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Differential expression of TIGIT and ICOS immune check points on lymphocytes in tissues of resected colorectal cancer compared with their counterparts in the peripheral blood and their relations to clinicopathologic characteristics

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Research Article

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Abstract

Purpose:

The current study was carried out to evaluate the expressions of ICOS and TIGIT, on T cells in tissues and peripheral blood of non-metastatic CRC patients, correlating them to different clinicopathologic characteristics of CRC.

Methods:

We recruited 31 cases of de novo colorectal carcinoma and 25 healthy controls over one year. Interleukin 21 (IL-21) levels were measured in the peripheral blood of both patients and healthy controls using ELISA. Flow cytometry investigated different subsets of T-cell and their expressions of TIGIT and ICOS in both peripheral blood and malignant tissue samples from patients, as well as from normal colonic tissues of CRC patients.

Results:

The percentages of CD4+ TIGIT+ T cells, CD8+ TIGIT+ T cells, CD4+CD8+ TIGIT+ T cells, CD4+ ICOS+ T cells, and CD8+ ICOS+ T cells were significantly higher in malignant CRC tissues compared to normal tissues. Moreover, TIGIT expression on CD4+ and CD8+ T cells was significantly elevated in the peripheral blood of CRC patients compared to controls. Conversely, ICOS expression on CD4+ and CD8+ T cells in peripheral blood was significantly higher in controls than in patients. Additionally, both ICOS and TIGIT expressions were markedly greater on CD8+ T cells, CD4+ T cells, and CD4+CD8+ T cells in CRC tissues compared to their counterparts in peripheral blood.

Conclusion:

There was upregulation of immunostimulatory molecules like ICOS, which has been associated with a favourable prognosis. However, the upregulation of immunoinhibitory molecules such as TIGIT predominates, which is linked to disease progression and metastasis.

Keywords: colorectal carcinoma, CD8+T cells, CD4+T cells, TIGIT, ICOS, SDG3

Introduction

Colorectal cancer (CRC) is one of the widely spread tumors in the world where more than 1.2 million patients with CRC are diagnosed annually [1], it is considered the second and third most commonly diagnosed cancer in females and males, respectively. CRC remains the fourth most common lethal disease after lung, prostate and breast cancers [2, 3].

Five-year survival rates have been declined from 90% in localized CRC to 15% in metastatic cases[4], for about 2 decades, the mainstay for treatment was chemotherapy with fluorouracil combined with either oxaliplatin or irinotecan. However, these regimens that directly target tumor cells often result in resistance mechanisms, and their toxicities can make it impossible to use them at therapeutically useful dosages [5].

The immune response to tumor cells involve innate and adaptive immune. Cellular and humoral components mediate adaptive anticancer immune responses, a subset of CRC patients can be treated with monoclonal antibodies against vascular endothelial growth factor (VEGF) or epidermal growth factor receptor (EGFR) that proved to expand the survival in metastatic cases [6], The emergence of cancer immunotherapy has expanded the available and effective treatment options for select individuals with CRC beyond the standard of care: surgery, chemotherapy, and radiation.

Immune checkpoint molecules are surface molecules expressed on T cells to control their antigen recognition in the process of immune response. Immune checkpoint are two types of receptors; Co-stimulatory molecules like CD28, (inducible co-stimulator) ICOS, and CD137 to stimulate the immune progress, and Co-inhibitory immune checkpoint molecules that inhibits immune progress, like PD1 (programmed death-1), V-domain Ig suppressor of T cell activation (VISTA), CTLA-4 (cytotoxic T-lymphocyte associated protein 4), and T cell immunoreceptor with Ig and ITIM domain (TIGIT) [7].

For treating cancer, there are many types of immunotherapies. These include monoclonal antibodies, T-cell transfer therapy, treatment vaccines, immune checkpoint inhibitors (ICI) and immune system modulators. Despite the proven advantage of immunotherapy, only a small group of patients gain benefits from the current immunotherapies, so the need for more inclusive immune-based therapies to house all CRC patients. Cancer immunotherapy is based on improved tumor antigen presentation and recognition; stimulation or amplification of an immune response; or disinhibition of immune cells to allow for an improved antitumor immune response, while there are several useful immunological checkpoints for immunotherapy, further specialized sites are always being investigated. PD-L1 expression level, microsatellite instability (MSI), and tumor mutation burden (TMB) are a

few examples of biomarkers whose expression is connected to the efficiency of ICIs (6). Blockade of CTLA-4 and PD-1/PD-L1 molecules are two approaches of checkpoint inhibition that have been widely used [8].

ICOS, a member of CD28 coreceptor family with significant homology to co-inhibitory receptor CTLA-4 and co-stimulatory receptor CD28, is expressed on CD8+T cells (CTL), CD4+T helper cells (Th1, Th2, Th17, Tfh, and Tregs), while its ligand ICOSL is expressed on APC cells including B cells, macrophages, dendritic cells, and somatic cells such as endothelial cells, lung epithelial cells, fibroblasts, mesenchymal stem cells and malignant cells [9, 10], in malignancy, ICOS is highly expressed in FoxP3+Tregs and have a higher immunosuppressive effect than those with low ICOS expression, also is able to convert CD4+CD25+T cells to Tregs with high proliferation rate (Ki67) [11]. $ICOS^{+high}$ Tregs infiltrating gastric adenocarcinoma was associated with short survival [12] while in hepatocellular carcinoma, it was correlated with intratumoral accumulation of pDCs [13], still its role in cancer requires further investigation.

T-cell immunoreceptor with immunoglobulin and immunoreceptor tyrosine-based inhibition motif domain (TIGIT), is composed of an extracellular immunoglobulin variable region (IgV) domain, a transmembrane domain, and an intracellular domain containing an immunoreceptor tyrosine-based inhibitory motif (ITIM) with an Ig tail-tyrosine (ITT)-like motif component [14, 15]. TIGIT is expressed on different immune cells including memory T cell, activated T cells, Tregs, follicular helper cells, NK, and NKT cells, also it is highly expressed on peripheral blood Tregs of cancer patients and upregulated in the tumor microenvironment (TME). CD112, CD155, Necl-5, and Tage 4 are its ligands and expressed on multiple tumor cells and APC cells [16].

IL-21 is a pleiotropic type I cytokine produced by many cells including CD4+T, CD8+I, B cells, macrophages, monocytes, NKT, and dendritic cells to bind with its receptor IL-21R inducing STAT3 activation in T and B cells, an effect important for their differentiation, in addition IL-21-induced T-helper (Th) 17 cell differentiation requires interferon regulatory 4 (IRF4). It was detected that ICOS signaling is critical for induction and maintenance of IL-21 expression, moreover T follicular helper cells (Tfh) are associated with high expression of IL-21.

The current study was carried out to evaluate the expressions of ICOS and TIGIT, on T cells in tissues and peripheral blood of non-metastatic CRC patients, correlating them to different clinicopathologic characteristics of CRC.

Patient and methods

Patients:

We introduced a case control study conducted on South Egypt Cancer Institute, Faculty of Medicine, Assiut University, and Faculty of Medicine, New Valley University, Egypt. It involved patients with denovo CRC without previous neoadjuvant treatment, aged 18 years and more, without any previous cancer treatments, also patients with concurrent infectious diseases, inflammatory bowel diseases, chronic kidney or liver diseases were all excluded, so we recruited 31 cases of CRC and 25 healthy controls of comparable age and sex, over a period of one year, the study fulfilled approval of ethical committee in faculty of medicine, Assiut university (IRB no 04-

2024-300483). Also, all methods were carried out according to the guidelines and regulations of Helsinki declaration. All participants have written informed consent.

All patients were subjected to full history taking and clinical examination including physical examination, imaging such as abdominal ultrasound, Chest x-ray, computed tomography scans (CT scan) of chest, and magnetic resonance imaging (MRI) of pelviabdomen, examination of the colon using two colonoscopy systems (Pentax EC 3890Lk & Olympus Cf-260AL) (appendix), tissue samples were taken for histopathology examination, tumor markers including CA19-9, and CEA. Detection of interleukin 21 (IL-21) by ELISA (Cat.no ELK2146) was done on peripheral blood of patients and healthy controls, also flow cytometric detection of T cell subsets and their TIGIT and ICOS expression in peripheral blood samples of patients and healthy controls, and in CRC tissue and normal tissue of colon in patients with CRC.

Surgical management

All patients in this study were surgically treated on radical basis. Radical total colectomy was done in one patient in whom cancer was in the sigmoid colon because of the presence of multiple polypi in the whole colon. Radical right hemicolectomy was performed in 11 patients; radical left hemicolectomy was done in 4 patients; 5 patients who had sigmoid cancer were treated by radical sigmoidectomy (two of them were operated laparoscopically); 4 patients who had rectosigmoid cancer were treated by radical anterior resection (Dixon) and 2 were treated by laparoscopic assisted Dixon; 4 patients had rectal mass, one of them was treated by radical abdominoperineal resection (Miles) operation; one had radical total proctosigmoidectomy; while the last two were treated by posterior pelvic exenteration.

Adjuvant treatments

Patients with stage III CRC received adjuvant 3-6 cycles of XELOX or FOLFOX regimens also those with stage II with poor differentiation or mucinous type, less than 12 LNs dissected, LVI, PNI, intestinal obstruction, tumor budding, or indeterminate safety margins received adjuvant 3 cycles of XELOX or 6 cycles of FOLFOX, for rectal carcinoma with pT3-T4, N+, low tumors within 4 cm from the anal verge, circumferential radial margin≤1 mm, LVI+, PNI+, or tumor budding (tier 3) received adjuvant concurrent chemoradiation (45-50.4 Gy/25-28 fractions/5-6 weeks) then received 4 cycles of adjuvant chemotherapy.

Samples:

Blood sample:

Ethylene diamine tetra acetic acid (EDTA) vacutainer tubes having labels of each participant's name, gender, age and date of collection, were used to collect three ml samples of whole blood.

Tissue samples from CRC patients:

From each patient included in this study, samples of CRC malignant tissues and adjacent normal tissue dissected as safety margins were considered controls were collected.

Cups having labels of each participant's name, gender, age and date of collection, were used to collect tissue samples that were taken at time of surgery in the operation rooms. Samples of adjacent safety margins suspected to have inflammatory bowel diseases or ulcerations were excluded, so only 25 normal tissue samples were collected.

Flow cytometric detection of T cell subsets and their TIGIT and ICOS expression

Samples from malignant CRC tissues and adjacent healthy safety margins of patients were collected, along with peripheral blood samples from both patients and controls.

Fresh CRC and normal colonic tissues of the same subjects were immediately collected in the operating rooms of South Egypt Cancer Institute and surgery department of Assiut university hospital (AUH), then they were placed in a cup containing saline and were moved to a petri dish, in which they were washed with 3 milliliters PBS (PH 7.4) to remove any residual salt solution. Tissues were then mechanically ground using a sharp scalpel for proper homogenization. The suspension was filtered through a 40-micron Falcon mesh positioned on a 50 ml polystyrene round-bottom Falcon tube to eliminate any fibers or residual tissue particles. After performing filtration, the cell suspension was moved to 5ml polystyrene round-bottom falcon tube. Then, centrifuging of the tube was done at 2500 rpm for one minute followed by discarding the supernatant.

Then the following steps were carried out on the peripheral blood and the filtered suspension of tissue.

1- One hundred µl blood sample or filtered suspension of tissue was mixed with 10 µl from each of phycoerythrin (PE) conjugated anti-ICOS (BD Bioscience) peridinin-chlorophyll-protein (PercP) conjugated anti-CD4 (BD Bioscience), V450 conjugated anti-TIGHT (BD Bioscience) and allophycocyanin (APC) conjugated anti-CD8 (Beckman coulter).

2- The mixture was subsequently incubated at room temperature for fifteen minutes in the dark.

3- After incubation, lysis of red blood cells was done with ammonium chloride lysis solution, followed by washing of these cells with phosphate buffer saline (PBS), then resuspended in PBS.

4- Analysis of Samples was performed a flow cytometer FACS Canto II (BD Bioscience-San Jose, CA, USA, serial no. V33896201978; 3 lasers, 8 colors). The flow cytometer was routinely subjected to daily quality control according to the manufacturer's recommendations. Collection of a minimum of 50.000 events was done to analyse each sample.

5- FACS DIVA software version 8.0.1 was used to analyze data. The lymphocyte population was identified by forward scatter and side scatter dot plot. Then the expression of CD8 and CD4 was explored on lymphocytes population to determine $CD8^+$ T cytotoxic, $CD4^+$ T helper and $CD4^+CD8^+$ double positive T cells which were gated after that for detection of TIGIT and ICOS expression as shown in Fig1.

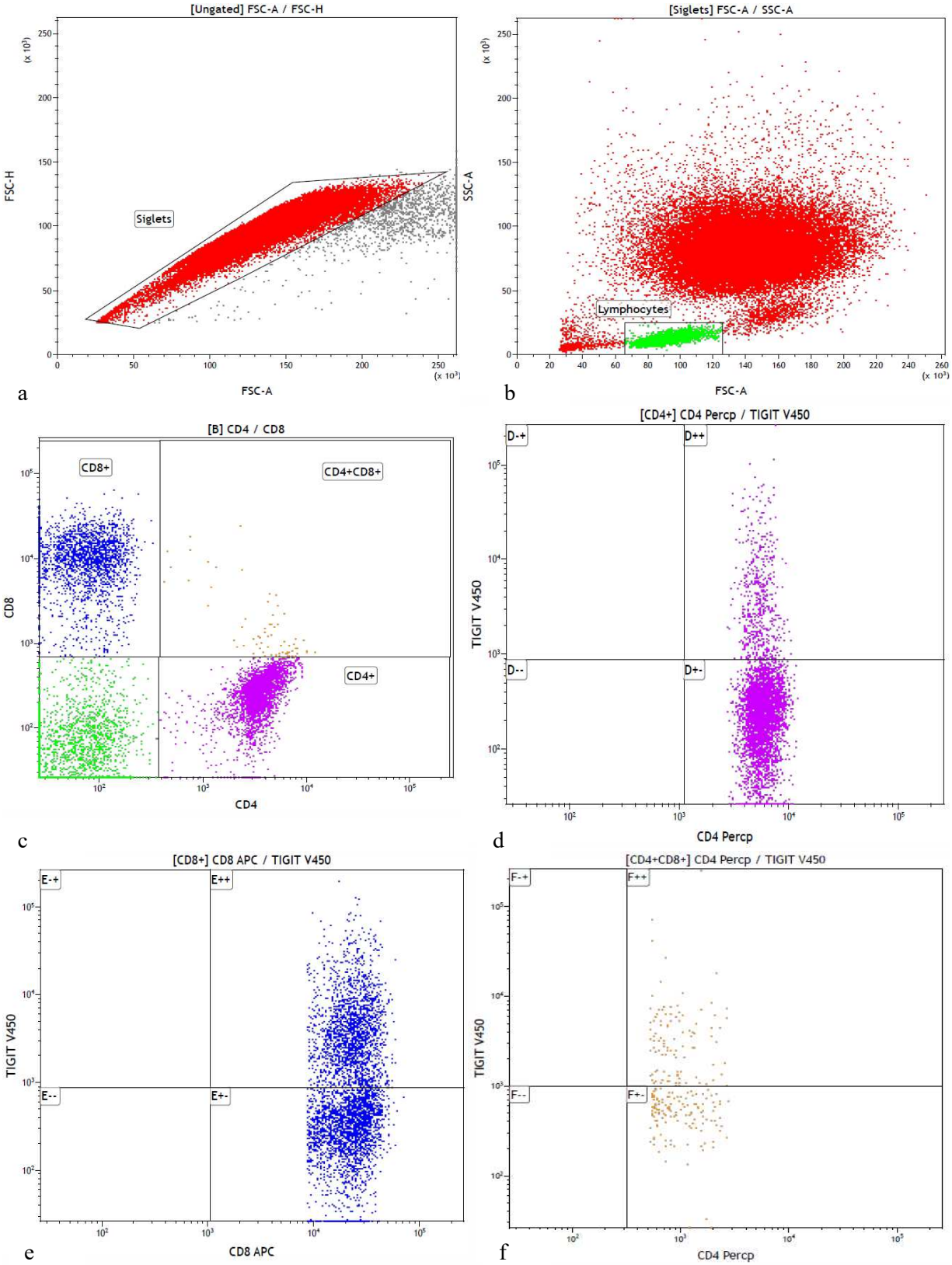
Statistics:

Data were analyzed using IBM SPSS version 27, all scale variables were of non-parametric distribution with Shapiro-Wilk test of <0.05, for descriptive stats, mean, median, standard error, and percentages were used, for inferential stats, Mann Whitney U test for comparison between scale data for two categorical variables, also Robust

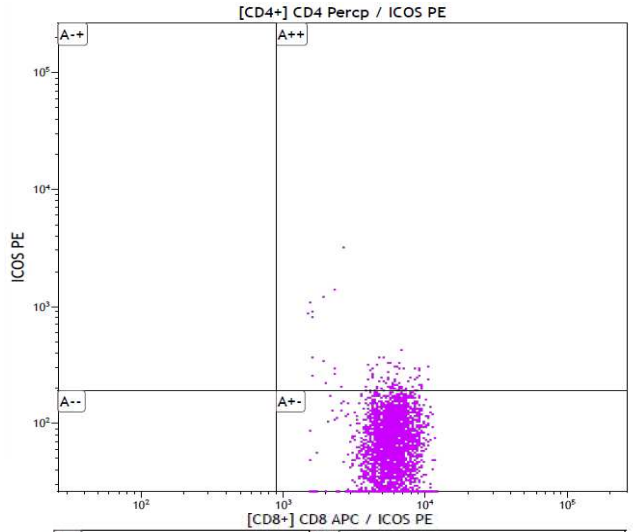
test (with correction of inhomogeneity of variances) and Kruskal Wallis test for more than two categorical variables, Wilcoxon matched pair-signed rank test for comparison between tissue and peripheral blood data of same patients, Spearman rho correlation for the relations between immune cells, GraphPad prism8 for graphing, and all results were considered significant at p-value <0.05. NLR was calculated by dividing total neutrophilic count over total lymphocytic count while PLR calculated by dividing platelet over lymphocytic counts.

Results:

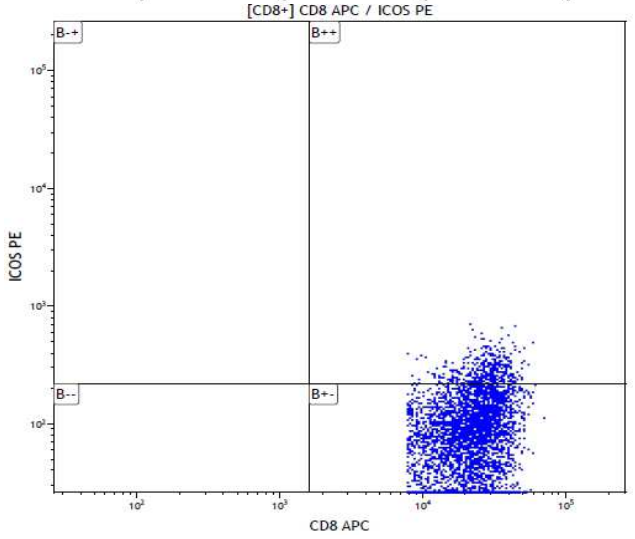
The mean age for patients and controls was 52.7± 2.4 with range of 30-81 and 51.2 ± 2.3 with range of 31-71 years respectively (*p*-value=0.65). Most of patients and controls were males with male to female ratio was 22/9 and 19/6 for patients and controls respectively (*p*-value=0.8). About one third of patients were smokers and complaining of weight loss, however only 9.7% of patients were underweight with comparable smoking and BMI to controls, table (1).


[Ungated] FSC-A / FSC-H
FSC-H
FSC-A
Siglets
a
[Siglets] FSC-A / SSC-A
SSC-A
FSC-A
Lymphocytes
b
[B] CD4 / CD8
CD8
CD4
CD8+
CD4+CD8+
CD4+
c
[CD4+] CD4 Percp / TIGIT V450
TIGIT V450
CD4 Percp
D-+
D++
D--
D+-
d
[CD8+] CD8 APC / TIGIT V450
TIGIT V450
CD8 APC
E-+
E++
E--
E+-
e
[CD4+CD8+] CD4 Percp / TIGIT V450
TIGIT V450
CD4 Percp
F-+
F++
F--
F+-
f

g


[CD4+] CD4 Percp / ICOS PE
A-+
A++
A--
A+-
ICOS PE

h


[CD8+] CD8 APC / ICOS PE
B-+
B++
B--
B+-
ICOS PE
CD8 APC

i

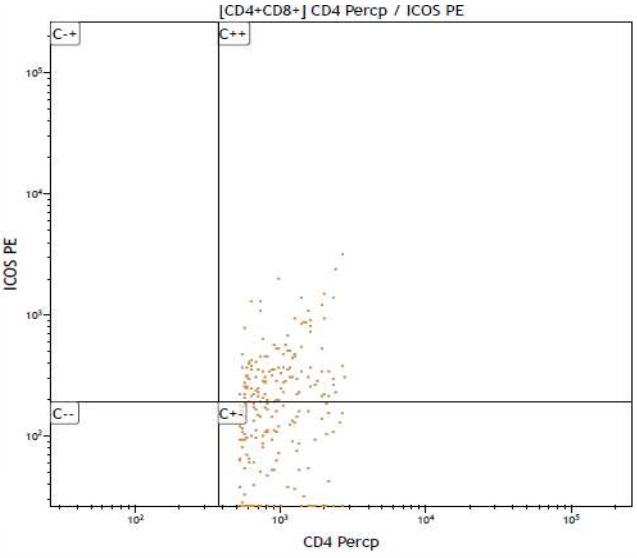

[CD4+CD8+] CD4 Percp / ICOS PE
C-+
C++
C--
C+-
ICOS PE
CD4 Percp

Fig1: Flow cytometric detection T lymphocyte subsets and their expression of TIGIT and ICOS

a: Forward scatter area and height to define singlets and exclude duplicates

b: Forward and side scatter dot plot was used to define lymphocyte population

c: The expression of CD4 and CD8 on lymphocyte population to detect $CD4^+$ T helper, $CD8^+$ T cytotoxic and $CD4^+CD8^+$ double positive T cells which were then gated for further detection of their TIGIT and ICOS expression

d, e, f: The expression of TIGIT on $CD4^+$ T helper, $CD8^+$ T cytotoxic and $CD4^+CD8^+$ double positive T cells

g, h, i : The expression of ICOS on $CD4^+$ T helper, $CD8^+$ T cytotoxic and $CD4^+CD8^+$ double positive T cells

Table 1: Demographic data of CRC patients

Data	Patients (no=31)	Controls (25)	p-value
Age (mean± SE)*	52.7± 2.4	51.2± 2.3	0.65
Range	30-81	31-71	
Sex M/F	22/9	19/6	0.8
Smoking	10/31 (32.3%)	10/25 (40%)	0.74
BMI (mean ±SD)*	26.1± 4.1	25.6± 5.2	0.66
BMI groups			
Underweight	2 (9.7%)	3 (12%)	
Normal weight	9 (29%)	7 (28%)	0.9
Overweight	12 (38.7%)	8 (32%)	
Obese	8 (25.8%)	7 (28%)	

Data expressed as number, percentages, and analysed by *independent sample t-test and Chi^2 test with continuity correction for p-value, BMI; body mass index.

The clinicopathological characteristics of CRC patients were mentioned in table 2, adenocarcinoma was the predominant pathology, more than half of the patients had locally advanced disease (stage III), and approximately 65% had left sided colonic carcinoma.

Table 2: Clinicopathologic data of the studied CRC patients

Data	Descriptive
Main complaint	
-Bleeding per rectum	11 (35.5%).
-Constipation	10 (32.2%).

-Abdominal pain	7 (22.6%).
-Anaemia	3 (9.7%).
Pathologic type	
-Adenocarcinoma	26 (83.9%)
-Mucinous carcinoma	5 (16.1%)
Pathologic grades	
Grade 2	26 (83.9%)
Grade 3	5 (16.1%)
Site	
Left colon	4 (12.9%)
Right colon	11 (35.5%)
Sigmoid	6 (19.4%)
Rectum and Rectosigmoid	10 (32.3%)
Pathologic stage	
I	4 (12.9%)
IIA	7 (22.6%)
IIC	2 (6.5%)
IIIA	5 (16.1%)
IIIB	13 (41.9%)
T staging	
T2	9 (29%)
T3	20 (64.5%)
T4	2 (6.5%)
N staging	
No	13 (41.9%)
N1	16 (51.6%)
N2	2 (6.2%)
Intestinal obstruction	4 (12.9%)
Perforation	2 (6.5%)
Tumor markers	
CEA (range)	5.8± 4.1 (0.5-126)
CA19-9 (range)	18.8± 3.4 (8.5-116)

Data expressed as mean± SE, range, number and percentages, CEA; carcinoembryonic antigen, CA; cancer antigen, T; tumor, N; lymph nodes.

Laboratory characteristics of the studied CRC patients and controls were illustrated in table (3) where platelet count, absolute neutrophilic, monocytic counts were significantly higher in patients, while absolute lymphocytic count and haemoglobin level were significantly lower. Moreover, NLR and PLR were significantly elevated in patients compared with controls.

Table 3: Hematologic data of CRC patients and their controls

Laboratory data	Patients (no=31)	Controls (no=25)	*p*-value
HB	10.1± 0.3	12.0± 0.2	0.017*
WBCs	10.7± 1.4	6.3± 0.4	0.006*
Absolute neutrophils	7.4± 0.7	4.8± 0.6	0.012*
Absolute monocytes	0.5± 0.1	0.1± 0.02	<0.001*
Absolute lymphocytes	1.3± 0.1	2.4± 0.2	<0.001*
Platelets	301.3± 21.2	233.7± 7.9	0.005*
LDH	194.4± 10.3	189.1± 4.3	0.6
NLR	8.3± 1.2	2.5± 0.2	<0.001*
PLR	30.4± 5.4	10.0± 0.8	<0.001*

NLR; neutrophil to lymphocytic ratio, PLR; platelet to lymphocytic ratio, Data expressed as mean± SE and analysed by Mann-Whitney U-test.

There were no significant differences in the percentages of CD4+, CD8+, and CD4+CD8+T cells between malignant CRC tissue and normal colonic tissue. The percentages of CD4+ TIGIT+T cells, CD8+ TIGIT+ T cells, CD4+CD8+TIGIT+T cells, CD4+ICOS+T cells, CD8+ICOS+T cells were significantly higher in malignant CRC tissues compared with normal tissues, while no significant differences in the percentage of tissue CD4+CD8+ICOS+T cells between malignant CRC tissue and normal colonic tissue (Fig2 and table 4).

Table 4: T cell subsets and their TIGIT and ICOS expression in CRC tissue and healthy colonic tissue

Tissue immune cells (%)	Malignant CRC tissue (m% ±SE)	Normal colonic tissue (m% ±SE)	*p*-value
CD4+T cells	17.2± 0.8	15.2± 2.1	0.6
CD8+T cells	23.1± 1.0	21.9± 0.7	0.3
CD4+CD8+T cells	1.7± 0.2	1.8± 0.2	0.6
CD4+TIGIT+T cells	71.7± 2.4	63.0± 3.3	0.038*
CD4+ICOS+T cells	20.7± 2.3	8.14± 0.8	<0.001*
CD8+TIGIT+T cells	79.0± 1.8	64.3± 2.1	<0.001*
CD8+ICOS+T cells	16.8± 2.6	7.0± 0.6	<0.001*
CD4+CD8+TIGIT+T cells	85.8± 1.1	73.7± 1.3	<0.001*
CD4+CD8+ICOS+T cells	41.0± 2.4	38.6± 2.3	0.5

Data expressed as mean percentage± SE and analysed by Man Whitney test, ICOS; inducible co-stimulatory, TIGIT; T cell immunoreceptor with Ig and ITIM domains.

Regarding their expressions in the peripheral blood, the total lymphocytic count and CD4+T cells were significantly lower in CRC patients compared with the healthy controls, while CD8+T cells were insignificantly higher in patients. The expressions of TIGIT on CD4+T cells and CD8+ T cells were significantly higher in CRC patients than the

controls. However, ICOS expression on CD4+CD8+T cell was significantly higher on controls compared with patients, there was no significant difference regarding the percentage of CD4+CD8+T cells, ICOS egression on CD4, and CD8+T cells, or TIGIT expression on CD4+CD8+T cells between patients with CRC and healthy controls, table (5).

Table 5: T cell subsets, their TIGIT and ICOS expression in peripheral blood of patients and healthy controls.

Peripheral blood immune cells	Patients	Controls	*p*-value
Lymphocytes	15.1± 1.6	39.6± 2.8	<0.001*
CD4+T cells	30.3± 1.3	43.9± 1.1	<0.001*
CD8+T cells	22.4± 1.3	19.3± 1.1	0.06
CD4+CD8+T cells	1.2± 0.13	1.01± 0.1	0.2
CD4+TIGIT+T cells	26.0± 1.1	20.8± 0.7	<0.001*
CD8+TIGIT+T cells	51.1± 2.4	42.0± 1.2	0.001*
CD4+CD8+TIGIT+T cells	34.2± 2.1	33.5± 1.6	0.8
CD4+ICOS+T cells	2.3± 0.4	2.1± 0.22	0.6
CD8+ICOS+T cells	5.3± 2.3	4.1± 0.2	0.6
CD4+CD8+ICOS+T cells	5.7± 0.7	13.3± 1.5	<0.001*

Data expressed as mean percentage± SE and analysed by Man Whitney test.

Differences in immune cell composition between tissue and PB compartments were illustrated in table (6) where the percentage of CD4+ T cells was significantly lower in malignant CRC tissues than peripheral blood in the same patients, $CD4^+CD8^+$T cells was significantly higher in malignant CRC than peripheral blood, with no significant difference in CD8 T cell between tissue and blood. The expression of both ICOS and TIGIT were significantly higher on CD4+T cells, CD8+T cells and $CD4^+CD8^+$T cells of malignant CRC tissues compared with peripheral blood.

Table 6: Differential expression of T cell subsets and their TIGIT and ICOS expression between CRC malignant tissues and peripheral blood of CRC patients.

Immune cells	Tissue (mean %± SE)	PB (mean%± SE)	*p*-value
Lymphocytes	31.1± 2.13	15.1± 1.6	<0.001*
Total $CD4^+$T cells	17.3± 0.8	30.3± 1.3	<0.001*
$CD4^+TIGIT^+$T cells	71.7± 2.4	26.0± 1.1	<0.001*
$CD4^+ICOS^+$T cells	20.7± 2.3	5.5± 2.5	<0.001*
Total $CD8^+$T cells	23.1± 0.95	22.4± 1.3	0.6
$CD8^+TIGIT^+$T cells	79.0± 1.8	51.11± 2.4	<0.001*
$CD8^+ICOS^+$T cells	16.9± 2.6	5.3± 2.3	<0.001*
Total $CD4^+CD8^+$T cells	1.7± 0.13	1.2± 0.12	0.037
$CD4^+CD8^+TIGIT^+$T cells	85.8± 1.1	34.2± 2.1	<0.001*
$CD4^+CD8^+ICOS^+$T cells	41.0± 2.4	13.8± 2.9	<0.001*

Data expressed as mean± SE and analyzed by Wilcoxon matched pair-signed rank test. * significant p value < 0.05

The mean and median IL-21 values were 1517.44 ± 92.3 and 1262.0 in patients and 608.39 ± 74.5 and 641.0 in controls, with a statistically significant difference ($p < 0.001$, see Fig2). We only observed significant negative correlation between IL-21 levels and CD8+ TIGIT+ T cells in malignant tissue ($r = -0.46$, $p = 0.009$).

In contrast, when examining the correlations between IL-21 and other immune cells across the entire study population (both patients and controls), we found significant positive associations between IL-21 and all tissue immune cells, including CD8+ TIGIT+ T ($r = 0.37$, $p = 0.006$), CD4+ CD8+ TIGIT+ T ($r = 0.54$, $p < 0.001$), CD4+ ICOS+ T ($r = 0.49$, $p < 0.001$), and CD8+ ICOS+ T cells ($r = 0.3$, $p = 0.024$).

Additionally, significant positive correlations were identified between IL-21 and peripheral blood levels of CD8+ T cells ($r = 0.3$, $p = 0.028$), CD4+ TIGIT+ T cells ($r = 0.57$, $p < 0.001$), and CD8+ TIGIT+ T cells ($r = 0.35$, $p = 0.009$). Conversely, negative correlations were found between IL-21 and peripheral blood levels of CD4+ T cells ($r = -0.6$, $p < 0.001$), CD8+ ICOS+ T cells ($r = -0.31$, $p = 0.02$), and CD4+ CD8+ ICOS+ T cells ($r = -0.64$, $p < 0.001$).

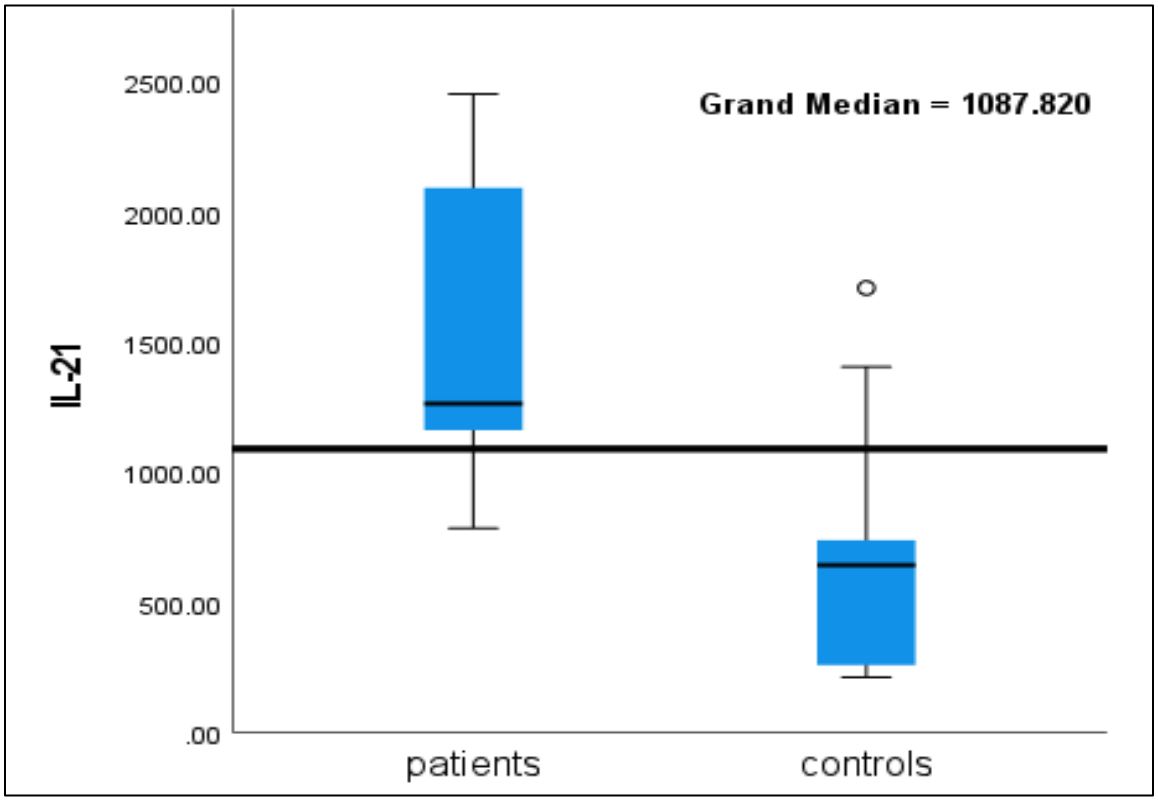


Fig2: Differential expression of IL-21 between patients and controls, Mann Whitney U-test, $p<0.001$.

Relation between malignant tissue immune cells and clinicopathologic features:

There was no significant association between tissue immune cells and different clinicopathologic features including LDH, NLR, PLR, pathological subtype, and stag. The age of patients was negatively correlated with

tissue CD4+CD8+T cells (r=-0.4, *p*=0.048). Tissue CD4+CD8+ICOS+T cells were significantly accumulated in females compared with males (48.5± 5.73 vs. 37.9± 2.3, *p*=0.043). Moreover, we found significant differences in tissue CD8+T cell level in different BMI groups (mean± SE= 34.3± 1.3, 24.3± 1.1, 20.8± 1.4, and 22.3± 1.8 for underweight, normal weight, overweight, and obese patients respectively, *p*=0.042). Also, patients with weight loss had a significantly elevated level of tissue CD4+TIGIT+T cells compared to patients without weight loss (80.2±1.8 vs. 67.6±3.1, *p*=0.011). Smokers had a significantly higher expression of CD4+TIGIT+T cells compared with non-smokers (78.5± 2.3 vs. 68.5± 3.13 *p*=0.048). For the grade, we found no significant impact of the grade on the level of different tissue immune cells except for CD4+CD8+ICOS+T cells which accumulated in G2 compared with G3 (42.6± 2.7 vs. 9.9± 3.5, *p*=0.032), also tissue CD4+CD8+TIGIT+T cells were increased in right colonic and sigmoidal carcinomas compared with left sided and rectal carcinoma (89.04± 1.2 vs. 87.4± 2.1 vs. 82.5± 3.1 vs. 82.7± 2.0, *p*=0.05), also CD4+CD8+ICOS+T cells were significantly accumulated in adenocarcinoma compared with mucinous carcinoma (43.4± 2.6 vs. 27.2± 2.02, *p*=0.001), Fig3.

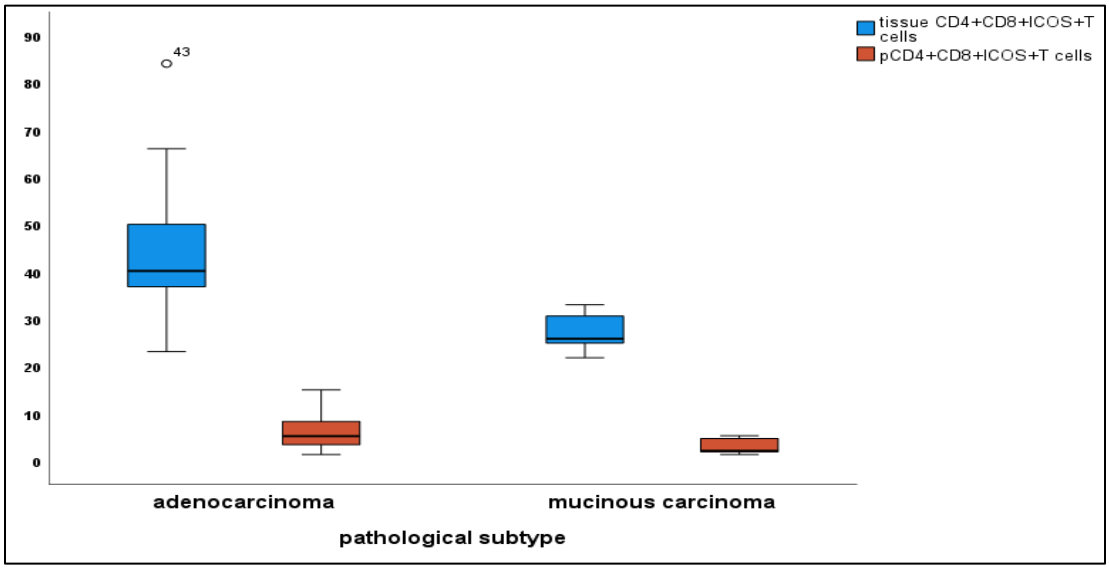


Fig3: differences of immune cells expressing ICOS according to pathological subtypes.

Relation between peripheral T cell subsets and their TIGIT and ICOS expression and clinicopathologic features

Regarding immune cells in peripheral blood, negative correlation between BMI and CD4+CD8+TIGIT+T cells (r=-0.36, *p*=0.049), also with CD4+CD8+T cells (r=-0.62, *p*<0.001), and positive correlation with CD4+CD8+TIGIT+T cells (r=+0.36, *p*=0.045). NLR exhibited significant positive correlation with CD4+TIGIT+T cells (r=+0.42, *p*=0.02), and negative correlation with CD4+TIGIT-T cells (r=-0.46, *p*=0.009).

Regarding BMI groups, only CD4+CD8+T cells were accumulated in underweight patients and progressively declined as BMI increased (2.8± 0.7, 1.4± 0.3, 1.1± 0.1, and 0.8± 0.11 for underweight, normal, overweight, and obese patients respectively, p<0.001),

We did not find significant associations between age, sex, smoking, grading, with different peripheral immune cells. Both CD4+CD8+TIGIT+T cells and CD4+ICOS+T cells showed variable distributions with different anatomic sites, the former accumulated in the sigmoid and rectum compared with right and left colon (46.6± 4.45, 34.1± 3.53 vs. 30.2± 2.4, 26.6± 5.5, p=0.013) while, the later cells was highly accumulated in the sigmoid compared with right colon, left colon, and rectum (5.0± 1.04 vs. 1.7± 0.42 vs. 1.4± 0.4 vs. 2.62± 0.73, adjusted p=0.015, 0.048, 0.15). Also, CD4+CD8+ICOS+T cells were significantly accumulated in adenocarcinoma compared with mucinous carcinoma (6.3± 0.4 vs. 3.1± 0.81, adjusted p=0.036)

Regarding N-stages, we only detected significant differences in peripheral lymphocytes (11.9± 1.64, 16.4± 2.45, &26.2± 1.6 for N0, N1, &N2, p=0.003), CD4+TIGIT+T cells (27.2± 2.0, 26.2± 1.23 &15.8± 1.93 for N0, N1, &N2, p=0.042), and CD4+TIGIT-T cells (72.3± 1.81, 73.8± 1.1 & 84.2± 1.9 for N0, N1, &N2, p=0.024), respectively, Fig4.

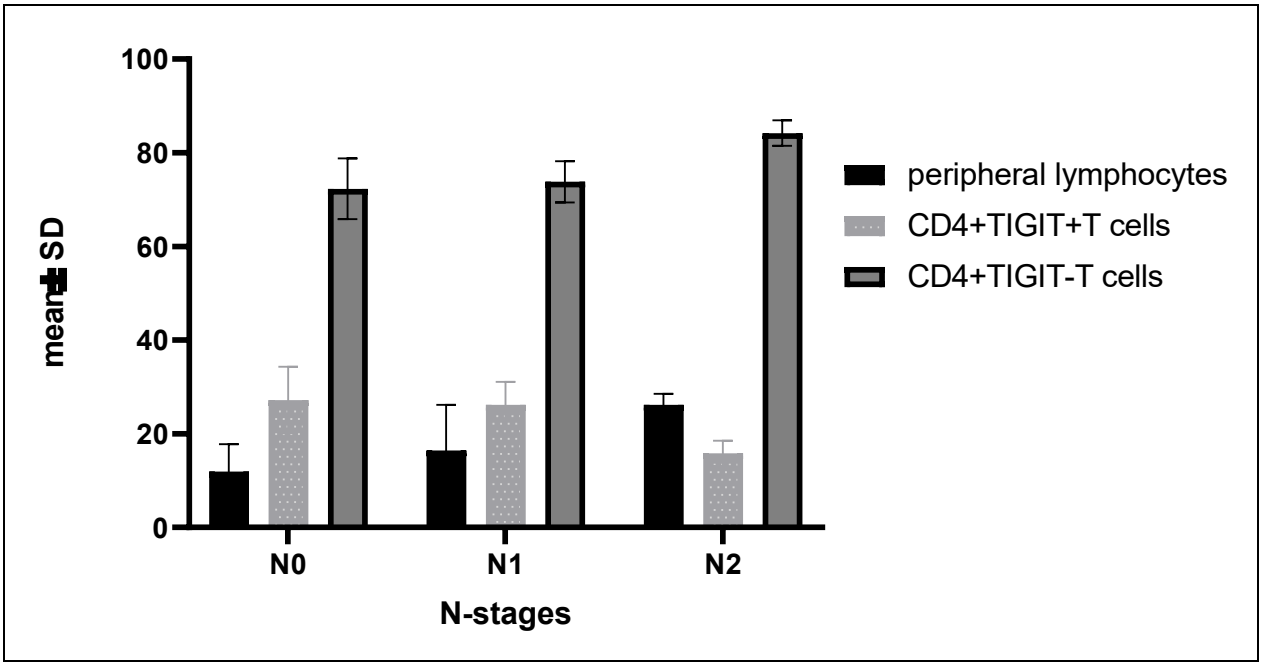


Fig4: Relation between peripheral T cell subsets and their TIGIT and ICOS expression and N-stages.

Regarding T-stages, the current results revealed significant accumulation of CD8+TIGIT+T cells in early T stages with T2, T3, &T4 had mean± SE of 59.9± 4.8, 48.6± 2.5, & 36.5± 4.1 respectively, p=0.024.

Conversely, CD8+TIGIT-T cells were accumulated with advancing T stages with mean± SE of 40.02± 4.8, 51.4± 2.5, & 63.5± 4.8 for T2, T3, &T4 respectively, p=0.03, figure (5).

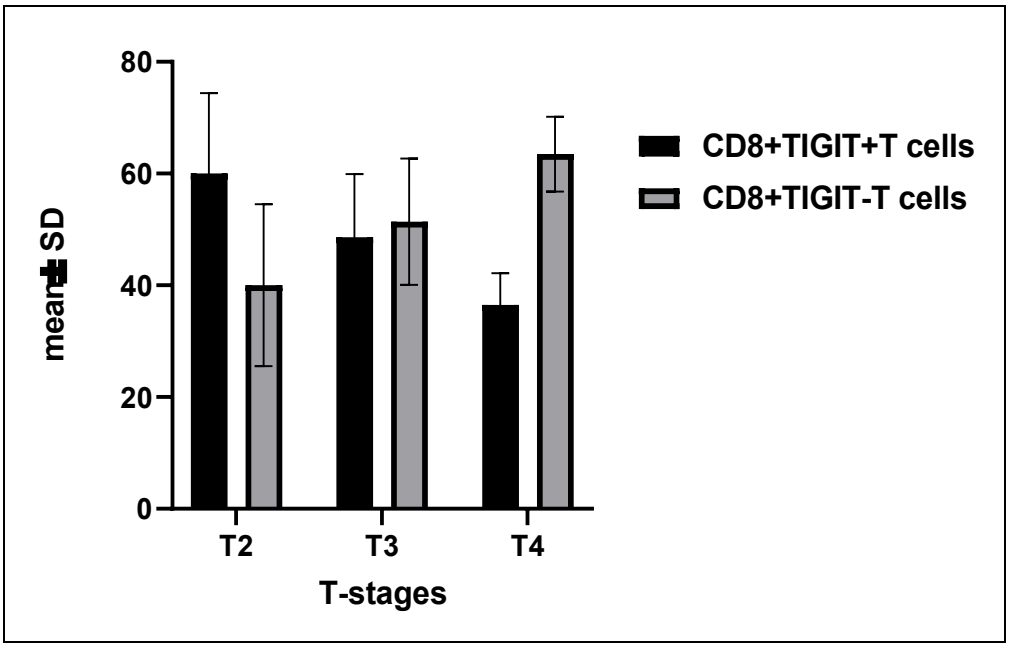


Fig5: Relation between peripheral T cell subsets and their TIGIT and ICOS expression and T-stages.

Summary of results

The study found no significant differences in CD4+, CD8+, and CD4+CD8+ T cell percentages between malignant colorectal cancer (CRC) tissue and normal colonic tissue. However, several T cell subsets (CD4+ TIGIT+, CD8+ TIGIT+, CD4+CD8+TIGIT+, CD4+ ICOS+, and CD8+ ICOS+) were significantly higher in malignant tissue. In peripheral blood, CRC patients showed lower total lymphocytic and CD4+ T cell counts compared to healthy controls, while CD8+ T cells were slightly higher. TIGIT expression was elevated on CD4+ and CD8+ T cells in CRC patients, but ICOS expression on CD4+CD8+ T cells was higher in controls. The composition of immune cells differed between tissues and blood, with CD4+ T cell percentages lower in CRC tissue and CD4+CD8+ T cells higher in malignant tissues. Additionally, both ICOS and TIGIT expressions were significantly higher in malignant CRC tissues compared to peripheral blood. The study found no significant associations between tissue immune cells and various clinicopathologic features like LDH, NLR, PLR, pathological subtype, and stage. Age negatively correlated with tissue CD4+CD8+ T cells, and females had higher levels of CD4+CD8+ICOS+ T cells than males. Significant differences in CD8+ T cell levels were observed among different BMI groups, while weight loss and smoking correlated with elevated CD4+TIGIT+ T cell levels. Tissue immune cell levels varied by grade and cancer type, with notable differences in adenocarcinoma versus mucinous carcinoma. In peripheral blood,

BMI negatively correlated with CD4+CD8+TIGIT+ and CD4+CD8+ T cells, while NLR showed positive correlation with CD4+TIGIT+ T cells. CD4+CD8+T cells were highest among underweight patients and decreased with increasing BMI. There were no significant associations between peripheral immune cells and age, sex, or smoking. Immune cell distributions varied by anatomical site, with CD4+CD8+TIGIT+ T cells higher in the sigmoid and rectum, while CD4+ICOS+ T cells were most abundant in the sigmoid. Peripheral lymphocytes and specific T-cell subsets differed significantly by N-stages, while CD8+TIGIT+ T cells accumulated in early T stages, inversely followed by CD8+TIGIT- T cells in advanced stages.

Discussion

CRC is not simply a single illness with unique genetic and epigenetic changes characterizing every patient disease, also include microsatellite instability, chromosomal instability and CpG island methylator phenotype, all contribute for different clinical, biological, and pathological behaviors of CRC [17], CRC microenvironment is developed as a result of several interactions between the host and the tumor as it gets progressed, this microenvironment comprises immune cells, myofibroblasts, stromal cells, vessels, inflammatory cytokines, and growth factors which in turn recruit more immune cells carrying multiple immune check point molecules that control the immune response to CRC [18].

Currently, we revealed that TIGIT and ICOS expressions on tissue infiltrating CD4+, CD8+, and CD4+CD8+T cells, in addition to TIGIT expression on the peripheral blood CD4+, CD8+, and CD4+CD8+T cells were significantly higher in patients compared with their controls. Moreover, the expressions of ICOS and TIGIT were significantly higher on malignant tissue infiltrating CD4+, CD8+, and CD4+CD8+T cells compared with their counterparts in the peripheral blood of patients.

ICOS was identified as a T-cell activation biomarker, played an important role in T cell proliferation and cytokine production with either proinflammatory or anti-inflammatory effects, however the role of ICOS pathway in CRC carcinogenesis remains to be defined, some studies demonstrated that ICOS induce antitumor effect through signaling on T effector and Tregs [19] while others demonstrated that ICOS/ICOSL pathway could potentiate immunosuppression mediated by CD4+Tregs [20], another study revealed that high levels of ICOS+CD4+ cells in CRC tissues or peripheral blood correlated with good prognosis [9], in our results ICOS expression on tissue and peripheral CD4+ and CD8+ were significantly higher in patients than controls and those on malignant tissue of patients were significantly elevated compared with those on peripheral blood.

ICOS stimulation results in inefficient production of IL-2 but adequately induce high levels of IL-4, IL-10, and IL-21 by activated T cells which indicate its role in regulating T helper cell differentiation [21], furthermore, marked ICOS expression on specific subset of CD4+ TIL namely T helper expressing transcription factor T-bet results in IFN-γ production in CRC cancer [9], in addition high levels of ICOS expression on TILs of CRC is associated with favorable outcomes, also its ligand is highly expressed on CRC tissue macrophages, in the current results we detected significant association of ICOS expression with female sex, low grade disease, and sigmoid location, while negative association between peripheral blood ICOS+T cells and IL-21.

Targeting ICOS with agonistic monoclonal antibodies potentiates the action of CTLA-4 inhibitors, moreover, concomitant use of ICOS stimulation and CTLA-4 inhibition induce a stronger antitumor effect compared with CTLA inhibition alone, also other studies have shown that combined CTLA-4 and PD-1 inhibition results in upregulation of ICOS expression on T cells sufficiently to consider its expression on T cells a biomarker for clinical response [22-24]. A component of CD4+TILs is FOXp3 Tregs which is well established in melanoma [11] hepatocellular carcinoma [13], and other tumors where ICOS expression on these cells is much higher compared with peripheral counterparts, and can induce profound immunosuppression and conversion of other CD4+CD25- non-Tregs cells into IL-10 expressing suppressive type 1 regulatory T cells. These cells are associated with poor prognostic features such as high proliferation Ki-67 rates, subsequently targeting ICOS alone seems to be less important than other pathways of cancer immunotherapies. Contrary to these findings, ICOS expression in the current study was found to have an association with good prognostic features in CRC.

It is hypothesized that ICOS:ICOSL axis plays important role in colorectal carcinogenesis as it was associated with progression from dysplasia to carcinoma, increasing tumor size, high grades, aggressive pathological subtypes, PD-1/PDL-1 expression, CD4+T cell accumulation, also ICOS interaction with its ligand induce the production of several cytokines including IL-21 [25] which induce a potent antitumor effect through regulation of a wide range of immune cells, however it is implicated in the pathogenesis of several immune and inflammatory disorders including ulcerative colitis and inflammatory bowel disease [26] suggesting it has a pro-tumor effect in the setting of colitis associated CRC through the promotion of T_h7 phenotype [27], our results were hardly aligned with these findings where negative association between ICOS+TILs and IL-21 level was detected, furthermore positive association of ICOS expression with pathologically less aggressive subtypes (adenocarcinoma not mucinous carcinoma) and lower grades (more indolent features) was elicited.

The development and progression of cancer depends to a great extent on immune disruption and dynamic interaction between tumor microenvironment and tumor cells that determine the characteristics and heterogeneity of cancers, CD8+T cells the prime line for defense against cancer, is continuously exposed to tumor antigens till being exhausted with upregulation of inhibitory receptors including PD-1 and TIGIT [28], blocking of PD-1 results in eradication of tumors especially in those resistant to chemoradiotherapy, moreover combined blockade of PD-1 and TIGIT leads to reversal of CTL exhaustion, in colorectal cancer the tumor tissue upregulate the expression of TIGIT on CD8+T, NK, APC cells resulting in aberrant immune response such as TIGIT+CD8+T cells to exhibit higher infiltration, exhausted phenotype, lower expression of proinflammatory cytokines and higher expression of inhibitory receptors like PD-1 and TIM3 compared with normal tissues and peripheral blood, furthermore high expression of these cells correlated with poor prognosis [29], our findings showed that CD8+TIGIT accumulated in CRC tissues and PB of patients compared with healthy controls and in patients' tissues compared with their PB but we failed to detect any association with clinicopathologic characteristics except for negative association of IL-21 level and tissue CD8+TIGIT+TILs.

Generally, TIGIT is overexpressed in solid tumors including colorectal cancer [30] and correlated with advanced stages, high grades, aggressive types, recurrence, and short survival [30, 31], however the poor prognostic impact

of TIGIT in CRC is inconsistent across studies [32], in the current study TIGIT expression on TILs was more accumulated in smokers and right sided lesion, moreover, TIGIT expression on peripheral immune cells were positively correlated with increased BMI, LDH, and NLR, however CD4+TIGIT+T cells accumulated in lower N stages and CD8+TIGIT+T cells accumulated in early T stages. But this discrepancy could be explained by small sample, heterogeneity of population, different locations with different immune profile.

IL-21, a multifunctional cytokines, is produced by different CD4+T cells specially T follicular helper and T helper 17 while its receptor is expressed mainly on activated B cells, different lymphatic tissues and non-lymphatic tissues including colonic one, in addition to its accusation in disturbed B cell function and antibody production, IL-21-IL-21R signaling activates different pathways including JAK/STAT, MAPK, and PI3K/AKT which are implicated in carcinogenesis including CRC

The role of IL-21 in CRC is confusing, it was reported to have antitumor and protumor effect as it facilitates the expansion of CD8+T cells and improves their expression of CD107 and INFϒ [33], however this effect could be suppressed by inhibitory receptors of T cells (PD1 and TIGIT) [34], comparable with these studies, we could not infer its actual role where it was negatively associated with increased expression of TIGIT on CD8+T cells on tissues of patients.

The small sample size is a significant limitation of this study, affecting the reliability of various statistical analyses, particularly in exploring the relationship between different stages of cancer and immune cells. In Egypt, GLOBOCAN 2022 reported a total of 5,940 new cases (3.9%) of colorectal cancer, with over one-third being metastatic. Additionally, our patient collection was limited to two institutions over just one year, focusing on non-metastatic colonic carcinoma and early rectal carcinoma cases that were not indicated for preoperative radiotherapy.

In conclusion, cancer hallmarks include epigenetic and genetic alterations that activate various immune cells, including T cells. This activation leads to the upregulation of immunostimulatory molecules, such as ICOS, which has been shown to have a positive prognostic impact. However, as T cells become exhausted, there is an increase in immunoinhibitory molecules like TIGIT, which have been associated with disease progression and metastasis.

Declarations:

Ethics approval: All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. The study was approved by the ethical committee in Faculty of Medicine, Assiut Universrity (IRB no 04-2024-300483).

Data availability statement: all data analyzed were included within the manuscript.

Compliance with ethical standards:

There is no conflict of interest. Research involves human participants and informed consent was obtained from all participants.

Consent to participate: Informed consent was obtained from all individual participants included in the study.

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Author contributions: All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by [Asmaa M. Zahran], [Amal Rayan], [Essam Eldeen M.O. Mahran], [Noha Abdel Malik], [Mohamed Farouk], [Amira Gamal El Dein Badary], [Maged AF Amine], [Mohamed Galal Elnaggar], [Salma G. Morsy], [Zeinab Albadry M. Zahran] and [Mohamed Ahmed Rizk]. The first draft of the manuscript was written by [Asmaa M. Zahran] and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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