

Disturbances in apoptosis of lamina propria lymphocytes in Crohn's disease

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Abstract

Introduction: The aim of this study was to assess the potential mechanisms providing resistance to apoptosis of lamina propria lymphocytes (LPL) directly in intestinal tissues from patients with Crohn's disease (CD).

Material and methods: Fifty CD patients were enrolled in the study. The control group consisted of healthy patients who underwent surveillance colonoscopy after endoscopic polypectomy. Each CD patient underwent colonoscopy with tissue sampling from inflamed areas of the colon with the assessment of immunohistochemical expression of active caspase 3, Fas, tumour necrosis factor receptor 1 (TNFR1), Bcl-2, Bax, CD4 and CD8. This was compared with healthy intestinal mucosa.

Results: The expression of active caspase 3 was significantly lower in LPL in CD (0.4 ± 0.3 vs. 2.8 ± 1.5 ; $p = 0.0002$). A statistically significant increase of CD4 and CD8 positive cells was noted in CD (2.3 ± 0.5 vs. 1.2 ± 0.2 , $p < 0.0001$; 2.1 ± 0.3 vs. 1.1 ± 0.3 , $p < 0.0001$, respectively). It was associated with a significant increase of the Bcl-2 (6.7 ± 2.7 vs. 2.9 ± 0.8 ; $p < 0.0001$) and a decrease of the Bax protein expression (3.4 ± 2.1 vs. 5.5 ± 1.8 ; $p < 0.0001$) in CD. The expression of Fas and TNFR1 did not differ between the study groups.

Conclusions: LPL in CD are resistant to apoptosis when compared with physiological conditions. This is probably due to an imbalance in Bcl-2 family proteins. TNFR1-related pathway is probably not involved in disturbances of LPL apoptosis in CD.

Key words: Bcl-2 family proteins, caspase 3, Crohn's disease, lamina propria lymphocytes.

Introduction

In healthy individuals the gastrointestinal immune system is still stimulated by millions of antigens [1]. This complicated system is able to differentiate physiological from pathological stimuli and eliminate the latter in the early phase of the immunological reaction. One of the most important populations of immune cells – lymphocytes – is engaged in this process [2, 3]. After having their protective role fulfilled in this phenomenon, lymphocytes are desensitized to further antigen stimulation

(energy) or eliminated in the programmed cell death mechanism. Disturbances in these processes can lead to an accumulation of inflammatory cells in the gut wall [4].

Crohn's disease (CD) and ulcerative colitis (UC) are inflammatory bowel diseases (IBD). The aetiopathogenesis of CD is still unknown. Numerous alterations in the local immunological status in the gut in patients with IBD have been described [5–7]. Disturbances in apoptosis of the immunoreactive cells in the gut wall are believed to be among the most important phenomena in the pathogenesis of IBD. However, the exact mechanisms leading to the impairment of programmed cell death are poorly understood [8, 9].

The two best described pathways leading to the execution of programmed cell death are called the extrinsic and intrinsic pathway. In the extrinsic pathway cellular apoptosis is induced by binding of some ligands, such as Fas ligand (FasL) and tumour necrosis factor- α (TNF- α), to their receptors: Fas and tumour necrosis factor- α receptor 1/2 (TNFR1/2), respectively [10–13]. The intrinsic pathway is induced as a result of the presence of several pathological triggering factors, such as ultraviolet radiation or oxidative stress [14]. Mitochondria play a central role in this type of apoptosis induction, together with a group of proteins belonging to the Bcl-2 family [15]. This family consists of a number of pro- (for example Bax, Bak) and anti-apoptotic proteins (Bcl-2, Bcl-x_L and others). As a result of the induction of programmed cell death both in the intrinsic and extrinsic pathway, the execution phase of this process is initiated. In this phase of the process, the central executive role is played by a cascade of enzymes called caspases, with the activation of a crucial caspase 3 [16]. This leads to the fragmentation of cellular membrane and the formation of apoptotic bodies, which are eliminated by phagocytic cells.

In the present study we investigated the mechanisms leading to resistance to apoptosis in lamina propria lymphocytes (LPL) in patients with CD. In order to examine these processes we analysed the expression of selected proteins engaged in the apoptotic pathways in biopsy specimens from

patients with CD by using immunohistochemical methods, and we compared it with the healthy intestinal mucosa.

Material and methods

Patients

The study group consisted of 50 patients with CD. Each patient underwent ileocolonoscopy with tissue sampling from inflammatory (macroscopically ulcerated) colonic lesions and with the assessment of endoscopic disease activity by calculating the Simple Endoscopic Score for Crohn's Disease (SES-CD) (Table I) [17]. Both the clinical (Crohn's Disease Activity Index – CDAI) and biochemical activity were also estimated [18]. The control group consisted of 10 healthy and asymptomatic patients (matched for gender and age) who underwent surveillance colonoscopy after endoscopic polypectomy. The screening endoscopy did not reveal any pathology in any patient; tissue samples were taken from normal colonic mucosa.

Immunohistochemistry

Tissues obtained during colonoscopy were immediately fixed in 10% neutral buffered formalin and embedded in paraffin. Paraffin sections (5 μ m thick) were dewaxed and then rehydrated in a typical manner. The tissue samples were then stained using the haematoxylin and eosin method. The immunohistochemical staining was performed in the Department of Histology and Embryology of the Poznan University of Medical Sciences as described previously [19]. The expression of selected proteins (CD4 – dilution 1 : 200, Dako; CD8 – 1 : 300, Dako; Bax – 1 : 2000, Dako; Bcl-2 – 1 : 500, Dako; active caspase 3 – concentration 1 μ g/ml, R&D; Fas – 1 : 200, Leica Monocasta; TNFR1 – 1 : 2000, Abcam) was estimated by using an immunohistochemical staining protocol (the ABC technique) in accordance with Hsu *et al.* [20]. In the case of CD4, CD8, Bax, Bcl-2, Fas and TNFR1, a high temperature antigen unmasking technique was also applied. The quality of used primary antibodies was tested by performing parallel immunohistochemi-

Table I. Simple Endoscopic Score for Crohn's Disease (SES-CD) [16]

Presence and size of ulcers	None 0 patients	Aphthous, < 0.5 cm, 1 patient	Large, 0.5–2 cm, 2 patients	Large, > 2 cm, 3 patients
Extent of ulcerated surface	0% 0 patients	< 10%, 1 patient	10–30%, 2 patients	> 30%, 3 patients
Extent of affected surface	0% 0 patients	< 50%, 1 patient	50–75%, 2 patients	> 75%, 3 patients
Presence and type of narrowings	None 0 patients	Single, can be passed, 1 patient	Multiple, can be passed, 2 patients	Cannot be passed, 3 patients

*Each variable is calculated separately in ileum and right colon, transverse colon, left colon and rectum. **SES-CD = raw sum of variables – 1.4 \times n (where n = number of affected segments).

cal staining with tissues having known expression of the investigated protein.

The immunohistochemical reactions were evaluated using semi-quantitative scales. A scale proposed by Remmele and Stegner was used in the case of active caspase 3, Bax, and Bcl-2. For the estimation of CD4, CD8, TNFR1 and Fas expression, a modified HercepTest was applied [21, 22]. In the case of proteins belonging to the Bcl-2 family, additionally, the pro-apoptotic Bax/Bcl-2 ratio was calculated according to Ina *et al.* [23].

Statistical analysis

Statistical analysis was performed using the GraphPad Prism version 4.0 software. The immunohistochemical expression of investigated proteins was compared in LPL from patients with CD and the control group. Statistical differences were assessed using Student's *t* test in the case of dependent probes, and using the Wilcoxon nonparametric test when the data did not concur with the Gaussian distribution. Statistical significance was defined as a level of *p* less than 0.05.

Ethical requirements

The Institutional Review Board at Poznan University of Medical Sciences approved the study (Decision 94/09).

Results

Patients

Demographic and clinical characteristics of the study group are presented in Table II.

Protein expression

The number of CD4 and CD8 positive LPL was significantly higher in mucosal samples taken from patients with CD when compared with the control group (2.3 ± 0.5 vs. 1.2 ± 0.2 for CD4, $p < 0.0001$; 2.1 ± 0.3 vs. 1.1 ± 0.3 for CD8, $p < 0.0001$) (Figures 1 and 2 A, B).

Fas was expressed in LPL with the mainly membrane staining pattern. The expression of Fas protein did not differ statistically between the study and the control group (1.2 ± 0.4 vs. 1.3 ± 0.4 ; NS) (Figures 1 and 2 C).

TNFR1 expression in LPL was weak and observed only incidentally. Strong staining for TNFR1 was observed focally in polymorphonuclear cells (granulocytes). There was no difference between the expression of TNFR1 in LPL in CD patients when compared with the control group (0.9 ± 0.4 vs. 1.2 ± 0.2 ; NS) (Figures 1 and 2 D).

Active caspase 3 positive LPL were noted only incidentally in samples from patients with CD, which was in contrast with healthy mucosal biop-

Table II. Patients' demographic and clinical data

Variable	Overall (N = 50)
Gender – male/female, n (%)	21 (42)/29 (58%)
Age, mean \pm SD [years]	32 \pm 12
Disease duration, mean \pm SD [years]	8 \pm 5
Disease phenotype, n (%):	
Inflammatory	29 (58)
Penetrating	21 (42)
Disease location, n (%):	
Colon	50 (100)
Ileum	33 (66)
CDAI, median (95% CI)	216 (205–262)
SES-CD, median (95% CI)	11 (10–15)
CRP, mean \pm SD [mg/l]	23.8 \pm 22.9
ESR, mean \pm SD [mm/h]	37 \pm 20
Medication used, n (%):	
5-Aminosalicylates	49 (98)
Steroids	20 (40)
Azathioprine	42 (84)
Antibiotics	18 (36)
Anti-TNF	10 (20)
Probiotics	41 (82)

CDAI – Crohn's Disease Activity Index, SES-CD – Simple Endoscopic Score for Crohn's Disease, CRP – C-reactive protein, ESR – erythrocyte sedimentation rate, anti-TNF – anti-tumour necrosis factor α antibodies.

sies, in which active caspase 3 expression in LPL was significantly higher (0.4 ± 0.3 vs. 2.8 ± 1.5 ; $p = 0.0002$) (Figures 2 E and 3).

The intensity of Bax expression in LPL in inflamed tissues was medium, and it was significantly weaker, according to the Remmele and Stegner scale, in comparison to samples from the control group (3.4 ± 2.1 vs. 5.5 ± 1.8 ; $p < 0.0001$) (Figures 2 F and 3).

In contrast, the expression of anti-apoptotic Bcl-2 protein was significantly higher in LPL in the

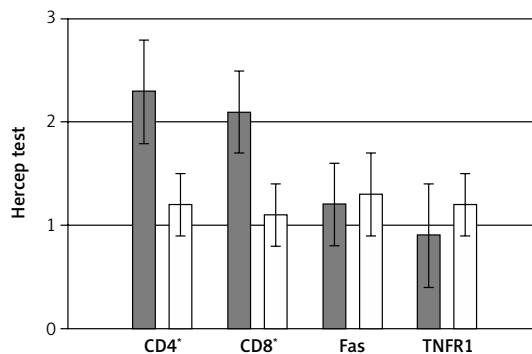


Figure 1. Immunohistochemical expression of CD4, CD8, Fas and tumour necrosis factor α receptor 1 (TNFR1) (HercepTest) in lamina propria lymphocytes. Grey columns – patients with Crohn's disease; white columns – control group; * $p < 0.05$.

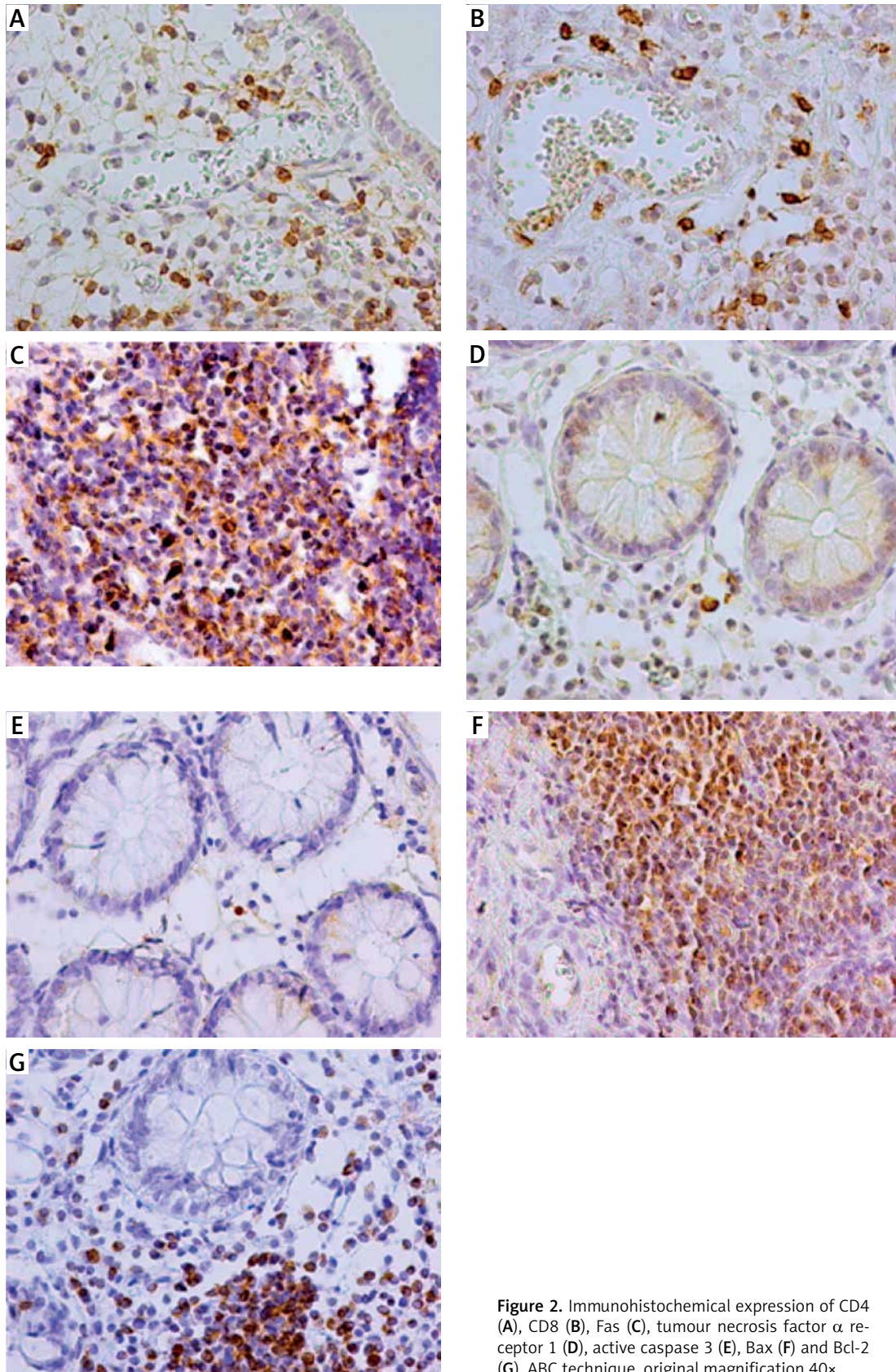


Figure 2. Immunohistochemical expression of CD4 (A), CD8 (B), Fas (C), tumour necrosis factor α receptor 1 (D), active caspase 3 (E), Bax (F) and Bcl-2 (G). ABC technique, original magnification 40x

inflamed tissues (6.7 ± 2.7 vs. 2.9 ± 0.8 ; $p < 0.0001$) (Figures 2 G and 3). The Bax/Bcl-2 pro-apoptotic ratio was significantly higher in LPL in healthy mucosa, when compared with samples taken from CD patients (1.9 ± 0.2 vs. 0.6 ± 0.3 , respectively; $p < 0.0001$).

Discussion

Different histological alterations are described in mucosal biopsies taken from patients with CD. One of the most important phenomena, from the pathological point of view, is infiltration with inflammatory cells [24]. The role of LPL in these processes is still not fully understood. A subgroup of CD4 LPL seems to be crucial for the induction of a variety of immunological phenomena [7]. In CD, a predominance of Th1 and Th17 CD4 LPL is well described in the literature. Th1 CD4 LPL produce several cytokines, such as interleukin-12 (IL-12) or interferon- γ (IFN- γ). On the other hand, the Th17-dependent immunological response generates cells producing IL-17 and IL-22 [25, 26]. This phenomenon leads to the amelioration of gut inflammation. Also CD8 LPL are involved in the pathogenesis of tissue destruction in the gut wall in IBD. The natural cytotoxic properties of the majority of CD8 positive T cells are impaired. As a result, the physiological apoptotic elimination of immunologically active cells is down-regulated, which leads to the propagation of inflammation [7].

In the current study we confirmed the importance of LPL as a main component of the inflammatory infiltrates in inflamed tissues taken from CD patients. A significant difference in the expression of CD4 and CD8 positive LPL between the study and the control group is the obvious reflection of the inflammatory character of CD. However, it remains unanswered what phenomena lead to these pathological alterations.

The present study showed that disturbances in the apoptotic elimination of LPL play a crucial role in the propagation of the inflammatory infiltration in CD. There was a significant difference between the expression of the most important executive active caspase 3 (an apoptotic marker) in LPL in healthy tissue, when compared with mucosal biopsies from patients with CD, where apoptotic LPL were only incidentally noted. These results are in accordance with previous studies showing that unstimulated lamina propria CD4 T cells from healthy people are more susceptible to apoptotic elimination when compared with unstimulated peripheral blood T cells [27]. This elimination is a result of Fas-mediated cell-to-cell interactions involving mainly intestinal memory CD45RO T cells. This phenomenon serves as a protective mechanism against uncontrolled expansion of intestinal CD4 T cells. Moreover, in normal intestinal tissue, lamina propria T cells undergo more

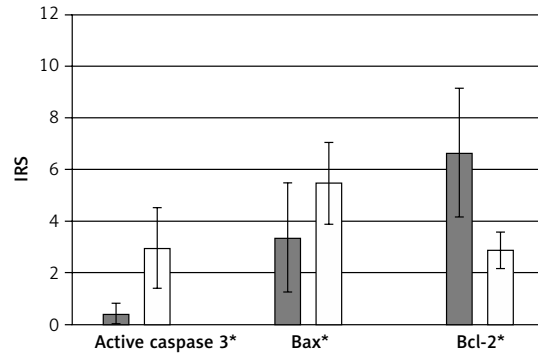


Figure 3. Immunohistochemical expression of active caspase 3, Bax and Bcl-2 (Immunoreactive Score – IRS) in lamina propria lymphocytes

Grey columns – patients with Crohn's disease; white columns – control group. * $p < 0.05$.

frequent, spontaneous programmed cell death, when compared with peripheral blood T cells [28]. Boirivant *et al.* also proved that the mechanism of apoptotic lamina propria T cell elimination in the healthy gut is additionally accelerated when these cells undergo immunological stimulation, even when compared with stimulated peripheral blood T lymphocytes [28, 29]. This shows that lamina propria T cells in physiological conditions have a natural, enhanced tendency for apoptotic elimination. In contrast, LPL from patients with IBD demonstrate resistance to programmed cell death. Boirivant *et al.* showed that LPL in IBD are more resistant to Fas-mediated apoptosis after CD2 stimulation [29]. Furthermore, in CD, LPL are also more resistant to spontaneous apoptosis or to apoptosis mediated by nitric oxide. This leads to the conclusion that resistance of LPL to programmed cell death, confirmed also in the current study, can be one of the driving forces of the presence of a chronic inflammatory infiltration in the intestinal wall. One can also conclude that disturbances in apoptotic LPL elimination are probably more severe in CD than in UC, which is why it is of great interest to investigate this phenomenon, especially in CD.

In the current study, we found that the expression of investigated Bcl-2 family proteins was significantly different in LPL in CD and in the healthy mucosa. The expression of anti-apoptotic Bcl-2 protein was significantly weaker, Bax was significantly higher, and the pro-apoptotic Bax/Bcl-2 ratio was significantly higher in normal LPL. In contrast, the expression of Fas and TNFR1 in CD and the control group did not differ significantly. The former is in accordance with the previous findings reported by Boirivant *et al.* [29]. It was proved that LPL in CD express the same amount of cell surface Fas as in controls. To the best of our knowledge, the expression of TNFR1 in this context has not been investigated so far.

Data from the current study show that probably one of the most important alterations in apoptotic mechanisms in LPL in CD concerns the intrinsic pathway, which is regulated, first of all, by Bcl-2 family proteins. The domination of one subtype (pro- or anti-apoptotic) of Bcl-2 family proteins in the cytoplasm regulates the structural and functional changes in mitochondrial membrane permeability, which is crucial for further executive apoptotic processes [9, 15]. The LPL in CD seem to be resistant to multiple pro-apoptotic stimuli due to a significant imbalance in the Bax/Bcl-2 ratio, promoting prolonged cell survival.

These data concur with the previous studies showing that disturbances in mitochondrial regulation of programmed cell death by Bcl-2 family proteins seem to be crucial for LPL resistance to apoptosis. However, to the best of our knowledge, the current study is one of the few studies to demonstrate this phenomenon directly in tissues from patients with CD [4, 23, 29, 30]. In the analysis performed so far, these processes were investigated, first of all, by using in vitro models. Boirivant *et al.* found that LPL in CD manifest increased expression of Bcl-2 after CD2 stimulation [29]. The authors speculated that this could result in resistance to Fas-mediated apoptosis; however, the link between Fas stimulation and Bcl-2 family protein functions still remains controversial. Itoh *et al.* proved that expression of Bcl-2 family proteins differs between mucosal intestinal and circulating T cells, and that CD is characterized by low expression of Bax and a high Bcl-x_L/Bax ratio in lamina propria T lymphocytes [30]. Ina *et al.* also observed disturbances in the intrinsic apoptotic pathway in lamina propria mononuclear cells, when compared with the control group [23].

One of the most important questions is what mechanisms lead to the disproportion of Bcl-2 family proteins in LPL in CD. The studies performed so far suggest that these phenomena are secondary to the presence of high amounts of proinflammatory cytokines in inflamed tissues [5, 7, 28]. One of the crucial cytokines promoting prolonged survival of LPL is IL-6 [31]. IL-6 binds to a complex of soluble IL-6 receptor (IL-6R) and gp130 protein on the CD4 T cell membrane. This activates the intracellular pathway mediated by the STAT3 transcription factor, which, in turn, influences Bcl-2 family protein expression, increasing the Bcl-2/Bax ratio. It was also demonstrated in animal studies that administration of anti-IL-6R antibodies down-regulated the inflammation in Th1-mediated colitides in mice models and increased cellular apoptosis [28, 32].

Atreya *et al.* presented a very interesting hypothesis regarding the IL-6-induced apoptotic resistance of lamina propria T cells [33]. It was

hypothesized that an important role in these processes is played by intestinal CD14 macrophages. These cells show very high transmembrane TNF (tmTNF) expression, and this protein binds to TNFR2 on CD4 lamina propria T cells, which activates TNFR associated factor 2 (TRAF2) and nuclear factor- κ B (NF- κ B) molecules. These processes stimulate IL-6 production by CD4 cells, which, in turn, increases the LPL resistance to apoptosis. In this model, the presence of co-stimulating CD14 macrophages is the crucial condition for inducing prolonged LPL survival via disturbed programmed cell death.

The role of other proinflammatory cytokines in regulating Bcl-2 family protein expression has been investigated to a lesser extent. IL-12 inhibits intestinal T cell apoptosis, but this process does not involve the induction of Bcl-2 or any other anti-apoptotic protein [28]. TNF- α , as a multifunctional cytokine, seems to be also a possible factor promoting prolonged LPL survival. It was shown that this cytokine is able to influence the Bcl-2 protein expression in an NF- κ B-mediated pathway [12]. However, the majority of proinflammatory processes are mediated by TNFR1. In our study, we did not find any differences in TNFR1 expression in LPL between patients with CD and the control group. This suggests that probably the TNFR1-dependent pathway is not involved in the hypothesized anti-apoptotic action of TNF- α . This is in accordance with the previous studies suggesting that TNFR1 plays an important role in the restitution of epithelial cells in the inflamed gut, rather than in the regulation of LPL functioning [33, 34].

In conclusion, it was found that LPL in inflamed tissues from patients with CD undergo programmed cell death significantly more rarely than control LPL. This is accompanied by the imbalance in Bcl-2 family protein expression, with a decreased pro-apoptotic Bax/Bcl-2 ratio. This, in turn, disturbs physiological mechanisms enabling maintenance of cellular homeostasis and provides resistance of LPL to multiple pro-apoptotic stimuli. These phenomena also have therapeutic implications, as it was shown that probably all of the drugs successfully used in CD can provoke the programmed cell death of inflammatory cells [35]. It also seems that anti-TNF antibodies, which strongly down-regulate the inflammatory infiltration in IBD, have the strongest apoptosis-regulating properties, which was also investigated by our group [19]. However, this phenomenon needs further studies in order to understand the pathophysiology of CD better. This, in turn, could have important implications in developing new, more successful therapeutic strategies targeting the disturbances of the apoptotic elimination of inflammatory cells in IBD [33].

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Conflict of interest

The authors declare no conflict of interest.

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