Original Article
Role of macrophages in progression of colorectal cancer: a contrast with the traditional paradigm

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Abstract: The phenotype of tumor-associated macrophages may be critical for tumor immunity, angiogenesis, and clinical disease outcome. Here, we elucidated the prognostic significance of the neovasculature and macrophages in colorectal cancer. We analyzed the effect of M2 macrophage density on the clinical behavior of 151 primary colorectal carcinomas using CD206 as a marker for type 2 macrophages. Triple immunohistochemical staining (ERG, SMA, and podoplanin) was used for microvessel evaluation. We found that M2 macrophages in colorectal cancer did not have a direct association with metastatic behavior. However, high numbers of CD206 tumor-associated macrophages correlated positively with recurrence-free interval duration (P=0.005). Fewer macrophages in the tumor microenvironment resulted in insufficient coverage of newly formed vessels by pericytes (P=0.011), and a high number of pericyte-impaired microvessels correlated with metastatic behavior (P<0.001). These results suggested that type 2 macrophages had a role in limiting the metastatic process by affecting vascular maturity and normalization. These findings contribute to understanding complex interactions in the tumor microenvironment and the clinical behavior of colorectal cancer.

Introduction

More than 100 years have passed since Ilya Mechnikov discovered the phenomenon of phagocytosis and the phagocytes with which his name will always be associated. However, knowledge about these cells, now called macrophages, remains incomplete. In the 1980s, researchers discovered that compared to interferon (IFN)-γ and lipopolysaccharide, the cytokine interleukin (IL)-4, has differential effects on macrophage gene expression [1]. In contrast to the classical activation of macrophages by IFN-γ, macrophage phenotypes induced by IL-4 are described as undergoing "alternative activation" [2, 3]. In 2000, Mills et al. proposed a new classification of macrophages as M1 (classical activation) versus M2 (alternative activation). This terminology is based on finding differential arginine metabolism of macrophages in different strains of mice with T helper type 1 (Th1) or T helper type 2 (Th2) backgrounds. Th1

mice with T cells that mainly produce IFN-γ have macrophage activation that produces nitric oxide (NO) from arginine. In contrast, ornithine production occurs in Th2 mice with T cells that produce IL-4 [4]. This finding led to the current paradigm that M1 macrophages have inflammatory and anti-tumor activity; M2 macrophages have anti-inflammatory and pro-tumor functions [5, 6]. Although the M1/M2 classification of macrophages is now considered an oversimplified approach that inadequately describes the spectrum of macrophage populations, it is commonly used in pathology in the context of tumor-associated macrophages (TAMs) [7]. Macrophages are often ascribed a prometastatic function because their absence leads to pronounced metastatic failure [8, 9]. Increased TAM numbers are associated with worse clinical outcomes for most malignancies, including breast, cervical, ovarian, prostate, and thyroid cancers, Hodgkin's lymphoma, hepatocellular carcinoma, lung carcinoma, and cutaneous

melanoma. Despite many studies on the pro-tumor functions of TAMs in several tumor types, their role in colorectal cancer (CRC) is controversial [10]. Some results suggest that in CRC, macrophages have anti-tumor activity and are associated with improved disease-free survival [11, 12]. Forssell et al. found that a dense macrophage infiltrate at the tumor front positively affects prognosis in patients with CRC and that direct macrophage-to-tumor cell contact is required to manifest anti-tumor activity [11]. Consistent with these results, Zhou et al. found that a high TAM density at the invasive front is associated with a lower rate of hepatic metastasis and improved prognosis in patients with CRC [13]. In contrast, other studies found that massive macrophage infiltration correlates with tumor progression, growth, and disease aggressiveness. Kang et al. found in vitro that TAMs increase CRC cell invasiveness and migration [14]. A recent study found that during the removal of apoptotic colon cancer cells TAMs change their own phenotype and have increased expression of TGFβ and IL-6. The latter has a crucial role in colon tumorigenesis and putatively contributes to the angiogenic process [15]. In summary, the role of TAMs in CRC remains inconclusive.

One key feature of TAMs is the ability to support tumor angiogenesis [16, 17]. M2 macrophages promote angiogenesis and tissue regeneration, whereas M1 macrophages are anti-angiogenic, although this strict categorization is controversial [18]. Because tumor cell numbers can only increase to one million cells before new blood vessels are required, cancers rely on M2 macrophages to provide angiogenic factors [19, 20]. They share these functions with M2-like macrophages induced in a physiological context during the vascular and matrix remodeling necessary for damage repair [21]. In this study, we examined the relationship between infiltration of type 2 macrophages in tumor tissue and metastasis of localized CRCs. We also evaluated the properties of the microvasculature in metastasizing and non-metastasizing carcinomas and analyzed correlations between type 2 macrophage infiltration, characteristics of the newly formed vessels, and metastases.

Materials and methods

Colorectal cancer sample analysis

We retrospectively analyzed 151 samples of CRC tumor tissue from patients who underwent surgical resection at St. Petersburg Clinical Research and Practical Center for Specialized Oncological Care between 2012 and 2015. Corresponding clinicopathologic records were obtained from the database. The tumors were located in different parts of the colon and rectum. Cases with different depths of invasion, from T1 to T4b, were included. In all cases, no regional lymph nodes were involved (N0). Approximately one-third of the samples were cases of synchronous metastasis (n=53), one-third with metachronous metastasis to distant organs (n=45), and one-third (n=53) without distant metastasis at the time of diagnosis and within at least a 5-year follow-up period after surgical resection (minimum 64 months, maximum 92 months). "Synchronous metastasis" was defined as the diagnosis of distant metastasis together with or within a 3-month interval after the diagnosis of primary colon cancer. "Metachronous" was defined as an occurrence after a 3-month postoperative period. Distant metastases were diagnosed using clinical imaging and confirmed using biopsy. Tumors were classified into one of three groups, according to location: right-sided colon carcinomas (including those in the cecum, ascending colon, and transverse colon), left-sided colon carcinomas (from splenic flexure to sigmoid), and rectal carcinomas. Cases of multiple primary tumors and unknown locations were excluded.

The study was approved by local ethics committee at "Military medical academy of S.M. Kirov" (No. 255 from October 26, 2021).
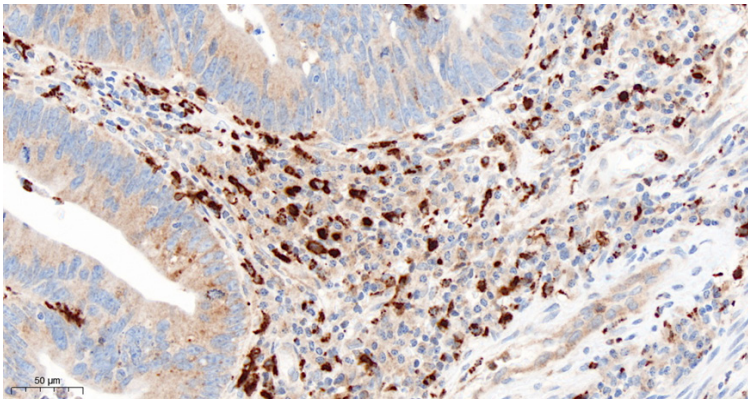
Immunohistochemistry

Triple immunohistochemical staining was performed to evaluate microvascular density (MVD) and pericyte-impaired microvessels (PIMs). Nuclear staining with ERG identified endothelial cells of blood and lymphatic vessels. Lymphatic vessels were differentiated using selective podoplanin immunoreactivity. Pericytes were considered present if α-Smooth Muscle Actin (α-SMA) immunoreactivity was visible anywhere along the vessel perimeter. Normal blood vessels showed ERG-expression and α-SMA; immature tumor microvessels lacked α-SMA immunoreactivity. MVD and PIMs were evaluated per square millimeter using a high-power microscopic field (400x).

Type 2 TAMs were identified using immunohistochemical staining with an antibody against

Table 1. Sources and dilutions of the immunohistochemical markers used in the study

Antigen	Distributor	Clone	Dilution
CD206	Sigma-Aldrich	polyclonal	1:1500
ERG	Epitomics	EP111	1:150
α-SMA	Dako	1A4	1:200
podoplanin	CellMarque	D2-40	1:50

Figure 1. Immunohistochemical detection of macrophages in colorectal cancer. Stromal M2 macrophages are clearly indicated by strong cytoplasmic staining with anti-CD206 antibody, 400 x.

the macrophage mannose receptor (CD206). The number of positive cells per square millimeter of tumor stroma was measured using a high-power microscopic field (400 x). Human placenta tissue was used as a positive tissue control because the functional profile of this cell population most closely resembles M2 macrophages [22]. Villous stromal macrophages (Hofbauer cells) demonstrated intense staining, in contrast to negative placental trophoblast cell staining.

Cut sections (2 μm) of formalin-fixed, paraffin-embedded tissue were used for the immunohistochemical studies. Heat-induced epitope retrieval, incubation with antibody, and chromogen visualization were performed using a Ventana BenchMark XT. The antibodies and staining conditions used are presented in **Table 1**.

Statistical analysis

Statistical analysis was performed using Stat-Tech v. 2.1.0 (Developer-StatTech LLC, Russia). Quantitative variables were tested for normality using Shapiro-Wilk tests (if the number of sub-

jects was less than 50) or Kolmogorov-Smirnov tests (if the number of subjects was more than 50). Quantitative variables that were not normally distributed were described using median (Me) and lower and upper quartiles (Q1-Q3). Mann-Whitney U tests were used to compare two groups with respect to a quantitative variable whose distribution deviated from the normal distribution. Comparisons of three or more groups during analysis of a quantitative variable whose distribution deviated from the normal distribution were performed using Kruskal-Wallis tests and Dunn's criterion with Holm's correction as a post-hoc method. The direction and strength of association between two quantitative variables were estimated using Spearman correlation coefficient analysis (when at least one variable was not normally distributed). Predictive modeling of the conditioning of quantitative variables on other quantitative variables was performed using simple or multivariable linear regression analysis.

Results

Immunohistochemical staining with CD206 revealed the presence of macrophages in the tumor stroma in all cases (**Figure 1**). Analysis of the subset of M2 macrophages in the tumor microenvironment found only a slight difference between metastasizing and non-metastasizing CRCs (**Figure 2**). The median value of CD206 positive cells for metastasizing CRC was 63, compared with 76 for non-metastasizing tumors (**Table 2**). A comparison of the dependence of metastasis on macrophages in those groups found the difference was not statistically significant (P=0.144).

When the group with metastasis was divided into two subgroups based on metachronous versus synchronous dissemination, a difference was revealed (**Figure 3**). The cases of synchronous metastasis had significantly lower densities of CD206 positive stromal cells - 56/
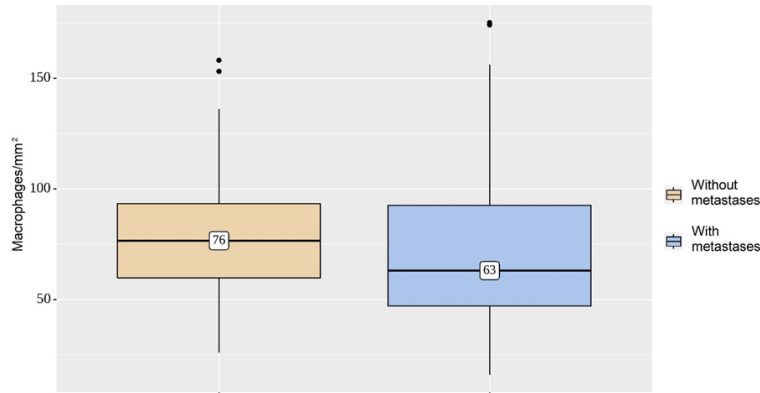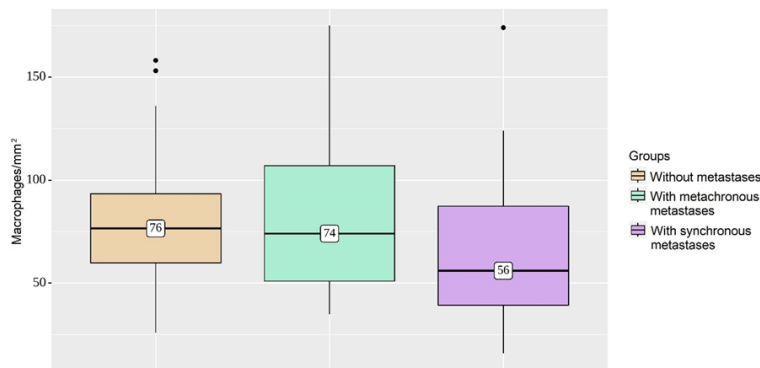
Figure 2. Density of macrophage infiltration per square millimeter. Mean numbers of M2 macrophages in the metastasizing and non-metastasizing groups are not different (P=0.144).

Table 2. Analysis of macrophages in groups with different metastatic behaviors

Group	Macrophages/mm^2		n	p*
	Me	Q$_1$-Q$_3$		
CRC without metastases	76	60-93	53	0.144
CRC with metastases	63	47-92	98	

*Mann-Whitney U-test. CRC: Colorectal Cancer.

Figure 3. Comparison of macrophage infiltration in three groups. The cases with synchronous metastasis have significantly lower M2 macrophage densities, compared to metachronous disease or local colorectal cancer (P=0.029).

Table 3. Analysis of macrophages in groups with different metastatic behavior

Group	Macrophages/mm^2		n	p*
	Me	Q$_1$-Q$_3$		
Without metastasis	76	60-93	53	0.029
With metachronous metastasis	74	51-107	45	
With synchronous metastasis	56	39-87	53	

*Kruskal-Wallis test.

mm^2, compared to metachronous disease or local CRC, 74/mm^2 and 76/mm^2, respectively (**Table 3**). The results indicated that the differences in the numbers of M2 macrophages in tumor stroma in the compared groups were significant (P=0.029).

The subsequent correlation analysis found a significant association between the number of macrophages in the CRC tumor microenvironment and the time to cancer recurrence (**Table 4**). The observed dependence of time to relapse from macrophages was described using a linear regression model:

$$Y_{\text{Time to relapse}} = 0.195 \times X_{\text{Macrophages}} + 1.221$$

The model indicated that with an increase in the number of macrophages in the tumor stroma by 10/mm^2, the recurrence-free time would increase by 1.95 months (P=0.005) (**Figure 4**).

The results of triple immunohistochemical staining of normal intestinal wall and CRC samples are presented in **Figure 5**.

Assessment of the microvasculature revealed that the average density of microvessels between groups did not differ significantly; it was 9/mm^2 in the group without metastases and 10-11/mm^2 in the group with metastases (P=0.158) (**Figure 6**).

In tumors with metachronous and synchronous metastases, capillaries without pericytes were more common among the newly formed vessels (i.e., median 7/mm^2 and

Table 4. Results of correlation analysis of the association between macrophages and time to relapse

Variable	Correlation characteristic		
	ρ Spearman	Strength of association assessed using the Chaddock scale	p
Macrophages-Time to relapse	0.340	Moderate	0.005
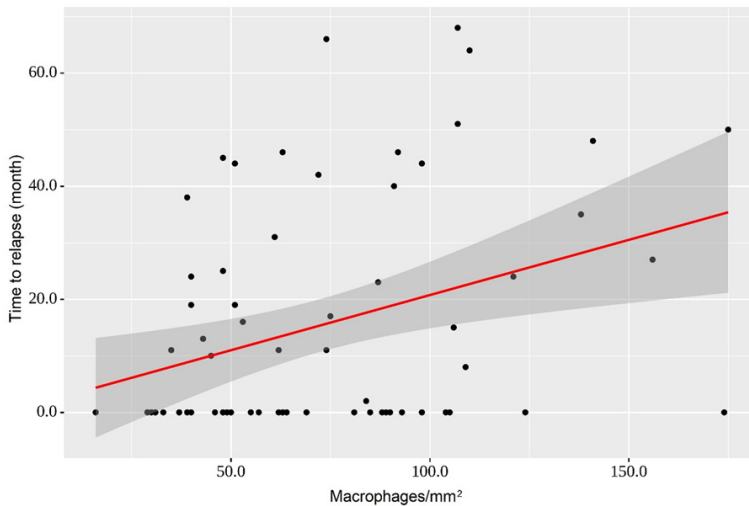
Figure 4. Regression curve characterizing the dependence of time to relapse from a stromal density of macrophages. With an increase in macrophage numbers in the tumor microenvironment in CRC by 10/mm^2, it is expected that the recurrence-free time will increase by 1.95 months (P=0.005).
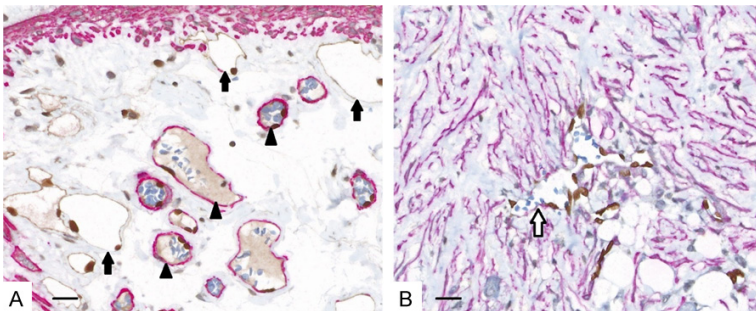
Figure 5. Triple immunohistochemical staining of microvessels in normal colon (A) and colorectal cancer (B). Nuclei of endothelial cells of the blood (arrowheads) and lymphatic capillaries (black arrows) are stained brown with ERG. Blood vessel contours appear red due to the presence of pericytes stained with α-SMA. Podoplanin staining defines the brown cytoplasm of endothelial cells of lymphatic vessels. Newly formed malformed blood vessels (white arrow) in the tumor stroma are devoid of pericytes and retain only the outline of their endothelial cells, which are recognizable by nuclei stained with brown anti-ERG. Note the striking desmoplastic change in the stroma in the tumor, with numerous activated myofibroblasts, detected using α-SMA. Scale bar = 200 µm, 400 x.

8/mm^2, respectively). At 2/mm^2, PIMs were quite low in non-metastasizing tumors (P< 0.001) (Figure 7).

A summary of results based on an analysis of microvascular characteristics in the studied groups of patients is presented in Table 5.

We then assessed the relationship between TAM density and microvasculature characteristics (Table 6). A relationship between M2 stromal presentation and overall MVD was not revealed in this group of samples (P=0.110). There was an inverse correlation between M2 macrophages and PIMs (P=0.011). A linear regression model described the observed variation in PIMs with respect to macrophage density:

$$Y_{PIMs} = -0.025 × X_{Macrophages} + 7.969$$

An increase in the number of macrophages by 100/mm^2 was accompanied by a decrease in PIMs by 2.5/mm^2 (Figure 8).

Discussion

Two distinct pathways link inflammation and cancer. Inflammatory conditions can promote the development of malignancies (extrinsic pathway). Activation of various oncogenes can promote an inflammatory tumor microenvironment (intrinsic pathway) [23]. TAMs are key players in cancer-related inflammation, and increased TAM density correlates with a poor prognosis and disease progression in most (but not all) human tumor types [24]. TAMs are included in the list of "cor-
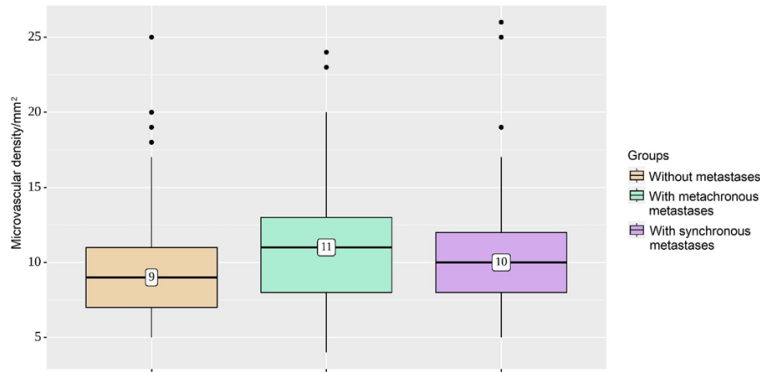
Figure 6. Microvascular density groups. The average density of microvessels in the group without metastases, compared with metastasizing tumors, is not significantly different (P=0.158).
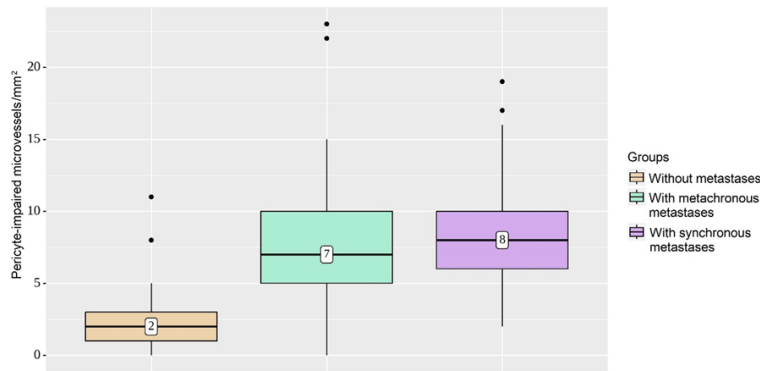
Figure 7. Characterization of pericyte-impaired microvessel densities in groups. In tumors with metachronous and synchronous metastases, capillaries without pericytes were more common among newly formed vessels, compared with quite low pericyte-impaired microvessels in non-metastasizing tumors (P<0.001).

Table 5. Microvascular characteristics of groups

Variable	Group	Values			p*
		Me	Q_1-Q_3	n	
MVD	Without metastasis	9	7-11	53	0.158
	With metachronous metastasis	11	8-13	45	
	With synchronous metastasis	10	8-12	53	
PIMs	Without metastasis	2	1-3	53	<0.001
	With metachronous metastasis	7	5-10	45	
	With synchronous metastasis	8	6-10	53	

*Kruskal-Wallis test. MVD: Microvascular Density; PIMs: Pericyte-Impaired Microvessels.

rupted" activated/recruited stromal cells that constitute the tumorigenic microenvironment and contribute to the hallmarks of cancer [25]. Plasticity is a well-known property of macrophages. They can integrate signals from the microenvironment and acquire distinct phenotypes (i.e., classical M1 or M2 macrophages) [26, 27]. According to the classical paradigm, M1 macrophages are involved in tumor suppression and immunostimulation. M2 macrophages promote immunosuppression, tumor growth, metastasis, and a more invasive phenotype [10]. In CRC, it was reported that an IFN-γ-driven tumor microenvironment exerts a better effect on patient survival and outcome than an IFN-α-driven microenvironment or a microenvironment without an IFN-associated immune response [28]. However, whether TAM exerts anti- or pro-tumor activity in CRC remains controversial. For example, Koelzer et al. found that high infiltration of CD163+ M2 macrophages is associated with fewer lymph node metastases and better clinical outcomes; iNOS+ M1 macrophages is not [29]. This difference indicates that there is heterogeneity in macrophage subsets and that full functional characterization is needed to predict clinical effects in CRC.

In the past, tumor angiogenesis was thought to be regulated mainly by cancer cells expressing proangiogenic factors. However, ample evidence now indicates that stromal cells in the tumor microenvironment are responsible for activating and maintaining chronic angiogenesis in many tumor types [25]. Conflicting results have been reported regarding associations between TAMs and tumor angiogenesis or lymphangiogenesis. TAMs express factors that are directly or indirectly involved in new vessel formation and sprouting. These factors include TGF-β, VEGF, PDGF, MMP-9, thymidine phos-

Table 6. Results of correlation analysis of association between macrophages and microvasculature

Variable	Correlation characteristic		
	ρ Spearman	Strength of the association assessed using Chaddock scale	p
Macrophages-MVD	-0.147	Weak	0.110
Macrophages-PIMs	-0.232	Weak	0.011

MVD: Microvascular Density; PIMs: Pericyte-Impaired Microvessels.
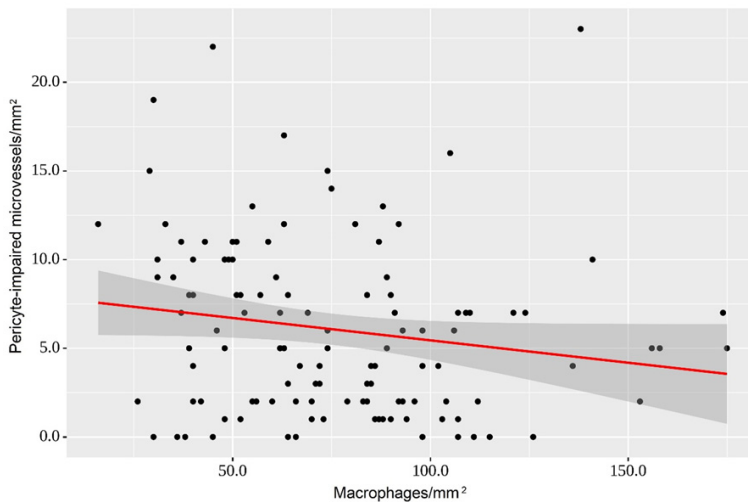
Figure 8. Regression curve demonstrates the observed variation in pericyte-impaired microvessels with respect to macrophage density. An increase in the number of macrophages of $100/mm^2$ is accompanied by a decrease in pericyte-impaired microvessels of $2.5/mm^2$ (P=0.011).

phorylase, and chemokines [28]. Hypoxia is an important stimulator of angiogenesis, and a hypoxic tumor microenvironment changes recruited macrophages into the M2 phenotype [30]. M1 macrophages secrete factors that initiate the process of angiogenesis. For example, VEGF stimulates endothelial cell sprouting and proliferation. Although induction of angiogenesis initially provides the tumor with more oxygen and nutrients, the ultimate response is poor because the continuously remodeled tumor vasculature is leaky and tortuous, causing irregular blood flow [31]. We found no difference in overall microvascular density between non-metastasizing and metastasizing CRC tumors; the average density of microvessels was $9/mm^2$ and $10-11/mm^2$, respectively. This result indicated that in CRC, the neoangiogenesis remained at the same level regardless of metastatic potential. However, we found that in metastasizing tumors, there was a significant predominance of the numbers of PIMs. In

tumors with metastases, the median density of capillaries without pericytes was $7/mm^2$, compared with $2/mm^2$ in non-metastasizing tumors (P< 0.001). This result suggested that factors were present that slowed the rate of maturation of blood vessels in the colorectal carcinomas prone to metastasis. We found no direct association between microvascular density in CRC with the number of CD206+ TAMs (P=0.110). However, we found a direct correlation between macrophages and PIM density (P=0.011). A high total number of macrophages detected using the CD206 marker appeared to be beneficial for maturation of growing blood vessels and remodeling of the vascular network by promoting pericyte recruitment. M2 macrophages secrete factors involved in later stages of angiogenesis, particularly PDGF-B and high levels of MMP-9, which suggests they have a role in vascular remodeling [18]. Recent study findings suggest tumor-type-specific involvement of multiple signaling pathways during recruitment of pericytes to the tumor vasculature. Spiller et al. found that supporting pericytes are recruited through PDGF-B secreted by M2 macrophages to stabilize the sprouts and develop into mature vessels [18]. Our results indicated that a higher proportion of PIMs in the total microvessel density was directly associated with tumor progression (P<0.001). Interestingly, tumors with high M2 density were associated with prolonged recurrence-free survival (P=0.005). Every 100 macrophages per one square millimeter of the tumor stroma postponed the manifestation of metastases by almost 2.0 months. This phenomenon can be explained by "vascular normalization", in which stabilized and mature vessels do not allow tumor cells to intravasate into the vasculature. Fewer circulating tumor cells reduce the likelihood that some will survive in the bloodstream and colonize distant organs.

In this study, we found that the presence and density of PIMs and the total number of M2 TAMs correlated with the clinical behavior of CRC. This finding was consistent with published studies that described a tendency toward a more favorable outcome in patients with CRC with increasing counts of M2 macrophages in the tumor microenvironment [29, 32].

High macrophage infiltration in colorectal tumors often correlates with a poor prognosis.

The opposite relationship has also been described, but the mechanism behind this phenomenon remains unclear. Here, we sought to reveal the role of alternatively activated TAMs in CRC progression. We found that M2 macrophages in CRC did not have a straightforward association with metastatic behavior. However, they had a direct relationship to the duration of the interval between tumor resection and the occurrence of metastasis. We also found a correlation between CD206+ TAM density and characteristics of the local microvasculature, namely, the degree of microvessel maturity. A lower number of macrophages in the tumor microenvironment was associated with insufficient coverage of the neovasculature by pericytes. Therefore, one hypothesis is that M2 TAMs have a role in limiting the metastatic process by affecting vessel maturation. Because metastasis is a critical prognostic factor, these findings partly explain why some studies found that increased TAM density is associated with an improved prognosis in patients with CRC. These results could assist in obtaining a deeper understanding of the mechanisms of the complex effects of macrophages on metastasis.

Acknowledgements

Disclosure of conflict of interest

None.

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