Distinctive profiles of tumor-infiltrating immune cells and association with intensity of infiltration in colorectal cancer

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Abstract. Tumor-infiltrating immune cells are heterogeneous and consist of characteristic compartments, including T helper (Th)1 and regulatory T (Treg) cells that exhibit distinctive biological functions. The present study investigated the profile of infiltrating immune cells from surgically removed tumor tissues from patients with colorectal cancer. The characteristic transcription factors of Th1 and Th2 cells, Treg cells, Th17 cells and T follicular helper (Tfh) cells were analyzed. The results demonstrated that a marked increased number of Treg cells presented in tumor infiltrates when compared with non-tumor adjacent tissues. An increased number of Th1 and Tfh cells existed in tumor infiltrates compared with non-tumorous adjacent tissues, while the infiltration of Th17 and Th2 cells was similar between tumor and non-tumor adjacent tissues. Furthermore, there were an increased number of Treg cells in tumors with low infiltration compared with those with high infiltration. The expression of CXC motif chemokine (CXC) receptor 3, CXC ligand (CXCL)L9 and CXCL10 was significantly increased on infiltrating T cells in tumors with high infiltration as compared with those with low infiltration. Macrophages exhibited a dominant M2 phenotype in tumor infiltrates of colorectal cancer, whereas a balanced M1 and M2 phenotype presented in macrophages from the peripheral blood. *In vitro* stimulation of macrophages isolated from tumor tissue of colorectal cancer with granulocyte macrophage colony-stimulating factor and lipopolysaccharide did not drive to an inflammatory phenotype. The results provide insights into the pattern of immune cell infiltration in Chinese patients with colorectal cancer. It may be beneficial that patients with colorectal cancer are screened for the defined profile along with the expression of CXCL9 and CXCL10 in order to achieve better efficacy in clinical applications of immune-based therapy, including anti-programmed cell death protein 1 therapy.

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Introduction

Colorectal cancer (CRC) is a common malignant disease, which has been intensely studied for tumor-immune interactions in order to develop successful immunotherapies. In particular, systemic T cell responses against tumor antigens and tumor-infiltrating T cells have been analyzed in detail in CRC (1-4). A number of studies have linked a high T cell infiltration to an improved survival in CRC (1-6). Patients with CRC as well as those with other malignant diseases are able to mount an antigen-specific T cell response without prior immunotherapy (7,8). Peripheral tumor-associated antigen-directed T cell responses were observed to have no survival benefit for patients with colorectal cancer despite of a limited number of patients studied (9). Various components, including the immune system, tumor stroma and tumor cells affect the induction and modulation of tumor-directed immune responses (10). Limited antitumor activity of spontaneous antigen-specific T cells at a clinical level in patients with CRC may be due to multiple factors. Investigating the profiles of infiltrating immune cells may help to understand the interaction between innate and adaptive immune response and improve immunotherapeutic approaches in CRC.

Traditionally, cluster of differentiation (CD)8$^+$ cytotoxic T cells have been considered as the key component of effective antitumor immunity, and breast tumors with higher levels of infiltrating CD8$^+$ T cells have been associated with improved patient survival (11,12). However, studies have also shown that CD8$^+$ T cells frequently fail to fully function *in vivo* if there is a lack of adequate assistance from CD4$^+$ T cells (13). Therefore, heterogeneous populations of infiltrating immune cells need to be clarified in order to understand the antitumor immune responses within tumor.

The current consensus is that interferon (IFN)-γ-producing CD4$^+$ T helper (Th)1 and CD8$^+$ T cells, along with mature dendritic cells (DCs), natural killer (NK) cells, M1 macrophages and type 1 NK T cells are able to generate effective but frequently attenuated anti-tumor responses, while CD4$^+$ Th2 cells and type 2 NK T cells in cooperation with CD4$^+$ Tregs (regulatory), myeloid-derived suppressor cells, immature DCs or M2 macrophages suppress antitumor immunity

and are able to promote tumor progression (14-16). However, this summarized observation comes with the caveat that variation exists among tumor types, with the pro-tumorigenic cells, including $CD4^+$ Th17, also shown to produce effective antitumor responses (17,18).

The present study was undertaken to characterize the immune cell subpopulations infiltrating human breast tumors in a direct *ex vivo* analysis of fresh tumor tissue short-term *in vitro* expansion. In the present study, a profile of tumor-infiltrating T cells and macrophages in human CRC was analyzed. A broad spectrum of markers was applied to distinguish two subsets of macrophages. In addition, it was examined whether tumor macrophages were prone to cytokine-driven conversion. In addition, the expression of CXC motif chemokine (CXC) receptor 3 (CXCR3), CXC ligand (CXCL)9 and CXCL10 was analyzed. These important molecules were associated with the intensity of infiltration. The results provided insights into the profile of infiltrating immune cells in human CRC and may be useful for further study of antitumor immune responses in human CRC.

Table I. Demographics of surgical patients with colorectal cancer.

Parameters	Degree of infiltration	
	With LN infiltration	No LN infiltration
Total, n	7	15
Sex, n		
Male	2	8
Female	5	7
Mean age, years	62.4	64.7
Location of tumor, n		
Ascending colon	0	5
Descending colon	0	3
Transverse colon	1	0
Sigmoid colon	2	2
Rectum	3	5

LN, lymph node.

Materials and methods

Patients and specimens. Subsequent to approval from the institutional review board of the First People's Hospital of Changzhou (Changzhou, China) and informed consent, surgically removed tissue blocks and peripheral blood mononuclear cells were collected from patients with colorectal cancer from the aforementioned hospital (n=22, 12 females and 10 males; age range, 52-79 years; median age 63 years; samples collected between April 2015 and March 2016). All analyses were performed in compliance with the Declaration of Helsinki. The demographic information of patients is described in Table I.

Isolation of infiltrating immune cells. Fresh tumor and non-tumorous tissue adjacent were harvested in sterile condition from patients during surgery and rinsed with cold PBS to remove blood clogs, fat tissue and surrounding necrotic tissue. The tissues were then dried with filter papers and weighed. The tissues were cut into small pieces (size, ~1 mm^3) in cold PBS. In total, ≥5 volumes of collagen IV (0.1 μg/ml in RPMI-1640) was added to 1 volume of tissue suspension and then incubated at 4˚C overnight. The tissue suspension was filtered through a nylon mesh (70-100 μm) to harvest single cells. Subsequent to washing with PBS, the mononuclear cells were isolated by gradient centrifugation with Percoll® Plus (GE Healthcare Life Sciences, Little Chalfont, UK) at 400 x g at room temperature for 25 min and counted with an Axiovert 100 inverted microscope (Carl Zeiss AG, Oberkochen, Germany) at x10 magnification. The results were expressed using a heat map for the intensity of infiltration with HemI software (HemI Illustrator; version 1.0.3.3; hemi.biocuckoo.org).

Isolation of macrophages and T cells. Mononuclear cells were suspended in pH 7.4 PBS at a density of >$5x10^5$ cells/ml and then incubated with anti-CD14 microbeads (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany; Cat#130-050-201) for 30 min at room temperature. Subsequent to washing, the resuspended cells passed through the MS cell separation column to separate macrophages and other cells according to the manufacturer's protocol. For T cell isolation, the cells were incubated with anti-CD3 microbeads at 4˚C for 30 min (Miltenyi Biotec GmbH; Cat# 130-050-101) prior to following the procedure as aforementioned.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from cells with an RNeasy Mini kit (Qiagen, Inc., Valencia, CA, USA) according to the manufacturer's instruction. cDNA was then synthesized with the iScript cDNA Synthesis RT kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA), according to the manufacturer's protocol. The specific primers were designed and purchased from Sangon Biotech Co., Ltd. (Shanghai, China). Gene expression profile was analyzed by RT-qPCR with customized primer sets as described in Table II. Briefly, PCR was performed using 10 ng cDNA, 500 nM forward and reverse primers, and SYBR Green master mix (Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA) in 20 ml reactions. Thermocycling conditions comprised an initial holding at 50˚C for 2 min, then 95˚C for 10 min. This was followed by a 2-step PCR program consisting of 95˚C for 15 sec and 60˚C for 60 sec for 35 cycles. Each sample was analyzed in triplicate, and SYBR Green fluorescence was detected using the Applied Biosystems 7900HT realtime PCR system. Data were analyzed with $2^{-\Delta\Delta Cq}$ method (19). The experiment was repeated at least three times.

Cell culture. $CD14^+$ macrophages were prepared from tissues and peripheral blood mononuclear cells by antibody-coated microbeads (Miltenyi Biotec GmbH), and the purity was routinely >90% as assessed with PE-labeled anti-CD14 antibody (cat no., 557154; BD Biosciences, Franklin Lakes, NJ, USA) by flow cytometry using FlowJo software (version 7.5; FlowJo LLC, Ashland, OR, USA). Macrophages were cultured *in vitro* in RPMI-1640 medium (Invitrogen; Thermo Fisher

Table II. Primer sequences for SYBR Green quantitative polymerase chain reaction.

Genes	Forward (5'-3')	Reverse (5'-3')
Tbx21	GGTTGCGGAGACATGCTGA	GTAGGCGTAGGCTCCAAGG
GATA-3	GCCCCTCATTAAGCCCAAG	TTGTGGTGGTCTGACAGTTCG
RORC	GTGGGGACAAGTCGTCTGG	AGTGCTGGCATCGGTTTCG
Foxp3	GTGGCCCGGATGTGAGAAG	GGAGCCCTTGTCGGATGATG
BCL-6	TGGTGACGCTTCAAAAGCCA	GCTAGAATAGACGATGTTTCCCG
CXCR3	CCACCTAGCTGTAGCAGACAC	AGGGCTCCTGCGTAGAAGTT
CXCL9	TGCAATGAACCCCAGTAGTGA	GGTGGATAGTCCCTTGGTTGG
CXCL10	TGAAATTATTCCTGCAAGCCAA	CAGACATCTCTTCTCACCCTTCTTT
GAPDH	GAAGGTGAAGGTCGGAGTC	GAAGATGGTGATGGGATTC
CD163	AGTCCCATCTTTCACTCTGC	GCATCTTCTATGTCCCAGTG
IL-10	GACTTTAAGGGTTACCTGGG	CTTGATGTCTGGGTCTTGGT
CD36	TTGCAGGTCAATCTATGCTG	CTGGGTTTTCAACTGGAGAG
IL-12β	CACAACGGAATAGACCCAAA	TTAAATAGCATGAAGGCCCA
IL-1β	CCACCCTCTATCACTGACTT	CAAGGCTCAGTACATGCTCA
IL-6	GATGCAATAACCACCCCTGA	TGACCAGAAGAAGGAATGCC
TNF-α	TGTACCTCATCTACTCCCAG	GAAGACCCCTCCCAGATAGA
β-actin	GCATCCACGAAACTACCTTC	GATCTCCTTCTGCATCCTGT

Tbx21, T-box 21; GATA3, GATA-binding protein 3; RORC, RAR-related orphan receptor c; Foxp3, Forkhead-box p3; BCL-6, B cell lymphoma 6 protein; CXCR3, CXC motif chemokine receptor 3; CXCL9, CXC motif chemokine ligand 9; CXCL10, CXC motif chemokine ligand 10; CD, cluster of differentiation; IL, interleukin; TNF, tumor necrosis factor.

Scientific, Inc.) supplemented with 10% fetal calf serum (GE Healthcare Life Sciences) and granulocyte macrophage colony-stimulating factor (GM-CSF; 50 ng/ml; R&D Systems, Inc., Minneapolis, MN, USA). Following stimulation at 15, 30 min, 2, 4 and 24 h, the cells were washed and stimulated with lipopolysaccharide (LPS; 100 ng/ml; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) at 37˚C for 16 h, and the culture cells were collected for the analysis of interferon responsive factor (IRF)5 expression.

Western blot analysis. Cell pellets were lysed in ice-cold buffer containing a protease inhibitor cocktail (Roche Diagnostics, Basel, Switzerland). The lysates (10 mg/lane) were fractionated by 8-10% gradient SDS-PAGE. The lysates were subsequently transferred onto polyvinylidene difluoride membranes and blocked with 10% non-fat milk in PBS at room temperature for 1 h and analyzed by immunoblotting with specific antibodies at room temperature for 1 h to IRF5 (Cat#13496; dilution 1:1,000; Cell Signaling Technology, Inc., Danvers, MA, USA) and β-actin (cat no., A1978; 1:2,000; Sigma-Aldrich; Merck KGaA). Subsequent to washing with 0.05% Tween-20 PBS, secondary horseradish peroxidase-conjugated antibodies (Cat#31430; dilution 1:10,000; Pierce; Thermo Fisher Scientific, Inc.) were added and the blots were incubated at room temperature for 1 h. The protein bands were visualized using enhanced chemiluminescence (Pierce; Thermo Fisher Scientific, Inc.).

Statistical analysis. Data are presented as the mean ± standard error. Statistical analysis was performed using two-tailed Student t-test for unpaired data and two-way analysis of variance for multiple comparisons with a post hoc Fisher's Least Significant Difference test. SPSS (version 19; IBM Corp., Armonk, NY, USA) was used for statistical analysis. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Infiltration profile of immune cells in tumor and non-tumorous adjacent tissues of colorectal cancer. The profiles of infiltrating immune cells isolated from tumor and non-tumorous adjacent tissues obtained from patients with colorectal cancer was analyzed by qPCR amplification of each characteristic transcription factor of Th1, Th2, follicular T helper (Tfh), Treg and Th17 cells. It was revealed that significantly increased quantity of forkhead-box p3 $(Foxp3)^+$ Treg cells, Th1 cells and Tfh cells were present in tumor tissues compared with the adjacent tissues (Fig. 1A-H). No statistical difference in the number of Th2 (GATA3; Fig. 1D) and Th17 cells (RORC; Fig. 1B) was observed between tumor tissues and the adjacent tissues. This indicated that the profile of immune cells is distinct in the tumor tissues from the adjacent tissues. In addition, the expression of CXCR3, CXCL9 and CXCL10 were significantly increased in T cells isolated from tumor tissues compared with the adjacent tissues. This indicated that high expression of those molecules is associated with infiltration in colorectal cancer.

Distinctive patterns of infiltrating immune cells in tumor tissues with low and high infiltration. The infiltrating lymphocytes in tumor tissues from patients with colorectal cancer

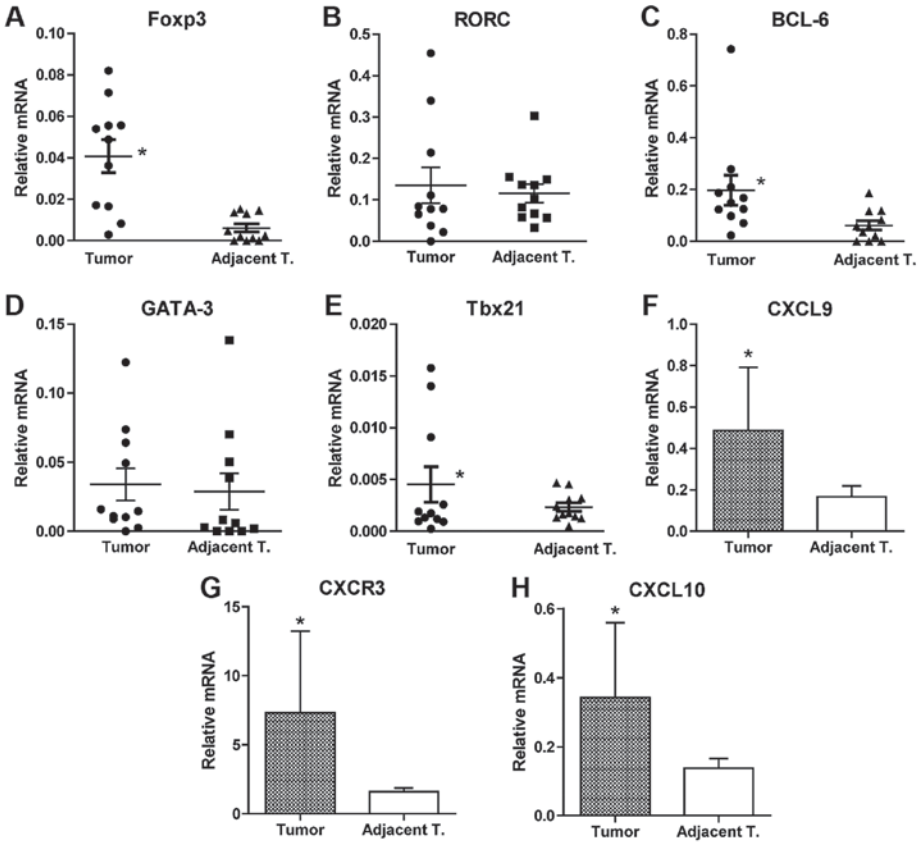

Figure 1. Analysis of the profile of infiltrating immune cells isolated from tumor and non-tumorous adjacent tissue. Immune cells were isolated from tissue blocks collected from selected patients with CRC during surgery via collagen IV digestion and gradient density centrifugation. Total RNA was extracted from the cells and subsequently reverse transcribed to cDNA. Specific primer sets were designed for transcription factors (A) Foxp3, (B) RORC, (C) BCL-6, (D) GATA-3 and (E) Tbx21 representing Treg, Th17, Tfh, Th2 and Th1 cells, respectively. qPCR was performed using the SYBR-Green method with specific primers to quantify the abundance of each subsets of infiltrating immune cells. GAPDH was amplified simultaneously for normalization. Data were analyzed using the $2^{-\Delta\Delta Cq}$ method and presented as relative values to GAPDH. T cells were isolated from tumor and non-tumorous adjacent tissue of 6 selected patients with CRC using T cell-specific microbeads. qPCR was performed on RNA isolated from T cells for quantification of (F) CXCL9, (G) CXCR3 and (H) CXCL10. Data are presented as the mean ± standard error of mean. Statistical analysis was performed using Student's t-test. $^{*}P<0.05$, tumor tissue vs. non-tumorous adjacent tissue. qPCR, quantitative polymerase chain reaction, Tbx21, T-box 21; GATA3, GATA-binding protein 3; RORC, RAR-related orphan receptor c; Foxp3, Forkhead-box p3; BCL-6, B cell lymphoma 6 protein; CXCR3, CXC motif chemokine receptor 3; CXCL9, CXC motif chemokine ligand 9; CXCL10, CXC motif chemokine ligand 10; Treg, regulatory T cells; Th, T helper; Tfh, follicular T helper.

were counted. Furthermore, the expression of each specific transcription factor Foxp3, GATA-binding protein 3 (GATA3), T-box 21 (Tbx21) and RAR-related orphan receptor C (RORc) for each different T cell population, including Treg, Th2, Th1, Tfh and Th17 cells, were analyzed. As shown in Fig. 2, there were relatively a greater number of Treg cells and fewer Th1, Th17 and Tfh cells in tumor tissues with low infiltration (<500 cells/mg tissue) compared with tissues with medium (500-1,000 cells/mg tissue) and high infiltration (>1,000 cells/mg tissue). By contrast, in tissues with high infiltration (>1,000 cells/mg tissue), there were an increased number of Th1, Th17 and Tfh cells and fewer Treg cells compared with tissues with low (<500 cells/mg tissue) and medium (500-1,000 cells/mg tissue) infiltration. The expression of CXCR3, CXCL9 and CXCL10 on T cells isolated from colorectal cancer tumor tissues was examined. As shown in Fig. 3, higher expression of CXCR3, CXCL9 and CXCL10 was observed on T cells isolated from tumor tissues with high infiltration compared with tumor tissues with low infiltration.

Characterization of tumor-infiltrating macrophages. To characterize the profile of macrophages in tumor tissue, the expression of major cytokines that are representative of M1 and M2 cells was analyzed by qPCR due to the limited number of isolated cells. The results revealed that tumor-infiltrating $CD14^{+}$ macrophages exhibited a dominant M2 phenotype as characterized by elevated expression of M2 marker genes, [interleukin (IL)-10, CD207, CD36 and CD163] compared with M1 marker genes [tumor necrosis factor (TNF)α, IL-6, IL-1β and IL-12β (Fig. 4A).

Response of isolated $CD14^{+}$ macrophages to GM-CSF stimulation. A total of three large tumor tissue blocks (>50 mg) obtained from surgical patients with colorectal cancer were selected for isolation of $CD14^{+}$ macrophages. Purified macrophages were stimulated with GM-CSF for different periods of time. It was demonstrated that macrophages from tumor tissues expressed markedly reduced IRF5, which is a characteristic transcription factor of M1 macrophages, compared with expression in peripheral macrophages isolated from peripheral blood of the same patient (Fig. 4B). Furthermore, tumor macrophages did not respond to the stimulation by GM-CSF, a driving cytokine for M1 macrophage differentiation, as measured by the expression of IRF5, whereas peripheral

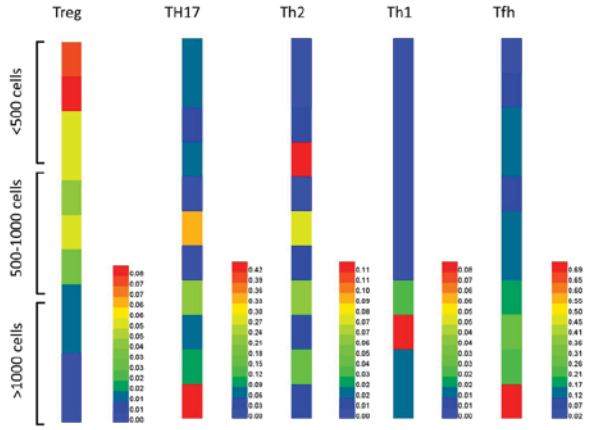

Figure 2. Association between the patterns of infiltrating immune cells with the intensity of infiltration in colorectal cancer tumor tissues. The numbers of infiltrating cells were enumerated using microscopy following isolation from tumor tissue with Percoll gradient centrifugation. The intensity of infiltration was set as low, medium and high with <500, 500-1,000 and >1,000 infiltrating cells per mg of tissue, respectively. The cells were subject to quantitative polymerase chain reaction analysis for each characteristic transcription factors for each type of cell. The relative expression of mRNA was plotted using a heat map to illustrate the relevance of each type of cell to the intensity of infiltration. Red indicates high expression of mRNA of each specific subset of cells, while blue indicates low expression of mRNA. Th, T helper; Tfh, follicular T helper.

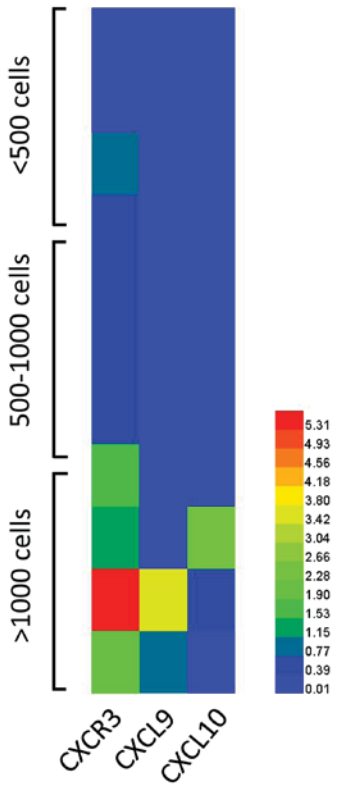

Figure 3. mRNA expression of CXCR3, CXCL9 and CXCL10 on T cells isolated from CRC tumor tissues. T cells were purified from post-digestion cell suspension of tumor tissue of colorectal cancer using anti-CD3 microbeads. The eluted cells were subject to lysis and purification of mRNA using a Qiagen RNA purification column. Quantitative polymerase chain reaction analysis was performed to determine the levels of RNA of each transcription factor, which represents each subset of immune cell. Data are presented as a heat map to associate the level of mRNA with the intensity of infiltration. CXCR3, CXC motif chemokine receptor 3; CXCL, CXC motif chemokine ligand.

macrophages exhibited a strong response to GM-CSF stimulation after 24 h of stimulation (Fig. 4C). The results suggested that tumor macrophages were less inflammatory and refractory to conversion driven by M1 stimulating agents in colorectal cancer compared with peripheral macrophages.

Discussion

The most common treatment for colorectal cancer is surgery. In the case of localized tumors, surgery may completely eliminate the cancer. When the cancer has invaded the bowel wall or the lymph nodes, chemotherapy and/or immunotherapy are required to achieve the best benefits. Colorectal cancer is one of the major cancer types for which new immune-based cancer treatments are currently in development (20). The understandings of antitumor immune responses are crucial to the design and implement of immunomodulation for treatment.

The evaluation of immune infiltrates is even more complex due not only to the numerous cell types that can be found in tumors (2,4), but also to the possibility that a given immune cell type can vary in terms of state of maturation and/or activation, and the fact that numerous diverse cell types can share similar markers (5). A CD4 T cell found in a tumor can be anergic, activated or regulatory. The same can be said for several other immune lineages (5). Tumor-infiltrating $Foxp3^+$ regulatory T cells have also been shown to have a strong prognostic significance in colorectal cancer. Salama *et al* (21) reported that the density of regulatory T cells in normal and tumor tissue to be independent prognostic indicators, but not the density of $CD8^+$ T cells. However, it has been reported elsewhere that $Foxp3^+$ Treg cells were independent indicators of the prognosis of colorectal cancer (20). Di Giorgio *et al* (22) also revealed that the presence of lymphocytic infiltration in the tumor was associated with an improved prognosis by multivariate analysis in patients with colorectal cancer resected between 1960 and 1978 (n=361; P<0.001). A number of studies have also emphasized the location of immune infiltrate in tumors; CD8+ T cell infiltrates in cancer cell nests often were associated with improved prognosis when compared with those in cancer stroma and marginal regions (1,4). Therefore, it will be more informative to describe a profile rather than emphasizing on a particular subset of immune cells in consideration of the complexity of immune infiltrates in colorectal cancer.

In the present study, the profiles of immune cells were analyzed, including Treg, Th1, Th2, Tfh and macrophages, and the profiles of low infiltration and high infiltration were compared. Profiles of tumor-infiltrating immune cells and immune cells in non-tumor adjacent tissues were also compared. However, these cells were also in different stages of differentiation, which was not addressed in the present study. The analysis of differentiation stages may provide further important information to define the profile of tumor-infiltrating immune cells. Notably, an increased number of Tfh cells were observed in the tumor tissue as compared with non-tumorous adjacent tissue, indicating significant involvement of B cell response in tumor tissues in colorectal cancer. The B cell response in tumor has been previously extensively studied in a number of types of cancer, including breast, ovarian and

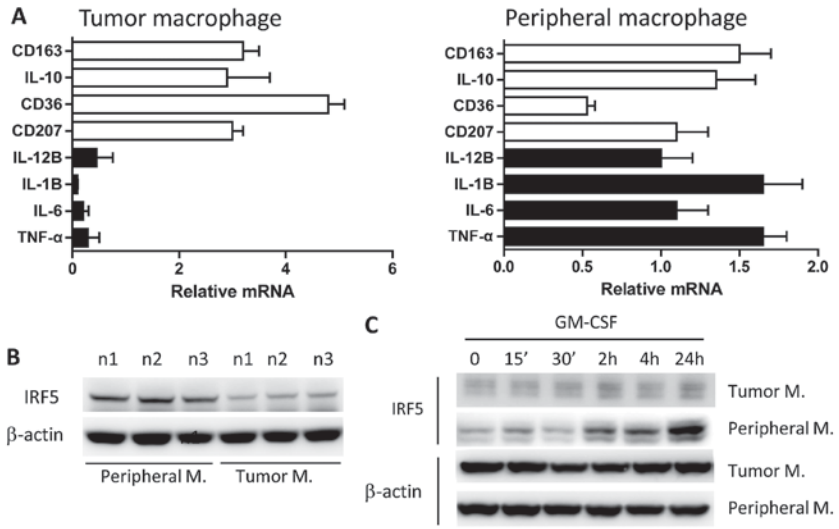

Figure 4. Phenotype of macrophages in tumor tissue of CRC and response of macrophages to GM-CSF stimulation *in vitro*. (A) Macrophages were purified from post-digestion cell suspension of tumor tissue and peripheral venous blood collected from patients with CRC using anti-CD14 microbeads. Total RNA was isolated from the purified cells and the mRNA levels of a panel of marker genes as indicated were determined by quantitative polymerase chain reaction. GAPDH was used for normalization. (B) A total of three specimens with sufficient number of infiltrating cells were selected, and the purified cells were lysed and subjected to the analysis of IRF5 at protein level with western blot analysis. (C) The purified macrophages were stimulated for different times as indicated in RPMI supplemented with 10% fetal calf serum and 50 ng/ml GM-CSF. Subsequently, the culture was stimulated with 100 ng/ml lipopolysaccharide at 16 h prior to western blot analysis of IRF5 expression. CD, cluster of differentiation; CRC, colorectal cancer; IL, interleukin; TNFα, tumor necrosis factor α; IRF5, interferon responsive factor 5; GM-CSF, granulocyte macrophage colony-stimulating factor.

non-small cell lung cancer (23). B cells exhibited evidence of somatic mutation and affinity maturation in breast cancer (23). In the present study, the increased number of Tfh cells indicated that local B cell differentiation occurred in tumor tissues. Consequently, it is likely the same scenario that occurred in colorectal cancer as that in breast cancer.

Macrophages are heterogeneous and comprise phenotypically and functionally distinct cell populations. With an increasing understanding of novel markers and differential roles of macrophages in the immune response, macrophages are characterized into different subsets. Different subsets require specific cytokine milieu for differentiation and maintenance and exhibit specific phenotypes and functions (24-30). Macrophage polarization is primarily determined by cytokines and ligands to pattern recognition receptors, including toll-like receptors (TLRs) on macrophages. Macrophages of the M1 phenotype are programmed to produce pro-inflammatory cytokines, including IL-12, IL-1β, TNFα and IL-6, and perform a crucial role in the initiation and perpetuation of inflammatory response, whereas macrophages of the M2 phenotype exhibit anti-inflammatory properties characterized by the production of IL-10 and IL-13 and prominent phagocytosis (26,28,30). Differentiation of M1 and M2 macrophages is driven by key cytokines, such as GM-CSF for M1 differentiation and M-CSF for M2 differentiation (28). By contrast, IFN-γ or IL-4 primes initially differentiated macrophages and promotes their polarization (31). In addition, activation by LPS through TLR-4 augments the production of cytokines by macrophages (26). It was previously reported that IRF5 and IRF4 are the putative lineage determining transcription factors for M1 and M2 macrophages (31,32). It has been shown that polarized M1 and M2 macrophages exhibit high plasticity and can be rendered to shift their phenotypes when the cytokine milieu changes. A balanced M1 to M2 ratio is required for the immune system homeostasis (27). In the present study, it was revealed that the macrophages of M2 phenotype isolated from tumor of colorectal cancer were refractory to *in vitro* converting to M1 phenotype, suggesting the defects of cells existed or the anergic state of cells. Current research to develop emerging immunotherapies that target the dysregulated M1/M2 macrophages is considered to make significant advances in cancer immunotherapy. Understanding the preferential accumulation of macrophages in a specific type of cancer would greatly support the future application of macrophage-directed immunotherapy. Although current agents such as Coley's toxins that stimulate the growth of M1 macrophages involve great side effects (33,34), new mediators that stimulate and maintain M1 macrophages will begin a new chapter in cancer therapy, and in such cases colorectal cancer may be a good candidate for macrophage-directed immunotherapy.

CXCR3, CXCL9 and CXCL10 were associated with the intensity of infiltration of T cells to tumor microenvironment. Zeste homologue 2 (EZH2)-mediated suppression of Th1-type chemokines CXCL9 and CXCL10 determine effector T cell trafficking to the tumor microenvironment (35). Treatment with epigenetic modulators such as EZH2 inhibitor removes the repression and increases effector T cell tumor infiltration, slows down tumor progression, and improves the therapeutic efficacy of programmed death-ligand 1 (PD-L1; also termed B7-H1) checkpoint blockade and adoptive T cell transfusion in tumor-bearing mice (35,36). In colorectal cancer, it was demonstrated that high expression of CXCR3, CXCL9 and CXCL10 on T cells was associated with high infiltration (>1,000 cells/mg). By analyzing the expression of these chemokines, the present results suggested the clinical specimens can be categorized into different groups that may be sensitive or insensitive to PD-L1 immunotherapy.

In the present study, it was identified that Th1 and Tfh cells, as well as M2 macrophages, are dominant cells in colorectal cancer tumors. The results of the present study suggest that the

analysis of the profile of intratumor immune cells may assist the prediction of prognosis.

Acknowledgements

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