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СУБПОПУЛЯЦИОННЫЙ СОСТАВ ИНТРАЭПИТЕЛИАЛЬНЫХ ЛИМФОЦИТОВ У ПАЦИЕНТОВ С БОЛЕЗНЬЮ КРОНА

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Болезнь Крона представляет собой аутоиммунное воспалительное заболевание кишечника, характеризующееся развитием хронического рецидивно-ремиттирующего воспаления, преимущественно в терминальных отделах тонкой кишки и толстой кишке, в результате генетической предрасположенности или действия факторов окружающей среды. Иммунопатогенез болезни Крона ассоциируется с нарушением бактериального клиренса и изменением иммунологических показателей, что приводит к дисбалансу экосистемы желудочно-кишечного тракта, сформированной микробиомом и такими неживыми компонентами, как слизь, пищевые метаболиты или добавки. Важную роль в мукозальном иммунитете играют интраэпителиальные лимфоциты желудочно-кишечного тракта, фенотип и функциональный профиль которых во многом определяется составом микробиома и воздействием на него различных экологических факторов (диета, курение, сезонные изменения, загрязнение воздуха, гигиена и др.). В данном исследовании у пациентов с болезнью Крона установлены фенотипические изменения интраэпителиальных лимфоцитов тонкой и толстой кишки, выявлена корреляционная зависимость между субпопуляциями интраэпителиальных лимфоцитов тонкой и толстой кишки и клинико-морфологическими показателями. Полученные данные могут являться биомаркером иммунного воспаления в желудочно-кишечном тракте и позволяют рассматривать интраэпителиальные лимфоциты в качестве терапевтической мишени при иммунорегуляции адаптивного мукозального иммунного ответа на аутоантигены.

Ключевые слова: экосистема; интраэпителиальные лимфоциты; факторы окружающей среды; проточная цитофлуориметрия; болезнь Крона; аутоиммунное воспаление.

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INTRAEPITHELIAL LYMPHOCYTES SUBSETS IN CROHN'S DISEASE PATIENTS

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Crohn's disease is an autoimmune inflammatory bowel disease characterized with the chronic relapsing-remitting inflammation, mainly in the terminal parts of the small intestine and colon, as a result of a genetic predisposition or environmental factors influence. It is associated with impaired bacterial clearance and changes in immunological parameters what resulted in disruption of the gastrointestinal tract ecosystem, formed by the microbiome and non-living components such as mucus, food metabolites or additives. Intraepithelial lymphocytes of the gastrointestinal tract play an important role in mucosal immunity and their phenotype and functional profile is largely determined by the microbiome composition and environmental factors affecting it (diet, smoking, seasonal changes, air pollution, hygiene, etc.). In this investigation the changes in intraepithelial lymphocytes phenotype of small and large intestine were shown in patient with Crohn's disease as well as the correlation of intraepithelial lymphocytes phenotype with clinical data was established. Obtained data may be used as a hallmark of immune inflammation in the gut and make intraepithelial lymphocytes as ideal candidate for targeting in further immunoregulation of mucosal adaptive immune response against autoantigens.

Keywords: ecosystem; intraepithelial lymphocytes; environmental factors; flow cytometry; Crohn's disease; autoimmune inflammation.

Introduction

The human gut can be considered an ecosystem comprised of microbes community and nonliving components such as mucus, food metabolites or food additives, and inflammatory bowel diseases (IBD) are increasingly associated with disruption of this ecosystem. Crohn's disease (CD) is a one of chronic autoimmune inflammatory diseases of the gastrointestinal tract refers to IBD and is characterized by an uncontrolled adaptive immune response against intestinal bacteria. Nearly 5 million individuals worldwide suffer from IBD, and the prevalence of disease continues to increase up to 70.000 new diagnoses each year [1].

Current investigations indicate that the etiology of IBD is multifactorial, with environmental, microbial, genetic, and immunological components contributing to the pathophysiology of disease. However, genetics explains a small fraction of risk, and the external environment plays a large and important role in disease pathogenesis. Environmental risk factors include childhood hygiene, air pollution, breastfeeding, smoking, diet, stress, exercise, seasonal variation, and appendectomy which may result in imbalance between regulatory and cytolytic effector lymphoid cells within the epithelium following a dysregulation of mucosal immunity, disturbances in composition of the intestinal microbiota and the generation of a pro-inflammatory microenvironment in IBD. The epithelial cytolysis leads to ulceration, allowing bacterial invasion of the mucosae and enhanced T-cell activation, along with the reduction in regulatory cells amplifying the pro-inflammatory immune response [2].

A basic principle of ecological restoration is that an ecosystem cannot be repaired until the underlying disturbance causing degradation has been removed. One of candidate for therapeutic target is intestinal intraepithelial lymphocytes (IELs) – a heterogeneous population of lymphoid cells, which localizes between the intestinal epithelial cells that form the intestinal mucosal barrier. The tissue-resident intraepithelial T-lymphocytes compartment is shaped by the local environment (oral antigen, microbial signals, region-specific cell–cell interactions) as can be appreciated by the different proportion of various cell subsets between the small and large intestine in human. The small intestine contains at least ten times more IELs than the colon. The classification of IELs includes two main subtypes: "induced" IELs that are phenotypically similar to conventional memory effector T cells and innate-like "natural" IELs that exhibit regulatory functions. Once IELs traffic to the intestine, these cells become tissue resident and do not recirculate [3; 4].

The location of IELs between epithelial cells, their effector memory, cytolytic and inflammatory phenotype, and their ability to destroy infected epithelial cells position them to kill infected epithelial cells and protect the intestine from pathogens. But IELs activation status and their close localization to the intestinal epithelium suggest that these cells may be involved into immunopathological responses and initiate or exacerbate IBD or promote cancer development and progression [5].

Nowadays there are limited data for a role of IELs in IBD. The conflicting results were reported: from the protective function of intestinal epithelium's barrier up to the contribution to gastrointestinal inflammation and

disease development [6]. So the data about IELs phenotype and functional characteristics as well as their role in preventing or reducing susceptibility to IBD remain under investigations.

In this article IELs subsets are detailed characterized in CD patients and their correlation with clinical and morphological features of disease are described what makes IELs as ideal candidate for immunobiomarker of autoimmune intestinal inflammation as well as potential target for further immunoregulation of mucosal adaptive immune response against autoantigens.

Materials and methods

Samples of peripheral venous blood as well as small intestine and colon mucosae tissue were obtained from CD patients (n=7) hospitalized in Minsk regional clinical hospital (Republic of Belarus) and healthy donors (control group, n=5) of the same age and gender ratio during scheduled surgeries. The clinical and demographic characteristics of investigated groups and materials are presented in the table 1.

Table 1

Clinical and demographic characteristics of investigated groups

Groups	n	Age, y.o.	Gender, m/f	Tissue sample size, cm^2	Disease duration, years	Disease severity
CD patients	7	31.0 (26.0–54.0)	5/2	20.7 (10.0–57.5)	5.0 (1.0–10.0)	A2L1-2B1-3P0-1
Healthy donors	5	30.0 (28.0–47.0)	3/2	23.5 (8.0–49.9)	–	–

The diagnosis was confirmed by histological examination of the tissue sample. Disease severity was established according to the Montreal classification of inflammatory bowel disease (ALBP) [7] presented in the table 2.

Table 2

The Montreal classification of inflammatory bowel disease

Grade	Age of diagnosis (A)	Location (L)	Behaviour (B)	Perianal disease (P)
1	A1 – below 16 y. o.	L1 – ileal	B1 – non-stricturing, non-penetrating	concomitant perianal disease is present
2	A2 – between 17 and 40 y. o.	L2 – colonic	B2 – stricturing	
3	A3 – above 40 y. o.	L3 – ileocolonic	B3 – penetrating	
4		L4 – isolated upper disease		

Samples collection procedure. Peripheral venous blood specimens and human tissues were obtained after patients or donors provided informed consent. Peripheral venous blood was collected aseptically by venipuncture into a blood collection tubes with anticoagulant (ethylenediaminetetraacetic acid, EDTA). Mucosa specimens from patients with Crohn's disease or donors were obtained from electively scheduled surgical resections. Tissue samples (small intestine or colon) were collected from the macroscopically least involved area of the mucosa. In all cases, diagnosis of Crohn's disease was confirmed by histopathological examination of the resected specimen. None of the patients was taking steroids or other immunosuppressive drugs at the time of the operation. Control specimens were obtained from patients undergoing surgery for large and small intestine.

IELs isolation method. IELs were isolated by a modification of a described protocol [8]. The specimens were cut into 1–5 mm^2 fragments and incubated for 1 h under intense shaking: the mucosal fragments were placed inside a 50 ml tube with culture medium RPMI 1640 (Gibco, Germany) contained 1 mM dithiothreitol and 1 mM EDTA (Sigma, Germany) supplemented with 10 % fetal calf serum (FCS) and antibiotic-antimycotic mixture (Gibco, Germany). The scheme of protocol steps presented at the fig. 1.

A single cell suspension was obtained by filtering through a 70 mm sterile filter (Carl Roth, Germany), washed in saline and layered onto 40–60 % Percoll gradient (Sigma, Germany). The gradients were spun at 1900 rpm for 30 min. The cell fraction between 40–60 % Percoll was the most enriched for IELs. The IELs layer was transferred to a new tube, diluted to 50 ml with saline, spun at 400 g for 8 min at RT, washed twice more with 50 ml saline, and counted.

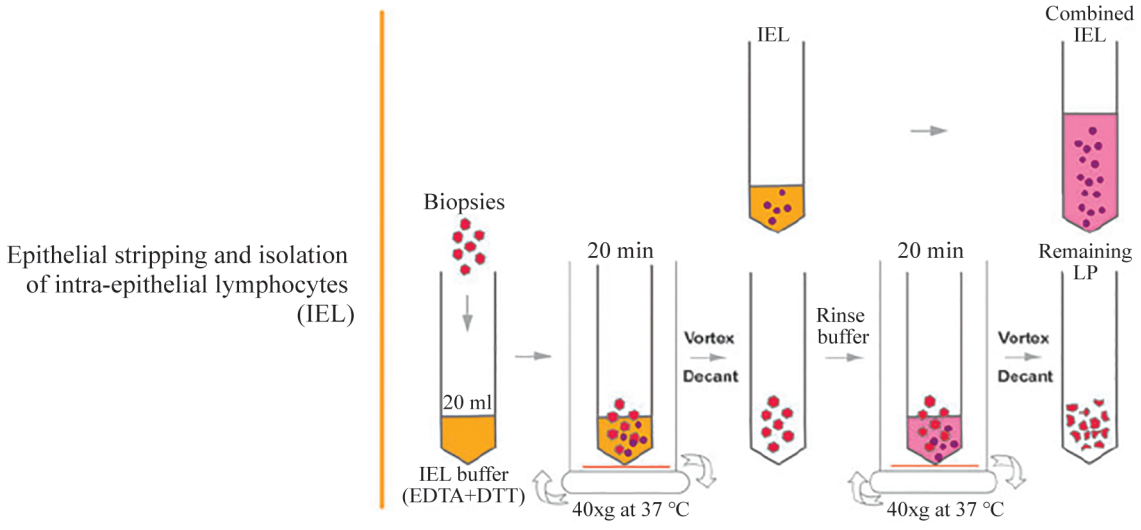

Fig. 1. IEL isolation scheme [8]

Flow cytometry method. Flow cytometry method was used for IELs viability assessment as well as characterization of surface markers expression on IELs. To assess cells apoptosis or necrosis IELs were labeled with Annexin A5-FITC/7AAD kit (Beckman Coulter, Germany) for 15 min at RT in HEPES-Ca^{2+} buffer. For the detection of apoptotic cells cell suspension was washed with ice-cold culture medium and centrifuged for 5 minutes at 500× g at 4 °C. Then supernatants were discarded, and cell pellets were resuspended in ice-cold 1× Binding Buffer to 5×10^6–10×10^6 cells/ml. Tubes were kept on ice. 10 µL of Annexin A5-FITC solution and 20µL of 7-AAD Viability Dye were added to 100 µL of the cell suspensions in the tubes and mixed gently. Tubes were kept on ice and incubated for 15 minutes in the dark. Then 400µL of ice-cold 1× Binding Buffer was added and mixed gently. Cells were analyzed within 30 minutes. Flow cytometry analysis of alive, apoptotic and necrotic IELs was done using biparametric histogram LOG FL1 (525nm) *vs* LOG FL4 (675nm) shows four distinct populations: the viable cells which have negative FITC and 7-AAD signal; the apoptotic cells which have a positive FITC and a negative 7-AAD signal; the secondary necrotic cells which have positive FITC and 7-AAD signal; the damaged viable cells with a negative FITC and a positive 7-AAD signal.

For immunophenotyping, 2×10^5 IELs were stained with 10 µl of CYTO-STAT tetra CHROME monoclonal antibodies panels (CD45-FITC/CD4-RD1/CD8-ECD/CD3-PC5 or CD45-FITC/CD56-RD1/CD19-ECD/CD3-PC5, Beckman Coulter, USA) and incubated at 20–25 °C for 15 min in the dark. CD161-PB antibody was used also for NK-cells identification; γδTCR-PC7 antibody was used for γδT-cells identification. Red blood cells were lysed with the VersaLyse Solution (Beckman Coulter, USA). The aliveness and the phenotype were measured on 10 000 IELs using flow cytometer Cytoflex (Beckman Coulter, USA). The algorithm of lymphoid cells analysis is presented at fig. 2.

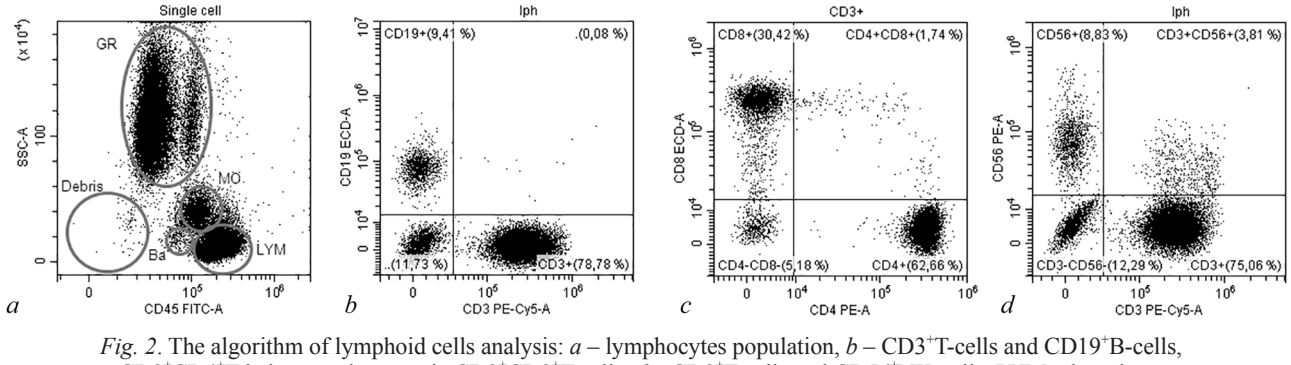

Fig. 2. The algorithm of lymphoid cells analysis: *a* – lymphocytes population, *b* – $CD3^+$T-cells and $CD19^+$B-cells, *c* – $CD3^+CD4^+$T-helpers and cytotoxic $CD3^+CD8^+$T-cells, *d* – $CD3^+$T-cells and $CD56^+$NK-cells. LYM – lymphocytes, GR – granulocytes, MO – monocytes, BA – basophiles.

According to the fig. 2, the gate (*a*) includes the lymphocytes which have bright $CD45^+$ FITC fluorescence and low SS (fig. 2 *a*). In this analysis, priority is given to including all lymphocytes while monocytes (lower CD4 expression and intermediate SS) and basophils (lower CD45 and low SS) were excluded as much as possible. The region boundaries were set on the two parameter histograms (plots) to bracket the double negative

populations ($CD3^-CD19^-$ and $CD3^-CD56^-$) and to tightly encompass the $CD3^+CD19^-$ and $CD3^+CD56^-$ populations as illustrated in fig. 2 *b*. The next region (fig. 2 *c*) boundaries were set on the two parameter histograms (plots) to bracket the double negative populations ($CD3^-CD4^-$ and $CD3^-CD8^-$) and to tightly encompass the $CD3^+CD4^-$ and $CD3^+CD8^-$ populations as illustrated in fig. 2 *d*.

Statistical method. Statistical analysis was made using *Statistica 8.0* software. The data did not correspond to the normal distribution (Shapiro-Wilk W test) and nonparametric methods were used for statistical analysis. The median and interquartile intervals (25 and 75 % percentiles) were used as descriptive statistic. The Mann-Whitney U test was used to compare two independent groups. Differences were considered statistically significant at $p < 0.05$. The correlation between the parameters was determined by the Spearman rank method and was presented as coefficient of correlation (R).

Results and discussion

The characteristics of isolated from gut tissues IELs number in Crohn's disease patients. For IELs isolation the gut tissue was rinsed by manually shaking, followed by removal of the muscular layer using scissors. The mucosal layer separation step is presented at fig. 3.

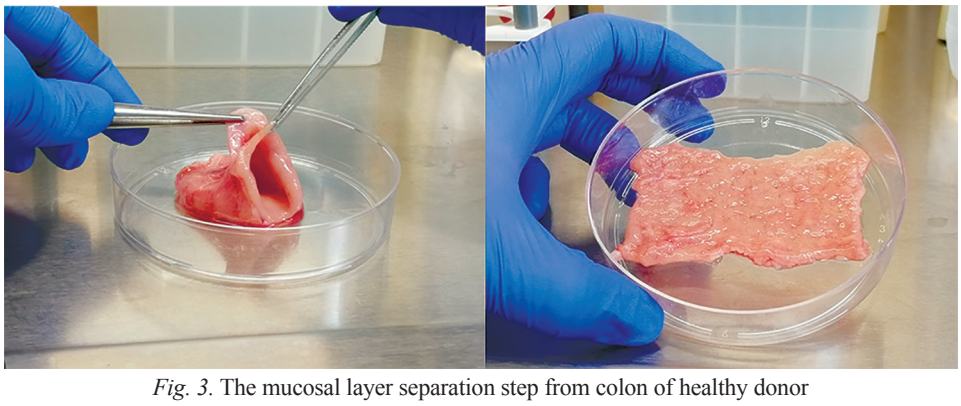

Fig. 3. The mucosal layer separation step from colon of healthy donor

To determine cell yield per cm^2, the size of the mucosal tissue was measured after removal of the muscular layer. The data are presented in the table 3.

Table 3

The tissue sample size and cell number per cm^2 in investigated groups

Groups	The part of gut	Tissue sample size, cm^2	The number of isolated IELs, $\times 10^6$	IELs number ($\times 10^6$) per cm^2
CD patients	Small intestine	17.50 (10.00–21.00)	24.50** (4.5–34.0)	1.10** (0.50–1.94)
	Large intestine	12.75 (10.10–40.00)	12.05* (8.0–52.0)	0.92* (0.37–1.30)
Donors	Small intestine	14.62 (9.80–18.50)	3.80 (2.50–5.90)	0.26 (0.10–0.54)
	Large intestine	8.47 (3.20–13.75)	7.65 (0.90–14.40)	0.25 (0.06–0.45)

Note. **$p<0.01$; *$p<0.05$; significances are indicated as compared to healthy donors.

After isolation, IELs quantity from CD patients small ($24.50\,(4.50 \div 34.00) \times 10^6$) and large ($12.05\ (8.00 \div 52.00) \times 10^6$) intestine were higher than from healthy donors ($3.80\ (2.50 \div 5.90) \times 10^6$ and $7.65\ (0.90 \div 14.40) \times 10^6$) as well as cells numbers per tissue cm^2: $1.10 \times 10^6/cm^2$ and $0.92 \times 10^6/cm^2$ – in small and large intestine of CD patients and $0.26 \times 10^6/cm^2$ and $0.25 \times 10^6/cm^2$ – in donors, respectively, $p < 0.01$ and $p < 0.05$.

The viability of intestinal IELs in Crohn's disease patients. The investigation of IELs viability after isolation in the both samples revealed that majority of cells was alive cells (92.1 % in CD patients and 95,8 % in healthy donors). For the determination of cell death types, IELs were stained with Annexin V conjugated to a fluorophore (phosphatidylserine externalization marker) and 7-amino-actinomycin D (7-AAD) that can only enter cells with compromised membranes (membrane integrity impairment dye). It is known that during apoptosis, a general view of cellular events involves the activation of caspases, programmed destruction of protein and DNA, loss of cell membrane asymmetry (external leaflet exposure of phosphatidylserine), and eventual loss of the integrity

of the plasma membrane [9]. The results of IELs viability and death types in investigated groups are presented in the table 4.

Table 4

IELs viability and death types (%) in investigated groups

Cells viability/death type	Phenotype	IELs number, %	
		CD patients	Donors
Alive	Annexin V^-7AAD^-	94.82 (92.35–97.02)	94.02 (90.08–96.43)
Primary apoptosis	Annexin V^+7AAD^-	2.35 (2.02–4.48)	3.58 (2.59–4.00)
Secondary apoptosis	Annexin V^+7AAD^+	0.71 (0.38–1.23)	0.56 (0.27–0.88)
Necrosis	Annexin V^-7AAD^+	2.12* (1.58–4.71)	1.84 (0.86–2.05)

Note. *p<0.05; significances are indicated as compared to healthy donors.

The investigation of IELs viability after isolation in the both groups revealed that majority of cells was Annexin $A5^-7AAD^-$ corresponding to alive cells (94.02 % in CD patients and 94.82 % in healthy donors). Meanwhile, the analysis of dead cells among IELs in CD patients showed the equal numbers of Annexin V^+7AAD^- primary apoptotic cells (2.35 %) and Annexin V^-7AAD^+ necrotic cells (2.12 %) while in healthy donors primary apoptotic cells (3.58 %) were dominated compared to necrotic ones (1.84 %). The percentage of Annexin V^+7AAD^+ secondary apoptotic cells was insignificant and made 0.71 % and 0.56 %, respectively, in CD patients and healthy donors.

The immunophenotype of intestinal IEL in Crohn's disease patients. For the investigation of IELs immunophenotype and their subsets the expression of T-, B- and NK-lymphoid cell markers were analyzed in cell suspension from small and large intestine in CD patients and healthy donors. The results of IELs phenotype using four-color flow cytometry analysis are presented in the table 5.

Table 5

IELs subsets (%) in Crohn's disease patients and healthy donors

Groups	Gut samples	T-cells	T-helpers	Cytotoxic T-cells	B-cells	NK-cells	γδT-cells
Crohn's disease	Small intestine	88.5* (85.8–93.9)	13.8 (13.4–31.5)	59.9 (29.8–68.2)	8.6* (6.3–14.2)	11.0 (8.6–13.3)	3,2* (2,8–3,5)
	Colon	69.9* (56.3–80.0)	32.9 (11.4–54.6)	49.1 (36.2–62.4)	18.0* (4.1–26.3)	16.9 (11.4–20.9)	12,8* (6,3–19,3)
Healthy donors	Small intestine	58.9 (58.6–67)	71.4 (70.7–72.1)	29.2 (28.3–30.0)	33.0 (30.4–35.6)	11.6 (10.7–12.5)	8,0 (3,7–13,1)
	Colon	50.2 (30.0–70.3)	36.6 (30.7–42.3)	63.8 (60.7–66.9)	44.1 (22.0–66.1)	18.4 (9.4–27.3)	17,4 (16,9–30,5)
p		p_{1-3}<0,001 p_{2-4}<0,05 p_{3-4}<0,01	p_{1-3}<0.05	p_{1-3}<0.05	p_{1-3}<0.05 p_{2-4}<0.01		p_{1-3}<0.05 p_{2-4}<0.05

Note. *p<0.05; significances are indicated as compared to healthy donors.

Four-color flow cytometry analysis of IELs showed that cells from CD patient were predominantly consisted of T-lymphocytes (88.5 % – in small intestine and 69.9 % – in large intestine) like in peripheral blood (table 5). But in CD patients the number of $CD3^+$IELs in small and large intestine were increased in 1,5 ($p < 0.001$) and 1,4 times (p<0.05), respectively, as compared to healthy donors. Moreover, $CD3^+$IELs were predominated in small intestine in CD patients (88.5 % versus 69.9 % in large intestine, $p < 0.01$) while nearly equal number of $CD3^+$IELs was detected in ileum (59.2 %) and colon (50.2 %) of healthy donors (table 5).

Moreover, the decrease of $CD3^+CD4^+$T-helper in small intestine as compared to healthy donors ($p < 0,05$) with a tendency to increase of $CD3^+CD8^+$cytotoxic T-lymphocytes in small intestine (immunoregulatory ratio = 0.31) were established in CD patients (table 5). It was demonstrated the equal numbers of $CD3^+CD4^+$T-helper and $CD3^+CD8^+$cytotoxic T-lymphocytes in the colon of CD patients with $CD4^+/CD8^+$ ratio = 0.76 but the increase of

cytotoxic T-cells IEL compared to T-helper IELs with $CD4^+/CD8^+$ ratio = 0.57 in healthy donor what corresponded to literature data.

Cytofluorometric investigation of $CD3^+CD4^-CD8^-\gamma\delta$T-cells revealed their decrease among IELs in the gut of CD patients (3.2 % – in small and 12.8 – in the large intestine) compared to healthy donor (8.0 and 17.4 %, respectively, $p < 0.05$) (table 5).

$CD19^+$B-cells were decreased in all gut samples from CD patients as compared to healthy donors (in 3.8 times – in small intestine, $p < 0.05$, and 2.4 times – in large intestine, $p < 0.01$). While there were no differences in the percent of neither $CD56^+$NK-cells (table 5) nor $CD161^+$NK-cells in investigated groups. These results suppose the involvement of T-cells as a major player in the pathogenesis of Crohn's disease.

For the comparative analysis the immunophenotype of peripheral blood lymphoid cells was estimated in Crohn's disease patients and healthy donors. The total number of lymphoid cells subsets in peripheral blood of CD patients and donors are presented in table 6.

Table 6

Lymphoid cells subsets (%) in peripheral blood of CD patients and donors

Groups	T-cells	T-helpers	Cytotoxic T-cells	B-cells	NK-cells	γδT-cells
Crohn's disease	68.2 (66.2–84.9)	60.1 (52.3–65.5)	33.4 (25.3–35.9)	12.7 (5.1–14.6)	12.8 (9.2–17.9)	8.5* (1.6–17.1)
Healthy donors	74.5 (70.0–79.0)	64.0 (55.1–73.0)	33.0 (31.0–35.0)	9.7 (9.4–10.0)	13.2 (12.4–14.0)	2.3 (1.9–2.8)

Note. *$p<0.05$; significances are indicated as compared to healthy donors.

The $CD3^+$ and $CD3^+CD4^+$T-helpers had tendency to decrease in CD patients as compare to healthy donors, at the same time there is no difference in cytotoxic $CD3^+CD8^+$T- and NK-cells in investigated groups, while there was the tendency to elevation of $CD19^+$B cells and the significant increase of γδT-cells in CD patients as compare to healthy donors.

According to literature data Crohn's disease are also generally thought to be driven by aberrant $CD4^+$IELs responses directed against the intestinal microbiota, aberrant differentiation and/or functions as major contributing factors to immunopathology at mucosal sites. Probably, the established decrease of $CD3^+CD4^+$T-helper in small intestine may be explained by apoptotic cell death as result of hyper stimulation and activation. Perhaps the most significant detrimental effect of $CD4^+$ IELs is their ability, in conjunction with $CD4^+$ T cells in the *lamina propria*, to promote the development of small intestinal inflammation in patients with IBD [10]. Although both Crohn's disease and ulcerative colitis share some important end-stage pathways of tissue damage, they represent immunologically different diseases with distinct effector $CD4^+$T-cell types involved. Crohn's disease is considered to be a classical Th1-cell-mediated inflammatory disorder that is characterized by elevated levels of IFNγ and IL-12. However, the more recent findings that inflamed colons from both mouse models and patients with Crohn's disease show considerable Th17 cell infiltrates, suggests a more complex disorder. In addition, IL-23, which promotes Th17 cell responses, seems to be a major player in IBD pathogenesis and genome-wide association studies in humans defined IL-23R as one of the major IBD susceptibility genes. Recent studies have also pointed to roles for thymic stromal lymphopoietin (TSLP) and the IL-17 family member IL-25 in the induction of $CD4^+$T cell-driven intestinal inflammation [11].

In humans, $CD8^+$IELs closely resemble systemic effector memory cells and exhibit cytolytic activity. It is thought that the intestinal microenvironment conditions $CD8^+$IELs to respond to non-classical major histocompatibility complex (MHC) class I molecules through the activation of natural killer receptors. These MHC class I ligands are upregulated in response to epithelial stress, infection or inflammation. Instead, it is thought the activation of antigen-specific conventional $CD8\alpha\beta^+TCR\alpha\beta^+$ IELs or recognition of epithelial stress ligands by these cells induces epithelial cytolysis. Animal studies suggest that autoreactivity is primarily a characteristic associated with the naturally occurring $TCR\alpha\beta^+CD8\alpha\alpha^+$IELs subset. This IELs subset was shown to be selected by self-antigens restricted by non-classical and classical MHC class I and II molecules during thymic development. The current line of thought is self-reactive T cells that failed to undergo negative selection are destined to preferentially migrate and expand in the intestine, where they acquire CD8αα and granzyme. In addition to having an autoreactive TCR, these naturally occurring innate-like lymphocytes express activating NK receptors, that enable them to recognize self-antigens induced under conditions of stress and inflammation. This latter autoreactivity is destined to recognize modifications of self that signal the presence of pathogens and transformed cells [12].

The correlation of IELs subsets immunophenotype and clinical parameters in CD patients. The correlation of the IELs subsets number in small and large intestine as well as peripheral blood lymphocytes and clinical data, such as the Crohn's disease duration, localization and severity was estimated in patients.

The negative correlation was established between the number of $CD3^+$T cells in small intestine and the duration of Crohn's disease (R=-0.8, $p > 0.05$) as well as perianal disease (R=-0.7, $p > 0.05$), mostly due to cytotoxic $CD8^+$T-cells and γδT-cells (table 7). All T-cells subsets with killer activity (CTL, γδT-cells and NK-cells) also inversely correlated with disease localization (R=-0.86, $p > 0.05$). At the same time, the number of T-helpers and B-lymphocytes was positively correlated with disease severity (R=0.94, $p < 0.01$) and the presence of perianal disease ($R = 0.70$, $p < 0.01$).

Table 7

The coefficient of correlation (R) of IEL subsets in small intestine and clinical parameters in CD patients

Parameter	T-cells	T-helpers	CTL	B-cells	NK-cells	γδT-cells
Age	-0.60	0.20	-0.60	0.60	-0.60	-0.60
Duration	-0.80*	0.40	-0.90*	0.80*	0.00	-0.80**
L	-0.57	0.00	-0.86*	0.57	-0.86*	-0.86*
B	-0.63	0.94**	-0.47	0.63*	-0.47	0.00
P	-0.70*	0.70*	-0.70*	0.70*	-0.70*	-0.35

Note. *p<0.05; **p<0.01.

In the colon of CD patients the less the number of γδT-cells was the more disease duration ($R = -0.90$, $p < 0.05$), area of localization ($R = -0.87$, $p < 0.05$) and perianal disease ($R = -0.65$, $p < 0.05$) were. The number of NK-cells also was inversely correlated with perianal disease ($R = -0.90$, $p < 0.05$) (table 8).

Table 8

The coefficient of correlation (R) of IELs subsets in colon and clinical parameters in CD patients

Parameter	T-cells	T-helpers	CTL	B-cells	NK-cells	γδT-cells
Age	0.37	0.37	0.14	0.48	0.08	-0.20
Duration	0.40	0.50	0.20	-0.10	-0.60	-0.90*
L	-0.09	0.48	0.09	-0.09	-0.29	-0.87*
B	0.44	0.17	-0.14	0.08	-0.08	0.17
P	0.39	0.39	-0.13	-0.13	-0.65*	-0.65*

Note. *p<0.05.

The number of lymphoid cells in peripheral blood was not significantly correlated with clinical parameters of CD patients.

Thus, the T-helpers and B-lymphocytes are involved in effector damage of gut tissue while T-cells subsets with killer activity (CTL, γδT-cells and NK-cells) probably play a protective or immunoregulatory role in CD immunopathogenesis.

Conclusion

IELs which are located between the epithelial cells of the intestinal mucosa close to the gut lumen have been suggested to be the first lymphocytes to encounter luminal antigens and therefore play an important role in mucosal immunity as well as in intestinal inflammation. In this article IELs number, viability and immunophenotype subsets were detailed characterized in CD patients. According to the obtained data the next conclusions were formulated:

1. The total IELs number is increased in 6,4 times in small intestine (p<0.01) and in 1,6 times in large intestine (p<0.05) of CD patients as compared to healthy donors, what indicates the active involvement of IELs in intestinal inflammation.

2. The majority of IELs after isolation from gut are Annexin $A5^-7AAD^-$ corresponding to alive cells in the both investigated groups (94.02 % in CD patients and 94.82 % in healthy donors) but the number of necrotic IELs among dead cells in CD patients is higher than in healthy donors (p<0.05) reflecting tissue damage process in inflamed gut.

3. The changes of IELs subsets in the gut of CD patients are revealed as compared to donors: the number of $CD3^+$IELs in small and large intestine are increased in 1,5 ($p<0.001$) and 1,4 times ($p<0.05$), respectively, characterizing with the prevailing of $CD8^+$IELs in small intestine (immunoregulatory ratio=0.31; $p<0.05$), while the numbers of $CD3^+CD4^-CD8^-\gamma\delta$T-cells ($p<0.05$) and $CD19^+$B-cells ($p<0.01$) among IELs are decreased in all parts of gut. There are no differences in the percent of NK-cells in investigated groups. These results suppose the involvement of T-cells as a major player in the intestinal inflammation maintenance and CD immunopathogenesis.

4. The established positive correlation of T-helpers and B-lymphocytes in small intestine with disease severity (R=0.94, $p<0.01$) and the presence of perianal disease (R=0.70, $p<0.01$) reflects the abberant effector T-cells function and indicates the involvement these subsets in the damage of gut tissue while lymphoid cells subsets with killer activity (CTL, γδT-cells and NK-cells) inversely correlated with clinical parameters (R=-0.86, $p>0.05$) and probably play a protective or immunoregulatory role in CD immunopathogenesis.

References

1. Orr M, Kocurek K, Bakos Y and McDowell R. A restoration ecology perspective on the treatment of inflammatory bowel disease. *Evolution, Medicine, and Public Health*. 2019;217–220. DOI:10.1093/emph/eoz031.

2. Guan Q. A Comprehensive review and update on the pathogenesis of inflammatory bowel disease. *Journal of Immunology Research.* 2019; article ID7247238. DOI: 10.1155/2019/7247238.

3. Van Kaer L and Olivares-Villagómez D. Developments, homeostasis and functions of intestinal intraepithelial lymphocytes. *Journal Immunology.* 2018;200:2235–2244. DOI: 10.4049/jimmunol.1701704.

4. Sumida H. Dynamics and clinical significance of intestinal intraepithelial lymphocytes. *Immunological Medicine*. 2019;42(3):117–12. DOI: 10.1080/25785826.2019.1658516.

5. Hu MD and Edelblum KL. Sentinels at the frontline: the role of intraepithelial lymphocytes in inflammatory bowel disease. *Current Pharmacology Reports*. 2017;23(1):321–334. DOI:10.1007/s40495-017-0105-2.

6. Cheroute H, Lambolez F, Mucida D. The light and dark sides of intestinal intraepithelial lymphocytes. *Nature Reviews Immunology*. 2011;11(7):445–456. DOI:10.1038/nri3007.

7. Satsangi J. Silverberg MS, Vermeire S. The Montreal classification of inflammatory bowel disease: controversies, consensus, and implications. *Gut.* 2006;55(6):749–753.

8. Trapecare M, Khan S, Roan N, et al. An optimized and validated method for isolation and characterization of lymphocytes from HIV+ human gut biopsies. *AIDS Research and Human Retroviruses*. 2017;33(S1):31–39. DOI:10.1089/aid.2017.0208.

9. Green D. Apoptosis and other cell death mechanisms. *Cold Spring Harbor Laboratory Press.* 2011:645–657.

10. Zhou C, Qiu Y and Yang H. CD4CD8aa IELs: they have something to say. *Frontiers in Immunology.* 2019;10:2269. DOI: 10.3389/fimmu.2019.02269.

11. Mayassi T and Jabri B. Human intraepithelial lymphocytes. *Mucosal Immunology.* 2018;11(5):1281–1289. DOI:10.1038/s41385-018-0016-5.

12. De Souza H and Fiocchi C. Immunopathogenesis of IBD: current state of the art. *Nature Reviews. Gastroenterology & Hepatology.* 2016;13:13–27. DOI:10.1038/nrgastro.2016.186.

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