Original Research Article

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Determination of a CD4+CD25+ Foxp3^{+}T cells subset in Egyptian Colorectal Cancer Patients

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Abstract

Human tumors including colorectal cancers (CRC) are often infiltrated by immune cells predominantly T lymphocytes especially regulatory T (Treg) cells expressing the forkhead box protein 3 (Foxp3). It has been suggested that CD25^{+}CD4^{+}Foxp3^{+} regulatory T cells (Tregs) might hamper effective immunosurveillance of emerging cancer cell. The aim of this study was to measure the frequency of total CD4+CD25+ Tregs & CD4+CD25+Foxp3^{+} subset of Treg cells in peripheral blood of Egyptian CRC patients and their correlation with the tumor stage, histopathology of the tumor and lymph node affection. A total of 31 CRC patients were enrolled in the study. The tumor was categorized using a TNM staging system. Peripheral blood samples were collected within the first 24 h of surgery. The frequency of total CD4+CD25+ Tregs & CD4+CD25+ Foxp3^{+} subset of Treg cells in peripheral blood mononuclear cells (PBMCs) were measured by flow cytometry and absolute count was determined. High frequency of Tregs was detected in cancer patients with distal margin involvement (44-48 cells/µL) compared with those with free distal margin (5-32 cells/µL). Similarly, higher frequency of Tregs were detected (16-44 cells/µL) in cancers with lymph node involvement compared with cancers without lymph node involvement (5-32 cells/µL). Higher frequency of CD4+CD25+Foxp3+ Tregs were found in mucinous adenocarcinomas than in other histopathological types, although both observations were statistically insignificant. The median value for total absolute lymphocyte count/ µL was 639, out of which CD4+CD25+ subset constituted 35 cells, and about half of this subset were Foxp3+Tregs. In conclusion, CD4+CD25+Foxp3+ Tregs may be a useful marker for predicting invasion, metastasis, and prognosis of colorectal cancer in Egyptian patients.

Keywords: CD4+CD25+FoxP3+ cells; Colorectal cancer; T regulatory cell

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Introduction

Colorectal cancer (CRC) is the third most common cancer worldwide[1] and one of the most common cancers in those under 40 years in Egypt.[2,3] Human tumors including colorectal cancer are often infiltrated by immune cells predominantly T lymphocytes and myeloid

cells[4]. Among these are the regulatory T (Treg) cells expressing the forkhead box protein 3 (Foxp3) transcription factor.[5] Regulatory T cells (Tregs) were previously named suppressor T cells. They express several markers like CD4, Foxp3, and CD25, but unfortunately both effector T cells and Tregs express CD4 and CD25, so it is difficult to differentiate between them therefore we used Foxp3 expression to detect suppressor activity.[6]

Tregs are potent immunosuppressive cells & the main mediators of peripheral tolerance.[7] Tregs mediate their action through controlling T cells, B cells, natural killer cells, dendritic cells (DCs), and macrophages via humoral and cell-cell contact mechanisms.[8] These include secretion of immunosuppressive cytokines, suppression of dendritic cells and direct cytolytic activity.[9] They also impede immune surveillance against cancer cells by promoting immunological tolerance of these cells since many tumor antigens recognized by autologous lymphocytes are normal or slightly mutated self-antigens.[10,11] Thus, Treg-dependent suppression limits antitumor immunity and allows immune evasion by the growing tumor resulting in tumor progression.[12]

On the other hand, Tregs may also suppress & control inflammation, which in some cancers can halt tumor progression and thus benefit the host.[5] Abundant Foxp3$^+$ Treg population in tumors has been associated with poor clinical outcomes in different types of cancer like ovarian, breast, renal, and pancreatic cancer.[13-16] For example, higher Foxp3 expression was considered an independent prognostic factor in ovarian cancer.[21] It was also correlated with recurrence in patients with non-small cell lung carcinoma at pathologic stage I.[22] However, the role of Foxp3$^+$Treg cells remains controversial in CRC, since high densities of these cells in CRC correlate with either better or worse outcomes.[17-19]

There is convincing evidence that several tumor associated antigen-directed responses were unmasked after successful invitro depletion of Tregs in CRC patients. But it remains to be conclusively clarified whether these impeding effects on immune responses exerted by Tregs are not (over-) compensated by their beneficial role on inflammation.[20] As a result, Tregs have been drawing attention in cancer immunotherapy and as a prognostic factor in recent years.[18]

Detection of CD4+CD25+Foxp3+ lymphocyte by flow cytometry has emerged as the premier technique for studying cellular activation signals at a cellular level. Multiparameter flow cytometry permits simultaneous detection of two or more markers within a single cell, allowing direct T_H1 versus T reg cells determination. This capability, combined with the high throughput inherent in the instrumentation, gives cell membrane marker staining an enormous advantage over existing single-cell techniques such as ELISPOT, limiting dilution, and T cell cloning.[23] This study aimed to measure the frequency of total CD4+CD25+ Tregs & CD4+CD25+Foxp3$^+$ subset of Treg cells in peripheral blood of Egyptian CRC patients and to assess their correlation with the tumor stage, histopathology of the tumor and lymph node affection.

Patients and Methods

Patients

From May 2019 to January 2020, 31 consecutive colorectal cancer patients were enrolled in the study, all were from the Surgery Department in Ain Shams University Hospital. Specifically, colorectal cancer patients were considered eligible for the study if they consented to donate a blood sample within the first 24 hours of surgery and had no concomitant allergic, infectious, inflammatory, and/or autoimmune comorbidities. Patients were sub-grouped according to The TNM staging system, which describes the extent of malignancies - primarily on their anatomy - and categorizes each malignancy by the status of the primary tumor (T), nodal involvement (N) and metastatic disease (M). TX: primary tumor cannot be assessed, T0: no evidence of primary tumor, Tis: carcinoma in situ or intramucosal carcinoma, T1: tumor has grown through the muscularis mucosa into the submucosa, T2: grown into the muscularis propria, T3: cancer has grown into the outermost layers of the colon or rectum but has not gone through them, T4 cancer shows

direct extension into adjacent organs/tissues. NX: nodes cannot be assessed, N0: no regional nodal metastasis, N1: cancer has spread to 1 to 3 nearby lymph nodes or into areas of fat near the lymph nodes, N2: cancer has spread to 4 - 7 nearby lymph nodes, N3: cancer has spread to more than 7 lymph nodes. M0: no distant metastasis, M1: distant metastasis present.

Sample

Blood was collected within the first 24 hrs of surgery. Ten-milliliter of blood were withdrawn, divided into two tubes, one for total lymphocytic count and the other for detection of Tregs, by flowcytometry.

Flowcytometry

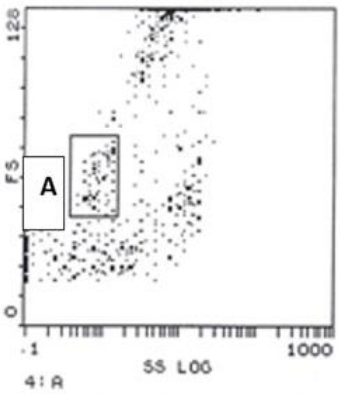
Determination of CD4+CD25+high, Foxp3 Tregs was done by detection of the combined expression of CD4+, CD25high and intracellular Foxp3 on peripheral blood lymphocytes using three colors EPICS XL flow cytometer (Coulter Electronics, Florida, USA). The test was done on lysed whole blood using the following monoclonal antibodies conjugated with different fluorescent dyes: Fluorescein isothiocyanate (FlTC)-conjugated anti-humanCD4, Phycoerythrin (PE)- conjugated anti-human CD25, Phycoerythrin- Cyanine 5 (PE-Cy5)-conjugated anti-human Foxp3, all were supplied by Miltenyi Biotec GmbH, Germany. According to the manufacturer's instructions: Fifty µL of EDTA anticoagulated whole blood was added to each tube which contain 1 mL lysing solution followed by wash with Phosphate buffered saline (PBS), then cell pellet was stained with combinations of the following antibodies (five µl each): anti-CD25-PE, anti-CD4-FITC in the test tube. Both tubes were then incubated in dark at room temperature for 20 minutes followed by single wash with PBS. The cell pellet was resuspended in 0.5 ml of freshly prepared fixation/permealization working solution and incubated for 30 minutes at 2-8°C in dark. This was followed by washing once with PBS then washing once again with 1ml of permealization buffer. Ten µL of anti-Foxp3-PE-Cy5 were added in test tube, and incubated for 30 minutes at 2-8°C in dark. This was followed by washing once with PBS then resuspended in 0.5 ml PBS for analysis. Lymphocytes were gated according to their forward and side scatter properties, and CD4 positive T cells were gated out of total lymphocytes. Tregs were identified by combined expression of CD4 + , CD25 and Foxp3[38]

Data acquisition and analysis were performed on EP-ICS XL flowcytometry using SYSTEM II version 3 software with a standard three-color filter configuration. Lymphocytes were identified according their size and complexity on the forward and side scatter plot Fig. (1-A). CD4+ T cells were identified based on CD4+ expression Fig. (1-B). CD25 high were identified relative to the intensity of CD25 on CD4cells dividing it into two parts on the plot, (CD4+CD25low) Fig. (1-G) and the (CD4 +CD25high) Fig. (1-F).

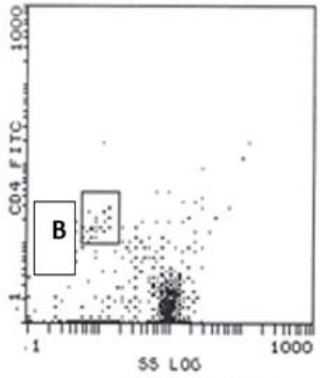
The absolute numbers of Foxp3+, CD25 and CD4 T cells were determined by multiplying the percentage of these cells in the lymphocyte gate by the number of circulating lymphocytes per microliter blood.[39]

Statistical analysis

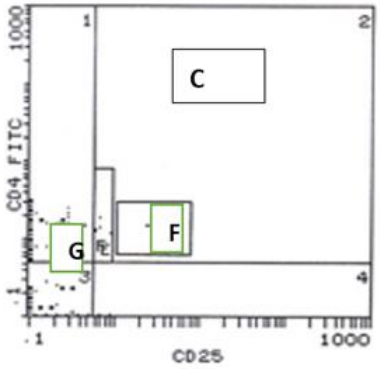
Data was collected, revised, coded and entered to the Statistical Package for Social Science (IBM SPSS) version 20. Qualitative data (like tumor staging , distal margin affection and type of surgery) presented as number and percentages while quantitative data were presented as mean, standard deviation and ranges.The comparison between two groups with qualitative data were done using *Chi-square test* and/or *Fisher exact test* was used instead of Chi-square test when the expected count in any cell was found to be less than 5. The comparison between two groups regarding quantitative data with parametric distribution was done by using Independent t-test. The confidence interval was set to 95% and the margin of error accepted was set to 5%. So, the p-value was considered significant at the level of < 0.05.



Lymphocyte gating using forward scatter versus side scatter



CD4+ cells gating



CD25·expression·on·CD4+·cells·CD4+·CD25·high·(region·F),·CD4+·CD25·low·(region·G)¶

Figure 1. Representative dot plots of flowcytometry and the gating strategy used (A to C): (A) show forward and side scatter to gate lymphocytes. (B) Show CD4 + cells were acquired after gating the lymphocyte population by forwardand side-scattered properties. (C), Gating approach for CD25+ cells and for discrimination between CD25high , CD25low on CD4+ cells. The gates for the CD25high and CD25low populations were set by comparing the CD25 expression levels of CD4 cells.

Results

The study included 31 colorectal cancer patients with ages ranging from 24 to 57 years old. The demographic data and characteristics of studied patients is provided in table (1). Most of the cancers were anatomically located in the lower rectum (14 patients) followed by the anal canal, caecum, mid-rectum & sigmoid colon (6, 5, 4, 2 patients respectively) as shown in Figure 2. A free distal margin was seen in 90% of tumors. Nineteen cases (61%) were T stage 3, 9 cases were stage 2 and 4 cases were stage 4. As regards N staging, most patients (65%) were N stage 0 where there was no lymph node metastasis.

Cancers were surgically resected by lower anterior resection (LAR) in 45% of cases, abdominal perineal resection (APR) in 39% & hemicolectomy in 16% of cases (Figure 3). Most of the CRC encountered in our patients were adenocarcinomas representing 45%, followed by moderately differentiated adenocarcinomas (23%), then mucinous & poorly differentiated adenocarcinomas (16% each) as shown in Figure 4.

Table 1. Demographic and laboratory characteristics in Egyptian colorectal cancer patients.

Variables		
Age (years)	Mean±SD	42.19 ± 14.85
	Range	24 – 67
TLC (Total leucocytic count)	Mean±SD	5.58 ± 2.47
	Range	3 – 13
HGB (hemoglobin)gm/dl	Mean±SD	10.19 ± 0.87
	Range	8 – 12
Platelets (Platelet /µL)	Mean±SD	279.32 ± 51.84
	Range	200 – 382
Tumor site	Anal canal	6 (19.4%)
	Caecum	5 (16.1%)
	Lower rectum	14 (45.2%)
	Mid rectum	4 (12.9%)
	Sigmoid colon	2 (6.5%)
Size of tumor	Mean±SD	4.26 ± 2.18
	Range	2 – 8
Distal margins	Free	28 (90.3%)
	Involved	3 (9.7%)
T staging	2	9 (29.0%)
	3	19 (61.3%)
	4	3 (9.7%)
N staging	0	20 (64.5%)
	1	4 (12.9%)
	2	7 (22.6%)
Operation	APR	12 (38.7%)
	Hemicolectomy	5 (16.1%)
	LAR	14 (45.2%)
Type of tumor	Adenocarcinoma	14 (45.2%)
	Moderately differentiated adenocarcinoma	7 (22.6%)
	Mucinous adeno	5 (16.1%)
	Poorly differentiated adenocarcinoma	5 (16.1%)

APR: Abdominal perineal resection LAR: Lower anterior resection

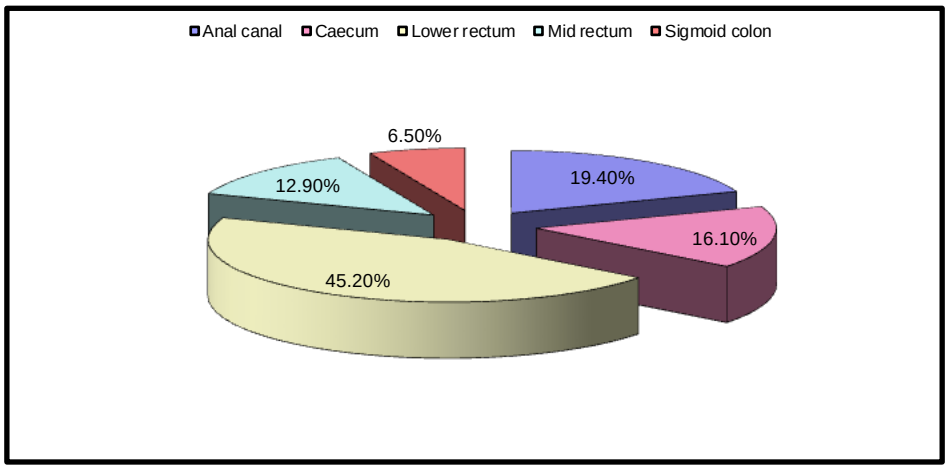


Figure 2. Anatomical distribution of tumor in Egyptian Colorectal Cancer patien**ts.**

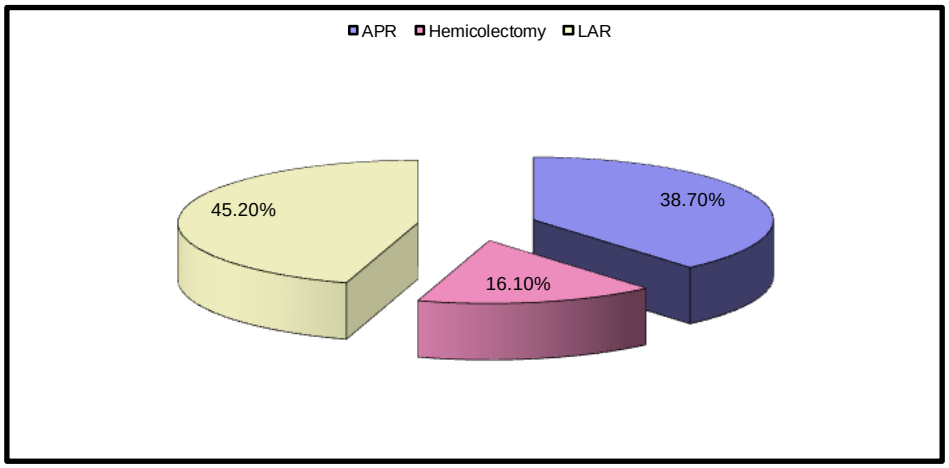


Figure 3. Surgical procedures used for resection of tumors.

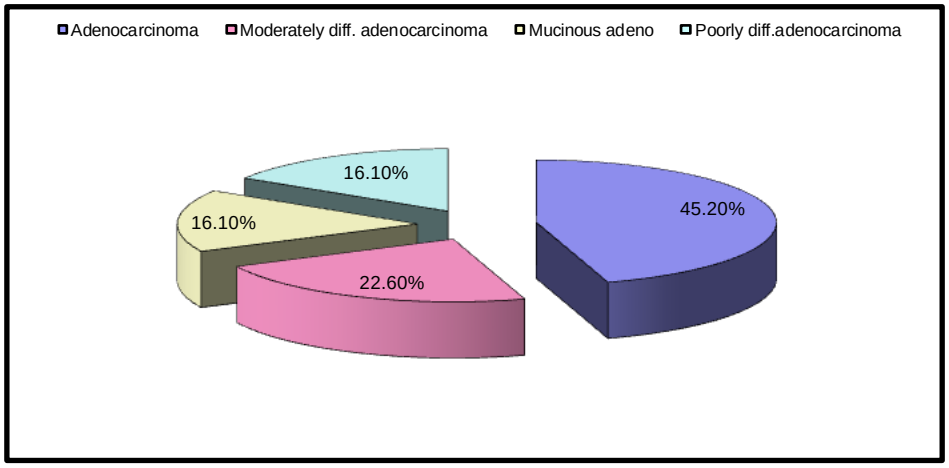


Figure 4. Histopathology of the tumor in Egyptian Colorectal Cancer patients.

The absolute lymphocytic count (ALC) and different Treg cells were measured in peripheral blood of CRC patients. Median of total absolute lymphocytes was 639, where CD4+/CD25+ Tregs constituted 35 (5.5%) of them, and half of these T regs were Foxp3+ (table 2).(Numbers were calculated by Flowcytometry).

Table 2. Absolute total lymphocytes and T reg subsets count in peripheral blood of CRC patients.

Cells/µL	Median (IQR) n=31	Range
Absolute lymphocytic count (ALC)	639 (190 – 889)	93 – 1340
CD4+CD25+	35 (13 – 63)	6 – 113
CD4+CD25+ Foxp3+	16 (5 – 32)	3 – 75

IQR: Interquartile range

Our study showed a strong positive correlation between the age of patients and the ALC in peripheral blood (Figure 5), a moderately positive correlation between hemoglobin level and absolute Treg cells with a slightly higher correlation with Foxp3^{+} Tregs (Table 3, Figure 6). We also found a strong positive correlation between the absolute counts of CD4+/CD25+ Tregs and CD4+/CD25+ Foxp3^{+} Tregs (Figure 7).

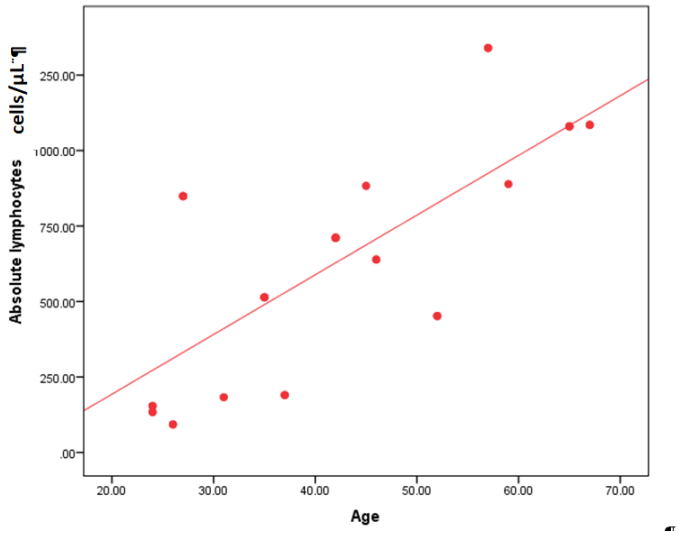


Figure 5. Correlation between Absolute lymphocytic count cells/µL & age (years).

Table 3. Relation of absolute number of different lymphocytes and treg subsets to other parameters in Egyptian Colorectal Cancer patients.

	Absolute lymphocytes		Absolute CD4+CD25+		Absolute CD4+CD25+FOXP3+	
	R	*P* -value	R	*P* -value	R	*P* -value
Absolute lymphocytes			0.217	NS	0.088	NS
AbsoluteCD4+CD25+	0.217	NS			0.954**	0.000
Absolute CD4+CD25+FOXP3+	0.088	NS	0.954**	0.000		
Age	0.778**	0.000	0.225	NS	0.102	NS
TLC	0.228	NS	0.047	NS	0.040	NS
HGB	0.347	NS	0.543**	0.002	0.613**	0.000
PLT	0.033	NS	0.300	NS	0.229	NS
Size of tumor	-0.292	NS	0.217	NS	0.137	NS

TLC: Total leucocytic count, HGB: Hemoglobin , PLT: Platelet. **P*>0.05 is not significant (NS).

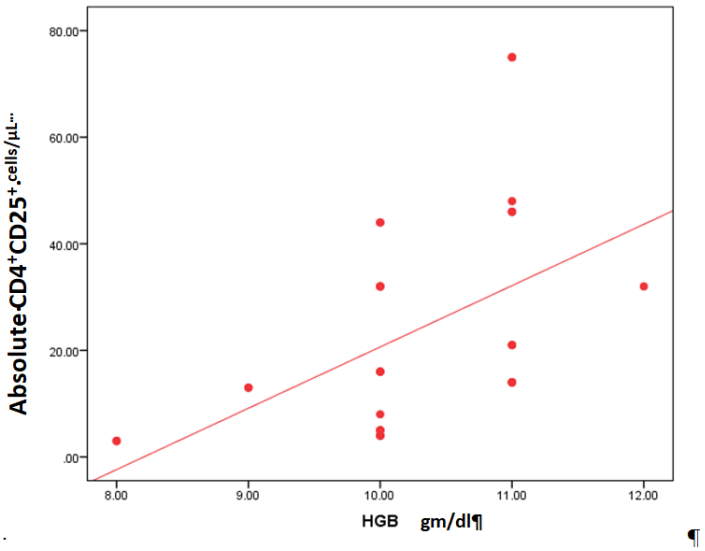


Figure 6. Correlation between CD4+CD25+FOXp3+ Tregs cells/µL and hemoglobin (gm/dl).

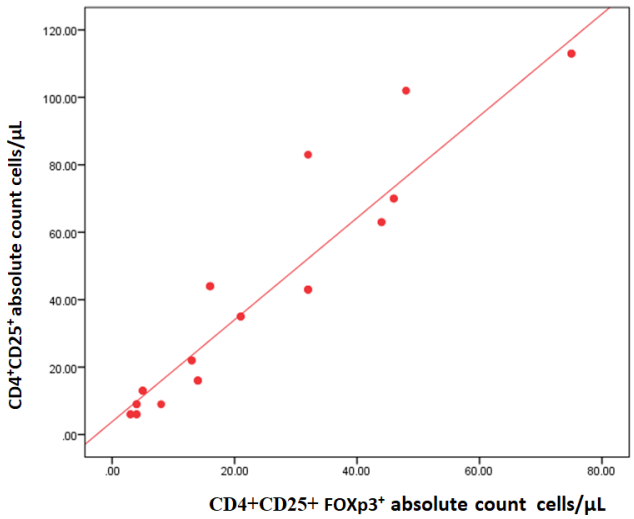


Figure 7. Correlation between absolute counts of CD4+CD25+ Tregs & CD4+CD25+FOXP3+ Tregs.

The relationship of other qualitative parameters to different lymphocytic counts showed a statistically significant difference only with the distal margin involvement, where higher Tregs (whether Foxp3$^+$ or -) were found in cases where the distal margins were involved (*P* <0.05) (Table 4).

It was also noticeable that relatively higher CD4+CD25+ were detected in patients with lymph node involvement, and higher CD4+CD25+ Foxp3$^+$ were found in mucinous adenocarcinomas than other histopathological types, although both observations were statistically insignificant (Table 4).

Table 4. Relationship between tumor characteristics and absolute lymphocyte and T-reg counts.

		ALC	*P*-value	Absolute CD4+CD25+ cells/µL	*P*-value	Absolute CD4+CD25+FOXP3+ cells/µL	*P*-value
		Median (IQR)		Median (IQR)		Median (IQR)	
Tumor Site	Anal canal	612.5 (514 – 711)	NS	29.5 (16 – 43)	NS	23 (14 – 32)	NS
	Caecum	1080 (154 – 1080)		44 (35 – 44)		16 (16 – 21)	
	Lower rectum	650.5 (190 – 883)		13 (6 – 83)		6.5 (4 – 44)	
	Mid rectum	716.5 (93 – 1340)		15.5 (9 – 22)		8.5 (4 – 13)	
	Sigmoid colon	639 (639 – 639)		70 (70 – 70)		46 (46 – 46)	
Distal margins	Free	675 (321 – 984.5)	NS	22 (11 – 44)	0.031	14 (5 – 32)	0.041
	Involved	134 (134 – 883)		63 (63 – 102)		44 (44 – 48)	
T staging	2	883 (849 – 1085)	0.000	13 (9 – 102)	NS	5 (5 – 48)	NS
	3	452 (154 – 639)		35 (16 – 63)		21 (13 – 32)	
	4	1080 (1080–1080)		44 (44 – 44)		16 (16 – 16)	

Table 4. (Continued).

		ALC	*P*-value	Absolute CD4+CD25+ cells/µL	*P*-value	Absolute CD4+CD25+FOXP3+ cells/µL	*P*-value
		Median (IQR)		Median (IQR)		Median (IQR)	
N staging	0	675 (483 –849)	NS	16 (11 – 43)	NS	13.5 (5 – 32)	NS
	1	518.5 (154 – 886)		59 (35 – 92.5)		26.5 (21 – 40)	
	2	190 (134 –1080)		44 (6 – 63)		16 (3 – 44)	
Operation	APR	612.5 (352 –797)	NS	29.5 (12.5 – 72.5)	NS	23 (11 – 40)	NS
	Hemicolectomy	1080 (639 – 1080)		44 (44 – 70)		16 (16 – 46)	
	LAR	452 (134 – 849)		17.5 (9 – 35)		9 (4 – 21)	
LN	Negative	675 (483 – 849)	NS	16 (11 – 43)	NS	13.5 (5 – 32)	NS
	Positive	190 (154 – 1080)		44 (35 – 63)		21 (16 – 44)	
Tumor type	Adenocarcinoma	536.5 (154 – 1080)	NS	28.5 (9 –44)	NS	14.5 (4 – 21)	NS
	Moderately differentiated. adenocarcinoma	452 (134 – 711)		16 (6 – 63)		14 (4 – 44)	
	Mucinous adeno	514 (514 – 639)		43 (43 – 70)		32 (32 – 46)	
	Poorly differentiated adenocarcinoma	849 (849 – 1085)		13 (13 – 113)		5 (5 – 75)	

ALC; Absolute Lymhocyte count APR: Abdominal perineal resection LAR: Lower anterior resection. **P*>0.05 is not significant (NS).

Discussion

Tregs are important cells in regulating the immune cell homeostasis by enforcing a dominant negative regulation on other immune cells. Induced Tregs which acquire CD25 (IL-2R alpha) expression outside of the thymus are induced by inflammation and disease processes, like autoimmunity or cancers.[24] In patients having cancer, Tregs tend to be upregulated and recruited to the site of the tumor. Studies have proved that Tregs suppress the tumor immunity mediated by the host defense.[25]

T-regulatory lymphocytes are investigated in cancer patients in several ways either through the identification of Treg cells within peritumoral infiltrate microenvironment or through the levels of circulating Treg cells in the peripheral blood. In addition, it can be measured repeatedly, allowing monitoring of cancer progression.[26]

It would have been more interesting to assess Tregs in both compartments (blood and tumor), but due to limited resources we focused on the blood only.

Our study involved 31 patients with CRC at different sites namely the anal canal, caecum, lower rectum, mid-rectum and sigmoid colon while other studies just focused on one tumor site as in the left colon, [26] which they considered to be a strength of their study as the study population will be more homogenous.

Methods of Treg identification has changed in different studies from one year to another so

it was not easy to compare our results with other formerly cited studies. The studies before 2007 or even before 2010, usually addressed T-cells with different characteristics.[27]

In our study the ALC and different Treg cells were measured in the peripheral blood of the studied patients. We found that the ALC was relatively higher in T 4 stage and lowest in T3 stage with a statistically significant value. Also, our study showed a strong positive correlation between the age of patients and the ALC in peripheral blood while Szczepanik et al. 2011[31] who assessed Treg counts in patients over 60 years vs. younger patients found no difference.

Treg counts were positively correlated with hemoglobin. Since Tregs are immune suppressors and anti-inflammatory, it may explain why an increase in their numbers can result in an increase in hemoglobin. These results are in line with a study by Vayrynen et al [(33)] which showed that low blood hemoglobin was associated with systemic inflammation in CRC patients.

The absolute counts of both CD4+CD25+ Tregs and CD4+CD25+Foxp3$^+$ Tregs tended to increase with increased T staging (more advanced disease) without a significant difference where more of both Tregs were found in stages 3 &4 as compared to stage 1. Our results are very similar to those by Ling et al, which showed that CD4+CD25+Foxp3$^+$ Tregs counts in the peripheral blood of CRC patients with early stages was lower than in those with more advanced stages but were not statistically significant.[29]

On the contrary, other studies showed that there was a tendency for lower peripheral blood CD4+CD25+ Foxp3+ Treg counts in later stages (III and IV) than in stage I & II but are of no statistical significance.[26] In another study, Treg cells were increased in stages II and III, but not in stage IV. Authors speculated that the reason may be Treg migration from tissue into the circulation.[30]

A very strong positive correlation between absolute CD4+CD25+ Treg counts and absolute CD4+CD25+ Foxp3$^+$ Treg counts was found. Interestingly a study by Scurr et al[36] studied different Treg populations including Foxp3$^+$ and Foxp3$^-$ in both the blood and tumors, they found increased proportions of CD4+ Foxp3$^+$ cells in the tumors as compared to blood. They also revealed the presence of a potent suppressive CD4+Foxp3$^-$ T-cell population within the colorectal tumors which could explain why results of studies are different and indicating immune suppression maybe potentiated by other Treg populations.

Our findings are contrary to those of Sellitto et al, where a linear relationship between the proportion of Tregs within CD4$^+$ population and the tumor stage was observed.[28] However, our study investigated the absolute count of Tregs, which does not always correspond to the mentioned above proportion. These differences might be due to differences in the study groups' size, characteristics and/or race.

We found that a significantly higher number of both types of Tregs were detected when the distal margins of the tumor were involved i.e. more advanced cancers. CD4+CD25+Foxp3$^+$ Tregs specifically were more abundant in mucinous adenocarcinomas which are known to have a poorer overall survival than other types of CRC. [34] Also, higher numbers of Tregs were found with lymph node involvement being higher in stage N1 than N2 although not statistically significant. So collectively, whenever there are signs for advanced CRC disease Tregs were found in higher numbers, confirming an anti-tumor suppressive role.

A meta-analysis published in 2015, which included eight studies on colorectal cancer patients concluded that the presence of Tregs within Tumor infiltrating Lymphocytes (TIL) is a positive prognostic factor.[32]

Our findings support the hypothesis that Foxp3$^+$ Tregs have a suppressive role on immunity allowing immune evasion and cancer spread in CRC, although their prognostic value remains controversial. Foxp3 expression by activated T cells may represent a valuable index in evaluating the degree of malignancy, clinicopathologic staging, and lymph node metastasis in colorectal cancer. With all the above-mentioned controversies, it is evident that Tregs are diverse and controversial regarding their prognostic value, posing a challenge in the immunotherapeutic intervention in colorectal malignancies.

However, we may conclude that CD4+CD25+Foxp3+ Tregs could represent a useful marker for predicting invasion, metastasis, and prognosis of colorectal cancer in Egyptian patients.

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Author Contributions

All authors made substantial contributions to the conception and design of the study, MS, YM acquisition of data, NM, MS analysis interpretation of data, KW, NM drafting the article and NM, MS, KW revising it critically for important intellectual content.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Ethical approval

The study protocol was reviewed and approved by the ethical committee of scientific research, Faculty of Medicine, Ain Shams University (Approval date: 15/1/2017).

Informed consent

A signed consent form was obtained from each study participant.

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