УДК 616.345-006.6:577.218:577.27
https://doi.org/10.20538/1682-0363-2025-4-78-86

Galectin-1 and Galectin-3 as Modulators of Systemic CD4+ T-lymphocyte Balance in Colorectal Cancer

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ABSTRACT

Aim. To evaluate the relationships between plasma levels of galectin-1 and galectin-3 and the composition of $CD4^+$ T-lymphocyte subpopulations (Th1, Th17, and Treg) in patients with colorectal cancer (CRC) and to determine a direct *in vitro* modulatory effect of tumor-associated galectin-1 and galectin-3 on the expression of key T-lymphocyte transcriptional factors (T-bet, RORC2, and Foxp3).

Materials and methods. The study included 26 patients with CRC and 17 healthy donors. Plasma concentrations of galectin-1 and galectin-3 were measured by enzyme-linked immunosorbent assay. Lymphocyte subpopulations were analyzed by flow cytometry. To assess the *in vitro* immunomodulatory effects of galectin-1 and galectin-3, a Transwell co-culture model of the colon adenocarcinoma cell line COLO 201 and peripheral blood mononuclear cells (PBMCs) from CRC patients was used, employing the selective galectin-1 inhibitor OTX008 and the galectin-3 inhibitor GB1107. The mRNA expression of target genes was evaluated by quantitative real-time polymerase chain reaction.

Results. Patients with CRC exhibited a decreased proportion of circulating Th1 and Th17 lymphocytes and an increased frequency of Treg cells, which is most pronounced in advanced disease stages. Plasma levels of galectin-1 and galectin-3 were also elevated. Galectin-1 concentration correlated negatively with Th1 and Th17 levels and positively with Treg levels. In contrast, the galectin-3 level was inversely associated only with the Th1 lymphocyte pool. Inhibition of galectin-1 and galectin-3 in the *in vitro* COLO 201/PBMC co-culture system induced increased mRNA expression of T-bet and RORC2 and decreased expression of Foxp3.

Conclusion. High concentrations of galectin-1 and galectin-3 in the blood of CRC patients are associated with systemic suppression of circulating $CD4^+$ T-lymphocytes. We demonstrated a direct *in vitro* modulatory effect of tumor-associated galectin-1 and galectin-3 on the differentiation of CRC patients' blood $CD4^+$ T-lymphocytes. These findings support the prospective use of targeted blockade of galectin-1 and galectin-3 in combination with existing immunotherapies for colorectal cancer.

Keywords: galectins, T-lymphocytes, immunosuppression, colorectal cancer, immunophenotype

Conflict of interest. The authors declare no obvious or potential conflicts of interest related to the publication of this article.

Source of financing. This research was carried out with support from the Russian Science Foundation, grant No. 25-25-20109, https://rscf.ru/project/25-25-20109/, and a subsidy grant allocated by the Department of Scientific, Technological Development and Innovative Activities of Tomsk Region, Agreement No. 02/3/2025 (experiments *in vitro*).

Conformity with the principles of ethics. All patients and donors signed an informed consenf to parficipafe in the study.

For citation: Poletika V.S., Reingardt G.V., Kurnosenko A.V., Kolobovnikova Yu.V., Urazova O.I. Galectin-1 and galectin-3 as modulators of systemic CD4+ T-lymphocyte balance in colorectal cancer. *Bulletin of Siberian Medicine.* 2025;24(4):78–86. https://doi.org/10.20538/1682-0363-2025-4-78-86.

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Галектин-1 и галектин-3 как модуляторы системного баланса CD4+ Т-лимфоцитов при колоректальном раке

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РЕЗЮМЕ

Цель. Оценка взаимосвязей между уровнем галектина-1 и галектина-3 в плазме крови и субпопуляционным составом $CD4^+$ Т-лимфоцитов (Th1, Th17 и Treg) у больных колоректальным раком (КРР), а также определение *in vitro* прямого модулирующего влияния опухоль-ассоциированных галектинов 1 и 3 на экспрессию ключевых транскрипционных факторов (T-bet, RORC2, Foxp3) Т-лимфоцитов.

Материалы и методы. В исследование включены 26 пациентов с КРР и 17 здоровых доноров. Концентрацию галектинов 1 и 3 в плазме крови определяли с помощью иммуноферментного анализа. Субпопуляции лимфоцитов анализировали методом проточной цитофлуориметрии. Для оценки *in vitro* иммуномодулирующего действия галектинов 1 и 3 использовали модель Transwell-сокультивирования клеточной линии аденокарциномы толстой кишки COLO 201 и мононуклеарных лейкоцитов крови больных КРР с применением селективных ингибиторов галектина-1 (OTX008) и галектина-3 (GB1107). Экспрессию мРНК изучаемых генов оценивали методом полимеразной цепной реакции в реальном времени.

Результаты. У больных КРР выявлено снижение доли циркулирующих в крови Th1- и Th17-лимфоцитов и увеличение уровня Treg-клеток, наиболее выраженных на поздних стадиях заболевания, а также повышение содержания галектина-1 и галектина-3 в плазме крови. Концентрация галектина-1 отрицательно коррелировала с содержанием Th1 и Th17 и положительно – с долей Treg, в то время как уровень галектина-3 был обратно взаимосвязан с содержанием Th1-лимфоцитов. Ингибирование галектина-1 и галектина-3 в *in vitro* совместной культуре клеток аденокарциномы толстого кишечника COLO 201 и мононуклеарных лейкоцитов больных КРР индуцировало повышение экспрессии мРНК T-bet и RORC2 и снижение экспрессии Foxp3.

Заключение. Высокие концентрации галектина-1 и галектина-3 в крови больных КРР ассоциированы с системной супрессией циркулирующих в крови $CD4^+$ Т-лимфоцитов. Показано прямое *in vitro* модулирующее влияние опухоль-ассоциированных галектинов 1 и 3 на дифференцировку $CD4^+$ Т-лимфоцитов крови пациентов с КРР. Полученные результаты обосновывают перспективы таргетного блокирования галектина-1 и галектина-3 в комбинации с существующими методами иммунотерапии колоректального рака.

Ключевые слова: галектины, Т-лимфоциты, иммуносупрессия, колоректальный рак, иммунофенотип

Конфликт интересов. Авторы декларируют отсутствие явных и потенциальных конфликтов интересов, связанных с публикацией настоящей статьи.

Источник финансирования. Исследование выполнено при финансовой поддержке РНФ (грант № 25-25-20109, https://rscf.ru/project/25-25-20109/) и гранта в форме субсидии, выделяемого Администрацией Томской области (Соглашение № 02/3/2025, работы *in vitro*.

Соответствие принципам этики. Все пациенты подписали информированное согласие на участие в исследовании. Исследование одобрено этическим комитетом СибГМУ (протокол № 8514/1 от 21.12.2020).

Для цитирования: Полетика В.С., Рейнгардт Г.В., Курносенко А.В., Колобовникова Ю.В., Уразова О.И. Галектин-1 и галектин-3 как модуляторы системного баланса CD4+ Т-лимфоцитов при колоректальном раке. *Бюллетень сибирской медицины.* 2025;24(4):78–86. https://doi.org/10.20538/1682-0363-2025-4-78-86.

INTRODUCTION

Colorectal cancer (CRC) remains one of the most socially sensitive oncological diseases [1]. Despite active development of modern anti-tumor therapies, including immune checkpoint inhibitors and other immunotherapeutic drugs, their clinical efficacy in CRC remains underwhelming [2]. Among the factors limiting the success of immunotherapy in CRC patients, tumor-induced systemic immunosuppression plays a key role [3].

Although the most significant alterations in the composition and functional state of immune cells occur within the tumor microenvironment, the immunosuppressive influence of the tumor extends far beyond its boundaries, affecting lymphoid organs and immune cells circulating in the peripheral blood. The latter leads to an imbalance of pro- and anti-inflammatory mediators, exhaustion of effector T-lymphocytes, and accumulation of myeloid-derived suppressor cells (MDSCs) and regulatory T-cells (Tregs) [4, 5]. This not only contributes to tumor progression but also significantly reduces the efficacy of immunotherapeutic drugs, as effector lymphocytes are rendered incapable of mounting a robust response even after immune checkpoint blockade [5, 6].

Alongside tumor-associated metabolic disorders and chronic inflammation, soluble mediators (modulators) secreted by malignant cells are considered to play a substantial role in the pathogenesis of systemic immunosuppression in CRC [7, 8]. Among these modulators are the β-galactoside-binding lectins galectin-1 and galectin-3, whose overexpression is characteristic of CRC cells [9, 10]. Galectins-1 and -3 are known to possess immunomodulatory potential towards cells of the adaptive immunity; however, the vast majority of studies on the immunotropic effects of these lectins have focused on their local action within the tumor microenvironment [11–13]. Despite significant progress in understanding the biology of carbohydrate–protein interactions, the question of how galectins-1 and -3 affect the subpopulations of circulating $CD4^+$ T-lymphocytes in CRC remains open. Elucidating the contribution of galectin-1 and galectin-3 to the development of systemic immunosuppression is of fundamental importance for developing new combined therapeutic strategies for CRC aimed at restoring immune homeostasis.

The aim of this study was to assess the relationships between the plasma levels of galectin-1 and galectin-3 and the subpopulation composition of $CD4^+$ T-lymphocytes (Th1, Th17, and Treg) in patients with CRC, as well as to determine the direct modulating influence of tumor-associated galectins 1 and 3 on the expression of key transcriptional factors (T-bet, RORC2, and Foxp3) in T-lymphocytes *in vitro*.

MATERIALS AND METHODS

The study included 26 patients with a histologically verified diagnosis of CRC (14 men and 12 women, mean age 62.9 ± 6.7 years). CRC staging was performed according to the international TNM classification (8th Edition, AJCC 2017). Of these patients, 15 were diagnosed with stage 0–II CRC (T0-4 N0 M0), and 11 patients had stage III–IV disease (T1-4 N1-2 M0-1). The control group consisted of 17 apparently healthy donors (11 men and 6 women, mean age 58.2 ± 3.1 years). Exclusion criteria were as follows: prior neoadjuvant chemotherapy or radiotherapy, the presence of other malignant neoplasms, and acute or chronic inflammatory diseases (in the acute phase).

Peripheral venous blood (20 ml) was collected from the cubital vein of all participants in the morning after an overnight fast. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll density gradient centrifugation on Ficoll. The plasma concentrations of galectin-1 and galectin-3 were determined by enzyme-linked immunosorbent assay (ELISA) using commercial reagent kits (BosterBio, USA).

The immunophenotyping of $CD4^+$ T-lymphocyte subpopulations (Th1, Th17, and Treg) in the PBMC suspension was performed using flow cytometry. Cells were stained with fluorochrome-conjugated monoclonal antibodies (Alexa Fluor 488, PerCP-Cy5.5, APC, PE; BD Biosciences, USA; RnD Systems, USA) against the surface antigen CD4 and the intracellular transcription factors T-bet, RORC2, and Foxp3. Analysis was conducted on an Accuri C6 flow cytometer (BD Biosciences, USA). Results were expressed as a percentage of the total lymphocyte count.

To determine the *in vitro* influence of tumor-associated galectin-1 and -3 on the expression of regulatory genes for T-lymphocyte differentiation, we developed a Transwell co-culture model using the human colon adenocarcinoma cell line COLO 201 (ATCC, USA) and PBMCs from CRC patients. Cells were cultured in complete RPMI-1640 medium (Elabscience, USA) supplemented with fetal bovine serum (Thermo Fisher Scientific, USA) and gentamicin (PanEco, Russia). The experiment utilized 24-well Transwell plates with semi-permeable membranes (0.4 μm, Sigma-Aldrich, USA). Tumor cells were seeded in

the lower chambers, while PBMCs isolated from patient blood, supplemented with phytohemagglutinin-P (10 μg/ml, PanEco, Russia), were placed in the upper chambers. Culturing was performed under the following conditions: an intact (control) co-culture and co-cultures with the addition of a galectin-1 inhibitor (OTX008, 2 μM) and a galectin-3 inhibitor (GB1107, 1 μM). Incubation lasted for 72 hours under standard conditions (37°C, 5% CO_2).

Following incubation, cells from the upper chambers were collected for molecular genetic analysis. Total RNA was extracted using the RNeasy Plus Mini Kit (QIAGEN, Germany), and RNA quality was assessed on a Multiskan Ex spectrophotometer (Thermo Fisher Scientific, USA); cDNA was synthesized by reverse transcription using the REVERTA-L kit (AmpliSens, Russia). The quantitative evaluation of mRNA expression for the target genes was performed by real-time polymerase chain reaction (RT-PCR) using the 5X qPCRmix-HS SYBR reaction mix (Eurogen, Russia) and specific primers (*tbx21*: F: 5'-CAGAATGCCGAGATTACTC-3'; R: 5'-AGGATACTGGTTGGGTAGGA-3'; *rorc*: F: 5'-CTGCTGAGAAGGACAGGGAG-3'; R: 5'-AGTTCTGCTGACGGGTGC-3'; *foxp3:* F: 5'-GCACATTCCCAGAGTTCCTC-3'; R: 5'-CAGTGGTAGATCTCATTGAGTGTC-3'; *β-actin*: F: 5'-TCGAGCAAGAGATGGCCAC-3'; R: 5'-AGGAAGGAAGGCTGGAAG-3'). The mRNA expression level of the *β-actin* gene was used for reference normalization. The relative quantity of cDNA in the samples was calculated using the ΔΔCt method and expressed in relative units (RU).

Statistical analysis was performed using IBM SPSS Statistics 27 software (IBM, USA). The normality of data distribution was assessed using the Shapiro–Wilk test. Quantitative measures were presented as the median and the interquartile range, *Me* (Q_1; Q_3). The Mann–Whitney *U* test was used for comparing two independent samples, and the Wilcoxon test was used for two dependent samples. Correlation analysis was performed by calculating the Spearman's rank correlation coefficient. Results were considered statistically significant at $p < 0.05$.

RESULTS

Flow cytometry analysis revealed significant alterations in the $CD4^+$ T-cell compartment in the peripheral blood of CRC patients. The percentage of Th1 lymphocytes ($CD4^+T\text{-}bet^+$) was 0.82% (0.24; 0.94), demonstrating a 1.5-fold decrease (p = 0.045) compared to the control group – 1.24% (0.48; 2.43). Furthermore, patients with CRC showed a 2.4-fold reduction (p = 0.005) in the proportion of Th17 lymphocytes ($CD4^+RORC2^+$) – 1.44% (0.19; 2.13) versus 3.51% (1.56; 4.79) in healthy donors. Conversely, the level of regulatory T-cells (Tregs, $CD4^+Foxp3^+$) in the blood of CRC patients was 1.19% (0.8; 1.48), which was 2.2 times higher (p = 0.011) than the corresponding value in the control group – 0.55% (0.23; 0.98) (Fig. 1).

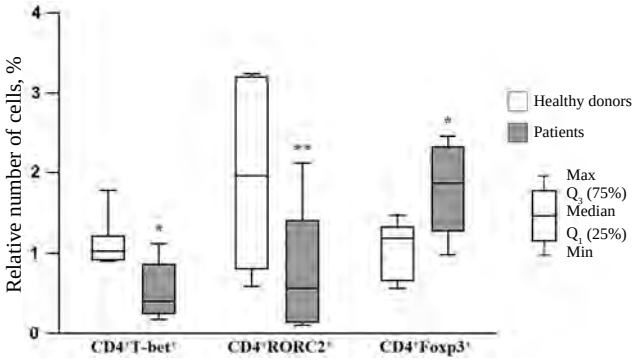

Fig. 1. Relative number of Th1, Th17, and Treg lymphocytes in the peripheral blood in patients with colorectal cancer and healthy donors, % of total lymphocytes: * $p < 0.05$; ** – $p <$ 0.01 compared to a similar indicator in healthy donors.

Stratification of CRC patients based on the tumor stage revealed that the relative content of Th1 1.03% (0.91; 1.23) and Th17 lymphocytes 1.96% (0.8; 3.21) was higher in patients with stage 0–II disease compared to those with advanced stages (III–IV) 0.4% (0.24; 0.86), p = 0.011 and 0.56% (0.13; 1.42), p = 0.038, respectively. In contrast, the proportion of Treg cells was 1.6 times lower (p = 0.017) in patients with stage 0–II CRC than in those with stage III–IV disease 1.19% (0.65; 1.34) vs. 1.87% (1.27; 2.34) (Fig. 2).

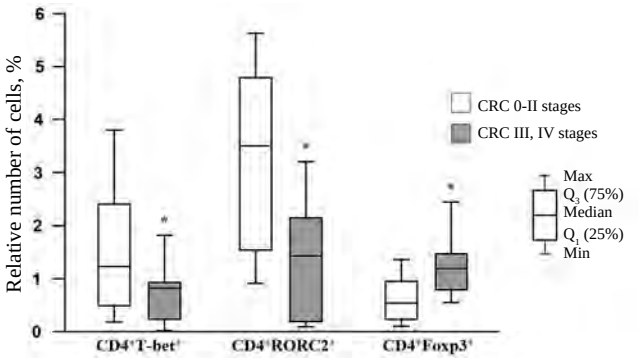

Fig. 2.– Relative number of Th1, Th17, and Treg lymphocytes in the peripheral blood in patients with colorectal cancer stratified by the disease stage, % of total lymphocytes: * $p <$ 0.05 compared to patients with stage 0–II CRC

According to ELISA, the plasma concentration of galectin-1 in CRC patients was 1.2 times higher (p = 0.003) than in the healthy donor group 16.17 (15.31; 17.10) vs. 13.74 (12.23; 14.79) ng/ml, respectively. Furthermore, the plasma level of galectin-1 in CRC patients correlated negatively with the content of Th1 (r = –0.56; p = 0.035) and Th17 lymphocytes (r = –0.59; p = 0.033) and correlated positively with the proportion of Tregs (r = 0.55; p = 0.035). The plasma concentration of galectin-3 was also elevated in CRC patients: 3.28 (2.30; 5.71) ng/ml compared to the control group 1.56 (1.19; 2.17) ng/ml in the control group (p = 0.006), but correlated only with the relative content of Th1 lymphocytes (r = –0.81; p = 0.001).

To test the hypothesis of a direct modulatory effect of tumor-derived galectins-1 and -3 on $CD4^+$ T-lymphocyte differentiation, we conducted an *in vitro* co-culture of the human colon adenocarcinoma cell line COLO 201 with PBMCs from CRC patients in the presence or absence of selective galectin-1 and galectin-3 inhibitors.

Blocking galectin-1 with the selective inhibitor OTX008 in *in vitro* co-cultures of COLO 201 cells and patient PBMCs led to a statistically significant increase in the mRNA expression of the key Th1 transcriptional factor T-bet from 1.23 (0.88; 1.60) to 2.28 (1.81; 2.58) RU, p = 0.012 and the Th17 marker RORC2 from 0.28 (0.23; 0.39) to 1.71 (1.22; 1.83) RU, p = 0.012 compared to control co-cultures. Conversely, the mRNA expression level of Foxp3, which regulates Treg differentiation, decreased from 6.25 (5.67; 7.45) to 3.48 (2.86; 4.11) RU, p = 0.012 (Fig. 3). The addition of the selective galectin-3 inhibitor GB1107 to the *in vitro* co-cultures induced unidirectional changes in the studied transcription factors: an increase in T-bet mRNA expression from 1.23 (0.88; 1.60) to 2.97 (2.83; 3.4) RU, p = 0.012 and RORC2 mRNA expression from 0.28 (0.23; 0.39) to 0.79 (0.57; 0.99) RU, p = 0.012, alongside a decrease in Foxp3 mRNA expression from 6.25 (5.67; 7.45) to 4.86 (4.26; 5.35) RU, p = 0.012 relative to the control co-cultures without inhibitors (Fig. 3).

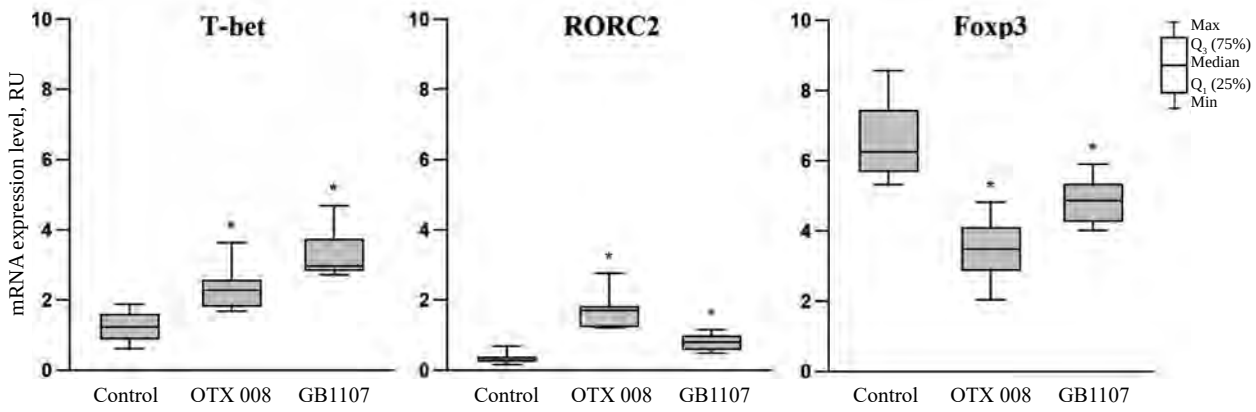

Fig. 3. mRNA expression level of the transcriptional factors T-bet, RORC2, and Foxp3 in *in vitro* cell cultures, RU: * $p < 0.05$ compared to the control co-culture

DISCUSSION

The results of our study confirm the presence of an imbalance in circulating $CD4^+$ T-lymphocytes in patients with CRC characterized by a reduction in effector subpopulations (Th1 and Th17) and an expansion of the immunosuppressive Treg cell pool.

The observed deficit in circulating $CD4^+$T-bet^+ Th1 lymphocytes, most pronounced in advanced-stage CRC, serves as a key indicator of systemic immune dysfunction and may contribute to immune evasion by malignant cells and tumor progression. Type 1 T-helper cells play a central role in orchestrating an effective anti-tumor immune response. Through the production of interferon (IFN)γ, as well as direct cell-to-cell contact, Th1 cells not only enhance the tumoricidal potential of tumor-infiltrating $CD8^+$ cytotoxic T-lymphocytes (CTLs) but also facilitate the presentation of tumor antigens by macrophages and dendritic cells and directly suppress cancer cell proliferation and tumor-associated angiogenesis [14, 15].

The relative content of $CD4^+$$RORC2^+$ Th17 lymphocytes in the blood of CRC patients was also reduced, particularly in patients with regional and distant metastases. Interpreting these results is complicated by the functional plasticity of the Th17

lymphocyte subset. Some researchers point to a predominantly pro-tumorigenic role for these cells (mediated by IL-17-dependent induction of tumor-associated inflammation and neoangiogenesis) in CRC pathogenesis [16–18]. However, under certain conditions, Th17 cells can exert anti-tumor effects by recruiting $CD8^+$ CTLs and neutrophils to the tumor site [19]. In the context of our study, the significant reduction in the Th17 pool in patients with metastatic CRC may indicate general insufficiency of the T-cell arm of adaptive immunity, despite the potentially pro-tumorigenic properties of these cells within the local tumor microenvironment.

Parallel to the deficit in effector Th1 and Th17 lymphocytes, we recorded an increase in the proportion of $CD4^+Foxp3^+$ Treg cells in the blood of CRC patients, reaching maximum values in patients with stage III and IV disease. According to current literature, Tregs are the primary inducers of immunological tolerance to tumor antigens. The tolerogenic potential of Treg cells is mediated by several complementary mechanisms, including the secretion of immunosuppressive cytokines (IL (interleukin)-10 and TGF (transforming growth factor)-β), direct cytolytic action on effector lymphocytes, expression of inhibitory molecules, such as PD (programmed cell death protein)-L1 and CTLA (cytotoxic T-lymphocyte-associated protein)-4, and the suppression of antigen-presenting cells [20, 21]. The expansion of Tregs in the peripheral circulation creates a barrier to an effective anti-tumor immune response and is a negative prognostic factor in CRC and other malignancies [20].

The mechanisms of systemic T-cell immune dysregulation in malignant colon tumors are multifaceted. Competitive consumption of glucose and amino acids by tumor cells disrupts the energy homeostasis and proliferative potential of effector T-lymphocytes [22], while lactate accumulation resulting from the Warburg effect in tumor cells suppresses type 1 and type 17 T-helpers while simultaneously stimulating Treg function [23]. Tumor-associated chronic inflammation, accompanied by elevated levels of circulating cytokines IL-1β, IL-6, and TNF (tumor necrosis factor)-α, leads to the expansion of myeloid-derived suppressor cells and Tregs against a background of CTL suppression and dysfunction of antigen-presenting cells, characterized by reduced MHC (major histocompatibility complex) II and co-stimulatory molecule (CD (cluster of differentiation)80/CD86) expression [24, 25]. Furthermore, malignant cells and elements of the tumor microenvironment secrete a wide spectrum of soluble mediators, including prostaglandin E2 (PGE2), adenosine, IL-10, and TGFβ, which modulate immune cell activity [26, 27]. The results of our study substantiate the involvement of galectin-1 and galectin-3, produced by malignant cells, in the development of tumor-induced immunosuppression in CRC.

The negative correlation between the plasma concentration of galectin-1 and the relative number of $CD4^+T\text{-}bet^+$ Th1 and $CD4^+RORC2^+$ Th17 lymphocytes and its positive correlation with the content of $CD4^+Foxp3^+$ Treg cells in the peripheral blood of CRC patients suggests a possible systemic tolerogenic influence of this lectin. Immunotropic action of galectin-1 is mediated by its binding to β-galactoside residues of membrane glycoproteins. For instance, the interaction of galectin-1 with CD45, CD43, CD7, and components of the T-cell receptor can induce apoptosis in activated lymphocytes [28]. Th1 and Th17 lymphocytes are most sensitive to the pro-apoptotic effect of galectin-1, which is associated with the specific glycosylation patterns of their membrane glycoconjugates that serve as ligands for this lectin [28, 29]. *In vitro* experiments have demonstrated other mechanisms for the immunomodulatory action of galectin-1, including the regulation of cytokine secretory activity, clonal expansion, and antigen-dependent differentiation of target lymphocytes [30]. The latter point is supported by our results, which show that selective inhibition of galectin-1 in a co-culture of PBMCs from CRC patients and COLO 201 colon adenocarcinoma cells led to increased expression of mRNA for the transcriptional factors controlling Th1 and Th17 development (T-bet and RORC2, respectively), and decreased expression of the Foxp3, which determines Treg cell differentiation.

In contrast to galectin-1, whose plasma level in CRC patients correlated with the relative content of all studied T-lymphocyte subpopulations, the concentration of galectin-3 correlated only with the number of $CD4^+T\text{-}bet^+$ type 1 T-helpers. Simultaneously, in co-cultures of COLO 201 cells and PBMCs from CRC patients, selective blockade of galectin-3 induced changes similar to those observed with galectin-1 inhibition (increased T-bet and RORC2 mRNA expression and suppressed Foxp3 expression). According to the literature, the biological activity of recombinant galectin-3 varies significantly depending on its local concentration [31, 32]. Furthermore, galectin-3-mediated

regulation of T-lymphocyte viability, function, and polarization is mediated not only through direct contact with the target cell but also via modulation of antigen-presenting cell activity in peripheral tissues [33, 34]. The relatively low level of galectin-3 (4.9 times lower than the concentration of galectin-1) in the blood of CRC patients appears insufficient for exerting a direct tolerogenic effect on the circulating pool of Th17 and Treg lymphocytes. On the other hand, our *in vitro* Transwell co-culture system replicated conditions approximating the tumor microenvironment, where the immunoregulatory activity of galectin-3 is more pronounced [35, 36].

CONCLUSION

This study demonstrates that in patients with colorectal cancer, elevated blood concentrations of galectin-1 and galectin-3 are associated with systemic suppression of T-cell immunity, manifesting as a reduced number of circulating Th1 and Th17 lymphocytes alongside a concomitant increase in Treg cells. The results of selectively inhibiting galectin-1 and galectin-3 in an *in vitro* co-culture of COLO 201 colon adenocarcinoma cells and peripheral blood mononuclear cells from CRC patients confirm the direct modulatory influence of soluble forms of galectins 1 and 3 on the expression of genes controlling the differentiation of $CD4^+$ T-lymphocytes towards either effector (Th1, Th17) or regulatory (Treg) phenotypes.

These findings contribute to the existing body of knowledge on the mechanisms of immune evasion in malignant colon tumors and substantiate the prospects of targeted galectin-1 and galectin-3 blockade in combination with existing immunotherapeutic strategies for colorectal cancer.

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Received on April 4, 2025;
approved after peer review on August 5, 2025;
accepted on September 9, 2025