

поведение молекулы в живом организме, включая биодоступность, транспортные свойства, сродство к белкам, реактивность, токсичность, метаболическую стабильность и многие другие.

Оценка биоактивности препаратов основывается на коэффициентах ресурса Molinspiration. Так, если значение коэффициента меньше 0 – это говорит о том, что активность соединения низкая; значение от 0 до 0,2 – средняя, от 0,2 и выше – высокая.

Анализ структуры метаболита прасургрела показал, что R-138727 имеет низкую активность в качестве ингибитора киназ и модулятора ионного канала, составляющие соответственно -0.24 и -0.13. Как лиганд рецепторов, сопряжённых с G-белком, ингибитор протеаз и ингибитор ферментов молекула имеет среднее значение, составляя 0.15, 0.19, 0.10 соответственно.

Из литературных данных известно, что антиагрегантный препарат прасургрел является пролекарством и быстро метаболизируется в печени до активного метаболита – R-138727. При молекулярном моделировании метаболита с P2Y₁₂ рецептором человека была подтверждена его способность связываться в активном центре фермента. Данное исследование подтверждает, что метаболит R-138727 обладает биоактивностью в организме человека, а также возможность использования прасургрела в качестве антитромботического препарата для профилактики атеротромботических осложнений, инфаркта миокарда, инсульта и других заболеваний сердечно-сосудистой системы связанных с повышенной реактивностью тромбоцитов.

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THE COMPARISON OF INTRAEPITHELIAL LYMPHOCYTES IN SMALL AND LARGE INTESTINE OF CROHN'S DISEASE PATIENTS

СРАВНИТЕЛЬНАЯ ХАРАКТЕРИСТИКА ИНТРАЗПИТЕЛИАЛЬНЫХ ЛИМФОЦИТОВ ТОНКОЙ И ТОЛСТОЙ КИШКИ У ПАЦИЕНТОВ С БОЛЕЗНЬЮ КРОНА

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The changes in intraepithelial lymphocytes phenotype of the small and large intestine were established in patients with Crohn's disease what may be used as a hallmark of immune inflammation in the gut and make intraepithelial lymphocytes ideal candidate for targeting in further immunoregulation of mucosal adaptive immune response against autoantigens.

У пациентов с болезнью Крона установлены фенотипические изменения интраэпителиальных лимфоцитов тонкой и толстой кишки, что может являться биомаркером иммунного воспаления в желудочно-кишечном тракте и позволяет рассматривать интраэпителиальные лимфоциты в качестве терапевтической мишени при иммунорегуляции адаптивного мукозального иммунного ответа на аутоантигены.

Ключевые слова: Интраэпителиальные лимфоциты, тонкая кишка, толстая кишка, Болезнь Крона, аутоиммунное воспаление.

Keywords: Intraepithelial lymphocytes, small intestine, colon, Crohn's disease, autoimmune inflammation.

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Introduction. Intestinal intraepithelial lymphocytes (IELs) are a heterogeneous population of lymphoid cells, which localizes between the intestinal epithelial cells that form the intestinal mucosal barrier. IELs distributed in the small intestine and large intestine; 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. Induced IELs express either CD8 $\alpha\beta$ + or CD4+T-cell receptor ($\alpha\beta$ +TCRs) phenotype and migrate to the periphery in response to antigenic stimulation via the upregulation of gut homing markers. Natural IELs exhibit an antigen-experienced phenotype in response to self-antigen during thymic maturation: either the $\alpha\beta$ + or $\gamma\delta$ +TCRs and are typically CD8 $\alpha\alpha$ +, but lack CD8 $\alpha\beta$ or CD4 co-receptors, and migrate to the intestinal epithelium. Moreover, CD8 $\alpha\alpha$ +IELs can develop extrathymically within cryptopatches or isolated lymphoid follicles in mucosa so the role of the thymus in natural IELs development remains controversial [1].

Once IELs traffic to the intestine, these cells become tissue resident and do not recirculate. The relative frequency of individual IEL subtypes differs in dependence on the intestine area. The number and proportion of IEL populations are differed between humans and influenced by housing conditions, depending on the level of antigenic stimulation in the intestine. IELs subsets are characterized with an antigen-experienced cytolytic effector phenotype, but the antigenic reactivity is regulated by their function within the intestinal epithelium under physiological or pathological (intestinal injury and inflammation) conditions [2].

The differentiation, activation and functional specialization of all IELs subsets are defined by interactions with other cell types and soluble factors as well as are influenced by ecological factors like dietary and microbial products in the gut. The dynamic interactions between environmental cues and the mucosal adaptive immune system help maintain a stable ratio and sustain barrier function. Addition to this, 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 inflammatory bowel diseases (IBD) or promote cancer development and progression [3].

Two chronic inflammatory diseases of the gastrointestinal tract – Crohn’s disease and ulcerative colitis – refers to IBD and are 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. Current investigations indicate that the etiology of IBD is multifactorial, with environmental, microbial, genetic, and immunological components contributing to the pathophysiology of disease. An imbalance between regulatory and cytolytic effector lymphoid cells within the epithelium results in a dysregulation of mucosal immunity 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 [4].

Nowadays there are limited data for a role of IELs in IBD. It is reported the correlation of the disease severity and the increase in the number of $\gamma\delta$ TCR+IELs in ulcerative colitis and Crohn’s disease. Recent paper defined a novel subset of human CD8 $\alpha\beta$ + $\gamma\delta$ T-cells expressing and reported that the numbers of this IELs correlate inversely with disease severity, and are restored to levels observed in healthy controls upon treatment, suggesting their role in mucosal regeneration in IBD. But there are also studies on IELs roles in the pathological conditions. So the data about IELs in preventing or reducing susceptibility to IBD remain under investigations [2, 4].

The aim of the study was to estimate intraepithelial lymphocytes phenotype in small and large intestine from patients with Crohn’s disease.

Materials and methods. Samples of small intestine and colon mucosa were obtained from CD patient (n=5) and healthy donor (n=3) during scheduled surgeries. CD diagnosis was confirmed by histological examination of the sample. The mucosal layer separation step is presented at figure 1.

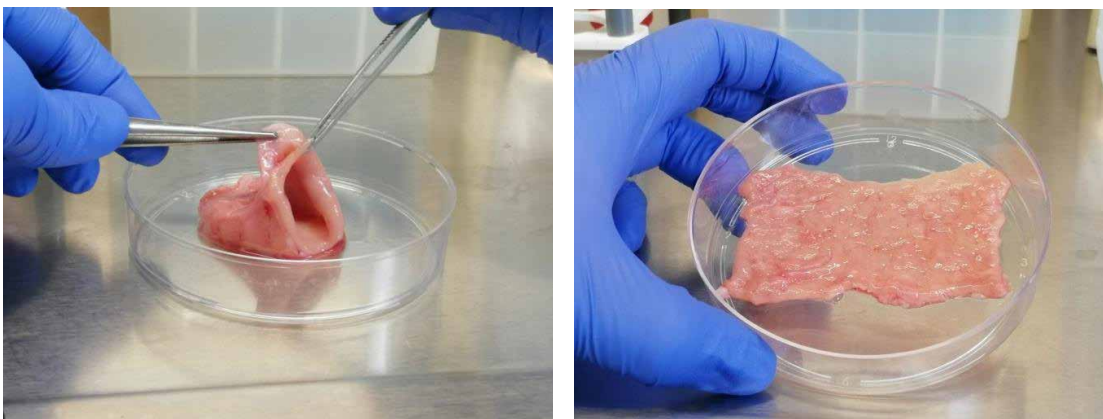


Fig. 1 – The mucosal layer separation step from colon of healthy donor

IELs isolation was performed according to Trapecare et al. [5]. Briefly, the specimens were cut into 1–5 mm² fragments and incubated for 1 h under intense shaking: the mucosal fragments were placed inside a 50ml tube contained in a larger tube that was taped to a rotor. The medium contained 2mM DTT and 5mM EDTA in RPMI 1640 (Gibco Life Technologies, Germany) supplemented with 10% fetal calf serum (FCS) and mixtures of antibiotics and antimycotic (Gibco Life Technologies, Germany). A single cell suspension was obtained by filtering through a 70mm sterile filter (Sarstedt, Germany), washed in phosphate-buffer saline (PBS) and layered onto the 40%-60% Percoll gradient. The

gradients were centrifuged at 1000 g for 20 min. The cell fraction between 40–60% Percoll was the most enriched for IEL and washed twice in PBS with 10% of FCS (figure 2).

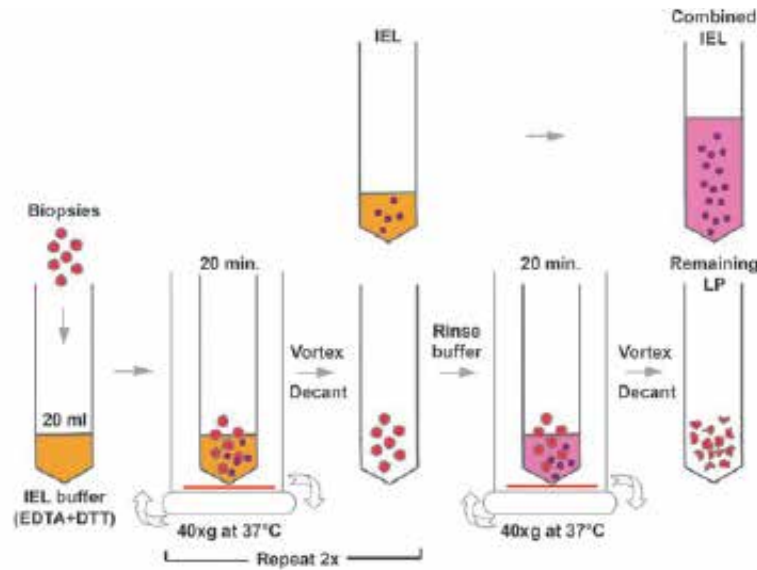


Fig. 2 – IELs isolation algorithm [5]

For immunophenotyping, 2×10^5 IEL were stained with 10 μ l 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) and incubated at 20–25°C for 15 minutes in the dark. The aliveness and the phenotype were measured on 10000 IEL using flow cytometer Cytoflex (Beckman Coulter, USA). The algorithm of lymphoid cells analysis is presented at figure 3.

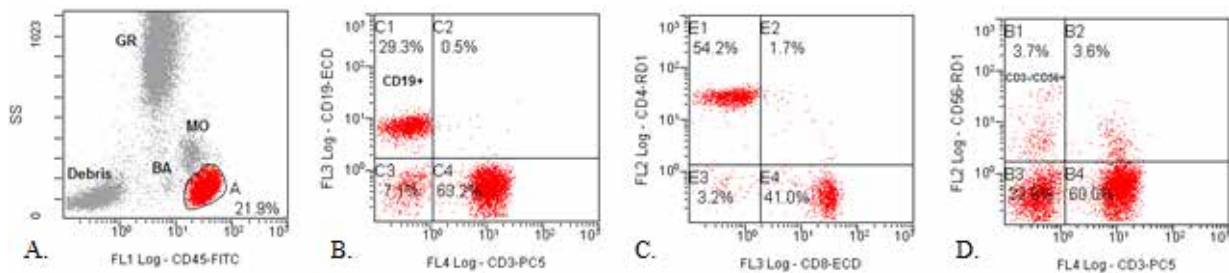


Fig. 3 – The algorithm of lymphoid cells analysis

Note: 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

Statistical analysis was made using Statistica 8.0.

Results. After isolation, IELs quantity from CD patient colon was higher than from healthy donor as well as cells number per tissue cm²: $8.72 \times 10^5/\text{cm}^2$ – in CD patients and $4.3 \times 10^5/\text{cm}^2$ – in donors. The investigation of IEL 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).

The results of IELs phenotype using four-color flow cytometry analysis are presented in the table 1.

Table 1 – Immunophenotype of IELs in Crohn’s disease patients and healthy donors

Groups	Diagnosis	Gut samples	n	CD3 ⁺ T-cells	CD4 ⁺ T-helpers	CD8 ⁺ T-cells	CD19 ⁺ B-cells	CD56 ⁺ NK-cells
Group 1	Crohn’s disease	Small intestine	5	87,3* (73,4÷90,2)	22,7 (8,4÷57,5)	62,1 (38,4÷66,2)	10,9* (6,2÷27,5)	10,1 (7,2÷12,2)
Group 2	Crohn’s disease	Colon	5	63,1* (54,3÷80,3)	47,9 (17,8÷54,6)	47,1 (36,2÷51,0)	21,1* (14,9÷26,3)	14,1 (11,8÷20,9)
Group 3	Healthy donors	Colon	3	53,2 (28,5÷69,1)	48,5 (30,7÷70,8)	60,7 (28,6÷66,3)	32,0 (22,6÷66,1)	9,9 (9,4÷26,6)

In CD patients the number of CD3⁺IELs in small and large intestine were increased as compared to healthy donors. Moreover, CD3⁺IELs were predominated in small intestine in CD patients. At the same time CD19⁺B-cells were decreased in all gut samples from CD patients as compared to healthy donors. While there were no differences in the percent of

CD56⁺NK-cells in investigated groups (table 1). These results suppose the involvement of T-cells in the pathogenesis of Crohn's disease.

The decrease of CD3⁺CD4⁺T-helper in small intestine as compared to large intestine with a tendency to increase of CD3⁺CD8⁺cytotoxic T-lymphocytes in small intestine (immunoregulatory ratio=0,4 (0,1÷2,7)) were established in CD patients. 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=1.2 in CD patients but the increase of cytotoxic T-cells IEL compared to T-helper IEL with CD4⁺/CD8⁺ ratio=0,73 (0,5÷2,4) in healthy donor what corresponded to literature data.

According to literature data Crohn's disease are also generally thought to be driven by aberrant CD4⁺ IEL and LPL responses, in this case 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⁺ induced 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. 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 IFN γ 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. Further studies are also needed to distinguish the exact contribution made by IELs in the inflamed intestine from that made by infiltrating systemic and lamina propria T cells.

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 (NKR). 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 $\alpha\alpha$ ⁺ IEL subset. This IEL 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 CD8 $\alpha\alpha$ 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.

Conclusion. In CD patients T-lymphocytes are involved in intestinal inflammation and play the major role in disease immunopathogenesis. Moreover, the disturbance of T-helper and cytotoxic cells balance was established in CD patients colon characterizing with increased number of CD4⁺IELs and decreased number of CD8⁺IELs what reflect the aberrant effector T-cell function.

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