

***In situ* analysis of FOXP3⁺ regulatory T cells and myeloid dendritic cells in human colorectal cancer tissue and tumor-draining lymph node**

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Received September 10, 2012; Accepted October 20, 2012

DOI: 10.3892/br.2012.35

Abstract. Forkhead box protein 3 (FOXP3) regulatory T cells (Tregs) are important in the maintenance of tumor immunity tolerance. Myeloid dendritic cells (mDCs) are antigen-presenting cells (APCs) specialized to initiate and regulate immunity. Tregs and mDCs are suspected of influencing the interaction between the tumor and immune system, and thus the course of tumors. However, the implication and interaction of their concurrent infiltration in colorectal cancer (CRC) remain unknown. The aim of this study was to determine FOXP3⁺ Tregs and CD11c⁺ mDCs infiltration in CRC and tumor-draining lymph node (TDLN) and to explore the clinical and pathological implication of suppressor and effector immune cell subsets. Immunohistochemical assay was conducted to assess FOXP3⁺ Tregs and CD11c⁺ mDCs infiltration in tumor tissue and in metastasis-free TDLN (mfTDLN) and metastatic TDLN (mTDLN). The results showed that FOXP3⁺ Tregs and CD11c⁺ mDCs infiltration was higher in tumor tissue compared to adjacent normal mucosa ($P<0.001$). FOXP3⁺ Tregs infiltration was associated with advanced tumor-node-metastasis (TNM) stage and lymph node metastasis ($P<0.001$ and $P<0.001$, for TNM stage and lymph node metastasis, respectively), whereas less CD11c⁺ mDCs infiltration of tumor *in situ* was associated with deeper tumor invasion, advanced TNM stages and lymph node metastasis ($P<0.05$, $P<0.001$ and $P<0.001$, for tumor invasion depth, TNM stages and lymph node metastasis, respectively). Compared to mfTDLN, mTDLN was significantly enriched in FOXP3⁺ Tregs ($P<0.001$) and reduced in CD11c⁺ mDCs ($P<0.001$). The statistical analysis demonstrated no significant correlations in

Tregs and mDCs infiltration. These results suggest that more FOXP3⁺ Tregs and less CD11c⁺ mDCs infiltration have stronger prognostic significance in CRC. The presence of tumor cells in mTDLN may contribute to a tolerogenic milieu and facilitate the survival of metastatic tumor cells.

Introduction

Colorectal cancer (CRC) is one of the most common gastrointestinal tumors and ranks third regarding cancer-associated morbidity. Clinically, due to preoperative medical imaging examination, surgical treatment technology and use of powerful chemotherapeutics, the CRC prognosis has evidently improved. Nevertheless, 40-50% of CRC patients suffer from recurrence and metastasis. In the various factors that affect the prognosis of CRC, a variety of immune cells in CRC microenvironment and draining lymph nodes play a crucial role.

Regulatory T cells (Tregs) are a unique CD4⁺ helper T cell subset and are thought to modulate the autoimmune response. Tregs are characterized by the CD4⁺CD25⁺ phenotype. Forkhead box protein 3 (FOXP3) belongs to the forkhead/winged-helix family of transcriptional regulators and acts as a master regulator of the development and function of Tregs. The most specific and reliable surface marker of Tregs has been identified (1-3). Limited anti-tumor activity of antigen-specific T cells at a clinical level may be limited in CRC patients for various reasons. One major reason for the clinical failure of tumor-directed immune responses might be the generation of the tolerogenic milieu in tumor tissue and tumor-draining lymph nodes (TDLN). Tregs are partly responsible for impeding immune escape against autologous tumor cells (4).

Dendritic cells (DCs) are powerful antigen-presenting cells (APCs) and the interaction of T cells with DCs is fundamental for a favorable immune response to tumors. DCs comprise two phenotypically and functionally distinct subpopulation, conventional myeloid dendritic cells (mDCs) and plasmacytoid DCs (pDCs). pDCs are type-I IFN-producing cells known for their ability to promote anti-viral innate and adaptive immune response. When compared with conventional DCs, pDCs are implicated in protective immunity as well as in tolerance

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Key words: colorectal cancer, tumor-draining lymph nodes, tumor metastasis, immune state, regulatory T cells, myeloid dendritic cells

induction (5-6). mDCs function as specific antigen-presenting cells, it can play a critical role in inducing and regulating T cell-mediated immune response, including TH1, TH2 and TH17 response (7). As the strongest APCs, mDCs are able to induce specific cytotoxic T-lymphocyte generation. For this reason, mDCs are closely correlated with tumor occurrence and development (8). Furthermore, mDCs have an important impact on selection of Tregs in thymus and differentiation, proliferation and function regulation in peripheral lymphoid tissues (9-12). *In vitro* studies also showed that mDCs have the ability to activate Tregs under certain circumstances (13). At present, whether or not the CRC intratumoral presence of mDCs affects the antigen-specific Tregs, clinical course or outcome of the patients has yet to be elucidated.

The aim of our study was to analyze the infiltration of CRC by FOXP3⁺ Tregs and CD11c⁺ mDCs in tumor *in situ* and TDLN, and to investigate whether or not there is a correlation with the clinicopathological characteristics of the disease. We applied an immunohistochemical method with FOXP3-Ab and CD11c-Ab to examine Tregs and mDCs infiltration and to identify the potential roles of Tregs and mDCs in CRC immunity.

Patients and methods

Patients and tissue specimens. Fifty two formalin-fixed paraffin-embedded (FFPE) CRC samples and 20 FFPE normal colorectal mucosa samples obtained from >5 cm from the tumor margin were used in this study from patients who underwent treatment at the General Hospital of CNPC in Jilin between 2008 and 2011. The investigation was approved by the Ethics Committee of CNPC. The patients (29 males, 23 females; mean age, 64 years, ranging from 47 to 82 years) were diagnosed as CRC without preoperative radiotherapy or chemotherapy. A number of clinicopathological characteristics, such as gender, age, location, histological type, tumor-node-metastasis (TNM), depth of tumor invasion, lymph node metastasis, and the differentiation degree were included in clinicopathological studies. According to the classification of CRC (WHO 2000), 47 samples were tubular adenocarcinoma and 5 were mucoid carcinoma. Regarding tumor differentiation, 6 samples were well-differentiated and 41 samples were moderately-poorly differentiated. Twenty of 52 patients had lymph node metastasis, while 32 of 52 patients had no lymph node metastasis. According to the Union for International Cancer Control (UICC 2002) criteria, 14 patients were in the grade T1+T2 group and 38 patients were in the grade T3+T4 group. Thirty patients were in the I+II tumor group, 22 were in the III+IV tumor group according to TNM staging criteria of the 2002 UICC staging system.

Immunohistochemical analysis. FFPE tissue sections (4 μ m) were prepared for immunohistochemistry using the Ready-to-Use Immunohistochemistry Hypersensitivity UltraSensitive™ S-P kit (Fuzhou Maxim, Fuzhou, China) according to the manufacturer's instructions. Tissue sections were first deparaffinized in xylene and rehydrated in a series of graded alcohols. After hydration, endogenous peroxidase activity was blocked using 3% H₂O₂ (v/v) for 10 min at room temperature. Standard antigen retrieval was then performed

using heat-induced epitope retrieval by heating the slides immersed in the boiling retrieval solution (EDTA, pH 8.0) in pressure boiler for 2 min. Gradually cooling at room temperature, the sections were washed three times with phosphate-buffered saline (PBS), then non-immune animal serum was dropped onto the sections for 10 min at room temperature and the serum was wiped off. The sections were subsequently incubated with monoclonal antibody against either FOXP3 (Wuhan Boster Biological Technology, Ltd., Wuhan, China), or monoclonal antibody against CD11c (Beijing Fir Jinqiao Biotechnology Company, Beijing, China), followed by a biotin-labeled secondary antibody and streptomycin anti-biotin peroxidase. Diaminobenzidine staining reaction was then performed, followed by hematoxylin counterstaining. The slides were then dehydrated, cleared and mounted as normal. Negative controls were performed by omitting the primary antibody instead of using PBS. Internal positive control was used for quality assurance. Ten randomly selected high-power fields (HPF; 1 HPF=0.237 mm²) were analyzed for Tregs and mDCs infiltration in the two tumors *in situ* and draining lymph nodes, and 10 HPF were averaged in each case.

Statistical analysis. Statistical analysis was performed using the Statistical Package for Social Sciences (SPSS) software, version 13.0. Correlation of Tregs and mDCs infiltration with the clinicopathological characteristics of the tumors was evaluated using the Student's t-test. Tregs and mDCs infiltration in metastatic TDLN (mTDLN) and in metastasis-free TDLN (mfTDLN) was also assessed using the Student's t-test. Correlation of Tregs infiltration with mDCs infiltration was analyzed using the Spearman's rank-order correlation analysis. For the comparisons, P<0.05 was considered to indicate a statistically significant difference.

Results

Tregs and mDCs infiltration in CRC. FOXP3 protein expression was found in the nucleus of Tregs. FOXP3⁺ Tregs were expressed in CRC tumor tissue and adjacent normal mucosa. However, the frequency of cells expressing FOXP3 protein in CRC tissue was much higher compared to adjacent normal mucosa (8.07/HPF vs. 0.96/HPF, P<0.01, Fig. 1A).

The expression of CD11c protein was also analyzed in CRC tumor tissue and adjacent normal mucosa, and was primarily expressed in the cell membrane of mDCs. CD11c⁺ mDC exhibited a higher infiltration in CRC, which was in variable shapes, irregular and varying in size, and certain cells had extended cell processes. CD11c⁺ mDCs mainly scattered in the tumor stroma, or in the surrounding edge of tumor cell clusters, and were often accompanied by infiltration with lymphocytes. CD11c⁺ DCs infiltration was significantly increased in CRC tumor tissue compared to adjacent normal mucosa (6.72/HPF vs. 2.32/HPF, P<0.01, Fig. 1B).

Clinical and pathological implication of Tregs and mDCs infiltration. The correlations between the clinicopathological characteristics of the tumor samples and Tregs or mDCs infiltration are summarized in Table I. Significant increase in Tregs infiltration was associated with tumor samples that had advanced TNM stages (9.56/HPF vs. 6.97/HPF, P<0.01) and

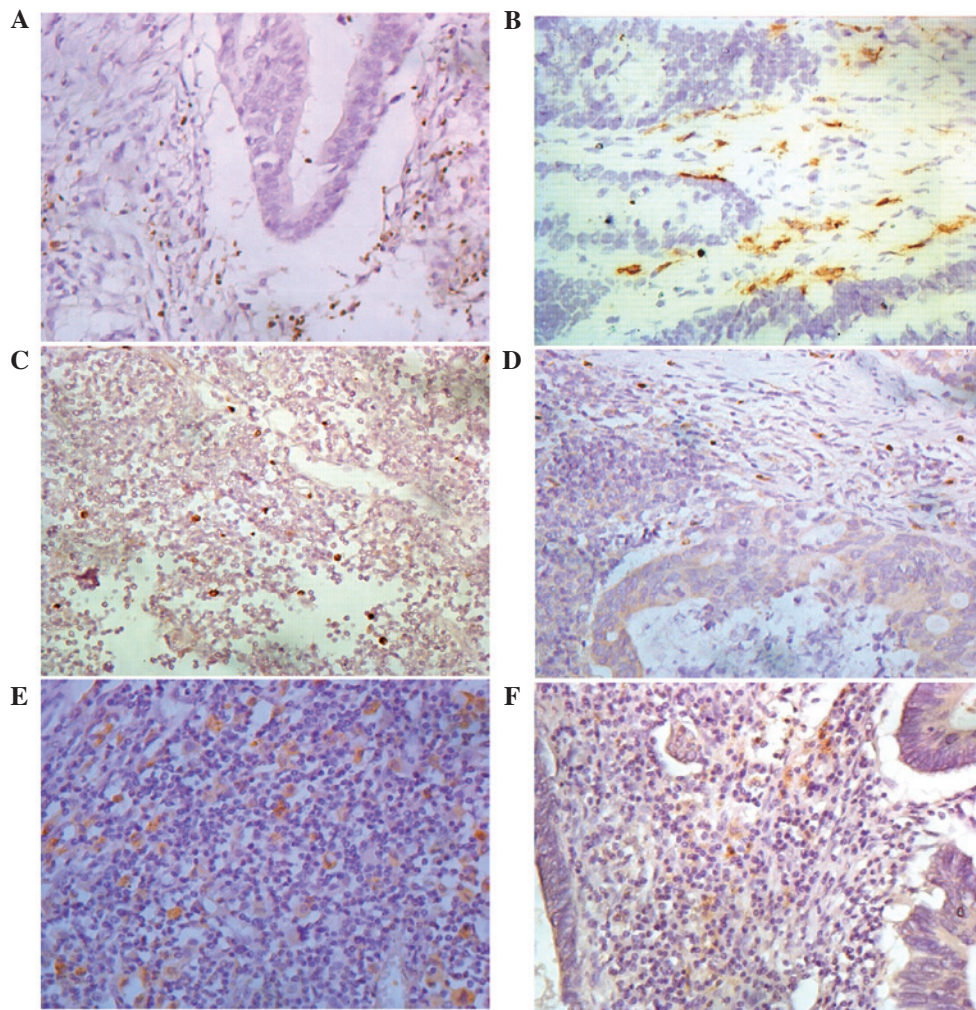


Figure 1. Immunostaining in colorectal cancer (CRC) tissues and tumor-draining lymph node (TDLN). Nuclear staining for (A) FOXP3 in CRC and membranous staining for (B) CD11c in CRC. Nuclear staining for FOXP3 in (C) metastasis-free TDLN (mfTDLN) and (D) metastatic TDLN (mTDLN) and membranous staining for (E) CD11c in mfTDLN and (F) mTDLN. Original magnification, x400.

lymph node metastasis (9.72/HPF vs. 7.04, $P < 0.01$). However, Tregs infiltration showed no statistically significant difference in patient age, tumor size, histology, invasion depth and tumor differentiation ($P > 0.05$). By contrast, tumors with the deeper tumor invasion were found to have lower infiltration of CD11c⁺ mDCs (5.81/HPF vs. 8.51/HPF, $P < 0.05$). The CD11c⁺ mDCs were significantly lower in advanced TNM stages showing signs of decreasing immunity against tumor (3.75/HPF vs. 9.69/HPF, $P < 0.01$). As expected, CD11c⁺ mDCs infiltration decreased in tumors with the presence of lymph node metastasis compared to the absence of lymph node metastasis (3.73/HPF vs. 8.99/HPF, $P < 0.01$). No statistically significant difference was found in various age, size, histology and differentiation groups with regard to CD11c expression ($P > 0.05$).

Tregs and mDCs infiltration in mTDLN and mfTDLN. We aimed to assess whether or not metastatic cancer cells had any impact on Tregs and mDCs infiltration in TDLN. Therefore, 52 CRC cases, including 20 cases with mTDLN and 32 cases with mfTDLN were detected. FOXP3⁺ Tregs were diffusely distributed in the paracortical areas of lymph node and metastatic carcinoma stroma. Tregs infiltration was significantly higher in mTDLN compared to mfTDLN (8.2/HPF vs. 5.5/

HPF, $P < 0.01$, Fig. 1C and D). The mDCs status was observed, and results showed that CD11c⁺ mDCs mainly were distributed in the T cell-rich zones of lymph nodes. They also accumulated in the vicinity of tumor nests, and CD11c⁺ mDCs infiltration decreased significantly in mTDLN compared to mfTDLN (3.55/HPF vs. 9.03/HPF, $P < 0.01$, Fig. 1 E and F).

Correlation of Tregs and mDCs infiltration. The correlation between FOXP3 and CD11c expression was also analyzed in CRC tissue and TDLN (Table II). The statistical analysis suggested that no significant correlation was observed in the Tregs and mDCs infiltrations.

Discussion

FOXP3, a member of the forkhead/winged-helix transcriptional factor family, has been demonstrated to be the master regulator of Tregs development in the thymus, as well as Tregs suppressive function (1-3,14). A series of studies demonstrated that Tregs was closely correlated with the tumor immune escape mechanism. In previous studies, increased proportions of CD4⁺CD25⁺ Tregs in the total CD4⁺ T-cell populations were detected in various types of cancer, including lung,

Table I. Correlation between Tregs and mDCs infiltration and clinicopathological characteristics in CRC.

Clinicopathological characteristics	No.	Treg/HPF \pm SD	P-value	mDC/HPF \pm SD	P-value
Age (years)					
≥ 60	33	7.77 \pm 2.47	0.165	6.81 \pm 4.30	0.430
<60	19	8.59 \pm 3.49		6.58 \pm 4.31	
Histology					
Tubular adenocarcinoma	47	8.11 \pm 3.51	0.369	6.97 \pm 4.25	0.135
Mucinous carcinoma	5	7.52 \pm 5.53		4.72 \pm 4.07	
Invasion depth					
T1+T2	14	7.25 \pm 2.82	0.109	8.51 \pm 4.39	<0.05
T3+T4	38	8.37 \pm 2.88		5.81 \pm 3.88	
Differentiation					
Well	6	8.12 \pm 3.96	0.485	8.26 \pm 4.61	0.198
Moderately-poorly	41	8.07 \pm 2.87		6.52 \pm 4.23	
Lymph node metastasis					
Yes	20	9.72 \pm 3.16	<0.001	3.73 \pm 1.55	<0.001
No	32	7.04 \pm 2.17		8.99 \pm 4.26	
TNM stage					
I+II	30	6.97 \pm 2.18	<0.001	9.69 \pm 4.01	<0.001
III+IV	22	9.56 \pm 3.10		3.75 \pm 1.56	

Values in bold indicate statistical significance. Tregs, regulatory T cells; HPF, high-power fields; SD, standard deviation; mDCs, myeloid dendritic cells; CRC, colorectal cancer.

Table II. Correlation between FOXP3⁺ Tregs and mDCs in CRC tissue and TDLN.

	R	P-value
CRC tissue	-0.29	0.063
TDLN	-0.30	0.137

R, Spearman's coefficient correlation; significant correlations (P<0.05). FOXP3; forkhead box protein 3; Tregs, regulatory T cells; CRC, colorectal cancer; TDLN, tumor-draining lymph node.

breast and ovarian tumors, and metastatic melanoma in lymph nodes (15-18). Tregs infiltrating human tumors were shown to be tumor antigen-specific as indicated by experiments showing that Tregs suppressed the proliferation of naïve CD4⁺ T cells and inhibited IL-2 secretion by CD4⁺ effector cells upon activation by tumor-specific antigens (19-20). Sato and colleagues found that ovarian cancer patients with a higher CD8⁺/Treg-cell ratio in tumor tissue had an increased survival chance (21). Their study suggested that Tregs negatively affects the antitumor immune response mediated by effector CD8⁺ T cells. This finding suggests that strategies aiming to enhance tumor-specific CD8⁺ T cells while decreasing Tregs may be effective in improving the outcome in cancer patients. Consistent with those studies, we found significantly higher numbers of Tregs in CRC compared to the adjacent normal

mucosa. Although numbers varied from patient to patient, there was an ~8-fold increase of Tregs infiltration in CRC compared to healthy mucosa.

To exhibit potential functions of Tregs in CRC, we analyzed the correlation between Tregs and clinicopathological characteristics. Results showed that Tregs infiltration was closely correlated with lymph node metastasis and TNM staging. Tregs in patients with lymph node metastasis were significantly more expressed compared to those without lymph node metastasis and Tregs infiltration showed an increase in TNM staging. Lymph node metastasis and TNM staging are important indications for judging the prognosis and survival time of cancer patients. Therefore, the results suggest that Tregs infiltration correlates with CRC metastasis and poor prognosis. Tumor cells have potentially more opportunity to be exposed to immune cells directly or indirectly with the progression of disease. Furthermore, tumor cells secrete factors such as transforming growth factor β (TGF- β), COX-2 and IL-10, thus recruiting and expanding Tregs in tumor tissue (22-23). In addition, tumor cells secrete chemokine CCL22, which can be combined with a high expression of CCR4 receptors on the surface of FOXP3⁺ Tregs. It also contributes to the recruitment of Tregs to tumor tissue (17).

mDCs are specialized APCs, characterized by the efficient induction of primary T-cell responses and are thus able to initiate body immune responses, and produce anti-tumor immunity (24). Of note, we found an ~3-fold increase of mDCs at the tumor site in CRC patients compared to normal mucosa patients. The mechanism of this phenomenon is

unclear. However, it underlines the importance of anti-tumor immunity responses in *in situ* analyses. We hypothesize that on CRC tumor-antigen stimulation, the body part of the mDCs migrates to tumor tissue. Previous studies have suggested that mDCs density ≥ 20 /HPF in tumor tissue was identified as high infiltration. According to this standard, mDCs infiltrated in CRC tumor tissue were 6.72/HPF and did not reach the level of anti-tumor immune response, which might be a reason for CRC local tissue immunodeficiency (25). Since the decrease in the frequency and function of mDCs was considered a negative factor in anti-tumor immunity (26), we performed an analysis of mDCs infiltration in correlation with the clinicopathological characteristics. The study showed that mDCs infiltration negatively correlated with lymph node metastasis and invasion depth. The mDCs infiltration in patients with lymph node metastasis was significantly lower than that in patients without lymph node metastasis, and a decrease in mDCs infiltration occurred with an increase in TNM staging. Therefore, the extent of infiltrated mDCs in CRC tumor tissue generally reflects the tumor immune state.

In tumor immunity, the presentation of tumor antigen to naive CD4⁺ T cells occurs in regional lymph nodes, known as TDLN, in principle the optimal environment for the generation of an effective anti-tumor immune response. However, TDLN are under the effect of tolerogenic factors produced by tumor cells (17,27,28). Tumor immune tolerance rather than activation is induced to hinder the immune attack and promote tumor progress. In other words, mfTDLN and mTDLN are under the effect of tolerogenic factors imported from the tumor site. It was thought that immune tolerance should be more evident in mTDLN, which are mostly exposed to tolerogenic factors locally produced by tumor cells. In the present study, we aimed to confirm whether or not mTDLN exposed to mostly tolerogenic factors were more immunosuppressed compared to mfTDLN. We analyzed Tregs and mDCs infiltration and found a significant excess of Tregs in mTDLN. By contrast, mDCs infiltration in mTDLN was much lower compared to mfTDLN. This observation suggests that in mTDLN the presence of tumor cells could break the *in situ* anti-tumor immune response down, while facilitating tumor progression.

The above results have demonstrated that Tregs and mDCs infiltration correlates with a malignant biologic phenotype in CRC. Additionally, studies have shown that mDCs were associated with Tregs selection in thymus and differentiation, proliferation and function regulation in peripheral lymphoid tissues (9-12). Increasing evidence suggests that intratumoral DCs are actively immunosuppressive and induce tolerance to tumor (29). However, understanding of the correlation of DCs and Tregs interaction, modulation of activity and function of various DCs subtypes at the tumor site is incomplete. Thus, we also analyzed the correlation between mDCs Tregs and in CRC tumor tissue and TDLN and did not find any significant correlation in the tumor tissue or in TDLN. As a tolerogenic DCs, pDCs may induce tolerance and promote Tregs development rather than tumor immunity (30-32). The shift of DCs subset in tumor tissue may tip the balance against anti-tumor immune response and facilitate the survival of tumor cells (33). The correlation and significance of pDCs and FOXP3⁺ Tregs in CRC is to be discussed in a future study.

In summary, FOXP3 are highly expressed in CRC tumor tissue and TDLN. Nevertheless, CD11c expression decreased in TDLN and in CRC tumor stroma with CRC development, and are strongly correlated with malignant tumor phenotypes. Thus, the concurrent increased expression of FOXP3 and decreased expression of CD11c may contribute to an efficient metastasis of CRC to the lymph node and to tumor progress.

Acknowledgements

This study was supported by grant no. 2011151 from the Department of Education of Jilin Province and National Natural Science Foundation of China (grant no. 81170632).

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