisping G, Lügering N, Lütke-Brintrup S, Pauels HG, Schürmann G, Domschke W, et al. Patients with inflammatory bowel disease (IBD) reveal increased induction capacity of intracellular interferon-gamma (IFN-gamma) in peripheral CD8+ lymphocytes co-cultured with intestinal epithelial cells. Clin Exp Immunol 2001;123:15-22.
16. Murch SH, Lamkin VA, Savage MO, Walker-Smith JA, MacDonald TT. Serum concentrations of tumour necrosis factor alpha in childhood chronic inflammatory bowel disease. Gut 1991;32:913-7.
17. Raddatz D, Bockemühl M, Ramadori G. Quantitative measurement of cytokine mRNA in inflammatory bowel disease: relation to clinical and endoscopic activity and outcome. Eur J Gastroenterol Hepatol 2005;17(5):547-57.
18. Komatsu M, Kobayashi D, Saito K, Furuya D, Yagihashi A, Araake H, et al. Tumor necrosis factor-alpha in serum of patients with inflammatory bowel disease as measured by a highly sensitive immuno-PCR. Clin Chem 2001;47(70):1297-301.
19. Mazlam MZ, Hodgson HJ. Peripheral blood monocyte cytokine production and acute phase response in inflammatory bowel disease. Gut 1992;33(6):773-8.
20. Wilde DB, Prystowsky MB, Ely JM, Vogel SN, Dialynas DP, Fitch FW. Antigen-reactive cloned helper T cells. II. Exposure of murine cloned helper T cells to IL 2-containing supernatant induces unresponsiveness to antigenic restimulation and inhibits lymphokine production after antigenic stimulation. J Immunol 1984;133(4):636-41.
21. Kaiser GC, Yan F, Polk D. Mesalamine blocks tumor necrosis factor growth inhibition and nuclear factor kappaB activation in mouse colonocytes. Gastroenterology 1999;116(3):602-9.
22. Gomez-Munoz A, O'Brien L, Salh B, Steinbrecher UP. 5-Aminosalicylate stimulates phospholipase D activity in macrophages. Biochim Biophys Acta 2001;1533(2):110-8.
23. Mitsuyama K, Tomiyasu N, Takaki K, Masuda J, Yamasaki H, Kuwaki K, et al. Interleukin-10 in the pathophysiology of inflammatory bowel disease: increased serum concentrations during the recovery phase. Mediators Inflamm 2006;2006(6): 1-7.
24. Fiorentino DF, Zlotnik A, Vieira P, Mosmann TR, Howard M, Moore KW, et al. IL-10 acts on the antigen-presenting cell to inhibit cytokine production by Th1cells. J Immunol 1991;146(10):3444-51.
25. Fiorentino DF, Zlotnik A, Mosmann TR, Howard M, O'Garra A. IL-10 inhibits cytokine production by activated macrophages. J Immunol 1991;147(11):3815-22.
26. Kucharzik T, Lügering N, Weigelt H, Adolf M, Domschke W, Stoll R. Immunoregulatory properties of IL-13 in patients with inflammatory bowel disease; comparison with IL-4 and IL-10. Clin Exp Immunol 1996;104(3):483-90.
27. Tacke F, Randolph GJ. Migratory fate and differentiation of blood monocyte subsets. Immunobiology 2006;211(6-8):609-18.

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