

# Tumour Lymphocytic Infiltration, Its Structure and Influence in Colorectal Cancer Progression

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## Abstract

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**BACKGROUND:** The role of the immune system in the control of tumour progression has been stressed, recently. Many studies indicate the fact that the immune system can prevent tumour progression in several types of human malignant neoplasms including colorectal cancer. According to some authors, a higher density of "tumour-associated lymphocytes" (TAL), in malignant neoplasms, correlate with prolonged survival of patients.

**AIM:** This study aims to determine the structure and the influence of the immune cells, TAL, in the progression of colorectal cancer (CRC).

**PATIENTS AND METHODS:** The study included 103 patients with CRC operated at the University Clinic of Digestive Surgery in Skopje, whose operative material was analysed at the Institute of Pathology, Medical Faculty in Skopje. The structure of tumor-associated cells and their density were determined and were correlated with neoplasm's grade, local growth (T), positive lymph nodes, lymphatic invasion and stage of the disease.

**RESULTS:** CD4+, CD8+ and CD20+ lymphocytes (Ly) were found in TAL. The density of TAL was significantly different in neoplasms with different T status, lymphatic invasion, patients with and without nodal metastasis and patients with a different stage of the disease. The density of CD4+, CD8+, and CD20+ cells were significantly different in neoplasms with different T. The density of CD8+ and CD20+ lymphocytes was lower in patients with nodal metastasis and higher stage.

**CONCLUSION:** The density of tumor-associated lymphocytes can anticipate the disease progression in patients with colorectal cancer, and the density of TAL influences the control of tumour progression.

## Introduction

Colorectal cancer (CRC) is the third most common malignant disease in the humans, with 570,000 new cases in the world each year. It is the second most common cause for the lethal outcome of malignancies in the West European countries and eighth in the developing countries. Despite the new surgical techniques and treatment with neoadjuvant and adjuvant chemotherapy, colorectal cancer is the most common tumour of the gastrointestinal tract which incidence and mortality continually increase [1]

[2]. Prognosis of colorectal cancer mostly depends on the stage of disease (local growth, positive lymph nodes and distant metastasis-TNM classification) and depends on many other factors such as lymphovascular invasion, histological grade, positive surgical margins, preoperative serum values of carcinoembryonic antigen (CEA) bowel obstruction and many others [3] [4] [5] [6]. It is considered that new biomarkers such as APC, K-ras, DDS, p53, BRAF, TGF- $\beta$ , CIMP and others can give more pieces of information for disease progression. The finding that some patients with certain mutations (KRAS), who have metastatic disease, do not respond

to the treatment with monoclonal antibodies represents the basis in the efforts for discovering personalised therapies [7] [8] [9] [10] [11]. This is a motivation for continued investigation of new prognostic factors to determine the disease progression and patient outcome as well as to improve the knowledge for developing new therapies. The role of the immune system in the control of disease progression is the basis for the development of immunotherapy in patients with malignant diseases and is a current topic for research in the field of oncology [9] [10] [11].

The idea that the immune system can recognise a neoplasm was introduced by Paul Ehrlich and was supported by Lewis Thomas and Macfarlane Burnet. Over the course of time, this idea was turned into a concept in which it is considered that the immune cells are capable of eliminating malignant cells. This is how the theory for "immune surveillance" was born. This theory supports hypotheses that the immune system is capable of surveillance of the newly appeared malignant cells and their elimination [12] [13] [14] [15] [16].

It is well known that colorectal cancer progression is influenced by inter-reaction between cancer cells and tumour microenvironment belonging to the patient [12] [13] [14].

It is considered that the immune cells present in tumour-associated stroma are in correlation with tumour progression and patient outcome. According to some authors, the high density of memory T-cells and cytotoxic T-cells in malignant neoplasms (tumour-associated lymphocytes-TAL) is in correlation with prolonged survival of patients [17] [18] [19].

Analysis of stromal inflammatory infiltrate, and tumour-infiltrating lymphocytes in CRC has not attracted the attention of researchers in the Republic of Macedonia recently, but new therapeutic approaches in the treatment of patients with CRC need new basic investigations to provide better management of these patients. According to that, we aimed to determine the density of TAL, the type of some immune cells, "tumour-associated lymphocytes", and their influence on CRC progression.

## Material and Methods

The study included 103 patients with CRC, 68 (66.2%) male, and 35 (33.98%) female with the age of the patients ranged from 33 to 97 years, the mean age  $64.57 \pm 11.5$  years. All of them underwent surgery in our institution, and their operative material was routinely analysed for histopathological diagnosis and pTNM staging at the Institute of Pathology and was

additionally analysed for TAL.

In this study the following parameters were examined: localisation of a tumour, local growth (T status), and grade of tumour differentiation (G), the presence of positive lymph nodes (LN) as well as the stage and the density of TAL.

The type of TAL was defined by immunohistochemical staining with antibodies against CD4 (Dako Monoclonal Mouse Anti-Human CD4, Clone 4B12); CD8 (Dako Monoclonal Mouse Anti-Human CD8, Clone C8/144B); CD20 (Dako Monoclonal Mouse Anti-Human CD20, Clone L26); with a standard procedure using Immunoperoxidase LSAB + system.

The density of TAL was determined semiquantitatively. The density of all cells (TAL) was determined in the first step in 4 grades and the density of positively stained cells from the total number of TAL for each marker individually was determined in the second step in the same manner as absent (-), scanty (+), moderate (++) and abundant (+++). To confirm consistency of grading, the cases were scored independently by two investigators. Examples of grading TAL and immune cell staining and their grading are shown in Figure 1.

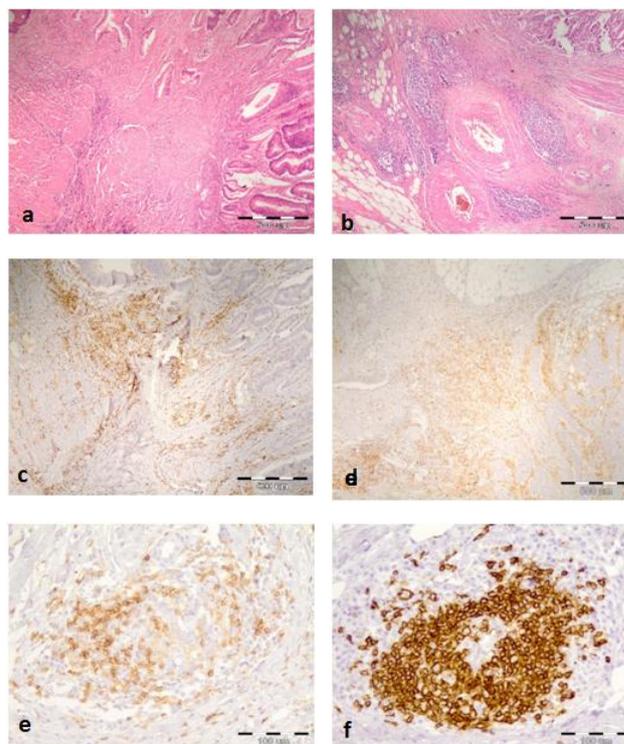


Figure 1: a) Scanty (+) Ly infiltration in the tumor invasive front (HE $\times$ 40) b) Abundant (+++) Ly infiltration in the tumor invasive front (Hex40) c) Moderate (++) CD4 Ly infiltration (x40) d) Abundant CD4+ Ly infiltration (x40) e) Higher magnification of CD4+ cells (x200) f) Higher magnification of CD20+ cells in the tumor invasive front of CRC (x200)

## Statistical analysis

Descriptive statistical methods were used for

statistical analysis of the data. Categorical variables are presented with absolute and relative numbers (%). Fisher's exact test was used for comparison of categorical variables. Spearman's correlation coefficient was used to determine the degree of correlation between analysed parameters. The statistical program SPSS for Windows, version 19.0 was used.

## Results

### Density of TAL

Distribution of patients and density of TAL according to the localisation of a tumour are shown in Table 1.

**Table 1: Distribution of patients and density of TAL according to the cancer localisation**

Localization	No of patients	Quantity of TAL		
		Scanty (No = 59)	Moderate (No = 32)	Abundant (No = 12)
Coecum	12 (11.65%)	8 (66.67)	3 (25)	1 (8.33)
Colon asc	9 (8.74%)	6 (66.67)	3 (33.33)	0
Flex hepat	3 (2.91%)	1 (33.33)	2 (66.67)	0
Colon trans	5 (4.85%)	2 (40)	2 (40)	1 (20)
Flex lienilis	3 (2.91%)	2 (66.67)	1 (33.33)	0
Colon des	3 (2.91%)	3 (100)	0	0
Sigma	29 (28.16%)	17 (58.62)	7 (24.14)	5 (17.24)
Rectosig	8 (7.77%)	5 (62.5)	2 (25)	1 (12.5)
Rectum	31 (30.09%)	15 (48.39)	12 (38.71)	4 (12.9)

Stage of the disease and local growth of CRC, i.e. T category according to the TNM classification for colorectal cancer (AJCC, 2010), number of patients with negative and positive lymph nodes, number of cases with and without lymphatic invasion, and the grade of differentiation in the analysed series are illustrated in Table 2. The largest number of examined patients was with a tumour in T3 and T4 local growth without metastatic deposits in the local lymph nodes, without lymphatic invasion and with moderately differentiated neoplasms. The density of tumor-associated lymphocytes about the parameters mentioned above is presented in the same Table.

**Table 2: Density of TAL according to the stage of disease, T category and nodal status and lymphatic invasion**

Stage	No of patients	Quantity (Density) of TAL			P-value
		Scanty	Moderate	Abundant	
I	11	1 (9.09)	5 (45.45)	5 (45.45)	<0.0001**
II	45	15 (35.71)	23 (22.33)	7 (16.67)	
III	41	37 (35.92)	4 (9.09)	0	
IV	6	3 (50)	3 (50)	0	
T category					
1	5	0	4 (80)	1 (20)	0.0018**
2	7	1 (14.29)	2 (28.57)	4 (57.14)	
3	60	35 (58.33)	20 (33.33)	5 (8.33)	
4	31	23 (74.19)	6 (19.35)	2 (6.45)	
N category					
N0	56	17 (30.36)	27 (48.21)	12 (21.43)	0.0001**
N1/N2	47	42 (89.36)	5 (10.64)	0	
L category					
L0	54	20 (37.04)	26 (48.15)	8 (14.81)	0.00005**
L1	49	39 (79.59)	6 (12.24)	4 (8.16)	

P (Kruskal-Wallis test) \*P < 0.05; \*\*P < 0.01.

The density of TAL was higher in patients with lower stage I and II of the disease, in tumours with less local invasion, in patients without nodal metastasis, and in tumours with no lymphatic invasion. On the contrary, in stage III and IV of the disease, the density of TAL was low in the most of the analysed cases and the tumour infiltrating lymphocytes was scanty in patients with advanced local growth, with nodal metastasis and with lymphatic invasion.

Statistical analysis did not show statistical differentiation in the quantity of TAL according to tumour differentiation (P > 0.05).

### Different cell types of TAL and their density in CRC

CD20, CD4 and CD8 positive cells in different quantity were found in all analysed cases.

### CD20+ Lymphocytes

The density of CD20+ tumour-associated lymphocytes about the stage of disease, T and N category, and lymphatic invasion is presented in Table 3.

**Table 3: Density of CD20+ cells according to the tumour stage, T and N category, and lymphatic invasion**

Stage	N° of patients	The quantity of CD20+ Ly			P-value	
		CD20+ Ly not present	Scanty	Moderate		Abundant
I	11	0	0	6 (54.55)	5 (45.45)	<0.0001**
II	45	1 (2.38)	24 (23.30)	16 (38.1)	4 (9.52)	
III	41	0	36 (34.95)	4 (9.09)	1 (2.27)	
IV	6	0	6 (100)	0	0	
T category						
1	5	0	0	3 (60)	2 (40)	0.0002**
2	7	0	0	4 (57.14)	3 (42.86)	
3	60	1 (1.67)	41 (68.33)	13 (21.67)	5 (8.33)	
4	31	0	25 (80.65)	6 (19.35)	0	
N category						
N0	56	1 (1.79)	25 (44.64)	21 (37.5)	9 (16.07)	<0.0001**
N1/N2	47	0	41 (87.23)	5 (10.64)	1 (2.13)	
L category						
L0	54	1 (1.85)	26 (48.15)	18 (33.33)	9 (16.67)	0.001**
L1	49	0	40 (81.63)	8 (16.33)	1 (2.04)	

P (Kruskal-Wallis test) \*P < 0.05; \*\*P < 0.01.

We found a significant difference in the density of CD20+ lymphocyte (Ly) in all analysed parameters.

Scanty CD20+ Ly infiltrates found in patients with advanced local growth, with nodal metastases, lymphatic invasion and with a higher stage of the disease (100% Stage IV, 88.64% Stage III, 50% Stage II and 0% of patients in Stage I). The density of CD20+ Ly infiltrate lower (scanty) in patients with poorly differentiated tumours but without a statistically significant difference (P = 0.089).

### CD4+ Lymphocytes

The density of CD4+ tumour-associated lymphocytes about the stage of disease, T and N

category, and lymphatic invasion is presented in Table 4.

**Table 4: Density of CD4+ tumour-associated lymphocytes about the stage of disease, T and N category, and lymphatic invasion**

Stage	No of patients	The quantity of CD4+ Lymphocytes			P-value
		Scanty	Moderate	Abundant	
I	11	0	10 (90.91)	1 (9.09)	P = 0.11 ns
II	45	10 (23.81)	26 (25.24)	9 (21.43)	
III	41	4 (9.09)	34 (33.00)	3 (6.82)	
IV	6	0	6 (100)	0	
<b>T category</b>					
1	5	0	4 (80)	1 (20)	P = 0.034*
2	7	0	7 (100)	0	
3	60	6 (10)	46 (76.67)	8 (13.33)	
4	31	8 (25.81)	19 (61.29)	4 (12.9)	
<b>N category</b>					
N0	56	10 (17.86)	36 (64.29)	10 (17.86)	P = 0.055 ns
N1/N2	47	4 (8.51)	40 (85.11)	3 (6.38)	
<b>L category</b>					
L0	54	11 (20.37)	37 (68.52)	6 (11.11)	P = 0.11 ns
L1	49	3 (6.12)	39 (79.59)	7 (14.29)	

P (Kruskal-Wallis test) \*P < 0.05; \*\*P < 0.01.

The low density of CD4+ Ly was detected in 100% of tumours with advanced local tumour growth (T4 category) and on contrary CD4+ low Ly density was not detected in tumours with T1. The statistical analysis confirmed that the density of CD4+ lymphocytes in the infiltrative front of a tumour significantly depended on the local growth of the disease.

Patients with regional lymph nodes metastasis had a lower density of CD4+ Ly infiltrate than patients without metastasis, but there was no a statistically significant difference in the density of CD4+ Ly according to the presence of lymph nodes metastasis. No statistical significance was found in the density of CD4+ Ly infiltrate according to the Stage, lymphatic invasion and grade also.

### CD8 Lymphocytes

The density of CD8+ tumour-associated lymphocytes about the stage of disease, T and N category, and lymphatic invasion is presented in Table 5.

**Table 5: Density of CD8+ tumour-associated lymphocytes about the stage of disease, T and N category, and lymphatic invasion**

Stage	No of patients	The quantity of CD8+ Lymphocytes			p-value
		CD8+ Ly not present	scanty	moderate	
I	11	0	0	11 (100)	0.0055**
II	45	1 (2.38)	12 (28.57)	28 (66.67)	
III	41	0	24 (54.55)	20 (45.45)	
IV	6	0	3 (50)	3 (50)	
<b>T category</b>					
1	5	0	0	5 (100)	0.006**
2	7	0	0	7 (100)	
3	60	1 (1.67)	22 (36.67)	37 (61.67)	
4	31	0	17 (54.84)	13 (41.94)	
<b>N category</b>					
N0	56	1 (1.79)	15 (26.79)	39 (69.64)	0.024*
N1/N2	47	0	24 (51.06)	23 (48.94)	
<b>L category</b>					
L0	54	0	18 (33.33)	35 (64.81)	0.36 ns
L1	49	1 (2.04)	21 (42.86)	27 (55.1)	

P (Kruskal-Wallis test) \*P < 0.05; \*\*P < 0.01.

We found a significant difference in the

quantity of CD8+ Ly in all analysed parameters except lymphatic invasion.

The density of CD8+ Ly significantly differs according to the local tumour growth. The low density of CD8+ Ly infiltrate significantly more often was present in patients with T4 and T3 status (54.84%, 36.67% respectively); there were no patients with T1 and T2 with a low density of CD8+ Ly infiltrate.

The density of CD8+ Ly was significantly lower in patients with nodal metastasis, lymphatic invasion and with an advanced stage of the disease.

We did not find a statistical difference in the density of CD8+ Ly according to the histological differentiation of a tumour.

The moderate density of CD8+ lymphocytes decreased when the grade of the differentiation decreased, but the difference was not significant.

### Correlations

The correlations of the density of tumour-associated lymphocytes with T tumour status, the involvement of the regional lymph nodes, lymphatic invasion, stage and the grade of differentiation were done using the Spearman's correlation test. Correlations were determined between the different T cell types also.

The results showed a significant negative correlation between the density of CD20+ Ly and T status, stage and G, i.e. the density of CD20+ Ly was lower in less differentiated tumours with advanced local growth and higher stage. The density of CD20+ Ly was in positive correlation with the density of CD8+ Ly and the density of TAL (Table 6).

The density of CD8+ Ly was lower in tumours with advanced local growth and higher stage of the disease. Correlations were not statistically significant except for the density of CD8+ Ly and the stage of the disease (Table 6).

**Table 6: Correlations of the quantity of TAL and all other analysed parameters**

	Spearman R	t-test, P-level
CD20 / CD8	0.367	3.97, P = 0.00013**
CD20 / TAL	0.436	4.87, P = 0.000004**
CD20 / T	-0.407	4.48, P = 0.00002**
CD20 / Stage	-0.564	6.87, P < 0.0001**
CD20 / G	-0.220	2.27, P = 0.025*
<b>CD4</b>		
CD4 / CD 8	0.387	4.21, P = 0.000055**
CD4 / CD 20	0.016	0.16, P = 0.87
CD4 / TAL	0.342	3.66, P = 0.00041**
CD4 / T	-0.155	1.57, P = 0.12
CD4 / Stage	-0.034	0.34, P = 0.74
CD4 / G	-0.091	0.92, P = 0.36
<b>CD8</b>		
CD8 / TAL	0.381	4.14, P = 0.000073**
CD8 / T	-0.271	2.83, P = 0.0056**
CD8 / Stage	-0.324	3.44, P = 0.00084**
CD8 / G	-0.107	1.08, P = 0.28

\*P < 0.05; \*\*P < 0.01.

Statistically, a significant correlation was found between CD4+ Ly and CD8+ Ly and the total

amount of TAL (Table 6). The density of CD8+ Ly increased when the density of Total amount of TAL increased and when the density of CD4+ Ly increased (Table 6). There was also a significant positive correlation between CD20+ cells and CD8+ cells.

## Discussion

Rudolf Virchow, 150 years ago, was the first who described inflammatory cells in tumours and established the theory that tumours arise at chronic inflammation sites. It is known that the risk of developing colorectal cancer is greater in patients with ulcerative colitis and those with Crohn's disease. Despite the knowledge that chronic inflammation is a risk for development of a malignant neoplasm, many clinical and experimental studies stress the protective role of immune cells in cancer progression, hence, the statements and cognitions that immune response plays a key role in fighting tumour growth and its dissemination [7].

Some authors consider that the type, density and location of the immune cells in colorectal cancer can be superior and independent prognostic factor for tumour progression [7].

Cancer progression is a complex process that is influenced by genetic, epigenetic and environmental factors, but at the same time, it is a process of interaction between cancer cells and their microenvironment, that is, stroma. Tumour stroma is built of different cells among which are inflammatory cells that belong to congenital and inherited immune system [20] [21] [22]. Former investigations have shown that lymphocytes that infiltrate the tumour-tumour infiltrating lymphocytes (TIL/TAL) are predominantly T lymphocytes among which two subtypes are identified CD4+ and CD8+ T lymphocytes. CD4+ T lymphocytes are T helper cells, whereas CD8+ T lymphocytes are cytotoxic cells capable of killing the "target cell", i.e. tumour cell which exposed antigens CD8+ T lymphocytes are capable of recognising. They are able to destroy tumor cells in direct cell interaction by releasing lytic components [22] [23] [24] [25] [26] [27]. CD4+ T lymphocytes are helper cells that regulate their proliferation and coordinate the immune response by stimulating the other immune cells. They are stratified in subgroups T helper 1 (Th1) and T helper 2 (Th2) cells. Th1 CD4+ T lymphocytes are responsible for the proliferation of cytotoxic T lymphocytes that participate in the killing of tumour cells.

In addition to T lymphocytes, B CD20+ lymphocytes arranged in aggregates have been found among TAL together with other immune cells. In lymph glands and the remaining lymphoid tissue, B lymphocytes receive signals for growth, differentiation

and affinity maturation from follicular T helper immune cells. They differentiate into plasma cells and memory cells with long lifespan [28]. The process of lymphoid neogenesis is described in infectious diseases, chronic inflammation processes, autoimmune diseases and chronic graft rejection [29] [30] [31]. In these conditions, T and B cells cooperate to create a strong immune response when B cells enhance T cell response by antigen presentation, costimulation and modulation of migration and function of dendritic cells [28], [29].

Lymphoid neogenesis is also described in malignant neoplasms, while the role of CD20+ lymphocytes in tertiary lymphoid structures that are found in these neoplasms has not been completely clarified and it is still in the phase of research [29]. Nowadays many patients with malignant neoplasms develop tumour-specific antibodies, and B cells have a significant role in the immune response to neoplasm both by exacerbating cancer-associated inflammation and by the effect they have on T lymphocytes, macrophages and myeloid stem cells.

We found a statistically significant correlation between the examined subtypes of lymphocytes with a negative trend between CD4+ and CD20+ cells and CD8+, CD20+ cells and also a positive trend between CD4+ and CD8+. The correlation implies mutual connection in the function of these cells in one common immune response to tumour antigens.

TAL-tumour infiltrating lymphocytes are concentrated in the stroma of an invasive tumour front. Many authors examined the role of TAL in tumor progression in relation to survival of patients with colorectal cancer [23] [24] [25] [26] [32] [33].

Even though for a long time it was considered that colorectal cancer is a bad immunogenic tumor, the new cognitions suggest that there is a substantial immune response to this disease and that the presence of the immune response is connected with a better prognosis of patients with colorectal cancer, that is, the increased presence of T cytotoxic lymphocytes is associated with absence of early metastases and absence of early recurrence, as well as that the type of density and location of the immune cells have prognostic significance in colorectal cancer [7] [33] [34].

Therefore, tumour-associated lymphocytes can provide significant prognostic information about patients with colorectal cancer, which could be used for treatment with adjuvant therapy in a different stage of cancer [29].

The results from the analysis of TAL in the invasive front of cancer in our group of patients with colorectal cancer showed the presence of T (CD4+ and CD8+) and B (CD20+) lymphocytes and their density significantly correlated with clinically-pathologic parameters for disease progression. The density of TAL was higher in patients with lower stage

(I and II) of the disease, in tumours with less local invasion, in patients without nodal metastasis, and in tumours with no lymphatic invasion. The density of CD8+ and CD20+ lymphocytes in a neoplasm was higher in patients without metastases in lymph nodes and with a lower stage of the disease. Lower density of CD20+ lymphocytes was found in neoplasms of patients with advanced local growth and lymphatic tumor invasion. Thus, it can be concluded that the density of tumor-associated lymphocytes can be a parameter for neoplasm progression in patients with colorectal cancer and the density of tumor-associated lymphocytes influences the control of tumor progression.

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