A Study on the Correlation Between M2 Macrophages and Regulatory T Cells in the Progression of Colorectal Cancer

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Research Article

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Abstract

Background: M2 macrophages and regulatory T cells (Tregs) can promote tumors and development by inhibiting the anti-tumor immune response. This study investigated the number of CD163-positive M2 macrophages and Foxp3-positive Tregs in the progression of colorectal cancer. It also investigated the correlation and of M2 macrophages and Tregs.

Methods: Postoperative tissue specimens and clinical data were collected from 197 patients with colorectal cancer who underwent initial surgical treatment in The Second Ward of Colorectal Surgery of the First Affiliated Hospital of Jinzhou Medical University from March 2020 to December 2020. Use immunohistochemical methods to detect the expression levels of CD163 protein-labeled M2 macrophages and Foxp3 protein-labeled Tregs in colorectal cancer tissues, matched paracancer tissues and lymph node tissues. Analyze the correlation between CD163 and Foxp3 in cancer tissues and lymph node tissues, as well as the relationship between clinicopathological characteristics and preoperative tumor markers.

Results: M2 macrophages and Tregs were significantly positively correlated in cancer and lymph node tissues, which significantly increased in cancer and metastatic lymph node tissues. Interestingly, M2 macrophages in non-metastatic lymph nodes also increased significantly in patients with metastatic lymph nodes. Tregs stage I+II is higher than stage III+IV in paraneoplastic tissues. In addition, both CD163 and Foxp3 were upregulated with increasing tumor TNM stage, depth of infiltration, lymphatic metastasis, and depth of infiltration, and both were positively correlated with CEA.

Conclusion: M2 macrophages and Tregs are important indicators of colorectal cancer progression and lymph node metastasis. There is a certain correlation between the two types of cells. It is possible that M2 macrophages, together with suppressor cells Tregs, promote an immunosuppressive environment.

Introduce

Colorectal cancer (CRC) is one of the most common malignancies in the world and is the fourth leading cause of death among cancer patients[1]. CRC is a complex cancerous disease involving multiple cellular pathways starting from the initial process of tumor transformation to metastasis[2]. The tumor microenvironment consists mainly of immune cells and extracellular matrix, with specific immune cells playing an even more critical role in tumor progression[3].

Tumor-associated macrophages (TAM) are one of the major tumor-infiltrating immune cell types and can be differentiated into "classically activated" M1 macrophages and "alternative activated" M2 macrophages[4]. M1 macrophages and M2 macrophages play opposite roles in the tumor microenvironment. Increased M2/M1 ratio in colorectal cancer is closely associated with enhanced tumor cell invasion[5, 6]. In solid tumors, including colorectal cancer, M2 macrophages are generally associated with poorer clinical outcomes[7]. CD163 is a member of the cysteine-rich superfamily of

scavenger receptors and is restricted to the monocyte/macrophage lineage. It is currently regarded as the most specific marker of M2 macrophages[8].

Regulatory T cells (Tregs) are another type of immune cell. In malignancies, Tregs promote tumor progression by suppressing effective anti-tumor immunity[9]. Tregs mostly have Foxp3 positive expression, and studies have shown that Foxp3 is the molecule that confers the suppressive activity of Tregs. This transcription factor plays a key role in the immunosuppressive effects of Tregs and is closely related to the development and the performance of immunomodulatory functions of Tregs[10, 11]. M2 macrophages and Tregs play an important immunomodulatory role in promoting CRC. M2 macrophages have the ability to induce the formation of Tregs[12]. Studies show a correlation between increased numbers of Tregs and M2 macrophages in several types of cancer[13-15]; however, to the best of our knowledge, this has not been investigated in colorectal cancer progression, especially in the lymph nodes.

In this study, we analyzed the correlation and clinical significance of the number of M2 macrophages and Tregs in the paracancer, cancer and lymph node tissues of 197 colorectal cancer patients by immunohistochemical methods, and further explored the mechanism of colorectal cancer infiltration and metastasis. Thus providing new ideas for CRC diagnosis and treatment.

1. Materials And Methods

1.1 Clinical data

Clinical data and postoperative paraffin specimens of patients undergoing radical colorectal cancer surgery at the Second Ward of the First Affiliated Hospital of Jinzhou Medical University were collected between March 2020 and December 2020. After inclusion and exclusion, a total of 197 cases were included. The clinical data of the patients are shown in Table 1. The tissue samples of patients with colorectal cancer are divided into cancer tissue, matched paracancer tissues and lymph node tissue. In addition, [16]they were divided into three groups according to the presence or absence of metastatic lymph nodes. Group A included 108 cases in which one normal lymph node was randomly selected from stage I and stage II patients. Group B included 89 cases in which one pathologically positive lymph node was randomly selected from stages III and IV patients. Group C included 89 cases in which one pathologically negative node was randomly selected from stages III and IV patients. Inclusion criteria were as follows. (1) The patient received a diagnosis of primary colorectal carcinoma. (2) First surgical treatment after diagnosis at our hospital. (3) Consent to participate in this study. The exclusion criteria were as follows. (1) Patients older than 86 years. (2) Young patients with a confirmed genetic background of the disease (familial adenomatous polyposis and hereditary non-polyposis CRC). (3) The patient had received chemotherapy or radiotherapy after the diagnosis. (4) The patient received targeted immune therapy after the diagnosis. (5) Intestinal malignant tumors with multiple sites or combined with other systemic malignancies. Clinical staging was according to the 2017 American Cancer Consortium (AJCC) staging criteria (8th edition). This study was approved by the Medical Ethics Committee of the First

Affiliated Hospital of Jinzhou Medical University (trial number: KYLL202090) All patients signed an informed consent form.

Table 1

Patient clinical data characteristics

1.2 Immunohistochemical analysis

Using immunohistochemistry streptomyces antibiotic protein-peroxidase linkage (SP) method: All specimens were formalin-fixed, paraffin-embedded, and cut into 4 um thick sections. Sections were placed on microscope slides, dewaxed in xylene and hydrated in a graded ethanol series, and subjected to antigen repair using ethylenediaminetetraacetic acid buffer (pH 9.0) at subboiling temperature for 20 minutes. Tissue sections were incubated with endogenous peroxidase blocker for 10 min at room temperature. Closed with goat serum (KIT-9710; Maishin Biologicals, Fuzhou, China) for 30 min. Sections were washed 3 times with phosphate buffered solution (PBS). Subsequently, anti-CD163 (1:500, ab182422, Abcam), anti-Foxp3 (1:500,ab20034,Abcam) were added and incubated overnight at 4°C. Biotin-labeled IgG polymer and Streptomyces anti-biotin protein-peroxidase (KIT-9710; Maesin Bio) were added sequentially. Finally, sections were developed using the diaminobenzidine (DAB) kit (Catalog No. DAB-0031; Maixin-Bio) and restained with hematoxylin.

Determination of the density of M2 macrophages and Tregs in colorectal cancer and lymph nodes using light biomicroscopy (Olympus BX43; Japan). The density of CD163 and Foxp3 was determined by two pathologists without knowledge of the patient's clinicopathological data by first randomly selecting five areas with M2 macrophage and Tregs infiltration at low magnification (×200). The number of M2 macrophages and Tregs (per mm²) was then calculated at high magnification (×400), and find the mean number of M2 macrophages and Tregs. For Tregs expression in lymph nodes was expressed as a percentage.

1.3 Statistical analysis

The SPSS version 26.0 software program and GraphPad Prism 8 were used to analyze the data. Measurement data were expressed as means ± standard deviation. The independent sample *t*-test was used to compare differences in M2 macrophages and Tregs in cancer tissues with different clinicopathological parameters, as well as differences within the M2 macrophage and Tregs groups. Spearman correlation analysis and linear regression analysis were used to analyze the correlation between two variables. A value of *p*<0.05 was considered significant.

2. Results

Clinicopathological parameters	Group A 例数例	Group B or C 例数例
	n=108(%)	(n=89(%))
Age		
≥65	65(60%)	48(54%)
<65	43(40%)	41(46%)
Gender		
Male	70(65%)	48(54%)
Female	38(35%)	41(46%)
Tumor site		
Colon	58(54%)	42(47%)
Rectum	50(46%)	47(53%)
TNM staging		
I	25(23%)	
II	83(77%)	
III		64(82%)
IV		25(18%)
N stage		
N0	108(100%)	
N1		60(67%)
N2		29(33%)
Differentiation		
High/moderate	93(86%)	61(69%)
Low	15(14%)	28(31%)
Tumor diameter (cm)		
≥5	63(58%)	40(45%)
<5	45(42%)	49(55%)
Distant metastasis		
M0	108(100%)	64(72%)
M1		25(28%)

Immunohistochemical results of CD163 and Foxp3 in different tissues

CD163 is mainly characterized by the presence of yellow or brownish-yellow medium granules on the membrane of M2 macrophages and weak staining in the cell matrix ((Fig.1A-D).Foxp3 is mainly characterized by the presence of brownish-yellow granules on the nuclei of Treg cells, distributed throughout the tissues of CRC patients (Fig.1E-1H). Cancer tissue (Fig.1B, 1F) stained significantly more than paracancerous tissue (Fig.1A, 1E), and metastatic lymph node tissue (Figure 1C, 1G) stained significantly more than non-metastatic tissue (Fig.1D, 1H).

2.2 The relationship between the expression of M2 macrophages and Tregs and the clinical characteristics of tumors

To clarify the infiltration characteristics of M2 macrophages and Tregs cells within colorectal cancer, we performed a statistical analysis of the mean number of M2 macrophages and Tregs in 197 colorectal cancer tissues with clinicopathological parameters (Table 2). The mean number of M2 macrophages and Tregs increased with increasing tumor stage and depth of infiltration ($p<0.05$). In addition, in the case of lymph node metastasis, vascular tumor thrombus, nerve invasion and distant metastasis, the more significant the increase in the mean number of M2 macrophages and Tregs expression ($p<0.001$); However, in patient age, gender, tumor diameter, tumor site and degree of differentiation, there were no significant differences between the two types of cells ($p>0.05$).

Table 2

Clinicopathological characteristics of 197 patients with nodal cancer

Clinicopathological parameters	cases	CD163+M2		Foxp3+Tregs	
		mean/HP	P	(mean/HP)	P
Gender			0.166		0.760
Male	118	18.45±5.85		23.04±6.89	
Female	79	19.67±6.33		22.72±7.67	
Age			0.156		0.544
≥65	118	19.44±6.15		23.17±7.09	
<65	79	18.19±5.89		22.53±7.38	
Tumor diameter (cm)			0.623		0.691
≥5	103	19.15±5.17		22.72±7.30	
<5	94	18.71±6.93		23.13±7.13	
Tumor site			0.541		0.927
Colon	100	19.20±5.88		22.96±7.46	
Rectum	97	18.67±6.26		22.87±6.65	
Lymph node metastasis			<0.001		<0.001
Have	89	22.57±4.76		26.73±6.06	
No	108	15.94±5.36		19.77±6.53	
TNM staging			<0.001		<0.001
I-II	108	15.94±5.36		19.77±6.53	
III-IV	89	22.57±4.76		26.73±6.06	
Differentiation			0.540		0.254
Medium + well differentiated	154	18.80±5.98		22.60±7.19	
Poorly differentiated	43	19.44±6.39		24.02±7.19	
Infiltration depth			<0.001		0.021
T1+T2	26	10.27±4.70		19.88±5.83	
T3+T4	171	20.26±5.07		23.37±7.29	
Vascular tumor thrombus			<0.001		<0.001
Have	85	22.48±4.86		26.00±6.47	
No	112	16.25±5.49		20.57±6.86	

Nerve invasion		<0.001	<0.001
Have	79	22.05±5.37	26.06±6.67
No	118	16.86±5.61	20.81±6.79
Distant metastasis		<0.001	<0.001
M0	172	18.02±5.70	21.76±6.55
M1	25	25.28±4.61	30.84±6.56

2.3 Different expressions of M2 macrophages and Tregs in different tissues

We observed (Fig. 2A and 2B) the mean number of M2 macrophages and Tregs in cancer tissues (18.94 ± 6.06) and (22.91 ± 7.20), significantly higher than the paracancerous tissues (7.08 ± 2.97) and (9.55 ± 2.82), ($p < 0.05$); In the paracancerous tissues (Figure 2C), the mean number of M2 macrophages in stage I+II (5.33 ± 2.38) was lower than that in stage III+IV (9.19 ± 2.12), ($p < 0.05$). However, (Fig. 2D) Tregs stage I+II (10.14 ± 2.72) is higher than III+IV stage (8.83 ± 2.79), ($P < 0.05$). To further investigate the progression of M2 macrophages and Tregs in colorectal cancer patients. We examined their lymph nodes and counted them. It was found (Fig. 2E and 2F) that the mean number of M2 macrophages and Tregs in group A (8.82 ± 3.28) and (9.99 ± 3.70) were significantly lower than those in group B (16.65 ± 3.60) and (18.07 ± 4.33), ($p < 0.05$); However, it is not clear whether the number of M2 macrophages and Tregs in the lymph nodes of patients with stage I+II is different from the number of M2 macrophages and Tregs in the non-metastatic lymph nodes of patients with stage III+IV. Therefore, we counted the M2 macrophages in group A and C, and found (Fig. 2G) that the mean number of M2 macrophages in group A (8.82 ± 3.28) was lower than that of group C (9.89 ± 2.47), ($p < 0.05$). (Fig. 2H) There was no difference in the mean number of Tregs (9.99 ± 3.70) and (10.42 ± 3.06) between group A and group C, ($p > 0.05$).

2.4 Correlation analysis of M2 macrophages and Tregs in cancer tissues and lymph nodes

By comparative analysis (Fig. 3) we found that M2 macrophages and Tregs were abundantly distributed in cancerous tissues and lymph nodes. In order to verify whether there is a certain correlation between the two in the development of colorectal cancer and lymph node metastasis, we used Spearman correlation analysis to find that: (Fig. 3a and 3b) M2 macrophages and Tregs are positively correlated in CRC and lymph nodes ($r = 0.269$, $p < 0.001$; $r = 0.541$, $p < 0.001$).

2.5 Correlation analysis of the number of M2 macrophages and Tregs with tumor markers in CRC

According to Table 3, Spearman correlation analysis was used to determine the relationship between the mean number of M2 macrophages and Tregs and preoperative CEA, CA199, and CA724 levels. It was found that the mean number of M2 macrophages and Tregs were found to be significantly and positively correlated with preoperative CEA levels, respectively (both $P < 0.001$).

Table 3

Correlation analysis between the number of M2 macrophages, Tregs and tumor markers

Tumor markers	The mean number of M2 macrophages		The mean number of Tregs	
	<i>r</i>	<i>P</i> value	<i>r</i>	<i>P</i> value
CEA level before surgery	0.464	<0.001	0.365	<0.001
CA199 level before surgery	0.170	0.017	0.089	0.211
CA724 level before surgery	0.095	0.182	0.050	0.486

3. Discussion

Immune cells play a crucial role in the development of tumors in the tumor microenvironment [17]. In this study, immunohistochemical staining was performed on paracancerous tissue, cancerous tissue and its surrounding lymph node tissue of 197 patients with colorectal cancer. The mean number of M2 macrophages and Tregs was found to be significantly higher in cancer tissue than in paracancerous tissue. The mean number of M2 macrophages was higher in stage III+IV than in stage I+II in paraneoplastic tissue, yet the mean number in stage III+IV Tregs was lower than in stage I+II. In lymph node tissue, the mean number of M2 macrophages and Tregs was significantly higher in metastatic lymph nodes. In addition, M2 macrophages also significantly increase in the non-metastatic lymph node tissue of patients with metastatic lymph nodes. Notably, our study found a positive correlation between M2 macrophages and Tregs in cancer tissues and lymph node tissues.

M2 macrophages are a kind of activated macrophages induced by Th2 cytokines interleukin (IL-4), interleukin-10 (IL-10) and interleukin 13 (IL-13). It promotes tumor angiogenesis and leads to tumor progression by suppressing T-cell-mediated anti-tumor immune responses [18, 19]. Our study found that the mean number of M2 macrophages was significantly higher in cancer tissue than in paracancerous tissue. The mean number of M2 macrophages was significantly greater in stage III+IV than in stage I+II in paraneoplastic tissue. With the rapid growth of tumor tissue leads to increased metabolism and uneven vascularization around it. Therefore, the lack of blood vessels in the tumor area causes tumor hypoxia. Hypoxia causes tumor cells to release factors such as HIF-1 α and HIF-2 α , which can increase the

expression of miRNAs through the PI3K/AKT/mTOR pathway and promote the polarization of M2 cells [20-22]. Lian et al [23] also noted that colon cancer cells secrete EGF, which can bind to EGFR on monocytes, activate the smad-PI3K-Akt-MTOR pathway and promote the differentiation of monocytes into M2 macrophages. We also found higher mean numbers of M2 macrophages in tumor tissue in the presence of more severe vascular cancer thrombosis, nerve invasion, and distant metastases. Meanwhile, the increase in the mean number of M2 macrophages was closely associated with the TMN stage, lymph node metastasis and depth of infiltration of the tumor. It indicates that M2 macrophages are involved in the formation of the immunosuppressive microenvironment in cancer tissues, which may be one of the important factors leading to the occurrence of colorectal cancer. The epithelial growth factor (EGF) secreted by M2 macrophages activates the factor receptor (EGFR) on tumor cells, which in turn up-regulates the VEGF/VEGFR signals in surrounding tumor cells to support tumor cell proliferation and migration [24]. Our study found that in lymph node tissue, the mean number of M2 macrophages not only increased significantly in metastatic lymph node tissue, but also in non-metastatic lymph nodes in patients with metastatic lymph nodes. It suggests that in patients with metastatic lymph nodes, there is more M2 macrophage infiltration in the part where tumor metastasis has not occurred. It shows that the microenvironment of the lymph node has changed before metastasis, and M2 macrophages are involved in it. This is the same result as our previous study [16]. Therefore, we speculate that M2 macrophages play an important role in the process of lymph node metastasis in colorectal cancer, which indicates a poor prognosis. Tacconi C et al [25] stated that VEGF-C promotes proliferation and expansion of lymphatic vessels, thereby increasing the pathway of tumor metastasis to lymph nodes. Therefore, M2 macrophages may change the tumor microenvironment and promote the lymph node metastasis of colorectal cancer [16, 26].

Tregs are a subset of T lymphocytes with immunosuppressive effects, which can induce tumor immune escape by inhibiting effector T cells, and induce tumor growth and immune tolerance[27]. To investigate the changes in Tregs during CRC progression, we examined the changes in Tregs in paracancerous, cancerous and lymph node tissues. CRC was divided into early (stage I+II) and late (stage III+IV) stages for analysis. It was found that Tregs in cancer tissues were significantly higher than those in paracancerous tissues, but in the late phases, the paracancerous tissues were lower than those in the early stage. We analyze tumor cells and microenvironmental macrophages to produce chemokine CCL22, which can bind to the CCR4 receptor highly expressed on the surface of FOXP3 + Tregs. This helps recruit Treg cells to tumor tissues[28, 29]. In addition, the dysregulation of colon inflammation is an important cause for the development of CRC. The increased inflammation of paracancerous tissues in the early stage of CRC promotes the continuous accumulation of Treg cells to inhibit inflammation [30, 31]. Märkl et al [32] analyzed 136 specimens of patients with early stage (stage I and II) colon cancer. It was found that Tregs infiltrated in cancerous tissues higher than in paracancerous tissues, and high Tregs in paracancerous tissues indicated a better prognosis, which just confirms our research. In addition, we analyzed the correlation between Tregs and clinicopathological characteristics. It is found that Tregs in CRC are closely related to TNM staging, lymph node metastasis and distant metastasis. TNM stage and

lymph node metastasis are important indicators of prognosis and survival time of tumor patients. Thus, our results suggest that Tregs infiltration is associated with CRC metastasis and poor prognosis.

Our research found that there is a certain correlation between M2 macrophages and Tregs in cancer tissues and lymph node tissues. M2 type macrophages can produce immunosuppressive cytokines and chemokines. These cytokines and chemokines are involved in recruiting lymphocytes and stimulating their development into Tregs [28, 33]. In addition, Tregs produce high levels of IL-10, IL-32 and TGF- β , which further inhibit the anti-tumor inflammatory response and stimulate M2 macrophages to increase the production of cytokines and chemokines, thereby being able to recruit additional Tregs[34]. Studies have pointed out [35] M2 macrophages and Tregs have synergistic effects in promoting proliferation, tumor angiogenesis and metastasis in ovarian cancer. Sun et al [15] studied 65 patients with laryngeal squamous cell carcinoma (LSCC) and found that Tregs and M2 type macrophages were positively correlated with each other in LSCC, and demonstrated that the two formed a positive feedback loop. In addition, we also preliminarily confirmed that M2 macrophages may induce the production of regulatory T cells by activating the TGF- β /Smad signaling pathway[36]. Therefore, we speculate that M2 macrophages in CRC may have the ability to induce the formation of Tregs and increase the expression of Tregs in tumor tissues. The interaction between the two may change the tumor microenvironment and promote the development of CRC and lymph node metastasis.

The specific mechanism of action of M2 macrophages and Tregs in CRC, especially the molecular mechanism of interaction in local lymph nodes, will be the next focus of our group's research. In addition, studies have pointed out [37, 38] that CD163 can be used as a potential prognostic biomarker for CRC patients, and Foxp3 can directly affect the prognosis of CRC patients. To further verify the impact of both on the monitoring and prognosis of CRC patients, our study analyzed the preoperative CEA, CA199 and CA724 levels in correlation with the number of M2 macrophages and Tregs. It was found that the level of CEA was significantly positively correlated with the mean number of M2 macrophages and Tregs. This is consistent with our previous findings [16]. We know that CEA plays an important role in monitoring the recurrence and metastasis of colorectal cancer. Therefore, we speculate that CEA has an important influence on the distribution of M2 macrophages and Tregs in colon cancer.

In summary, the presence of M2 macrophages and Tregs in colorectal cancer patients is an important indicator of colorectal cancer progression and lymph node metastasis. Our results provide some insights for M2 macrophages and Tregs in the progression of colorectal cancer and its lymph node metastasis. There is a certain correlation between the two. The relationship between M2 macrophages and Tregs may affect the progression of colorectal cancer and lymph node metastasis. This could be a potential future target for immunotherapy and provide important clinical value for colorectal cancer diagnosis, treatment, and prognosis.

Declarations

Ethics approval and consent to participate

The study was conducted in accordance with the Ethics Committee of the First Affiliated Hospital of Jinzhou Medical University and the 1964 Helsinki Declaration. Informed consent was obtained from all participants included in the study.

Consent for publication

Not applicable

Availability of data and materials

The analyzed data sets generated during the study are available from the corresponding author on reasonable request. Inquiries for data access may be sent to the following e-mail address: shifengqiao2020@163.com.

Competing interest

The authors declare that they have no competing interests.

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Authors' contributions

Y-LC, Y-G, X-QM, and C-YY performed the experiments. Y-LC, Y-PW, J-HL and C-FB contributed to the statistical analysis and drafted the manuscript. Y-LC, X-YS, Y-F and Y-S participated in collecting the data contributed to the statistical analysis and manuscript writing. S-FQ conceived the present study and helped revise the manuscript. All authors read and approved the final manuscript.

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Figures

Figure 1

Immunohistochemical results of CD163 and Foxp3 in different tissues

Immunohistochemical staining of colorectal cancer and lymph nodes as shown:(A) paracancerous tissues and (B) cancer tissues are membrane staining of CD163, (E) paracancerous tissues and (F) cancer tissues are nuclear staining of Foxp3; (C) Metastatic lymph nodes and (D) non-metastatic lymph nodes are membrane staining of CD163, (G) metastatic lymph nodes and (H) non-metastatic lymph nodes are nuclear staining of Foxp3. Original magnification ×400.

Figure 2

Different expressions of M2 macrophages and Tregs in different tissues

(Fig. A and B) M2 macrophages and Tregs were significantly higher in cancer tissue than in paracancerous tissue, with statistically significant differences (both *** $p < 0.001$). In paracancerous tissues (Fig. C) M2 macrophages stage I+II are lower than stage III+IV, (Fig. D) Tregs cells stage I+II are higher than stage III+IV, the difference is statistically significant (*** $P < 0.001$; ** $P < 0.05$). (Fig. E and F) M2 macrophages and Tregs in group A were significantly lower than those in group B, and the differences were statistically significant (both *** $P < 0.001$); In the non-metastatic lymph node tissue (Figure G), the mean number of M2 macrophages in group A was lower than that in group C, and the difference was statistically significant (** $p < 0.05$). (Fig. H) There was no significant difference in Tregs between group A and group C, and the difference was not statistically significant (* $p > 0.05$).

Figure 3

(Fig. a) Correlation of M2 macrophages and Tregs in cancer tissue ($r=0.269$, $p<0.001$); (Fig. b) Correlation of M2 macrophages and Tregs in lymph node tissue ($r=0.541$, $p<0.001$).