

## Review

# Macrophages in Tumor Microenvironment: From Molecular Aspects to Clinical Applications

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## Abstract

Macrophage cells are a part of the immune system, which are also involved in tumor microenvironment (TME). Specific macrophages are activated by different chemokines like IL-4, IL-13 and IFN- $\gamma$  and subsequently are able to differentiate into M1 and M2. Moreover, Ms2 are divided into 4 sub-phenotypes: M2a, M2b, M2c and M2d. We have analysed the relationship between macrophages, the role of micro RNAs and their importance in angiogenesis and lymphangiogenesis. Furthermore, we investigated how macrophages interact in the pathogenesis of breast, gastric and colorectal cancer. We reported several studies regarding the involvement of these cells in tumoral angiogenesis and proliferation. This review represents a starting point to better comprehend the molecular and biological steps regarding the involvement of Ms in cancer, focusing the attention on their effects in specific tumors.

**Keywords:** Breast Cancer (BC), ColoRectal Cancer (CRC) Angiogenesis, Gastric Cancer (GC), Lymphangiogenesis, Macrophages, TAMs (tumor-associated macrophages), TME (Tumor MicroEnvironment)

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Macrophages are innate immune cells, deriving from circulating monocytes, which extravasate in response to various stimuli in order to differentiate into tissue-resident macrophages.<sup>[1]</sup>

Their involvement in maintaining normal tissue homeostasis and responding to pathogens is well established, and different phenotypes have been identified based on the means of activation, defined by the microenvironment: macrophages 1 (M1) are known as “classically activated”, while macrophages 2 (M2) are “alternatively activated”.<sup>[2]</sup>

The former differentiate in response to interferon- $\gamma$  (IFN- $\gamma$ ), bacterial lipopoly-saccharide (LPS) and/or cytokines such as Tumor Necrosis Factor  $\alpha$  (TNF- $\alpha$ ), whilst the latter are further divided into subsets: M2a induced by IL-4 and IL-13, M2b in response to TLR activation and/or immune complexes (ICs), and M2c stimulated by IL-10, trans-forming growth factor  $\beta$  (TGF- $\beta$ ) or glucocorticoids, M2d engendered by NF- $\kappa$ B and HIF- $\alpha$ .<sup>[3,4]</sup>

All these phenotypes play different roles, based on their respective cytokine profiles. Depending on several molecular patterns, monocytes are switched into two subtypes of macrophages: M1

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and M2. M1 are characterized by high concentration of colony stimulating factor -2 (CSF-2), IFN- $\gamma$  and TLR agonist, and are able to produce IL-1 $\alpha$ , IL-1 $\beta$ , IL-12, TNF- $\alpha$  and GFAP; whereas IL-10 is under expressed. An abundance of M1 macrophages in the environment contributes to a primarily pro-inflammatory role; they also express high levels of MHC class I and class II molecules, which aid phagocytosis.

On the other hand, M2, stimulated by IL-4 and IL-13, generally produce IL-4, IL-6, IL-10, IL-13, which promote pro-tumorogenic functions, especially neo-angiogenesis and metastasis proliferation.<sup>[5-9]</sup> (Table 1)

The macrophages found in tumor microenvironment (TME) are known as Tumor Associated Macrophages (TAMs). Evidence suggests that in the early phases of tumor progression, they primarily express an M1 phenotype that inhibits tumor growth and angiogenesis. In later stages, however, various stimuli deriving from the TME, such as hypoxia, promote the shift towards an M2 phenotype.<sup>[10,11]</sup>

In addition, the transition from TAM M1 into TAM M2 type is strictly correlated to the interaction between CSF-1/CSF-1 Receptor (CSF-1R), which promotes the survival, proliferation and chemotaxis of macrophages. Furthermore, observing the microenvironment in detail, TAMs proliferate in tumoral regions where hypoxia is prevalent. The phenomenon is driven by an upregulation of macrophage chemo-attractants like endothelin-2 and vascular endothelial growth factor (VEGF), which can recruit M2 types.<sup>[12-14]</sup> Subsequently, the increase of macrophages in blood vessels causes a specific event called "intravasation", by which M2s are able to spread into the circulation system and sustain metastases proliferation.<sup>[15-18]</sup>

TAMs are also involved in the production of chemokines like CCL2, CCL17, CCL22 and in the spreading of proteases such as plasmin, urokinase plasminogen and matrix metalloproteases, which degrade extracellular matrix promoting angiogenesis.<sup>[19]</sup>

For instance, Matrix Metalloprotease 9 (MMP-9) has also been found to stimulate tumor growth, and its production is upregulated in response to TAM derived IL-23.

The production of IL-10 in large amounts by M2 types promotes immune suppression by inhibiting Th1 and natural killer (NK) cells.<sup>[15]</sup> M2s also express an abundance of TGF- $\beta$ : a growth factor

that inhibits the cytotoxic activity of NK and CD8+ cells, induces apoptosis of dendritic cells (DC) favouring immune escape and promotes polarization of TAMs towards an M2 phenotype.<sup>[20,21]</sup> (Fig. 1).

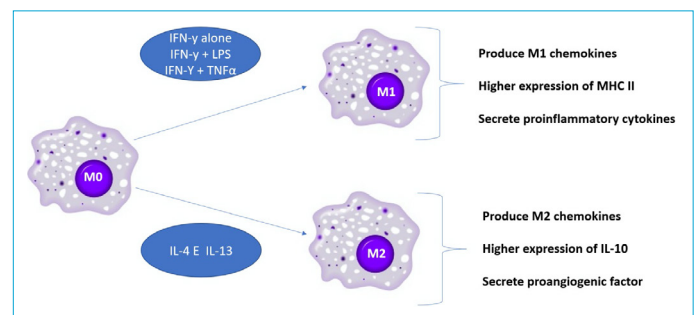
The role of macrophages in tumor progression is well established; nevertheless, there is still an actual debate regarding their effects on specific tumours like breast cancer (BC), gastric cancer (GC) and colorectal cancer (CRC). Recent research has improved the production of novel drugs that inhibit specific molecular pathways and stop tumour proliferation.

### TAMs and Breast Cancer

Breast cancer (BC) is the most common malignant tumor in women, and distal metastasis of highly invasive breast cancer cells is the major cause of death in these women. BC could be divided into three groups: BC expressing hormone receptor, estrogen receptor (ER+) or progesterone receptor (PR+), BC expressing human epidermal receptor 2 (HER2+) and triple-negative breast cancer (TNBC) (ER-, PR-, HER2-). Regarding BC, a strong correlation with M2 type macrophages has been proven in murine models,<sup>[22]</sup> while *in vitro* studies have shown that TAMs co-cultivated with breast cancer cells upregulate the production of matrix metalloproteases, stimulating tumor growth and angiogenesis.<sup>[23]</sup>

The preliminary data suggests, intuitively, that an abundance of TAMs would have a certain negative prognostic role in BC; however, this correlation remains controversial.<sup>[24]</sup>

Zhao et al. have conducted a meta-analysis on nineteen studies, concluding that TAMs infiltration was associated with an aggres-



**Figure 1.** The picture shows the "polarization" of Macrophage 0 (M0) to M1 and M2 towards the production of specific chemokines.

**Table 1.** Table summarizes the principal classes of macrophages, drawing the attention on the sub phenotypes of M2, which are mostly involved both in the tumour development and in angiogenesis process

Phenotype	Environment	Products	References
M1	CSF-2, IFN- $\gamma$ , TLR-agonist	IL-1 $\alpha$ , IL-1 $\beta$ , IL-12, TNF- $\alpha$ , GFPA	[4]
M2	IL-4, IL-13	IL-4, IL-6, IL-10, IL-13	[4]
Subphenotypes M2	Products	Functions	References
M2a	IL-10, IL1-RA, TGF- $\beta$	Pro-fibrotic, inhibition Th1	[3-5]
M2b	IL-10, IL-1, TNF- $\alpha$	Immune regulation	[3-5]
M2c	IL-10, TGF- $\beta$	Tissue repair, matrix remodelling, immuno-suppressive behaviour	[3-6]
M2d	HIF1- $\alpha$ , NF-kB	Angiogenic process, metastases proliferation, tumor growth	[3-5, 9]

sive behaviour, in the form of reduced overall survival (OS), disease free survival (DFS) and relapse free survival (RFS).

However, inconsistencies emerge from the use of different biomarkers to identify TAMs population: CD68 was deemed more accurate than CD206 or CD163 in this regard.<sup>[25]</sup>

Moreover, Qiu X et al. have demonstrated that high density of M2 type in TNBC is associated with poor prognosis and increased risk of metastasis. Immunohistochemical studies have shown that specific markers like CD 136 and CD 204, which can be used like target during chemotherapy, characterize M2 population.<sup>[26]</sup>

Moreover, Chen et al. have found that M2 phenotype promotes metastasis both in breast cancer and in gastric cancer in murine models via an increase in chitinase 3 like 1 protein (CHI3L1).

So, CHI3L1 interacts with interleukin-13 receptor  $\alpha 2$  (IL13R $\alpha 2$ ) on the membrane of cancer cells, promoting the production of matrix metalloproteases via the activation of mitogen activated protein kinase (MAPK) pathway.<sup>[27]</sup>

### TAMs and Gastric Cancer

Although GC also originates from chronic inflammation or *Helicobacter pylori* infection,<sup>[28-29]</sup> M2 type macrophages play a crucial role in GC development, because their presence and density modify the prognosis of tumour and the resistance to treatment. Different studies have already described the relationship between GC and macrophage infiltration; for example, Sammarco et al. have demonstrated how TAM infiltration changes the prognostic factor in surgically resectable GCs.<sup>[30]</sup>

Furthermore, the treatment of GC, angiogenesis has become the cornerstone of chemotherapy. Novel therapeutic agents are prepared to reduce neo-angiogenesis, such as ramucirumab, or others that target CSF-1R such as emactuzumab.<sup>[31]</sup>

In addition, Eum et al. have shown that the macrophages found in the malignant ascites of advanced gastric cancer patients express an M2 phenotype, and have associated this finding with a worsened prognosis.<sup>[32]</sup>

Macrophages play also a prognostic role, according to a study conducted by Svensson MC et al.<sup>[33]</sup> In 148 patients with resectable Esophageal and Gastric (EG) adenocarcinoma, an immunohistochemical analysis was conducted, highlighting that M2 type CD68<sup>+</sup>/CD163<sup>+</sup> determine a poor prognosis, instead of the presence of CD68<sup>+</sup>/CD163<sup>-</sup>, despite the use of neoadjuvant chemotherapy (NAC).

For locally advanced EG Adenocarcinoma, it has been shown that FLOT scheme in NAC in CD68<sup>+</sup>/CD163<sup>-</sup> cluster promotes the overall survival, the regression in size of the primary tumor and the reduction of distant metastases. Moreover, Wang et al. have already demonstrated that high M2 type and total TAMs density were correlated to low overall survival (OS), whereas, a high M1 type density with increased OS.<sup>[34]</sup>

The progression of EG adenocarcinoma depends on specific cluster, not only on Macrophage's type.

Another interesting aspect regards the relationship between exosomes and TAMs and their correlation in GC progression. GC re-

lated exosomes, recruiting PD1<sup>+</sup> TAM and inhibiting CD8<sup>+</sup> T cells, are capable to increase tumoral progression. Furthermore, GC exosomes transfer ApoE into GC cells, by PI3K/Akt pathway, and can model the cyto-skeleton, promoting tumoral cell migration to distant sites.<sup>[35,36]</sup>

### TAMs and Colo-rectal Cancer

Similar controversies emerge regarding CRC, as some studies highlight a positive prognostic role of TAMs, while others associate an abundance of TAMs with a worsened prognosis.<sup>[37-40]</sup>

For instance, Ammendola et al., in several studies have underlined the role of TAMs in locally advanced colorectal cancer, describing an unfavorable prognostic role despite early surgery.<sup>[41,42]</sup>

Furthermore, Ye et al. have shown in 1,008 CRC biopsies that the number of TAMs does not differ between CRCs treated with chemotherapy and CRCs that have not been treated.<sup>[43]</sup>

On the other hand, it is pivotal to expand upon this field of research, particularly about the impact of TAMs in hepatic metastasis due to their involvement in the promotion of angiogenesis.

Takasu et al. studied the effect of TAMs in hepatic secondary lesions in 71 patients, who underwent curative surgery (R0) for CRC and were diagnosed with liver metastasis. According to this study, TAM density is high in small tumors and is correlated with less aggressive features.<sup>[44]</sup>

Several studies have shown the different impact of M1 and M2 type on CRC. M1 macrophages demonstrate to have a poor correlation with tumoral progression; meanwhile M2 macrophages are strictly correlated with the presence of liver metastases and dedifferentiated tumors. Besides, it was hypothesized that the M1/M2 ratio could be used to predict liver metastases in CRC. For example, in a cohort of 360 patients a simple blood test was performed, analysing peripheral blood mononuclear cells. The results show a rise of these cells in CRC. Hence, the ratio M1/M2 may be used like a novel biomarker for the treatment and its prognostic value in CRCs.<sup>[45,46]</sup>

### TAMs, Angiogenesis and Lymphoangiogenesis

Angiogenesis and lymphangiogenesis are phenomena that occur mainly during embryogenesis, because their presence is reduced during growth when they start limited their presence to sites of wound healing and inflammation. The role of angiogenesis is significant in cancer, as it is known to drive tumor growth.<sup>[53]</sup> Various stimuli deriving from innate immune cells can drive the angiogenic process during tumor growth, primarily the production of pro-angiogenic factors within the TME. Tumor lymphangiogenesis plays a fundamental role in the development of metastasis and may occur both within the primary tumor and/or in the tumor periphery. Angiogenesis and lymphangiogenesis are driven by both stimulatory and inhibitory signals. Vascular endothelial growth factor (VEGF)-A is a known agonist of VEGFR2 found on blood endothelial cells (BECs). VEGF-C and VEGF-D play a key role in the survival of lymphatic endothelial cells (LECs), along with their proliferation and migration, through the engagement of VEGFR3. VEGF-A, VEGF-B, VEGF-C, VEGF-D and placenta growth



factor (PIGF) bind to three endothelial receptors: VEGFR1, VEGFR2 and VEGFR3. VEGF-A promotes the survival, proliferation, sprouting and migration of BECs, increases endothelial permeability and has a proinflammatory role. It is also involved in lymphangiogenesis: both directly, by binding to VEGFR2/VEGFR3 heterodimer receptor, and indirectly by stimulating the production of VEGF-C and VEGF-D by immune cells (e.g., macrophages, mast cells). PIGF and VEGF-B bind to VEGFR1 on BECs, along with various immune cells and pericytes. Angiopoietins (ANGPT1 and ANGPT2) bind with Immunoglobulin-like and EGF-like domains-1 (TIE1) and TIE2 receptors and modulate angiogenesis and lymphangiogenesis through the engagement of Tyrosine Kinase. ANGPT1, expressed by pericytes, encourages BEC survival, whereas ANGPT2, secreted by BECs, acts as an autocrine and paracrine TIE2 ligand. Numerous studies show that the pro- or anti-tumorigenic function of immune stromal cells is cancer specific regarding different solid tumors (breast, prostate, pancreas, gastric and colorectal), and depends on the stage of the tumor and on their localization within the microenvironment. Evidence shows that certain subsets of these cells may play a protective role whereas other types may have a pro-tumorigenic function. Single-cell mapping of peri-tumoral and intra-tumoral immune cells might aid in defining the roles of different subtypes of immune stromal cells in the onset and progression of various solid tumors.

Angiogenesis is a key component of cancer as it plays a crucial role in tumor growth (Fig. 2).

Furthermore, lymphangiogenesis, defined as the development of new lymphatic vessels, is involved in the metastatic process of many kinds of tumor.

Many innate immune cells can stimulate tumor growth by encouraging angiogenesis, mainly by producing angiogenic molecules within the TME.

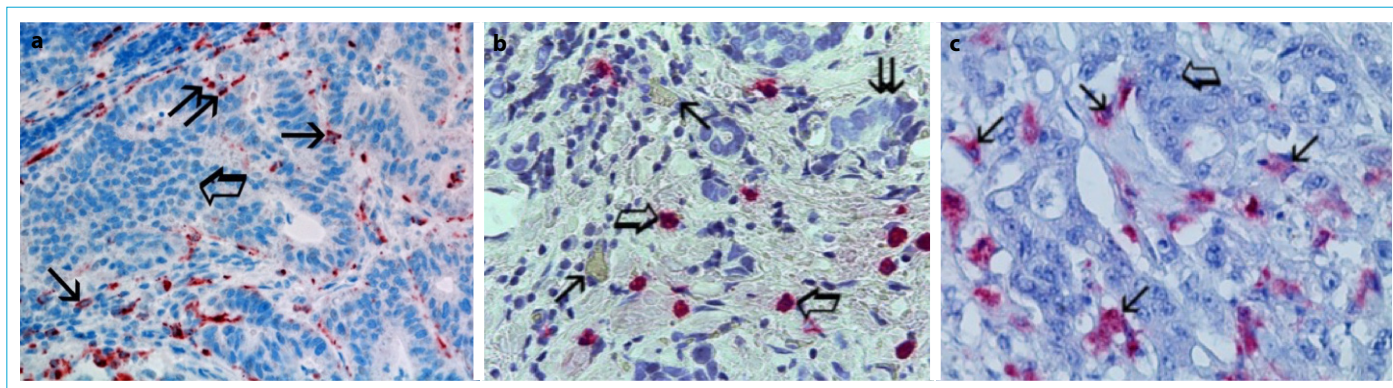
For example, macrophages are involved in the production and secretion of metalloproteinase-9 (MMP-9) which degrades the extracellular matrix, releasing the VEGF stored within.

The angiogenic process is characterized by two different biological pathways: the first one is the MyD88-dependent

pathway leads to the activation of nuclear factor kappa (NF- $\kappa$ B) and mitogen-activated protein kinase (MAPK). The second one is the TIR-domain-containing adapter-inducing interferon- $\beta$  dependent pathway causes the activation of serine/threonine-protein kinase-1 and receptor-interacting serine/threonine-protein kinase 1. These intracellular cascade signals, in the end, stimulate TAMs to secrete various pro-angiogenic factors, such as VEGF, TP, FGF-2, TNF- $\alpha$ , IL-1, -6, -8. In fact, an increased expression of TLRs can be found in tumor cells, cell lines, and tissues. Additionally, angiogenesis is controlled by both stimulatory and inhibitory signals.

### TAMs and MicroRNAs

Deregulation of microRNAs (miRNAs) can drive oncogenesis, tumor progression, and metastasis by acting cell-autonomously in cancer cells. Several miRNAs are implicated in the modulation of macrophage activation and function in tissues.<sup>[48]</sup> They are important regulators of various cellular activities including cell growth, differentiation, development and apoptosis.<sup>[49]</sup> Relevant miRNAs include miR-155, miR-125a/b, miR-146a, miR-21, and miR-19a. For example, miR-21 and miR-146 are selectively enriched in exosomes and are elevated in the plasma of patients with breast cancer<sup>[50]</sup> and higher serum expression of exosomal miR-19a or reduced exosomal miR-548c-5p levels indicates poorer colorectal cancer prognoses.<sup>[51]</sup> MiR-155 is a proinflammatory miRNA because it enhances the production of proinflammatory cytokines in macrophages and other immune cell type. The mechanism of action of miR-155 on transcripts encoding inflammatory mediators is unknown; allegedly it acts on transcriptional targets indirectly. KH-type splicing regulatory protein (KSRP) controls the expression of these same inflammation factors by controlling the biogenesis of miR-155. The mechanism of regulation of miR-155 expression in Ms treated with Lipopolysaccharide (LPS) probably gives us more information on a possible model



**Figure 2.** (a) Gastric cancer tissue, (b) breast cancer tissue and (c) colorectal cancer tissue red-stained macrophages to the anti-CD68 antibody. Arrows indicate single macrophages near micro vessels (x40 magnification).

of post-transcriptional regulation followed by a microRNA. Yang et al. Provided in a recent study the first evidence suggesting that macrophages can transfer miRNA via exosomes to breast cancer cells. They found that the vesicular miRNA is responsible for macrophage-promoting breast cancer cell invasion, providing a rationale for therapeutically targeting miR-223 in M2 macrophages or exosomal miR-223 from M2 macrophages.<sup>[54]</sup> Nevertheless, the ability of miRNAs to control macrophage differentiation, activation, and function in cancer remains limited. This lack of information may reflect the current limited availability of genetic models that target individual miRNAs in subsets of TAMs.

## Conclusion

Tumor development is a multistep process, during which different genetic and epigenetic alterations are involved in the initiation and progression phases. The stromal microenvironment is fundamental in maintaining the homeostasis of normal tissues or, otherwise, promoting tumor development. A plethora of immune cells (i.e., lymphocytes, macrophages, mast cells, monocytes, myeloid-derived suppressor cells, dendritic cells, neutrophils, eosinophils, natural killer (NK) and natural killer T (NKT) make up the tumor microenvironment (TME). Regarding the organs and tissues that are normally in contact with the external environment, such as skin and gastrointestinal system, immune stromal cells help to defend the organism from pathogenic insults and cancers cells. When this dynamic balance tends to favor cancer cells, counterintuitively, immune stromal cells aid the growth and proliferation of tumor cells: some of these cells, or the molecules re-leased by such cells, could stay within the stroma, but may also reach the lumen of the new blood and lymphatic vessels, causing metastasis.

The density of immune stromal cells is increased in cancer, and is correlated with angiogenesis, with the amount of metastatic lymph nodes and with the rates of patient survival. These cells have a pro-tumorigenic effect in cancer, being involved in the release of classical and non-classical angiogenic (VEGF-A, CXCL-8, MMP-9, Tryptase, Chymase) and lymphangiogenic factors (VEGF-C, VEGF-F and PDPN). They also express pro-programmed death ligands (PD-L1 and PD-L2), widely regarded as immune checkpoints in cancer. Numerous clinical trials are focusing on targeting immune checkpoints as an innovative therapeutic strategy in cancer. In order to further define the role of different subsets of cells in various human cancers, many studies will be needed, and should not be limited to the assessment of cell density and micro-localization.

Tumor microenvironment macrophages play several important roles within the tumor microenvironment (TME).

Two types of Macrophages, M1 and M2, can be found in the TME. M1 play a role in inflammation and exert immune activity against tumor. M2, on the other hand, are known as tumor-associated macrophages (TAMs) and have a pro-angiogenic effect. Evidence within the scientific literature shows that M2 produce well know pro-angiogenic factors such as: VEGF, TP, FGF-2, tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), and interleukins (IL-1, 6, and 8). After being secreted by M2, these molecules stimulate the proliferation, differentiation, survival, migration and vascular permeability of Endothelial Cells (ECs), thus leading to the formation of new microvessels.

Additionally, macrophages induce angiogenesis via the production and release of metalloproteinase-9 (MMP-9) which can degrade the extracellular matrix, causing the release of VEGF stored within.

On another note, it has been found that TAMs are linked via Toll-like receptors (TLRs). Recent pilot data published by our group has shown that TAM density increases along with angiogenesis. TLRs are defined as a family of membrane-spanning, non-catalytic receptors expressed by immune stromal cells, including TAMs.

TLR4, it has been found to be over-expressed in human and murine colorectal neoplasia; moreover, TLR4-deficient mice are refractory to colon carcinogenesis, highlighting that the higher TLRs density on tumor cells fosters tumor development directly or indirectly through angiogenesis.

In this review, we have queried PubMed, free online biomedical database, developed by the National Center for Biotechnology Information (NCBI) at the National Library of Medicine, for the following keywords: Macrophages, Gastric Cancer (GC), Breast Cancer (BC), Colorectal Cancer (CRC) Angiogenesis, Lymphangiogenesis, TAMs (tumor-associated macrophages), TME (Tumor MicroEnvironment). We have subsequently collected reviews and systematic reviews of the last years and we have analysed the main clinical studies.

Cancer is a heterogeneous disease, characterized by a multistep development process deeply involving the tumor microenvironment.

Nowadays, a fundamental diagnostic and prognostic role is played by histology and immuno-histochemistry, but in recent years the importance of circulating biomarkers has been clearly established.<sup>[47]</sup>

In this rapidly developing landscape, the identification of biomarkers produced by TME, and TAMs may provide a new gold standard for the initial diagnosis, the prognostic evaluation, the identification of new chemotherapy targets and the subsequent diagnosis of recurrences.

## Future Prospective

The relationship between Macrophages and tumor poses a huge debate in oncology. Some aspects remain still unknown, for example, the effects of NK cells or the role of CCL5 in CRC. We have described the importance of macrophages in three distinct forms of tumors, due to their respective frequency, such as CRC and breast cancer, or for its worse prognosis, like GCs. We tried to collect the main studies regarding this topic, proving the direct interaction between M2 macrophages and their function in the progression, evolution and treatment of tumors. In literature, a high correlation of Ms density with local angiogenesis and lymphangiogenesis was shown. Although much work has been done to characterize soluble factors present in the TME that recruit and influence Ms to promote angiogenesis and lymphangiogenesis, less is known about how the mechanical properties of TME instruct these cells to carry out these deleterious functions.

Immune stromal cells density is increased in cancer and there is a correlation with angiogenesis and lymphangiogenesis.<sup>[52]</sup> These cells exert a pro-tumorigenic role in cancer through the release of classical and non-classical factors so, high proliferation index associated with a neoangiogenic/lymphangiogenic process and a greater presence of Ms in tumor tissue is related to tumor progression and therefore a worse prognosis. Thus, immunohistochemical analysis of endoscopic diagnostic biopsies or serum levels of novel biomarkers could guide us on the state of the disease before medical or surgical treatment.

We also speculate the possibility to predict surgical radicality and survival improving the parameter N (Node) of TNM (Tumor- Node-Metastasis) classification used in clinical practice for staging of cancer patients.

So, in the future, it will be possible to explore the role of Ms in promoting angiogenesis and lymphangiogenesis within the tumor. Special attention may be directed to the mechanical stimuli sensed by these cells within the TME. Combining transcriptional profiling of Ms, retrospective analysis of newly defined prognostic biomarkers in tissues of patients, search for newly defined circulating biomarkers and investigation of cellular mechanisms, we aim to enhance the comprehension of tumor metastasis and relapse and to discover new therapeutic targets inhibiting Ms and their releasing factors, inhibiting angiogenesis and lymphangiogenesis therefore tumor progression. Moreover, initial studies have shown that interfering with miRNA activity may reprogram the cell activation state by targeting critical molecular checkpoints that tune the balance between pro- and antitumoral macrophage functions. These results should encourage the development of pharmacological

formulations that either suppress or enhance the activity of selected miRNAs to reprogram TAM phenotype.

## Disclosures

**Peer-review:** Externally peer-reviewed.

**Conflict of Interest:** None declared.

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