Медицинская иммунология
2023, Т. 25, № 5,
стр. 1103-1110

Medical Immunology (Russia)/
Meditsinskaya Immunologiya
2023, Vol. 25, №5, pp. 1103-1110

ФЕНОТИПИЧЕСКАЯ ХАРАКТЕРИСТИКА ИНТРАЭПИТЕЛИАЛЬНЫХ ЛИМФОЦИТОВ ТОЛСТОЙ КИШКИ У ПАЦИЕНТОВ С БОЛЕЗНЬЮ КРОНА

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Резюме. Интраэпителиальные лимфоциты (IEL) играют важную роль в поддержании иммунного гомеостаза и обеспечивают первую линию защиты слизистых оболочек желудочно-кишечного тракта от антигенов, а также быстро реагируют на повреждение эпителия. В последнее время IEL рассматриваются в качестве ключевых медиаторов аберрантного иммунного ответа, характеризующегося стойкой иммунной активацией, воспалением и нарушением барьерной функции слизистых оболочек, что наблюдается при болезни Крона. В данном исследовании впервые приводится сравнительная характеристика субпопуляционного состава IEL толстой кишки у пациентов с болезнью Крона и здоровых доноров для последующего определения их потенциальной роли в патогенезе заболевания.

Материалом исследования явилась ткань толстой кишки и периферическая кровь 10 пациентов с болезнью Крона и 6 здоровых доноров. IEL выделяли из ткани методом клеточной диссоциации; циркулирующие лимфоциты получали из периферической венозной крови путем центрифугирования на градиенте плотности. Фенотип лимфоидных клеток оценивали с использованием моноклональных антител и метода проточной цитометрии.

Большинство IEL толстой кишки идентифицировалось как $CD3^+$Т-лимфоциты, однако статистически значимые различия в их количестве отсутствовали в исследуемых группах. При этом установлены изменения субпопуляционного состава Т-клеток: у пациентов с болезнью Крона соотношение $CD3^+CD4^+$IEL и $CD3^+CD8^+$IEL составило 1:1 и коррелировало с процентом Т-лимфоцитов в периферической крови ($R = 0,7$; $p < 0,05$), в то время как в донорской ткани выявлялось ожидаемое преобладание $CD3^+CD8^+$Т-киллеров (соотношение достигало 1:2, $p < 0,05$). Наряду с этим повышение неклассических γδIEL (преимущественно за счет $V\delta1^+$Т-клеток) и $CD161^+$Т-клеток в сочетании со снижением TNK-клеток наблюдалось как в толстой кишке ($p < 0,01$), так и в периферической крови ($p < 0,05$) пациентов с болезнью Крона относительно контрольной группы. При этом количество

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Д.Б. Нижегородова, А.Ч. Шулейко, А.М. Старостин, М.И. Ванслав, Г.И. Иванчик, А.В. Величко, М.М. Зафранская «Фенотипическая характеристика интраэпителиальных лимфоцитов толстой кишки у пациентов с болезнью Крона» // Медицинская иммунология, 2023. Т. 25, № 5. С. 1103-1110. doi: 10.15789/1563-0625-PCO-2839

For citation:

D.B. Nizheharodava, A.Ch. Shuleika, A.M. Starastsin, M.I. Vanslau, G.I. Ivanchyk, A.V. Vialichka, M.M. Zafranskaya "Phenotype characteristic of colonic intraepithelial lymphocytes in patients with Crohn's disease", Medical Immunology (Russia)/Meditsinskaya Immunologiya, 2023, Vol. 25, no. 5, pp. 1103-1110. doi: 10.15789/1563-0625-PCO-2839

DOI: 10.15789/1563-0625-PCO-2839

γδIEL коррелировало с локализацией (R = -0,6; p < 0,05) и течением заболевания (R = 0,7; p < 0,01) согласно Монреальской классификации.

У пациентов с болезнью Крона выявлены изменения состава IEL толстой кишки, которые характеризуют вовлечение субпопуляций Т-хелперов, γδТ-лимфоцитов и мукозально-ассоциированных CD161$^+$Т-клеток в патогенез аутоиммунного воспаления, что требует дальнейшего исследования для установления их патогенетической роли.

Ключевые слова: интраэпителиальные лимфоциты, проточная цитометрия, болезнь Крона, толстая кишка, аутоиммунное воспаление, мукозальный иммунитет

PHENOTYPE CHARACTERISTIC OF COLONIC INTRAEPITHELIAL LYMPHOCYTES IN PATIENTS WITH CROHN'S DISEASE

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Abstract. Intraepithelial lymphocytes (IEL) play a critical role in maintaining the immune balance of the gut and provide the first line of mucosal defense against luminal antigens as well as rapidly respond to epithelial injury. Recently, IEL have received a lot of attention as key mediators of aberrant immune response resulted in persistent immune activation, inflammation and altered intestinal barrier function, seen in Crohn's disease (CD). This study describes for the first time subsets of colonic IEL in CD patients as compared to healthy controls aimed at characterization of altered IEL contribution to the pathogenesis of Crohn's disease.

The peripheral venous blood and colon tissues were obtained from 10 CD patients and 6 donors. IEL were isolated from the mucosa by incubation the tissue in a predigesting solution. Lymphoid cells phenotype was investigated using monoclonal antibodies and flow cytometry.

The majority of colonic IEL was identified as CD3$^+$T lymphocytes and no significant differences were found in their numbers in investigated groups. However, changes in T cell subsets composition have been shown: the ratio of CD3$^+$CD4$^+$IEL and CD3$^+$CD8$^+$IEL was 1:1 in colon of CD patients and correlated with T cells in peripheral blood (R = 0.7; p < 0.05) while donor tissues were characterized by expected CD3$^+$CD8$^+$T killers prevalence and the ratio reached 1:2 (p < 0.05). The increase of unconventional γδIEL (mainly due to Vδ1$^+$T cells) and CD161$^+$T cells in association with TNK cells decrease were revealed in colon (p < 0.01) as well as in peripheral blood (p < 0.05) of CD patients as compared to donors. Moreover, the number of colonic γδIEL was correlated with disease location (R = -0.6; p < 0.05), and disease behavior (R = 0.7; p < 0.01) according to Montreal classification.

The observed data indicates changes in colonic IEL composition in CD patients that may provide valuable insight into the contribution of T helpers, γδT cells and mucosa-associated CD161$^+$T cells in autoimmune intestinal inflammation but need further possible mechanisms discussion.

Keywords: intraepithelial lymphocytes, flow cytometry, Crohn's disease, colon, autoimmune inflammation, mucosal immunity

Introduction

The mucosal immune system is the largest (about 400 m^2) autonomous immune structure, which has evolved as the first line of a body defense and represented by organized compartments and isolated lymphoid cells, classifying into intraepithelial lymphoid cells (IEL) and lamina propria lymphoid cells [8]. As 80% of the mucosal immunocompetent tissue is localized in the gut, intestinal IEL have received a lot of attention as key mediators of aberrant immune response seen in Crohn's disease (CD), a chronic autoimmune inflammatory bowel disease characterized by multifactorial aetiology and an uncontrolled adaptive immune reaction against intestinal microbiota [5]. Experimental data suggest that an imbalance between regulatory and cytolytic effector mechanisms results in a dysregulation of mucosal immunity, persistent immune activation, a

generation of a pro-inflammatory microenvironment, altered intestinal barrier function or even promotion of cancer development in CD patients [14].

On the one side, the location of IEL between epithelial cells, their effector memory and inflammatory phenotype as well as ability to destroy infected epithelial cells place them to protect the intestine from pathogens. On the other side, IEL-mediated epithelial cytolysis leads to ulceration, allowing bacterial invasion, and enhanced T cell activation in combination with regulatory cell reduction. Moreover, the interaction with non-immune epithelial cells and fibroblasts initiates tissue reorganization, including epithelial proliferation and fibrosis in gastrointestinal tract [3, 11].

As IEL immunobiology goes beyond the scope of classical immunology, limited data were reported about a role of IEL subsets in CD pathogenesis. It has not yet been clarified in what manner IEL subpopulations are changed during the initiation and development of adaptive antigen-specific autoimmune response and what are distinctive features of IEL distribution in CD patients. This article will focus on colonic IEL exhibiting properties of innate and adaptive immunity and describe for the first time IEL subsets in CD patients as compared to donors aimed at characterization of altered IEL contribution to immune dysregulation in intestinal mucosae.

Materials and methods

The peripheral venous blood samples and colon tissues were obtained from 10 CD patients, aged 28.0 (22.7-36.8) years, of which 6 men and 4 women, during electively scheduled surgical resections in Minsk Regional Clinical Hospital (Republic of Belarus). The "Crohn's disease" diagnosis was confirmed by histopathological examination of the resected specimen. Patients had the following disease location: terminal ileum (n = 4), colon (n = 3), ileocolon (n = 3). Of these, 3 patients had an inflammatory disease behavior, 4 patients – stricturing and 3 patients – a penetrating/fistulous phenotype; 2 patients were also diagnosed perianal disease. None of the patients was taking steroids or other immunosuppressive drugs at the time of the operation. Control specimens were obtained from age- and sex-matched 6 donors undergoing surgery for large intestine. Written informed consent was provided by all individuals.

Tissues were processed within 2 h after resection. After removal of the muscular layer mucosal tissue was cut into pieces of 0.5 cm^2. IEL were isolated from the mucosa by shaking the tissue in a predigestion solution (Hank's balanced salt solution without Ca^{2+} and Mg^{2+} containing 10 mM HEPES, 5 mM EDTA, 5% fetal bovine serum, 1 mM dithioerythritol (Gibco, Germany)) for 20 min at 37 °C under continuous rotation followed sample application onto cell strainer (100 μm). Cell suspension containing IEL was centrifuged at 300 g for 10 min and resuspended to the required volume for immunophenotyping.

Peripheral blood lymphocytes (PBL) were isolated from heparinized venous blood samples by Histopaque-1077 (Sigma, Germany) density gradient centrifugation. Cells viability was determined by trypan blue exclusion.

The lymphoid cells subsets of IEL and PBL were determined using monoclonal antibody panels: CD45-FITC/CD4-RD1/CD8-ECD/CD3-PC5, CD45-FITC/CD56-RD1/CD19-ECD/CD3-PC5/CD5-PC7, γδTCR-FITC/αβTCR-PE/Vδ1TCR-PC7/CD3-AF750/Vδ2TCR-PB/CD45-KrO, CD127-FITC/Lin-PE/CD3-APC/CD161-PB/CD45-BrV (Beckman Coulter, USA). Monoclonal antibody reagents were added according to the manufacturer's instructions to 100 μL of cell suspension, and reaction mixtures were incubated at 20-25 °C for 15 min in the dark. Results were analyzed on 20000 lymphocytes using a 10-channel flow cytometer Cytoflex (Beckman Coulter, USA).

Statistical data processing was performed using Statistica 8.0. The median (Me), 25^{th} and 75^{th} percentiles were used as descriptive statistics of the studied groups. Significant differences between investigated groups were determined by nonparametric criteria Mann–Whitney U-test; p-values < 0.05 and $p < 0.01$ were considered as statistically significant. The correlation was estimated using Spearman's rank coefficient (R).

Results and discussion

The comparative analyses of IEL and PBL numbers, cell viability and phenotype were done in investigated groups. After isolation IEL viability was not less than 86% in CD patients and not differed from one's in donor's tissue. But IEL yield per 1 g of tissue was significantly higher in colon of CD patients as compared to control group: 30.1 (27.9 ÷ 39.0) × 10^6 *versus* 19.4 (15.6 ÷ 24.7) × 10^6, respectively, indicating the activity of the autoimmune inflammation. An increase of colonic IEL may disrupt the balance between immune tolerance and immune response to self-antigens, including microbiota, which can result in damage of the intestinal epithelium. At the same time there were no significant differences in PBL total numbers in investigated groups.

As lymphoid cells are a highly heterogeneous population the following subsets have been investigated in colon and peripheral blood: NK cells, TNK cells, γδT cells (Vδ1$^+$T, Vδ2$^+$T and Vδ3$^+$T subpopulations), mucosa-associated innate lymphoid Lin$^-$CD161$^+$ cells and B_1 cells were identified as innate immunity cells while T helpers, T killers and B_2 cells – as the main

TABLE 1. CHARACTERISTIC OF LYMPHOID CELLS IN COLON AND PERIPHERAL BLOOD OF CD PATIENTS AND CONTROL GROUP, Me ($Q_{0.25}$÷$Q_{0.75}$) ‰

Lymphoid cells	IEL		PBL		p-value
	CD patients	Control group	CD patients	Control group	
	1	2	3	4	
$CD3^+$T cells, %	74.7 (65.1 ÷ 80.1)	70.4 (50.2 ÷ 78.1)	75.2 (66.3 ÷ 78.3)	72.1 (67.9 ÷ 70.3)	n. s.
$CD3^+CD4^+$ T helpers, %	47.9 (17.9 ÷ 54.7)	30.8 (25.2 ÷ 36.6)	63.2 (59.8 ÷ 65.6)	60.2 (57.6 ÷ 62.7)	n. s.
$CD3^+CD8^+$ T killers, %	47.1 (36.3 ÷ 51.1)	66.9 (63.9 ÷ 70.5)	33.4 (25.5 ÷ 33.7)	33.3 (27.3 ÷ 39.3)	$p_{1-2} < 0.01$
γδTCR$^+$T cells, %	24.0 (17.3 ÷ 35.6)	13.5 (9.9 ÷ 16.4)	8.5 (2.5 ÷ 10.6)	3.1 (2.1 ÷ 5.5)	$p_{1-2} < 0.01$ $p_{3-4} < 0.05$
$CD19^+$B cells, %	21.1 (14.9 ÷ 26.3)	12.6 (7.9 ÷ 17.3)	12.8 (11.8 ÷ 14.7)	13.1 (10.8 ÷ 15.3)	n. s.
$CD19^+CD5^+$ B_1 cells, %	5.6 (3.7 ÷10.0)	11.1 (10.0 ÷12.2)	16.8 (14.9 ÷ 19.7)	20.8 (17.1 ÷ 24.5)	n. s.
$CD56^+$ NK cells, %	14.1 (11.8 ÷ 19.8)	27.3 (18.4 ÷ 35.7)	14.3 (12.8 ÷ 17.9)	10.1 (7.2 ÷ 13.1)	n. s.
$CD3^+CD56^+$ TNK cells, %	13.7 (3.8 ÷ 19.3)	26.6 (24.9 ÷ 33.3)	0.2 (0.1 ÷ 0.3)	5.8 (7.2 ÷ 13.1)	$p_{1-2} < 0.01$
$CD161^+$ cells, %	24.1 (20.7 ÷ 34.7)	5.4 (2.6 ÷ 15.0)	19.7 (16.3 ÷ 23.1)	9.6 (5.8 ÷ 13.5)	$p_{1-2} < 0.01$ $p_{3-4} < 0.05$

Note. Me ($Q_{0.25}$÷$Q_{0.75}$), median, 25th and 75th percentiles; IEL, intraepithelial lymphocytes; PBL, peripheral blood lymphocytes; n, patients' number in a group; p-value, statistically significant test result; n. s., not significant; CD, cluster of differentiation; TCR, T cell receptor; NK cells, natural killer cells; TNK cells, natural killer T cells.

population of acquired immunity cells. The results of lymphoid cells phenotype are presented in Table 1.

The majority of colonic IEL was determined as $CD3^+$T lymphocytes and no significant differences were found in their numbers in investigated groups. However, changes in T cell subsets composition have been shown: the ratio of $CD3^+CD4^+$IEL and $CD3^+CD8^+$IEL was 1:1 in colon of CD patients and correlated with T cells in peripheral blood ($R = 0.7$; $p < 0.05$) while donor tissues were characterized by expected $CD3^+CD8^+$T killers prevalence and the ratio reached 1:2 ($p < 0.01$).

Currently the generally accepted approach to the immune status assessment is the characterization of lymphoid cells in the peripheral blood. However, the investigation of local mucosal gut immunity under immunopathological conditions remain highly relevant as well as its following comparison with circulating lymphoid cells for the identification the relationship between local reactions and systemic effects.

The obtained results are consistent Hu et al. (2017), indicating the absolute majority of $CD3^+$T lymphocytes among IEL and a decrease in their percentage in lamina propria, but at the same time demonstrating the active involvement of T lymphocytes of both compartments in inflammatory bowel diseases pathogenesis [5]. IEL localization contributes to their rapid response to antigenic structures, including microbiota components that come into contact with the gut epithelium. Mucosal T lymphocytes have a predominantly effector phenotype, exhibit various cytotoxic activities, including alloreactive and virus-specific, provide assistance to B cells, play a role in maintaining tolerance, and regulate the function of epithelial cells, thereby fulfilling the role of immune surveillance or the first line of defense. However, the presence of autoreactive T cells clones in CD patients leads to the fact that instead of the first line of defense, epithelial damage occurs as a result of an imbalance between immunological tolerance and effector immune response [12].

In healthy gut tissues αβT lymphocytes are mainly represented by cytotoxic $CD3^+CD8^+$T cells, which play an important protective role in the detection and elimination of damaged epithelial cells and anti-infective protection. While in CD patients there is an infiltration of the epithelial layer by $CD3^+CD4^+$T helpers what was also confirmed in this study as T helpers were mainly determined in the colon of CD patients (Table 1). This presumably is associated with chronic antigenic stimulation by opportunistic bacteria in the gut and indicates an autoimmune inflammatory response. Autoimmune inflammation in CD patients is mainly associated with increased activation and aberrant proliferation of

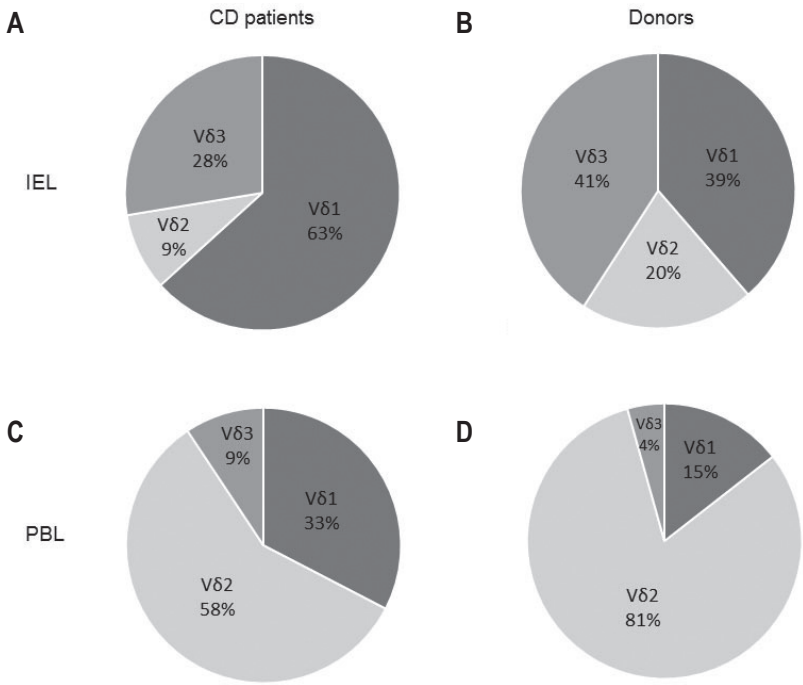

Figure 1. γδT cells subsets (%) in colon and peripheral blood of CD patients and control group

Note. (A) γδIEL subsets in colon of CD patients. (B) γδIEL subsets in colon of control group. (C) γδT cells subsets in peripheral blood of CD patients. (D) γδT cells subsets in peripheral blood of control group.

T helper types 1 and 17, the effector reactions of which are mediated by the production of pro-inflammatory cytokines (IL-12, IL-23, IFNγ and IL-17). T helpers are the main mediators of cellular immunity and play a key role in the activation of other immune cells such as B cells and cytotoxic T cells by modulating an antigen-specific immune response [6].

However, a growing number of evidence points to the role of cytotoxicity mechanisms in intestinal tissue injury: T killers realize their effector reactions through the appearance of such highly active biomolecules as perforin, granzymes, granulisin, Fas-ligand and tumor necrosis factor α. Moreover, pro-inflammatory cytokines released by T killers may be involved in disrupting the epithelial barrier by inducing epithelial cell apoptosis and increasing intestinal permeability. High levels of cytotoxicity support the existing concept that CD affects the entire gastrointestinal tract [1].

The investigation of T cells subsets based on the type of T cell receptors (αβTCR or γδTCR) expression revealed a statistically significant increase in the number of γδT cells in colon ($p < 0.01$) as well as in peripheral blood ($p < 0.05$) of CD patients as compared to donors (Table 1). Moreover, the number of colonic γδIEL was correlated with disease location ($R = -0.6$; $p < 0.05$), and disease behavior ($R = 0.7$; $p < 0.01$) according to Montreal classification what confirmed the involvement of unconventional γδT lymphocytes in CD immunopathogenesis.

Due to the high heterogeneity of γδT cells, a detailed subsets analysis of circulating and resident unconventional T cells was carried out. As seen in Figure 1, $Vδ1^+$T cells subset was prevailed (> 60%) among colonic γδIEL (Figure 1A) and significantly elevated in peripheral blood among CD patients (Figure 1C) in combination with decreased $Vδ2^+$T cells numbers as compared to control group (Figure 1B and D, $p < 0.05$).

Unlike αβT lymphocytes, γδT cells combine cells' properties of innate and acquired immunity: their receptor apparatus is partially similar to that of antigen-presenting cells and NK cells in expression of pattern recognition and killer/inhibitor receptors, however, this population is able to recognize antigenic structures via a specific T cell receptor, similarly to classical T lymphocytes [2]. An established persistent increase in γδT lymphocytes rate in colon and peripheral blood of CD patients is consistent with Regner et al. [12] characterizing the non-thymic origin of some lymphoid cells of the gut mucosa, at times reaching up to 50% of intestinal lymphoid cells. Experimental studies are rather contradictory in confirming the involvement of γδT lymphocytes in CD pathogenesis which is partly due to the high heterogeneity of this population, and therefore their clinical significance has not been fully elucidated [10].

Taking into consideration the predominantly cytotoxic $Vδ1^+$T cells profile, γδT cells can be assumed as one of the potential mechanisms of mucosal barrier

damage and gut inflammation contributing to the chronicity and systemic reactions to antigens of the gastrointestinal microbiota. Most authors support the hypothesis of aberrant activation of γδT lymphocytes due to inflammatory microenvironment formed by changes in αβT cells subsets in the intestinal mucosa, as well as microbial stimulation what resulted in their active homing through the intestinal epithelium via an occludin-dependent mechanism and involvement in disease progression by realizing their cytotoxic effector reactions. In addition, excessive epithelial regeneration mediated by γδT cells may contributes to the formation of pseudopodia, atypical serrated surface, and the development of serious disease complications, such as colorectal cancer [9].

Besides γδT cells, CD patients also display a significantly higher percentage of mucosa-associated innate lymphoid CD161$^+$ cells in colon and peripheral blood than healthy individuals (Table 1). CD161 is a C-type lectin-like type-II transmembrane protein that is expressed on the majority of natural killer (NK) cells, innate T cells (mucosal-associated invariant T cells, invariant natural killer T cells, γδT cells), some adult peripheral blood αβT cells as well as on innate lymphoid cells, restricting to those cells with a secretion of interleukin-17, and therefore to type 17 phenotype [7]. The reasons behind the preferential accumulation of CD161$^+$ cells in the intestine are not completely understood. Apart from the presence of CD161$^+$ cells in the gut, a number of studies demonstrated that CD161 was preferentially expressed on circulating T cells that exhibited gut-homing properties like expression of chemokine receptor 9 or integrin α4β7. In fact, CD161 was proposed to be involved in lymphocyte transendothelial migration and thereby could facilitate trafficking of CD161-expressing T cells to the intestine [4].

The interesting revealed fact was the decrease of circulating and colonic TNK cells reflecting a possible reduction of immunoregulatory mechanisms as this population has a critical role in peripheral tolerance. TNK cells represent a minor subset of T lymphocytes that share cell-surface proteins with conventional T cells and NK cells. A lack of TNK cells may result in a defective regulation of luminal bacteria and as a consequence overt bacterial invasion into the intestine and chronic inflammatory responses. Accordingly, in the presence of TNK cells, dendritic cells were found to produce more IL-10 and lose the ability to produce IL-12. It is unclear what percentages of IEL are in fact TNK cells in human colon, presumably, it may vary from 17 to 45%. The biological function of TNK cells is paradoxical, because these cells can rapidly produce large amounts of T helper type 1 (Th1), Th2, and regulatory cytokines. TNK cells may thus promote or suppress cell-mediated immunity in different conditions. But the exact function of TNK cells in the intestine and whether they may also have regulatory functions in intestinal inflammation remain uncertain [13].

Conclusion

In colonic IEL composition the changes in major (T helpers) and minor (γδT cells, TNK cells and mucosa-associated CD161$^+$ cells) populations of intraepithelial T lymphocytes have been revealed in CD patients, while B lymphocytes and NK cells may be not so pathogenetically significant and further research is required to establish their role in disease immunopathogenesis. Taking into account that the intestinal environment is tolerant to most foreign harmless antigens, the identified changes in the quantitative composition of lymphoid subpopulations may be trigger mechanisms for disruption of mucosal barrier integrity, translocation of intestinal bacteria, and an increase in the number of local and systemic inflammatory reactions. The established correlation suggests the migration of mucosal lymphoid cells and their circulation in the peripheral blood what can be used as diagnostically significant markers of disease progression and opens up new possibilities for the use of mucosal lymphoid cells in targeted therapy and preventive medicine.

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Поступила 15.04.2023
Принята к печати 26.04.2023

Received 15.04.2023
Accepted 26.04.2023