



PERIOPERATIVE CHANGES IN LYMPHOCYTE SUBPOPULATIONS IN PATIENTS UNDERGOING SURGERY FOR COLORECTAL CANCER

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SUMMARY – Surgical procedure has immense impact on the immune balance. However, little is known about perioperative changes in T regulatory and Th17 lymphocyte subpopulations in patients undergoing colorectal resection. Patients with resectable colon cancer were enrolled in the study. Blood samples were obtained on two occasions, i.e. before the procedure and two days after the surgery. We also recruited a control group of young, healthy individuals. Lymphocyte subpopulations were analyzed with the use of flow cytometry. Investigated subpopulations consisted of total lymphocyte count, CD4+, CD8+, T regulatory Foxp3+ (Tregs), Th17 lymphocytes and white blood cell (WBC) count. There were significant differences in immune cell levels before and after the surgery. Reduction was recorded in the CD4+, CD8+, Tregs and total lymphocyte counts ($p=0.002$, $p=0.01$, $p=0.008$ and $p=0.001$, respectively). Increase was observed in total WBC and Th17 cells, however, Th17 lymphocytes did not reach statistical significance ($p=0.01$ and $p=0.5$, respectively). In conclusion, surgical intervention caused changes in all lymphocyte subpopulations investigated in patients undergoing surgery for colorectal cancer. However, it seemed to be an effect of perioperative trauma. Further studies are needed to investigate the impact of surgical intervention on lymphocyte subpopulations.

Key words: *Lymphocyte subsets; T-lymphocytes, regulatory; Th17 cells; Lymphocyte count; Colorectal surgery*

Introduction

Colorectal cancer affects 1.4 million people worldwide, causing 694,000 deaths. Novel treatment methods are improving long-term survival; however, the outcomes are still not satisfying. In the vast majority of cases, colorectal cancers are treated surgically followed by adjuvant therapy, which currently includes usage of monoclonal antibodies such as anti-VEGF bevacic-

zumab. There is continuing search for optimal therapy and currently involves checkpoint immunotherapies. Cancer development is accompanied by tissue inflammation. The tumor is infiltrated with different cells of the immune system. The composition of immune cells in peripheral blood of patients with colorectal cancer is under constant investigation¹. Interaction between different subtypes of immune cells seems to have crucial influence on overall survival². A special role is assigned to regulatory lymphocytes with expression of Foxp3+ molecule (Tregs)³. High concentration of Tregs in tumor tissue was demonstrated to worsen clinical outcome in pancreatic and hepatobiliary neoplasms⁴. Surgical procedure has a huge impact on immune bal-

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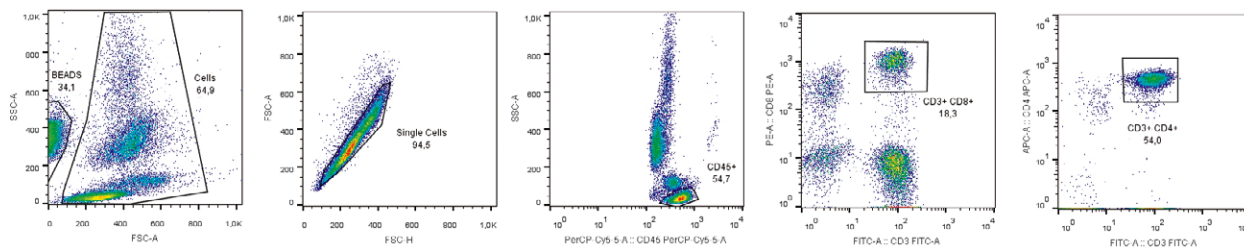


Fig. 1. Gating strategy for CD3+ CD4+ and CD3+CD8+ T cell identification in peripheral blood of colorectal cancer patients and healthy individuals.

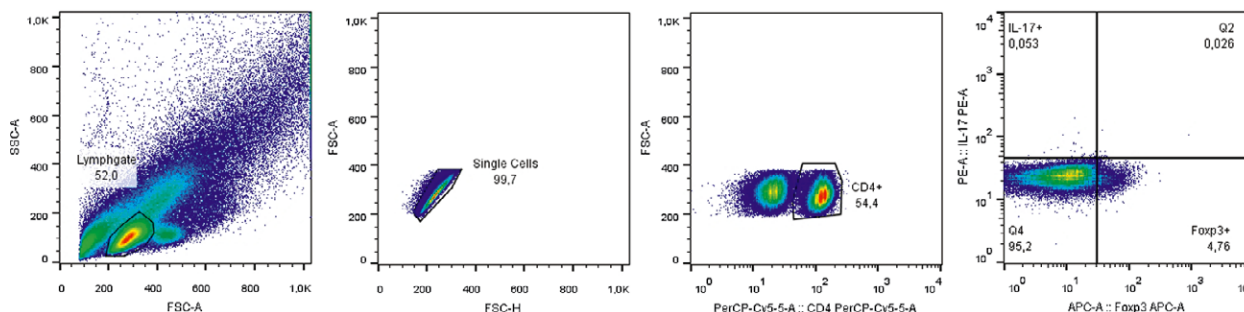


Fig. 2. Gating strategy for CD4+ Foxp3+ Tregs and CD4+ IL-17+ Th17 cell identification in peripheral blood of colorectal cancer patients and healthy individuals.

ance^{5,6}. However, studies focusing on perioperative changes in T regulatory and Th17 lymphocytes subpopulations are scarce.

The aim of the study was to assess the number of lymphocyte subtypes in perioperative period in patients with colon cancer.

Materials and Methods

Patients with resectable colon cancer were enrolled in the study. Patients had to fulfill the following inclusion criteria: age above 18 years, histologically proven colon cancer, and resectable lesion. Exclusion criteria were as follows: patients with distant metastases, patients with immune deficiencies, patients with colon cancer associated with inflammatory bowel disease, patients who had been treated with neoadjuvant chemo- or radiotherapy, and patients requiring blood transfusion during surgical procedure or in the postoperative course. The inclusion criteria were met by 14 patients. Blood samples were obtained on two occasions, i.e. before the procedure and two days after the surgery. We also recruited control group consisting of young, healthy subjects. Lymphocyte subpopulations were analyzed with the use of flow cytometry.

Isolation of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMC) were isolated by standard Ficoll-Paque (Pharmacia, Uppsala, Sweden) density gradient centrifugation from whole peripheral blood samples collected from colorectal cancer patients and healthy control subjects into EDTA containing tubes (Vacutainer System, Becton Dickinson, San Jose, CA, USA).

Determination of absolute number of CD3+CD4+ cells

Whole peripheral blood samples (25 μ L) were incubated in TruCount tubes (BD Biosciences, BD Biosciences, San Jose, CA, USA) with the following monoclonal antibodies: anti-CD3-FITC, anti-CD8-PE, anti-CD45-PerCP and anti-CD4-APC (BD Multitest TM, BD Biosciences, BD Biosciences, San Jose, CA, USA) for 30 minutes at 4 °C. Then the samples were treated with 250 μ L of FACS Lysing Solution (BD Biosciences, San Jose, CA, USA) and after erythrocyte lysis, 10,000 of CD3+CD4+ cells with beads was acquired on a FACSCanto flow cytometer using FACSDiva software. The absolute numbers of CD3+CD4+ and CD3+CD8+ lymphocytes were calcu-

lated on the basis of beads and lymphocyte counts, according to the formula below:

$$\left[\frac{\text{number of events in region containing CD3}^+\text{CD4}^+ \text{ or CD3}^+\text{CD8}^+ \text{ cells}}{\text{number of events in absolute count bead region}} \right] \times \left[\frac{\text{number of beads per test}}{\text{test volume}} \right] = \text{absolute number of CD3}^+\text{CD4}^+ \text{ or CD3}^+\text{CD8}^+ \text{ cells.}$$

Determination of Treg and Th17 cell count

For Tregs and Th17 cell analysis, PBMC were stained using Th17/Treg Human Phenotypic Kit (BD Pharmingen™, Canada) with the following mAbs: anti-CD4-PerCP, anti-IL-17A-PE and antiFoxp3-APC, according to the manufacturer's instructions. In parallel, a control sample was prepared to determine nonspecific cell binding of antibodies (isotype control). Samples were analyzed in FACSCanto flow cytometer using FACSDiva software. The absolute numbers of Treg cells were calculated on the basis of the CD4⁺Foxp3⁺, whereas Th17 cells were calculated as CD4⁺IL-17A⁺ cells both as percentage of CD4⁺ and absolute number of CD3⁺CD4⁺ cells. Gating strategies are presented in Figures 1 and 2.

Statistical analysis and ethical consideration

All data were analyzed with Statistica version 13.0 PL (StatSoft Inc., Tulsa, OK, USA). The results were expressed as mean and standard deviation (SD) and median. The χ^2 -test of independence was used to evaluate categorical variables. The Shapiro-Wilk test was used to check for normal distribution of data and the Student's *t* test was used for normally distributed quantitative data. For non-normally distributed quantitative variables, the Mann-Whitney *U* test was used. The level of statistical significance was set at $p < 0.05$.

All procedures were performed in accordance with ethical standards of the 1964 Declaration of Helsinki and its later amendments (Fortaleza 2013). The study was approved by the local Ethics Committee. Each patient signed an informed consent prior to inclusion in the study.

Results

Fourteen patients were eventually included in the analysis. Patient characteristics are presented in Table 1. Preoperative counts of CD4⁺, CD8⁺ and Tregs were

Table 1. Patient characteristics

General characteristics	
Age (years)	66 (38-80)
Weight (kg)	73.5 (55-97)
Height (cm)	165 (150-185)
Length of stay (days)	6 (3-11)
Morbidity	2
1-year recurrence	4
Tumor localization	
Localization	Number of patients
Cecum	4
Ascending colon	1
Transverse colon	0
Descending colon	1
Sigmoid colon	2
Sigmoid rectal junction	6
Tumor stage	
Stage	Number of patients
I	2
IIA	9
IIIB	2
IIIC	1

Table 2. Baseline number of immune cells in cancer group and control group

Cell type	Control group	Cancer group	p value
CD4 ⁺	672 (247-975)	818 (150-1491)	0.78
CD8 ⁺	320 (120-611)	336 (49-998)	0.98
T reg	47.8 (14.5-73.0)	47.16 (12.33-109.72)	0.84
Th 17	1.72 (0.74-5.28)	0.17 (0-4.47)	0.001

similar in the cancer group and healthy controls ($p=0.78$, $p=0.98$ and $p=0.94$, respectively). Only Th17 count was significantly higher in healthy controls than in cancer group ($p=0.001$) (Table 2).

All patients had colorectal cancer confirmed on postoperative pathological examination. There were no statistical differences in tumor staging and immune cell count (CD4⁺ $p=0.92$; CD8⁺ $p=0.96$; Th17 $p=0.42$; Treg $p=0.95$). No statistical significance was found regarding local recurrence at 1-year observation (CD4⁺ $p=0.62$; CD8⁺ $p=0.72$; Th17 $p=0.25$; Treg $p=0.72$).

Table 3. Number of immune cells before and after surgery

Cell type	Number before surgery (median, range)	Number after surgery (cells/uL, median, range)	p value
Cd4+ (cells/uL)	818 (150-1491)	321 (160-986)	0.002
Cd8+ (cells/uL)	336 (49-998)	260 (24-992)	0.01
Treg (cells/uL)	47.16 (12.33-109.72)	27.1 (9.44-123.25)	0.008
Th17 (cells/uL)	0.17 (0-4.47)	0.33 (0-10)	0.5
WBC ($10^3/\text{mm}^3$)	4.7 (2.4-10.3)	8.1 (4.9-13.3)	0.01
Lymphocyte count ($10^3/\text{mm}^3$)	1.35 (0.4-3.2)	1.1 (0.3-2.3)	0.01

There were significant differences in immune cell levels before and after the surgery. We observed reduced counts of CD4+, CD8+, Tregs and total lymphocyte ($p=0.002$, $p=0.01$, $p=0.008$ and $p=0.001$, respectively). We observed an increase in total WBC and Th17 cell count; however, Th17 lymphocytes did not reach statistical significance ($p=0.01$ and $p=0.5$, respectively). The results are summarized in Table 3.

Discussion

In our study, we found that cancer patients had a significantly lower level of Th17 lymphocytes than healthy controls. However, many authors report increased levels of Th17 cells in colorectal cancer patients^{7,8}. On the contrary, Ling *et al.* obtained results similar to ours. Not only were the levels of Th17 cells higher in healthy controls than in colorectal cancer patients, but also the number of Th17 cells correlated inversely with colorectal cancer stage⁹. Although Th17 lymphocytes are believed to enhance anti-tumor immunity, they may induce adverse reaction¹⁰. Th17 cells, as well as Tregs, accumulate in premetastatic niche, where they produce pro-tumorigenic factors such as VEGF-A¹¹. Moreover, Amicarella *et al.* report that Th17 cells may secrete pro- and anti-tumorigenic factors, depending on their localization, thus the final role of Th17 cells requires further investigation¹². Lee *et al.* report that higher levels of Th17 might be useful as markers of lymph node invasion¹³. In our group, only two patients had lymph node invasion, thus it was too small to verify this hypothesis.

Surgical removal of the tumor changed the composition of immune cells. We observed a significant increase in Th17 cell count, in comparison with the figure recorded in healthy controls. All of our patients

received minimally invasive approach, thus their immune system might have been less affected. However, Ling *et al.* obtained similar results, i.e. colorectal cancer resection caused Th17 lymphocyte count elevation⁹. An interesting study by Zhang *et al.* investigated the number of Tregs and NK cells in patients undergoing video-assisted thoracoscopic surgery (VATS) lobectomy or classic thoracotomy due to non-small cell lung carcinoma. In their research, the number of Tregs was lower after than before the surgery in both groups. However, at 90-day follow up, patients having undergone VATS lobectomy had lower Tregs count than thoracotomy group¹⁴. This may favor minimally invasive approach in cancer surgery. All our patients were operated with minimally invasive techniques, which are believed to cause less trauma to the patient. However, large studies including meta-analyses found no difference in long-term oncologic outcomes comparing open surgery and laparoscopic approach¹⁵⁻¹⁷. A small number of researches comparing open and laparoscopic surgery may stimulate novel study designs for perioperative immunoregulation.

Removal of the tumor also caused drop in the number of Foxp3 regulatory lymphocytes. However, their number after the surgery was lower than in healthy controls. The role of Tregs remains controversial. It is worth mentioning that we also found an increased number of Foxp3 cells in tumor microenvironment in early stages of colorectal carcinoma development, such as adenoma with high-grade dysplasia¹⁸. Waniczek *et al.* identified intense Tregs infiltration in tumor stroma as an independent factor of shorter disease-free survival and overall survival¹⁹. As mentioned before, lymphocyte infiltration in tumor environment rich in Foxp3 lymphocytes is a negative prognostic factor in many neoplasms. On the contrary, a recent meta-analysis including 3811 patients assessed the

prognostic value of Foxp3 lymphocytes in colorectal cancer microenvironment. Tregs infiltration in tumor stroma and epithelium was found to correlate positively with 1-, 3-, 5- and 10-year survival. However, this finding did not refer to disease-free survival²⁰. The study was also focused on tumor microenvironment, and Tregs number in peripheral blood was not assessed.

We are also aware that our study had some limitations. Firstly, study group had only 14 patients. More cases would significantly increase the study value and statistical significance of our results. Long-term control of lymphocyte subtypes would also be valuable, however, most of our patients underwent adjuvant chemotherapy or radiotherapy, which severely interferes with immune cell composition. Nevertheless, there are some data supporting the hypothesis that radiotherapy does not affect Tregs population²¹.

Conclusion

Surgical intervention caused changes in all lymphocyte subpopulations investigated in patients undergoing surgery for colorectal cancer. However, it seemed to be the effect of perioperative trauma. Additional studies are needed to investigate the impact of surgical intervention on lymphocyte subpopulations.

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Sažetak

PERIOPERACIJSKE PROMJENE SUBPOPULACIJA LIMFOCITA U BOLESNIKA OPERIRANIH ZBOG KOLOREKTALNOG KARCINOMA

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Kirurški zahvat ima golem učinak na ravnotežu imunog sustava. Međutim, malo se zna o perioperacijskim promjenama u subpopulacijama T regulatornih i Th17 limfocita u bolesnika podvrgnutih resekciji kolorektuma. U istraživanje su bili uključeni bolesnici s resektabilnim karcinomom kolona. Uzorci krvi prikupljeni su dva puta: prije zahvata i dva dana nakon operacije. Uključena je bila i kontrolna skupina sastavljena od mladih i zdravih osoba. Subpopulacije limfocita analizirane su protočnom citometrijom. Istraživane su sljedeće subpopulacije: ukupan broj limfocita, CD4+, CD8+, T regulatorni Foxp3+ (Tregs), Th17 i bijela krvna slika. Utvrđene su značajne razlike u razinama imuno stanica prije i nakon operacije. Snižene razine zabilježene su za CD4+, CD8+, Tregs i ukupni broj limfocita ($p=0,002$, $p=0,01$, $p=0,008$ odnosno $p=0,001$). Povišenje je zapaženo za bijelu krvnu sliku i Th17 stanice, međutim, Th17 limfociti nisu postigli statističku značajnost ($p=0,01$ odnosno $p=0,5$). Zaključujemo da je kirurški zahvat uzrokovao promjene u svim istraživanim subpopulacijama limfocita kod bolesnika podvrgnutih operaciji kolorektalnog karcinoma. Međutim, čini se da je to bio učinak perioperacijske traume. Potrebna su daljnja istraživanja kako bi se ispitaio utjecaj kirurškog zahvata na subpopulacije limfocita.

Ključne riječi: *Subpopulacije limfocita; T-limfociti, regulacijski; Th17 stanice; Broj limfocita; Kolorektalna kirurgija*