

# MACROPHAGES IN COLORECTAL CANCER: FROM NORMAL MUCOSA TO DISTANT METASTASIS: BEYOND THE M1/M2 PARADIGM

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**Supported by** the SALVAGE project (OP JAK; reg. no. CZ.02.01.01/00/22\_008/0004644) - co-financed by the European Union and the state budget of the Czech Republic.

## Abstract

Colorectal cancer (CRC) is the third most common malignancy and a leading cause of mortality worldwide. The tumor microenvironment (TME) strongly influences CRC growth, immune evasion, and metastasis. Among various immune cells, tumor-associated macrophages (TAMs) act as key regulators of cancer progression. Although traditionally classified as M1 (pro-inflammatory, anti-tumor) or M2 (anti-inflammatory, pro-tumor), single-cell RNA sequencing and spatial transcriptomics reveal that macrophage phenotypes exist along a continuum, challenging the classic dichotomy.

This review examines macrophages throughout CRC development, from normal mucosa to adenoma, primary tumor, and liver metastasis. Early adenomas feature M1-like macrophages that drive local inflammation, whereas advanced adenomas and invasive CRC show M2-like macrophages promoting angiogenesis, extracellular matrix remodeling, and immunosuppression.

TAMs are crucial in CRC metastasis, particularly to the liver. M2-polarized Kupffer cells express CD206 and CD163, secrete hepatocyte growth factor, and activate PI3K/AKT, thus aiding extravasation, survival, and proliferation of metastatic cells. They also foster lymphangiogenesis and immunosuppression through IL-10 and TGF- $\beta$  release.

CRC's consensus molecular subtype (CMS) influences TAM composition: CMS1 (microsatellite instability-high) tumors typically harbor M1 macrophages, while CMS4 (mesenchymal) tumors are enriched with M2-like TAMs, facilitating stromal remodeling, angiogenesis, and unfavorable prognosis.

Spatial distribution also matters. Abundant M1 macrophages at the invasive margin correlate with better outcomes, whereas M2 macrophages in tumor centers and metastatic sites drive progression. Some CD206<sup>+</sup> macrophages, however, support vascular normalization, which can limit metastasis. These findings underscore the complexity of TAMs in CRC and highlight the necessity of multi-marker phenotyping.

Given the limitations of the M1/M2 paradigm, advanced techniques such as spatial transcriptomics and single-cell RNA sequencing offer novel insights into TAM heterogeneity. Future therapeutic strategies targeting TAMs, including metabolic reprogramming, epigenetic modulators, and immune checkpoint inhibitors, hold promise for improving CRC patient outcomes by shifting the balance toward an anti-tumor immune response.

**Keywords:** colorectal cancer, tumor-associated macrophages, M1/M2 markers, tumor microenvironment, normal mucosa, adenoma-colorectal cancer liver metastasis sequence, prognostic significance.

## **Background**

Colorectal cancer (CRC) is the third most common malignancy worldwide and a leading cause of cancer-related deaths. Its progression from normal mucosa through adenoma to invasive cancer and distant metastasis often to the liver depends not only on the intrinsic genetic/epigenetic alterations within tumor cells but also on a complex tumor microenvironment (TME). Among the various cellular players in the TME, tumor-associated macrophages (TAMs) have emerged as key regulators of CRC progression.

Traditionally, TAMs were classified into two dominant polarization states: M1 (classically activated, pro-inflammatory, and anti-tumor) and M2 (alternatively activated, anti-inflammatory, and generally pro-tumor). However, mounting evidence indicates that this M1/M2 paradigm is overly simplistic. Cutting-edge techniques such as single-cell RNA sequencing and spatial transcriptomics have revealed that macrophage phenotypes exist on a continuum often with overlapping markers challenging the conventional binary framework. Recent research has also shown that TAM subtypes can simultaneously express both M1- and M2-associated markers and display unique functional properties depending on tumor stage, anatomical location (tumor center vs. invasive margin vs. lymph nodes), and consensus molecular subtype (CMS) of CRC.

**Aims of the Article.** The central aim of the article is to examine how macrophages contribute to CRC development and progression at each key step: from normal colon mucosa, through adenoma, to advanced primary tumors, and ultimately to metastases. The authors seek to underscore how different

macrophage polarization states or hybrids thereof are distributed across these stages; highlight the molecular drivers (e.g., cytokines, transcription factors, epigenetic regulators) that steer macrophage plasticity, and) evaluate how these findings reconcile the conflicting data on prognostic relevance of M1 versus M2 macrophages. A particular focus is placed on how macrophages establish pre-metastatic niches, regulate angiogenesis, and support epithelial-to-mesenchymal transition and lymphovascular invasion.

**Summary of the Literature.** In preparing this review, the authors surveyed a broad set of peer-reviewed studies spanning molecular biology, immunology, and clinical oncology. They draw upon extensive *in vitro* and *in vivo* research on macrophage polarization, single-cell analyses, and immunohistochemical profiling of TAMs in CRC specimens. Additionally, key clinical studies are referenced to clarify the often-contradictory data on the prognostic impact of TAM subtypes. While many reports link increased M2-type macrophages (often defined by CD163 or CD206 staining) to worse patient survival, other investigations highlight that certain M2-related markers can support vessel “normalization,” improving the anti-tumor immune response. Such inconsistencies emphasize the need for refined classification methods beyond M1/M2.

**Issues Under Discussion.** The chief issue tackled is the mismatch between long-standing macrophage polarization models (M1 vs. M2) and the heterogeneous reality observed in clinical CRC samples. Rather than two starkly polarized subsets, macrophages display a broad phenotypic spectrum with multiple transitional states. This complexity is heightened by regional factors (e.g., tumor center vs. invasive margin), genetic and epigenetic influences (such as DNMT and TET enzyme activity), and interactions with other cells (e.g., cancer associated fibroblasts, T regulatory cells). The review ultimately argues that a clearer understanding of macrophage diversity in CRC may explain contradictory findings regarding their prognostic significance and could open new avenues for targeted immunotherapy. By integrating advanced spatial transcriptomic techniques, the authors propose next-generation strategies to more accurately profile TAM heterogeneity and identify actionable therapeutic targets that harness or reprogram macrophages for better CRC outcomes.

## **1. Introduction**

CRC is the third most common type of cancer and the second leading cause of cancer-related mortality in the world [1]. Distant CRC metastases, which most frequently appear in the liver, drastically worsen survival[2]. Emerging evidence suggests that both adaptive and innate immune cells in TME play a critical role in CRC development and progression [3].

Macrophages are common immune cells in TME of CRC. As antigen-presenting cells (APCs), macrophages present tumor antigens on their surface through the major histocompatibility complex class II (MHC II), promoting the activation and differentiation of T cells, interconnecting innate and adaptive

immunity. As effectors, generation of reactive oxygen species and then destroy cancer cells by enzymatic degradation via lysosomal enzymes and the generation of reactive oxygen species [4]. In addition, they produce cytokines and chemokines to slow down tumor progression either directly or by modulating other immune cells [4].

The concept of TAMs was introduced to describe the role of macrophages [5] in tumor-induced immunosuppression, invasion and metastasis. TAMs include M0 non-polarized macrophages, which, in response to signals from TME, acquire M1, M2 and other more complex phenotypes [4]. Unlike other macrophages, TAMs have a higher proliferative capacity and are characterized by both M2 (predominantly) and M1-like transcriptional profiles. TAMs can exhibit both pro-tumor (M2-like) and anti-tumor (M1-like) activities depending on their polarization states and the signals they receive from the TME [6, 7, 8, 9]. This diversity is reflected in the expression of various surface markers and functional characteristics, which ultimately shape their biological roles [4,5].

Considering the phenotypic diversity of TAMs and the presence of transitional forms between individual types, the classification based on the M1/M2 dichotomy is somewhat outdated, making it challenging to describe the diverse biological functions performed by TAMs. This may explain the contradictory information regarding the anti-tumor and pro-tumor effects of M1 and M2 macrophages. In this regard, it is relevant to study the biological role of specific macrophage molecules, their expression levels, as well as the potential co-expression with other markers to describe the effects of TAMs [10, 11].

Widespread use of single-cell RNA sequencing in recent years has contributed to accumulation of data illustrating cellular heterogeneity of tumor tissue. New associations were established between molecular diversity of TAMs and their role [12, 13].

The current review focuses on the phenotypically driven diversity of TAMs biological effects in CRC development, progression, and metastasis. Additionally, this review aims to analyze data concerning the specificity of individual markers for different macrophage phenotypes and their biological roles in various regions of primary tumors and metastases.

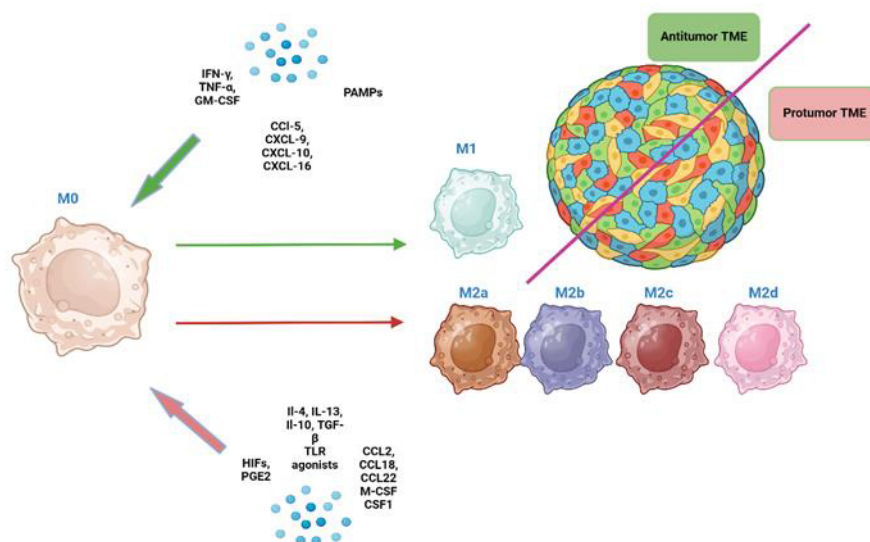
## **2. Diversity of macrophages**

Macrophages are highly plastic cells, which are capable of acquiring different phenotypes in response to signals from TME [7]. The 1st and still the most known TAMs classification considers non-polarized (M0), M1 (classically activated, pro-inflammatory, anti-tumor) and M2 (alternatively activated, anti-inflammatory, pro-tumor) macrophages [7] (Fig1).

M1 macrophages are characterized by high phagocytic and antigen-presenting activity and are induced by cytokines (IFN- $\gamma$ , TNF- $\alpha$ , granulocyte-macrophage colony-stimulating factor (GM-CSF), lipopolysaccharide (LPS), chemokines (CCL5, CXCL9, CXCL10, CXCL16) and other factors [14, 11, 15].

In turn, they produce a spectrum of pro-inflammatory cytokines (e.g., IFN- $\gamma$ , IL-12, TNF- $\alpha$ , IL-1 $\beta$ , and IL-6). Through IFN- $\gamma$  they stimulate T-cell-mediated killing of tumor cells. Additionally, M1 macrophages can recruit and activate other immune cells, including natural killer cells and dendritic cells, bolstering the anti-tumor immune response [16, 17].

M2 macrophages exhibit low phagocytic and antigen-presenting activity and are induced by cytokines (IL-4, IL-13, IL-10, transforming growth factor-beta (TGF- $\beta$ ), growth factors (macrophage colony-stimulating factor (M-CSF or CSF1), chemokines (CCL2, CCL18, CCL22) and other factors (prostaglandin E2 (PGE2), hypoxia-inducible factors (HIFs) [18]. They produce matrix metalloproteinases (MMPs) that degrade extracellular matrix and secrete growth factors and cytokines such as VEGF, FGF, IL-6, and IL-12, promoting angiogenesis, tumor growth, invasion, and metastasis. Additionally, M2 macrophages release immune-suppressive cytokines like IL-10 and TGF- $\beta$ , which can induce fibrosis by activating fibroblasts and promoting collagen production, as well as facilitate epithelial-mesenchymal transition (EMT) [16, 19]. Moreover, by producing proteins like collagen, which shield tumor cells from damage, M2 macrophages can create a favorable environment for tumors to survive and grow [20]. Additionally, collagen can interact with integrin receptors on tumor cells, activating signalling pathways that promote cell survival and resistance to death, thereby enhancing tumor cell viability and proliferation [21].

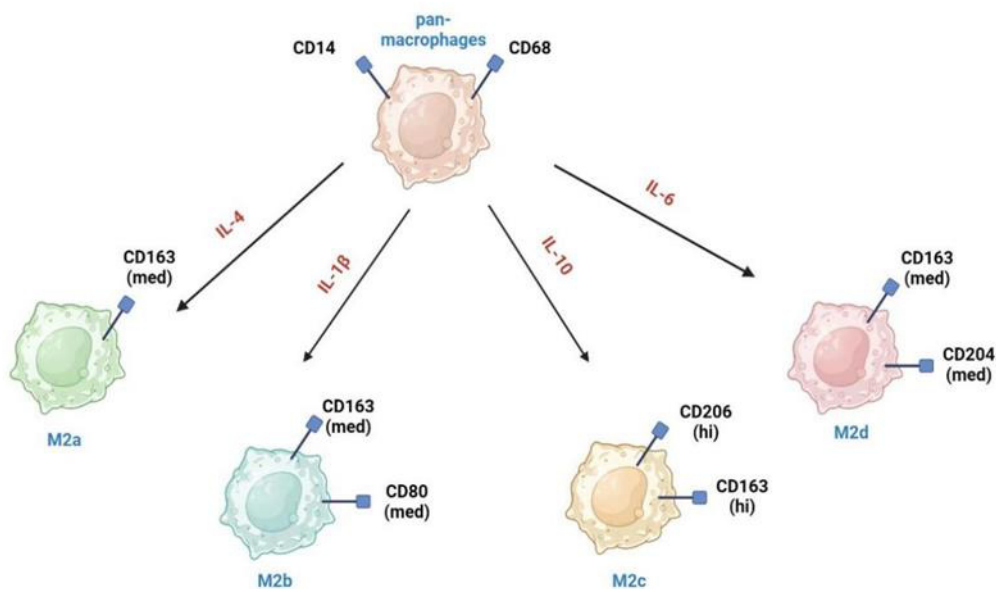


**Figure 1.** The direction of macrophage differentiation in response to various signals from the TME (the classical paradigm of M1/M2 macrophage dichotomy)..

Green arrows indicate the direction of macrophage polarization toward M1, and red arrows indicate the direction of polarization toward M2

Counterintuitively, M1 macrophages can also promote tumor progression through activation of NF- $\kappa$ B signaling, production of pro-inflammatory mediators and matrix remodelling [22, 23]. M2 macrophages can also have anti-tumor effects, in particular by contributing to vascular maturation and “normalization” in CRC, which can limit metastasis and improve prognosis of CRC patients. [24-27]. These findings suggests that M1 and M2 macrophages may play a more complex role in CRC progression than was previously thought [24, 25].

Subtypes of the classical M1/M2 classes are discussed below. The chemokine system in diverse forms of macrophage activation and polarization. M2 macrophages *in vitro* can differentiate into several subtypes (M2a, M2b, M2c and M2d) in response to different signals, [26] (Fig 2). Currently, unique surface marker signatures for each of these subtypes have been identified, paving the way for exploring the potential biological effects of these TAMs. [25].



**Figure 2.** The main phenotypic characteristics of M2a, M2b, M2c, M2d macrophages. In addition to the macrophage markers presented in the figures, these macrophages co-express other markers, albeit at lower levels.

\* med - medium level of expression    hi - high level of expression

M2a macrophages are induced by IL-4 or IL-13, secrete high levels of IL-4 and IL-13 and express markers such as CD206, CD163, CD209, CLEC7A (CD36), CD204, FCGR3A (CD16a), MSR1, and TIMD4 [26, 27]. They are involved in tissue repair and remodelling, and they promote tumor growth by enhancing angiogenesis and suppressing anti-tumor immune response [28]. M2b macrophages are induced by immune complexes and Toll-like receptor (TLR) agonists and express high levels of IL-10 and TNF- $\alpha$ . They have a mixed pro- and anti-inflammatory profile and support tumor progression by promoting immunosuppression and facilitating tumor cell invasion and metastasis [29]. M2c

macrophages are induced by IL-10, TGF- $\beta$  and glucocorticoids and are characterized by high expression of MARCO, CD206, CD163, CD209, CD204 [30]. M2c contribute to tumor progression by promoting an immunosuppressive environment and aiding in tissue repair mechanisms that tumors exploit for growth. M2d macrophages are induced by IL-6 and adenosine and exhibit high levels of VEGF, iNOS, and IL-10. They are strongly associated with angiogenesis and tumor growth [31]

The discovery of several subsets of M2-macrophages has illustrated the oversimplification of the the M1/M2 paradigm seems as an, since „pure” M1 and M2 forms can only be obtained under *in vitro* conditions [32]. *In vivo* TAMs frequently express a combination of M2 (CD163, CD206), M1 (CD80, CD86, and CD32) and M0 (CD68) markers, which reflects TAMs complexity and plasticity within TME [11, 27, 33].

Majority of TAMs display an overlap between M1 and M2 and within M2 subtypes, so terms “M2-predominant” phenotype seems more appropriate [28, 34].

Table 1 shows the markers that are strongly associated with either the M1 or M2 type, and points out others that may be expressed throughout different polarization states. CD68, M0 marker, is expressed by majority of both M1 and M2 TAMs, which probably mirrors polarization process (Supplementary file, Table 1). It is important to note that TAMs can simultaneously co-express M1-associated phenotypic markers (e.g., CD86, CD80, iNOS,) and M2-associated markers (e.g., CD163, CD206, VEGF). This phenotypic plasticity likely underpins the diverse and at times contradictory roles these cells play in tumorigenesis and cancer progression [34, 35]. Also, co-expression of M1 and M2 markers by a single cell could reflect transitional states between these two types and illustrate significant plasticity of macrophages and dynamic nature of macrophage activation in response to various TME stimuli. It is hypothesized that TAM are characterized by a unique phenotype that does not exist under normal physiological conditions and carries both M1 and M2 surface markers [36]. Other macrophages, eg. CD169+ (SIGLEC-1) do not fit neatly into the M1 or M2 categories [34,37–39]. CD169+ macrophages were found in various types of cancer in human, as well as mouse cancer models, including CRC with high microsatellite instability [39]. In primary CRC, CD169 macrophages can exhibit pro-tumor effects, whereas in metastasis to LNs their effect is mainly antitumor [38].

The above-presented concept of hybrid M1 and M2 phenotypes may explain the contradictory results regarding the biological effects of M1 and M2 macrophage subtypes. This phenotypic ambiguity can lead to mixed immune responses within tumors, potentially influencing cancer progression in unpredictable ways and complicating prognostic assessments [40]. Nevertheless, the classification of macrophages into M1 and M2 remains relevant for characterizing specific macrophage markers and their biological effects [41].

Given the limitations of the early classification of macrophages, a new one, which is based on comprehensive scRNA-seq analyses in several human cancers, including CRC, has been recently

suggested [42, 43]. Based on specific gene signatures and functions the following types of TAMs are distinguished: interferon-primed tumor-associated macrophages (IFN-TAMs), immune regulatory tumor-associated macrophages (Reg-TAMs), inflammatory cytokine enriched tumor associated macrophages (Inflam-TAMs), lipid-associated tumor-associated macrophages (LA-TAMs), pro-angiogenic tumor-associated macrophages (Angio-TAMs), RTM-like tumor-associated macrophages (resident-tissue macrophages (RTM-TAMs), glycolytic TAMs (supplementary file, ST2) [42, 44].

This new classification has improved our understanding of the different types and functions of TAMs beyond the traditional M1/M2 polarization paradigm. Thus, in accordance with the current state of the field, the phenotypic diversity of TAMs extends beyond the traditional M1/M2 polarization paradigm. From Table 2, it can be inferred that the biological effects of TAMs are apparently driven by unique combinations of both M1 and M2 markers [45] (Table 2). Notably, TAMs that predominantly exhibit M1 markers often show pro-tumor effects (e.g., angio-TAMs, LA-TAMs), while expression of M2 markers is associated with anti-tumor properties. In our view, this is due to the unique biological properties of these markers, which are not directly related to the M1/M2 macrophage dichotomy paradigm. This underscores the need for a more nuanced understanding of the biology of individual macrophage markers, taking into account their specific functional properties and contextual factors, rather than relying solely on their phenotypic classification. In this regard, in our opinion, the classification presented in ST2, which highlights only 7 types of TAMs, is somewhat simplified, as individual markers can have specific biological roles in cancer development, as well as prognostic and diagnostic significance, as demonstrated in ST1.

**Table 2**

**Classification units of TAMs based on single-cell sequencing data**

<b>Types of TAMs</b>	<b>Protein markers</b>	<b>Cytokines</b>	<b>Functions/Effects</b>
Interferon-primed TAM	CD14, CD86, CD163, MHC II, CD64 (FcγRI)	PD-L1, TNF-α, IL-12	Exhibit either a pro-tumor M2-like phenotype or an anti-tumor M1-like phenotype.  Pro tumor effects: Enhance tumor proliferation, T cell exhaustion, Immunosuppression, Decreased Immunosuppression, Suppressed T cell activation Antitumor effects: IFN-γ enhances the anti-tumor immune response by promoting the activation of M1-like macrophages, which produce pro-inflammatory cytokines such as IL-12, TNF-α. This

			activation can lead to increased phagocytic activity and improved tumor cell killing
Immune regulatory TAM	CD206, CD40, CD80, PD-L1, Arg1	IL-10, TGF- $\beta$ , VEGF, CCL22, PDL-1	Can either promote or inhibit tumor progression Effects: Promoting Tumor Cell Survival and Proliferation, Suppressing Anti-tumor Immunity, Promotes inflammation, T cell suppression
Inflammatory cytokine-enriched TAM	CD80, CD86, CD68, MHCII	Il-1 $\beta$ , CXCL1/2/3/8, CCL3, CCL3L1	Pro-inflammatory activity, regulation of WNT-signaling
Lipid-associated TAM	CD36, CD163, CD206, CD80, CD40	Arg-1, XBP1, TREM2, FABP5, APOE	Promotion of Tumor Growth, Antigen processing and presentation pathways, ATP biosynthetic processes protumor effects, promotion of metastasis
Pro-angiogenic TAM	VEGF, CD206, CD163	VEGFA, PDGF, TGF- $\beta$ , MMP2, MMP9, and MMP12.	Angiogenesis, Promotion of HIF pathway in tumor cell; Activate NF-kB, Notch, VEGF signaling. Protumor effects
Rresident-tissue macrophages-like TAM	CD68, CD163, Tim4, CD206	Arg1, PDL-1	Immune tolerance, Immunosuppression, promotion of tumor invasiveness
Glycolytic TAMs	CD163, CD206, Arg1	IL-1 $\beta$ , TNF- $\alpha$ , IL-6, IL-10, HIF-1 $\alpha$	Immunosuppression, promotion of tumor invasiveness, M2-like polarization

### 3. Macrophages in normal mucosa (NM) of the colon: understanding the distribution and functional implications

During the first three weeks of postnatal life, embryonic macrophages in the colon are replaced with macrophages of monocytic origin, which is related to the formation of the microbiota. This process involves microbiota-induced production of CSF2 and chemokines, which attract circulating monocytes

to the intestinal mucosa where they differentiate into specialized macrophages. The replacement is crucial for developing tolerance to the new microbiota, maintaining intestinal homeostasis, and ensuring appropriate immune responses in the unique gut environment [46].

Resident macrophages can be found in various layers of the colon, however, the largest population of macrophages is observed in the subepithelial portion of lamina propria. They are strategically positioned at the first line of defence against pathogens that occasionally penetrate the epithelial layer [47].

M2 type, which expresses CD163, CD206, CD36, and TREM, and encompasses all their subtypes constitutes the majority of resident macrophages in healthy NM. [48] Their anti-inflammatory phenotype ensures tolerance against food antigens and commensal microorganisms [49]. One of such mechanisms is involvement of regulatory T helper (Treg) cells [49]. Also, M2 macrophages secrete growth factors that promote proliferation and differentiation of epithelial cells [50] and therefore are involved in repair and remodelling of the mucosa. Besides, M2 macrophages help to regulate the composition of the gut microbiota and prevent dysbiosis [51].

M1 macrophages, while present in smaller numbers, play important roles in maintaining intestinal homeostasis. They monitor for potential pathogens, using their pattern recognition receptors and present antigens to T cells, contributing to immune surveillance and tolerance to commensal bacteria [52]. They are also highly phagocytic, clearing debris and potentially harmful microbes without triggering excessive inflammation. Besides, M1 macrophages participate in maintaining the integrity of the epithelial barrier [52, 53].

Furthermore, NM harbors transitional macrophages between M0 and M2 states, which can be recognized by co-expression of CD68+ and CD163+ [54, 55]. Most transitional macrophages located in the subepithelial layer of NM also express CX3CR1, which is crucial for maintaining gut homeostasis by controlling aberrant intestinal inflammation [56]. Absence of CX3CR1 increases infiltration by pro-inflammatory macrophages and Th17 lymphocytes in the colon [57].

Macrophages in mucosa-associated lymphoid follicles, which are the most numerous on the border of mucosa and submucosa, help to maintain epithelial barrier function, contribute to immune tolerance to commensal bacteria and promote tissue repair. They predominantly express M2 markers: CD163+, CD206+ and CD169+ [58, 59]. However, due to high plasticity they can adopt pro-inflammatory (M1-like) phenotype in response to microenvironmental signals [60, 47, 59]. Tingible body macrophages (TBM) are found in germinal centers of secondary lymphoid follicles [59] They phagocytize apoptotic B-cells during the germinal center reaction [59]. This process, known as efferocytosis, is crucial for maintaining tissue homeostasis and preventing autoimmune responses. Also, TBMs may help to downregulate germinal center reactions by releasing prostaglandins, which can reduce B-cell induction of IL-21 [61, 59]. Recent research suggests that TBMs originate from pre-existing lymph node-resident

precursors, which enter lymph node follicles in a germinal center-dependent manner. Intravital imaging has shown that TBMs are stationary cells that use highly dynamic cytoplasmic protrusions to capture and phagocytose migrating dead cell fragments. The presence of nearby apoptotic cells can trigger the activation and maturation of follicular macrophages into classical TBMs, even in the absence of germinal centers [59].

High density of M2d macrophages (iNOS+, MSR1+, VEGF+) was identified in the lamina propria of the mucosa in close proximity to submucosal blood vessels. Through hypoxia-inducible factor 1-alpha (HIF-1 $\alpha$ ), these cells can activate endothelial cells' genes associated with angiogenesis, such as Tnfaip2, Ecm, Mmp2, and Mmp14. In this way, macrophages likely contribute to the growth and restoration of blood vessels, supporting the vascular system of the lamina propria [62, 63].

#### **4. Macrophages in colorectal adenoma**

Colorectal adenoma (CA) carcinoma sequence is the most common pathway for CRC initiation. At the early stages of adenomas, M1 macrophages accumulate inside the tumor over M2 macrophages, possibly as a response to the disruption of the epithelial barrier integrity [64]. M1 population in adenomas is characterized by expression of CCL3, CCL4, CXCL2, and CCL19, as confirmed by the research of Wierzbicki J. et al [65]. With an increase in the malignant potential from hyperplastic through tubular and tubulo-villous to villous polyps, the expression of CCL3, CCL4, and CCL19 in lesions decreased. Similar dynamics were observed not only in polyps but also in adjacent normal mucosa [65].

As the lesion size increased, the expression of CCL3, CCL4, and CCL19 decreased, whereas the expression of CXCL2 increased in the unaffected parts of the colon. [65]. Additionally, CXCL2, a chemoattractant for myeloid-derived suppressor cells (MDSCs), suppresses the expansion and activity of anti-tumor effectors, such as T and NK cells. [65, 66, 67]. There are conflicting data regarding expression of CXCL2 by M1 - M2- or both types of macrophages [68, 69] or by both types of macrophages [70].

There is an increased production of MMP9 and MMP2 by macrophages in CA, which are involved in extracellular matrix remodelling and promote transformation of CA into CRC. MMP+ macrophages share markers of M1 and M2 types and probably belong to transitional cells between them [71].

Furthermore, in the case of adenoma progression towards CRC, there is a predominance of F4/80-High MHCII-Low macrophages (characteristic of embryonic tissues, decreasing in the post-embryonic period) over the F4/80-High MHCII-High. F4/80-High MHCII-Low macrophages share expression of M2 markers (eg., ARG1) with many glycolytic genes typical for M1 macrophages and consistently demonstrate simultaneous expression of M1 and M2 macrophage markers [72, 73].

Late-stage adenomas are characterized by predominance of M2 macrophages over M1 type, however, both types of macrophages contribute to creating a favourable pro-tumor microenvironment [74].

CCR2-independent subset of macrophages (predominantly M2c subtype) in CA become the dominant subset among resident macrophages and even more abundant after transformation into tumor. Analysis of CA has shown that CSF1 from neighbour cells of the adenoma microenvironment, is a key factor in macrophage self-renewal [72]. Thus, the microenvironment creates an isolated niche for tissue-resident M2 macrophages, which promotes their survival and self-renewal [72, 75].

The transformation of adenoma into CRC is driven by genetic chromosomal instability (CIN), microsatellite instability (MSI), and epigenetic methylation alterations, including and changes in epithelial cells [76]. CIN promotes an anti-cancer M1-like macrophage phenotype, enhancing immune responses [77], while the IL-1 $\alpha$ /IL1R/MyD88/TET2 axis regulates DNA methyltransferases (DNMT) such as DNMT1 and DNMT3b methylation in macrophages, influencing their direct of polarization [78]. Overexpression of DNMTs in tumor cells downregulates tumor suppressor genes, promoting tumor progression, while M2 macrophages can induce DNMT1 overexpression, further silencing tumor suppressors [79; 80]. Late-stage adenoma progression is marked by dysregulated DNMT methylation/demethylation, shifting macrophages toward an M2 phenotype that enhances (vascular endothelial growth factor) VEGF and TGF- $\beta$  production, supporting tumor survival and metastasis [81]. Non-coding RNAs, including miR-155 and miR-145, also regulate adenoma-to-CRC transformation [82]. miR-155 promotes M1 macrophage polarization, potentially inhibiting CRC progression [83], while miR-145 drives M2 polarization by enhancing IL-10 production via HDAC11 targeting and is transferred to macrophages via extracellular vesicles (EVs) [84].

## 5. Macrophages in primary CRC

The anti-cancer response of macrophages is triggered by the recognition of cancer cells, with a key role played by TLRs, particularly TLR4, which recognize danger-associated molecular patterns released by cancer cells. At this stage, macrophages can phagocytize both apoptotic and live cancer cells, followed by antigen presentation to T-cells [85]. Phagocytosis of apoptotic tumor cells (efferocytosis) is primarily associated with M2 macrophages, able to recognize "eat me" signals on apoptotic tumor cells [86]. Phagocytosis of viable tumor cells is regulated by both pro-phagocytic "eat me" (calreticulin and SLAMF7) and anti-phagocytic "don't eat me" signals (CD47, PD-L1, and  $\beta$ 2-microglobulin) on the tumor cell surface. CD47 is a key "don't eat me" signal that inhibits phagocytosis by binding to SIRP $\alpha$  on macrophages [87, 88]. Blocking CD47 or other anti-phagocytic signals can enhance the clearance of viable tumor cells by macrophages, which is being explored as a anticancer immunotherapy strategy [89, 88]. Some viable tumor cells can evade phagocytosis through enhanced expression of these anti-

phagocytic signals. [87]. Phagocytosis of viable tumor cells by macrophages can sometimes lead to the formation of tumor hybrid cells, which may acquire survival advantages and contribute to cancer progression [90].

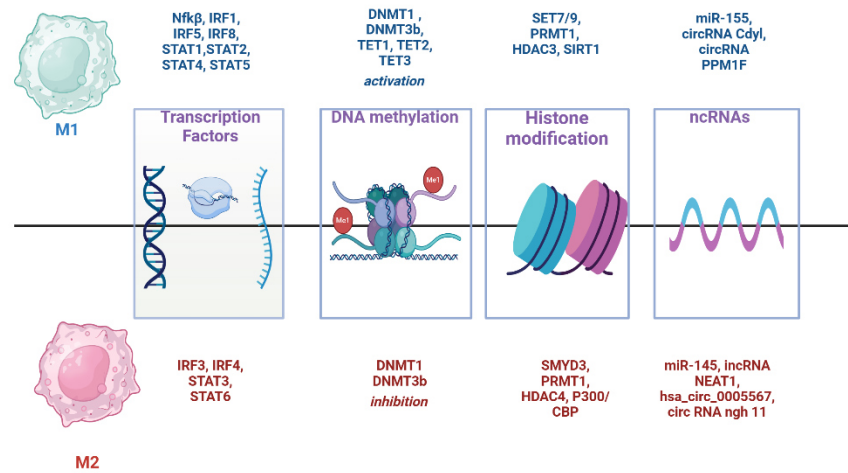
Majority of TAMs present antigen in the context of MHC II molecules to CD4+ T cells, whereas some macrophages, particularly of the M1 phenotype, can cross-present tumor antigens to CD8+ T cells via MHC class I molecules [91].

#### *Drivers of macrophage polarization in CRC*

The recognition of tumor cells triggers macrophage polarization, which is influenced by both macrophage-intrinsic genetic and epigenetic factors and is extensively regulated by the TME [92]. Depending on the molecular cues these modifications can either suppress or promote CRC progression [93]. In CRC, factors such as the IL-1 $\alpha$ /IL1R/MyD88/TET2 axis, PAD4, MMP14, MMP9, MMP2, VEGF, HIF-1 $\alpha$ , arginase-1, cytokines (IL-4, IL-5, IL-13), and chemokines (CCL2, CCL3, CCL4, CCL5, CXCL12) contribute to macrophage polarization [94, 95]. These signals modulate transcriptional activity in macrophages through DNA methylation, histone modifications, and miRNAs, playing a critical role in shaping macrophage function within CRC [93, 78].

#### Transcription factors (TF)

Key transcription factors involved in M1 polarization include IRF1 and IRF5, which mediate the production of inflammatory cytokines TNF- $\alpha$ , IL-6, and IL-12, and also NF- $\kappa$ B and IRF8 (Fig. 3) [96, 97, 98]. Members of STAT-family of TF (STAT1, STAT2, STAT4 and STAT5) are also important for M1 polarization [99]. STAT1 is activated by IFN- $\gamma$ , promoting the expression of pro-inflammatory genes [100]. Contrary, IRF-3 and IRF-4 promote M2 polarization [101]. Also STAT3 and STAT6 proteins are central to M2 polarization. STAT6 is activated by IL-4/IL-13, promoting the expression of M2 markers like the mannose receptor and PPAR $\gamma$ . IRF4 competes with IRF5 and upregulates STAT6 [102, 103].



**Figure 3.** Transcription and epigenetic factors determining the direction of M1/M2 polarization of macrophages.

### DNA methylation and demethylation

Two types of DNA methyltransferases (DNMTs), enzymes which are responsible for methylation, promote macrophage polarization into M1 type: DNMT1 and DNMT3b (Figure 3). DNMT1 plays a crucial role in M1 activation by suppressing the expression of Krüppel-like factor 4 (KLF4) and suppressor of cytokine signaling 1 (SOCS1), enhancing the secretion of proinflammatory cytokines such as TNF $\alpha$  and IL-6 [79, 104]. DNMT3b targets and inhibits the PPAR $\gamma$  promoter, a positive regulator of M2 macrophage polarization [105]. Knockdown of DNMT3b can promote M2 polarization [80]. Demethylation is equally important and is controlled by ten-eleven translocation (TET) enzymes, specifically TET1, TET2, and TET3, which oxidize 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC), leading to the eventual removal of the methyl group [106]. The demethylation leads to M2 macrophage polarization [107]. The balance between DNMT and TET activity is crucial for maintaining macrophage M1/M2 plasticity [108]. DNMT inhibition may also promote an immunosuppressive environment by shifting macrophages toward the M2 phenotype, which can support tumor growth [109]. On the other hand, demethylation can also facilitate the activation of pro-inflammatory genes, promoting M1 macrophage polarization in response to certain inflammatory signals. In M1 macrophages, increased TET activity can lead to the expression of key cytokines and inflammatory markers, contributing to their anti-tumor function. DNMT inhibitors, such as azacitidine and decitabine, promote demethylation and reactivation of silenced genes, making them useful in anticancer therapy [110]. These inhibitors could also enhance the M1 macrophage response, potentially improving the effectiveness of immune-based cancer treatments [80].

### Histone modifications

Histones are subjected to post-translational modifications such as methylation, acetylation, phosphorylation, and ubiquitination. These modifications can influence chromatin structure, affecting gene expression and altering the accessibility of different DNA sites for transcription factors. Histone methyltransferases (HMTs) play a specific role in macrophage polarization (Fig. 3). For example, SET7/9 activates NF- $\kappa$ B, promoting M1 polarization by inducing TNF- $\alpha$  production. SMYD2, another HMT, inhibits M1 polarization by regulating pro-inflammatory cytokines [108]. In contrast, SMYD3 promotes M2 polarization by activating pathways that lead to M2 macrophage phenotypes [111]. PRMT1, an arginine methyltransferase, is involved in both M1 and M2 polarization through different mechanisms [108]. Histone acetyltransferases (HATs), such as P300/CBP, and histone deacetylases (HDACs) also influence macrophage polarization [112]. P300/CBP is crucial for M2 macrophage polarization [112], while HDAC3 supports M1 activation [113].

### ncRNA

Non-coding RNAs (ncRNAs) can regulate gene expression and maintain the balance between M1 and M2 macrophages by modulating transcription, translation, and mRNA splicing. miR-155 and miR-145 are particularly important in regulating the direction of M1/M2 macrophage polarization [114, 115] (Fig. 3). Various other ncRNAs, including circRNAs and lncRNAs, also play significant roles in macrophage polarization by influencing key signaling pathways such as NF- $\kappa$ B and the miR-224-5p/IL-33 axis. These regulatory networks contribute to the complex dynamics of macrophage polarization within the tumor microenvironment [116, 108].

Epigenetic regulation plays a crucial role in shaping macrophage function within the tumor microenvironment, influencing their polarization and secretory profiles. Among the key targets of this regulation are ECM-modifying enzymes, including MMPs and ADAM family proteases. ncRNAs such as miRNAs, circRNAs, and lncRNAs modulate the expression of genes involved in ECM remodeling by directly or indirectly regulating MMP activity [117].

Through epigenetic mechanisms, macrophages can fine-tune the expression of proteolytic enzymes, thereby influencing tumor invasion, metastasis, and immune evasion. Interestingly, numbers of MMP+ and ADAM8+ TAMs in CRC are correlated, with both populations secreting disintegrin and metalloprotease domain 8 (ADAM8). ADAM8 plays a key role in activating MMPs, which leads to matrix remodelling and promotes tumor invasion. Additionally, ADAM8 proteins are involved in cell migration, adhesion, and membrane shedding, processes that are critical for metastatic spread. Inhibiting the activity of ADAM8 and MMPs has been shown to impede the invasive and migratory capabilities of drug-resistant CRC cells. As such, ADAM8 may serve as a potential macrophage-related biomarker in CRC, warranting further investigation. [118]

IL-10 and TGF- $\beta$  also contribute to creating an immunosuppressive environment by inhibiting function of cytotoxic T cells, enhancing the recruitment of MDSCs and promoting regulatory B cells. [119].

TAMs of M1 phenotype, albeit less numerous, display mainly anti-tumor effects and have been associated with a favorable prognosis in CRC [120, 121].

Presence of mixed M1/M2 macrophages (especially CD68+, CD80+, MHC-II+) in the TME is associated with reduced frequencies of liver metastasis [122, 123]. High infiltration of CD68+ TAMs has been suggested as a favorable prognostic marker in CRC [124].

Besides, in the early stages of CRC, M1 macrophages synergize with M2 macrophages to participate in angiogenesis through secretion of VEGF and iNOS. Then density of CD206+, CD163+ M2 macrophages, which secrete PDGF-B and high levels of MMP-9, gradually increases, which is necessary for the maturation of growing blood vessels and remodelling of the vascular network. PDGF-B facilitates the recruitment of pericytes, which contribute to stabilization of sprouts and their evolution into mature vessels [125, 126]. Overall, increased angiogenesis in tumors is often considered an unfavorable prognostic factor, contributing to the progression and metastasis of CRC. Several studies have identified CD206 as a potential biomarker associated with poor prognosis in CRC [127, 128, 125]. However, other studies have demonstrated that a high density of certain M2 phenotypic markers, particularly CD206+ and CD163+ TAMs, positively correlates with a prolonged relapse-free interval in CRC patients. Additionally, it predicts lower tumor malignancy and a reduced number of lymph node metastases in CRC patients. [126, 40]. In experimental tumor growth models and in other cancer types have observed a similar trend [129]. For instance, an increased presence of CD206+ TAM in tumor tissue has been associated with a reduction in tumor burden in syngeneic mouse tumor models. Additionally, a high density of CD206+ macrophages has been linked to improved overall survival in patients with cutaneous melanoma. This phenomenon can be explained by "vascular normalization," where in stabilized and matured vessels prevent tumor cells from infiltrating the vascular network [130, 131]. This explains the conflicting data regarding the prognostic role of M2 in CRC.

Recent studies have identified a subset of secreted phosphoprotein 1 (SPP1), also known as osteopontin)-positive macrophages within the tumor tissue, which possess unique characteristics and immunosuppressive properties [131]. SPP1 is expressed by both the M2- and M1-macrophages. SPP1+ TAMs are primarily found at the invasive front in close proximity to both CRC cells and fibroblasts, where they contribute to EMT, angiogenesis, tumor growth, invasion and metastasis [131, 132]. Targeted downregulation of SPP1+ in TAMs was tested as a potential therapeutic strategy [133]. It was found that SPP1+ TAMs differentiate from THBS1+ TAMs, which are also markers of poor prognosis in CRC. Typically, THBS1+ TAMs are associated with the M2 phenotype. THBS1 is highly expressed in the

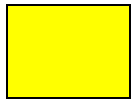
stromal areas of both primary and metastatic CRC lesions, it is responsible for exhaustion of cytotoxic T-cell and impaired vascularization, correlating with the aggressiveness of the disease [131].

The aforementioned indicates that using a single marker does not allow differentiating between TAMs of different phenotypes, often antagonistic (Table 3). In Table 3, the cross-expression of M1 and M2a–d macrophage markers is demonstrated. Multiplex staining using a set of markers should be used to characterize TAMs [128]. Wang et al., 2023 have demonstrated recently that markers of M1 macrophages (NOS2, CXCL10, CD11c) were weakly expressed both in the invasive front and in the tumor center (TC), while markers of M2 (CD163, CD206, CD115) were primarily expressed in the invasive front in primary CRC. CRC patients with low expression of M1 markers or high expression of M2 markers demonstrated poor prognosis. Noteworthy, the prognostic value of a combination of different markers (NOS2/CXCL10/CD11c or CD163/CD206/CD115) was higher than that of a single marker [128].

Table 3

**Overlapping markers between M0 and M1- or M2-macrophages and between M2-subphenotypes**

<b>M0*</b>	ADGRE1	CCR2 (CD192)	CD14	CD68	CSF1R (CD115)	ITGAM (CD116)
	SPP1	PPARG	CXCR1	F4/80		
<b>M1</b>	CD11c	FCGRIA (CD64)	CD80	CD86	iNOS	CD40
	CXCL10	MHC-II	TLR-2	TLR-4	TLR-8	MRP8
	IL-10R	IDA	SPP1	VEGF	IL1R1	
<b>M2a</b>	MHC-II	SPP1	THBS1	IDA	CD163	CD206
	CD200R	CLEC7A (CD301)	CD36	CD209	FCGR3A (CD16)	MSR1 (CD204)
	TIMD4	CD206	FIZZ1	Arg1	SPP1	CD155
<b>M2b</b>	CD86	MHC-II	IL1R1	IL-10R	CD209	MSR1 (CD204)
	IDA	PDCD1LG	THBS1			
<b>M2c</b>	TLR-2	TLR-4	TLR-8	CD163	CD206	FCGRIA (CD64)
	MSR1 (CD204)	THBS1				
<b>M2d</b>	iNOS	VEGF	MSR1 (CD204)			



Overlap between M1 and M2



Overlap between M2 subtypes

\* The overlap of between M0 and M1 or M2 phenotypes was not analysed due to the presence of transitional forms

### *Macrophages and tumor microarchitecture*

Beyond the complexity of the various polarization states, macrophages in different regions of CRC also can play opposing roles [134, 40, 94]. The plasticity of TAMs allows for dynamic transitions between M1 and M2 types in response to microenvironmental signals. In the tumor center, which is characterized by a high degree of genetic instability, cellular proliferation and hypoxia, TAMs are mostly polarized into an M2-like type [134]. The ratio of M1 to M2 macrophages may influence the progression and metastasis of CRC and is regarded as a prognostic marker [94].

The tumor invasive margin (TIM) is the area where cancer cells actively invade the surrounding tissue, thus exhibiting a more aggressive and invasive phenotype. Pinto et al. detected dominance of CD163+ TAMs in the invasive front and CD80+ TAMs in intratumoral region (IT) and invasive front [135]. Compared to TAMs in the tumor core, they often display a more complex, mixed (M0, M1 and M2) phenotype, which reflect their adaptability to the dynamic environment at the tumor-host interface [136]. Several studies have established a correlation between types of TAMs and their abundance in invasive front with the clinical and pathological features of CRC. E.g., the number of M2 TAMs, predominantly CD163-positive, in TIM increases as tumor advanced. High numbers of M2 TAMs in TIM correlated with low histological differentiation, lymphovascular invasion and lymph node metastasis. Additionally, it was found that a higher M1/M2 ratio in the TME, particularly at the invasive front, correlates with a better prognosis and reduced metastatic risk [137, 138]. Inner and outer layers of TIM have different tissue architecture and different immune landscape [139]. Moreover, immune cells (CD4, CD8, CD20, FoxP3, CD45RO, pan-cytokeratin (pan-CK), a NK/macrophage panel encompassing CD56, NKp46, CD3, CD68, CD163, and pan-CK, a dendritic cell panel with CD3, CD1a, CD208, CD123, CD68, CD15, which studied by Artur Mezheyski et al., 2021 of the single type, being located in inner and outer layers may show different prognostic associations [140, 141]. Tumor stage stratification revealed that TAMs, especially CD163+, were more abundant in TIM of T3 tumors, whereas CD80+ macrophages predominated in less invasive T1 tumors. Particularly in stage T3-T4 CRC, a higher CD68/CD163 ratio and a lower CD80/CD163 ratio were associated with decreased overall patient survival [135].

The peritumor zone (PT) is adjacent to the invasive margin. Determined by the depth of the invasion PT region may include submucosa, muscularis propria and subserosal fat. Immune cells in this region play a crucial role in tumor progression, invasion, and metastasis, as well as in shaping the immune response against the tumor. Tertiary lymphoid structures, which are enriched in CD68+

macrophages are frequently seen in PT [142]. TAMs in the PT can interact with fibroblasts, endothelial cells, and other immune cells to modulate angiogenesis, immune responses, and support the growth of the tumor stroma [143]. TAMs in PT area can co-express a range of both M1 and M2 markers, including CD68, CD163, and CD206, reflecting their involvement in both pro- and anti-inflammatory activity.. [144, 136].

To summarize, TAMs in TC and PT predominantly exhibit M2-like phenotype, promoting tumor progression and might enhance immunosuppression or support tumor cell survival under hypoxic conditions. TAMs in invasive front may demonstrate a more complex phenotype that supports both tumor growth and anti-tumor immunity, which is crucial for invasion and metastasis of tumor cells [135, 145].

Conflicting data regarding predominant phenotype of TAMs phenotypes in CRC can be explained from the perspective of CMS, which are based on differential gene expression in the TME. The CMS in CRC are recognized according to genetic modifications and intratumoral immune profiling: CMS1 (MSI immune), CMS2 (canonical), CMS3 (metabolic), CMS4 (mesenchymal) [146, 147]. CMS1 is characterized by microsatellite instability followed by immune activation and dense infiltration by CD8 T-cells, CD4 memory T-cells, Th1, follicular helper T-cells,  $\gamma\delta$  T-cells, as well as activated DCs and NK cells [146, 147]. Macrophages in this CRC subtype are predominantly polarized towards the M1 phenotype [145]. CMS2 includes tumors with activated WNT and MYC signaling pathways, while CMS3 is characterized by profound metabolic dysregulation and frequent KRAS mutations. They are characterized by weak immune cells infiltration. Mixed M1/M2 macrophage phenotypes prevail in these CMS2 and CMS3 subtypes. The pro- and anti-tumor effects of macrophages are determined by the apparent M1/M2 ratio and might vary depending on the metabolic environment [145]. CMS4 includes tumors characterized by EMT, associated with matrix remodeling, strong stromal activity, TGF- $\beta$  pathway activation, and angiogenesis. CMS4 exhibits fewer CD8 and CD4 T-cells and more Tregs, monocytes, eosinophils, myeloid cells, and resting dendritic cells (DCs) compared to CMS1 tumors. Macrophages predominantly have an M2 phenotype creating a pro-tumor microenvironment [148]. It is also necessary to consider the presence of mixed CRC subtypes and the potential for one subtype to transit into another. In such cases, it becomes challenging to assess the dominance of M1 or M2 macrophages and their contribution to prognosis. Therefore, studying the biological role of specific macrophage markers and their impact on tumor progression or regression appears to be particularly relevant [148].

## **6. Role of macrophages in regional lymph node (RLN) metastases**

RLN metastasis occurs in several stages: (1) initiation of lymphangiogenesis in the primary tumor, (2) migration of first tumor cells along with immune cells through lymphatic channels toward draining

lymph nodes, (3) initiation of lymphangiogenesis in the RLN, (4) formation of a pre-metastatic niche in RLN, (5) proliferation of tumor cells in the RLN parenchyma. At each of these stages, macrophages play a specific role in regulating, initiating, or suppressing the aforementioned processes [149, 150].

The role of M2 macrophages (especially M2d) in the production of VEGF-C, which in turn is an inducer of lymphangiogenesis, is well known [151]. Also, VEGF-C can bind to VEGFRceptor3 on lymphatic vessels, inhibiting vascular endothelial cadherin (CD144) expression and damaging the endothelial barrier, facilitating tumor cell entry into lymphatic vessels [152]. A high number of M2 macrophages and A low number of M1 at the tumor invasive front correlate with lymphovascular invasion, poor histological differentiation [141]. It has also been shown that the M2/M1 ratio is a more significant predictor of the risk of RLN metastasis than the number of pan-, M1, or M2 macrophages at the invasive front (IF). These findings suggest that M2 TAMs at the invasive front may play a role in CRC progression from stage II to stage III [152, 141].

Hydrodynamics play a key role in the spread into RLNs. Blood vessels in the tumor typically have abnormal permeability and blood flow, causing plasma to accumulate in the extracellular spaces and impairing drainage due to compression of local lymphatics [153, 154]. This leads to elevated intratumor interstitial fluid pressure (IFP), creating an IFP gradient that promotes tumor cell migration to the RLN [154]. It has been shown that interstitial flow polarizes macrophages towards an M2-like phenotype (CD163+, CD206+) through integrin/Src-mediated mechanotransduction pathways involving STAT3/6 [155]. In accordance with this flow-induced polarization, M2 macrophages exhibit a higher migration rate. In addition, interstitial flow attracts M2 macrophages to tumor masses, promoting cancer cell invasion through the secretion of MMPs and growth factors, such as TGF- $\beta$ , which degrade the ECM and support the invasive and metastatic potential of cancer cells. Additionally, macrophages release immunosuppressive cytokines (e.g., IL-10), which suppress anti-tumor immune responses, further contributing to tumor progression [152, 155].

Macrophages, mainly of M2 subtypes, are naturally present in mesenteric lymph nodes [156]. Additionally, M2 macrophages infiltrate non-metastatic RLNs prior to metastasis [157, 158]. M2 macrophages are regarded as a source of angiogenic factors (VEGF-C, iNOS), which induce lymphangiogenesis and angiogenesis in the RLNs, multiplying routes for metastasis [141]. Also, M2 macrophages play a significant role in the formation of the premetastatic niche in RLN by ECM remodelling via secreted MMP-9 [159]. In addition, M2 macrophages create an immunosuppressive environment in lymph nodes before metastasis occurs, secreting cytokines like IL-10 and recruiting regulatory T cells (CCR-6+Tregs) to the premetastatic niche [160]. Research by Yanping Wang et al., 2021, demonstrated an increased number of M2b, M2c, and M2d macrophages (CD163+, CD206+, VEGF+, iNOS+) in exposed RLN. Furthermore, higher numbers of M2 macrophages were observed in RLNs with macrometastases compared to those with micrometastases [152].

Anti-tumor effects of CD169+ macrophages in RLNs were also demonstrated. It was established that the density of CD169+ macrophages in RLN is positively correlated with the density of infiltrating T- or NK-cells in tumor tissues, indicating the significance of CD169-positive macrophages in anti-tumor immune responses [161].

## **7. Role of macrophages in distant hematogenous metastases**

Macrophages can modulate distant CRC metastasis at all sequential stages of this process: invasion of tumor cells, angiogenesis in pCRC, intravasation and survival of tumor cells in the circulatory system, formation of pre-metastatic niches in distant organs, extravasation of tumor cells, colonization of tumor cells to establish micro-metastases, and proliferation of tumor cells at the secondary sites [162].

### *Invasion of CRC cells*

During EMT, epithelial-like, early proliferating cancer cells lose their intercellular adhesions and acquire a fibroblast-like phenotype with invasive and migratory properties, which is a prerequisite for metastasis [163]. EMT can confer stem cell-like characteristics on cancer cells, enhancing their ability to initiate new tumors and resist therapies [164].

M2-macrophages, as cells secreting MMPs, TGF- $\beta$  and IL-8 play the key role in EMT [165, 94]. However, M1 macrophages as a source of IL-6, TNF, and IL-1 are also necessary [166]. The cytokines produced by M1 and M2 macrophages can work synergistically to create a pro-EMT environment. For example, TNF- $\alpha$  (from M1) and TGF- $\beta$  (from M2) can cooperate to enhance EMT and stem cell-like properties in CRC cells through the NF- $\kappa$ B/Twist axis [167, 168].

### *Angiogenesis in primary tumor*

Angiogenesis plays a crucial role in providing nutrients and oxygen to support tumor growth and progression. M2a and M2d macrophages (iNOS+, VEGF+ CD204+, CD163+) can secrete pro-angiogenic factors that influence formation of new blood vessels in primary tumor, their maturation and stabilization [68].

M1 macrophages also contribute to angiogenesis through secretion of VEGF-A and FGF2, which are essential for capillary sprouting [169]. For instance, in a single-cell atlas of tumour-infiltrating myeloid cells, a cluster of TAMs with an M1-like phenotype coexists with a macrophage subpopulation with angiogenic properties associated with poor prognosis [170, 171]. Several studies have shown the joint contribution of M1 and M2 macrophages to angiogenesis and tumor progression [172, 126]. Contrastingly, Bi Y. et al. 2020 reported that M1 macrophages may inhibit angiogenesis and tumor growth by promoting the production of CXCL9, CXCL10, and CXCL11 in CRC, and their predominance may be considered as markers of favorable prognosis in CRC [173].

### *Intravasation, circulation and extravasation of cancer cells*

Intravasation and extravasation are pivotal stages of the metastatic cascade. M2 macrophages play a leading role in these processes, with minor contribution of M1 and transitional M1/M2 macrophages [174, 133]. During intravasation, tumor cells detach from the primary tumor and permeate into blood vessels. MMPs remodel the stromal ECM and degrade the basement membrane, making the tumor stroma and endothelial barrier more permissive for the intravasation of CRC cells [175]. Epidermal growth factor (EGF), predominantly secreted by CD206+ TAMs, can promote the invasion and mobility of CRC cells, and activation of EGFR on tumor cells is necessary for intravasation to persist [176]. VEGF produced by M2 like TAMs can facilitate the intravasation and extravasation of tumor cells by altering the permeability of blood vessels [177].

Importantly, TAMs can directly "guide" cancer cells to blood vessels (migratory macrophages) and help them enter the bloodstream (sessile perivascular macrophages) [177]. The following sequence of events has been described: TGF- $\beta$ , produced by tumor tissue, induces expression of CXCR4 by TAMs. At this stage, CXCR4+ TAMs interact with cancer cells and induce the expression of actin regulators, ultimately leading to the formation of podosomes in TAMs and invadopodia in tumor cells. These specialized structures facilitate extracellular matrix degradation and enhance metastatic spread. Those mechanisms have been studied in lung, ovarian, breast, and prostate cancer metastases. [178, 179]. Also, chemokine CXCL12 expressed by perivascular fibroblasts can attract CXCR4+ TAMs together with mobile cancer cells to blood vessels. Upon entering the blood vessel, migratory TAMs differentiate into perivascular macrophages (predominantly M2), promoting vascular permeability and intravasation of tumor cells [180, 179].

Research suggests that macrophages may help CRC tumor cells to survive in the bloodstream by releasing cytokines and chemokines. Eg., IL-6 from both M1 and M2 macrophages can activate the JAK-STAT3 pathway, supporting tumor cell survival and growth [181]

In the liver, M2-type macrophages, characterized by CD163 and CD206 markers, can produce hepatocyte growth factor (HGF) that binds to the c-Met tyrosine kinase receptor on the surface of migrating tumor cells, potentially promoting their extravasation into the liver through activation of various signalling pathways, including JAK/STAT3, MAPK, PI3K/AKT, and NF- $\kappa$ B [182, 181, 183].

#### *The premetastatic niche (PMN) in the liver*

In CRC, the liver serves as a primary site for distant metastases. The development of the PMN and the imprinting of macrophages within this niche depend on EVs, which originate from the primary tumor and circulate in the blood. Integrins on the surface of tumor-derived exosomes are responsible for organotropism, as their patterns correlate with sites of metastasis [184]. EVs transport RNA, lipids, metabolites, or proteins, which they can transfer to other cell types to modulate their phenotype and functions. Due to their diverse molecular profile, EVs influence the pre-metastatic niche by modulating immune cells, remodelling the ECM, and promoting angiogenesis. In the liver, for example, tumor-

derived exosomes containing miR-934 and miR-135a-5p bind to a subpopulation of CD206+ resident Kupffer cells (KC) [184, 185, 186, 187]. This leads to enhanced expression of the fatty acid transporter CD36 and polarization of KC towards an anti-inflammatory M2 phenotype, which contributes to the formation of premetastatic niches by suppressing PTEN and activating the PI3K/AKT signalling pathway. [188, 189].

KCs are present in both the metastatic TME and the normal liver. Their main differences are presented in Table 3 (supplementary file table 3). It is important to note that the phenotypic and functional differences between KCs in the TME of metastasis and normal liver are not absolute, and there is heterogeneity within the KC population. Additionally, the specific roles of KCs may vary depending on the stage of metastasis, the presence of other immune cells, and the overall microenvironmental cues [190, 191].

It is considered that the main types of macrophages in liver metastases (LM) of CRC are M2 macrophages (CD206+, CD163+, CD86+) and KCs, although pan-macrophages (CD68+) and M1 macrophage phenotypes (primarily CD86+) as well as transitional forms were also reported [174, 187]. The dominance of M2 macrophages is associated with poor prognosis [192] (Supplementary file, Table 4).

M2 macrophages are pivotal in recruiting MDSCs to the liver, which is a key step in PMN formation. CRC cell-derived VEGF-A triggers M2 macrophages (especially CD163 and CD206) to produce CXCL1, attracting CXCR2-positive MDSCs to the pre-metastatic site, promoting liver metastasis [193, 194]

Also, macrophages can contribute to PMN formation by fusing with tumor cells, forming "hybrids that promote distant metastases. In mouse studies, macrophage-melanoma hybrids injected into mice led to metastatic lesions in the pancreas [195]. These hybrids may aid in premetastatic niche formation, though further research is needed to clarify their role in extravasation and colonization for therapeutic targeting.

#### *Colonization of liver by tumor cells to establish micro-metastases*

In models using the human breast cancer cell lines MDA-MB-435, MDA-MB-231, T47D, and MCF7, stimulation of the CCL2/CCR2 axis in monocytes and macrophages increased CCL3 gene transcription and protein production, which in turn promoted their retention at metastatic sites [196]. Moreover, in colorectal cancer LM, the CCL3/CCR1 axis mediates direct binding between macrophages and tumor cells, partly via an  $\alpha 4$  integrin dependent mechanism, while CCR2 positive M2 macrophages drive metastatic progression; accordingly, CCR2 mediated regulation of CCL3 is highlighted as a potential therapeutic target [197].

CCR2 macrophages at the metastatic site are capable of supporting the following pro-metastatic processes: CCR2+ macrophages secrete factors like VEGF-A that increase vascular permeability, facilitating tumor cell extravasation; they create a supportive niche for metastasizing tumor cells,

promoting their survival and proliferation; CCR2<sup>+</sup> macrophages contribute to immunosuppression in the metastatic microenvironment [198, 199]. Additionally, as noted above, cancer-associated fibroblasts (CAFs) polarize TAMs to perform pro-tumor functions in the primary tumor. Specifically, Zhang et al. (2019) demonstrated that CAFs secrete IL-8, attracting circulating monocytes via the CXCR2 pathway, and also produce IL-6, which upregulates VCAM-1 expression on tumor cells—together enhancing monocyte adhesion and driving M2-like macrophage polarization that promotes immune suppression. [200]. In the case of metastasis, CAFs and macrophages interact, increasing the metastatic potential and supporting the colonization of incoming tumor cells [200, 174].

#### *Proliferation of tumor cells at the LM*

Macrophages continue to support the development of metastases and the proliferation of tumor cells after the migration of tumor cells has occurred. It was shown that predominantly M2 macrophages in CRC LM (either free or in complex with tumor cells) produce HGF, which through c-MET triggers multiple downstream signaling cascades in tumor cells via MAPK, ERK1/2/MAPK, and RAS pathways that stimulate cancer cell proliferation [201, 202].

In parallel with this, M2 macrophages are recruited into the LM through binding of the matricellular protein SPON2. The expression of SPON2 positively correlates with M2-TAM infiltration in CRC tumors and is associated with poor prognosis in CRC patients. Furthermore, SPON2 promotes cytoskeletal remodeling and transendothelial migration of monocytes by activating the integrin  $\beta$ 1/PYK2 axis. SPON2 may indirectly induce M2 polarization by upregulating cytokines, including IL-10, CCL2, and CSF1 expression in tumor cells. Blocking M2 polarization and depleting macrophages suppressed SPON2-induced tumor growth and invasion. Moreover, inhibition of the SPON2/integrin  $\beta$ 1/PYK2 axis impairs transendothelial monocyte migration and TAM functions that support cancer progression in vivo. to  $\alpha$ 4 $\beta$ 1 integrin on M1 macrophages [203].

When studying the phenotypic profile of macrophages in metastasis, it is essential to consider the CMS subtypes of metastases. Some studies have shown that CMS subtype in metastases can differ from primary CRC [204]. Metastatic propensity is higher in CMS4-mesenchymal primary tumors and lower in CMS1 and, to a lesser extent, CMS3 subtypes. 90% of liver metastases belong to one of two subtypes, either CMS2-canonical or CMS4-mesenchymal.

Figure 4 shows the dominant macrophage phenotypes in the adenoma-colorectal cancer-liver metastasis sequence, considering the CMS subtypes. At the same time, adenoma-colorectal cancer-liver metastasis sequence are characterized by significant heterogeneity of macrophages, including mixed and transitional forms, which must be taken into account when assessing their biological effects and disease prognosis [204].

**Conclusions** Despite the diversity of macrophage phenotypes, including their mixed forms observed in both adenoma and colorectal cancer, as well as its liver metastases, the following phenotypic portrait of macrophages can be outlined in the sequence: normal mucosa (M2) – early adenoma (M1) – late adenoma (M2) – colorectal cancer and liver metastases (the predominance of M1/M2 is determined by CMS subtypes) (Fig. 4)

Macrophages play a complex and multifaceted role in CRC initiation, progression, and metastasis. Their plasticity allows them to exert both pro- and anti-tumor effects depending on the specific TME and stage of disease. The traditional M1/M2 paradigm is oversimplistic, as macrophages in the TME often display mixed phenotypes. Also, macrophage phenotypes and functions evolve throughout CRC progression, from normal mucosa through adenoma to invasive carcinoma and metastasis. The spatial distribution of macrophages within tumors (e.g. tumor center vs invasive margin) impacts their functional roles and prognostic significance. Macrophages are active players in the metastatic cascade, including the stimulation of angiogenesis, intravasation, and extravasation of tumor cells, as well as the creation of pre-metastatic niches.

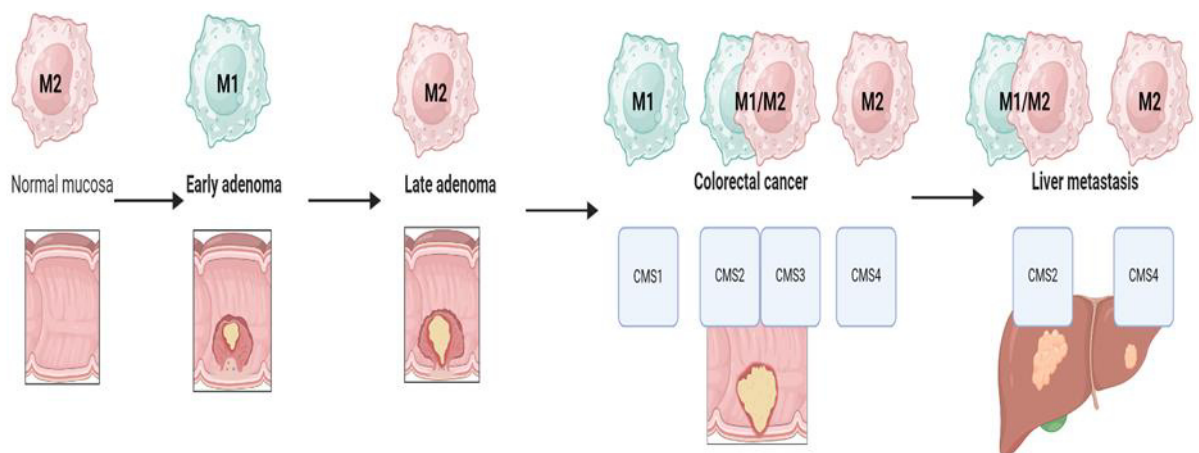


Figure 4. Macrophage M1/M2 phenotypes in the adenoma-colorectal cancer-liver metastasis sequence. (Cytokines influencing M1-M2 macrophage polarization are indicated above; transcriptional and epigenetic factors are shown below the images).

The complexity and diversity of macrophages in liver metastasis underscore their pivotal roles in cancer progression and highlight the potential for macrophage-targeted therapies to improve patient outcomes [205]

Given the heterogeneity of TAMs and the existence of heterogeneity advanced techniques that enable spatial visualization of distinct macrophage phenotypes a needed. One of the most promising methods currently is spatial transcriptomics (ST) [206, 131]. ST allows simultaneous measurement of

thousands of proteins and genes while maintaining spatial context and enables unbiased discovery of novel macrophage subtypes, provides insights into cell-cell interactions, and reveals functional states of macrophages in different tumor regions. Integration of spatial transcriptomics with other omics data and advanced computational methods will be crucial for fully elucidating macrophage biology in colorectal cancer and developing more effective targeted immunotherapies.

Despite the considerable body of research on macrophage involvement in CRC, several gaps and inconsistencies remain unresolved. First, the prognostic significance of M2 macrophages continues to spark debate. While numerous studies associate elevated densities of CD163<sup>+</sup> or CD206<sup>+</sup> adverse patient outcomes, others studies showed that M2 macrophages can promote vessel “maturation” and potentially improve prognosis by contributing to vascular normalization. Such divergence likely stems from the fact that M2 cells encompass multiple subtypes (e.g., M2a, M2b, M2c, M2d) and often exhibit mixed phenotypes with overlapping M1/M2 markers *in vivo*. In this regard, future research should focus on expanding standard immunohistochemical panels beyond CD163/CD206 and establishing uniform criteria for subclassifying macrophage populations both at the protein and transcriptome levels.

Second, conflicting data persist regarding the role of M1 macrophages. Traditionally viewed as anti-tumor effectors, M1 cells can also foster chronic inflammation, promote epithelial-to-mesenchymal transition, and facilitate tumor dissemination. This “dual face” phenomenon may result from contextual factors such as tumor region (center vs. invasive margin vs. regional lymph nodes) and the specific molecular subtype of CRC (CMS1 through CMS4). Future work must incorporate molecular subtyping and spatial transcriptomics to clarify how distinct TAM niches interact with host tissue, potentially guiding novel prognostic tools and targeted therapeutic strategies.

A further challenge lies in reconciling genetic with epigenetic determinants of macrophage polarization. Whereas some data suggest that DNMT1/3b suppression skews cells toward an M2-type, other models implicate these enzymes in fostering M1-related inflammatory cascades. Elucidating the full scope of DNA methylation, histone modifications, and non-coding RNA regulation of TAM subsets will require longitudinal studies in patient-specific organoids and *in vivo* models, integrating methylome, transcriptome, and proteome analyses of both tumor and microenvironment.

Lastly, the translational potential of reprogramming macrophages remains underexplored in CRC. Therapeutic approaches aimed at blocking “don’t eat me” signals (e.g., CD47, PD-L1) or at converting M2 to M1 phenotypes *in situ* have yet to be systematically evaluated in the context of different TAM subtypes. Moving forward, combining multi-marker phenotyping, spatial biology, and advanced epigenetic profiling offers a promising route to resolve current controversies, refine patient stratification, and pave the way for more precise therapies that harness the plasticity of macrophages to combat colorectal cancer.

## **Competing interests**

The authors declare that they have no competing interests in this section.

## **Abbreviations**

ADAM8 – A disintegrin and metalloproteinase domain 8

Angio-TAMs – pro-angiogenic tumor-associated macrophages

APCs – antigen-presenting cells

CA – colorectal adenoma

CIN – chromosomal instability

CMS – consensus molecular subtype

CRC – colorectal cancer

CSF – colony-stimulating factor

DNMT – DNA methyltransferases

EGF – epidermal growth factor

ECM – extracellular matrix

EMT – epithelial-mesenchymal transition

GM-CSF – granulocyte-macrophage colony-stimulating factor

glyc-TAMs – glycolytic tumor-associated macrophages

HDACs – histone deacetylases

HGF – hepatocyte growth factor

HIFs – hypoxia-inducible factors

HMTs – histone methyltransferases

IF – invasive front

IFN-TAMs – interferon-primed tumor-associated macrophages

IFP – interstitial fluid pressure

Inflam-TAMs – inflammatory cytokine-enriched tumor-associated macrophages

IT – intratumoral region

KCs – Kupffer cells

KLF4 – Krüppel-like factor 4

LA-TAMs – lipid-associated tumor-associated macrophages

LPS – lipopolysaccharide

M-CSF – macrophage colony-stimulating factor

MSI – microsatellite instability

ncRNAs – non-coding RNAs

PGE2 – prostaglandin E2

PMN – premetastatic niche  
PT – peritumor zone  
Reg-TAMs – immunoregulatory tumor-associated macrophages  
RLN – regional lymph node  
RTM-TAMs – resident tissue macrophage-like tumor-associated macrophages  
scRNAseq – single-cell RNA sequencing  
SPP1 – secreted phosphoprotein 1  
TAMs – tumor-associated macrophages  
TBM – tingible body macrophages  
TGF- $\beta$  – transforming growth factor-beta  
TIM – tumor invasive margin  
TLRs – Toll-like receptors  
TME – tumor microenvironment  
TF – transcription factors  
VEGF – vascular endothelial growth factor

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### **Contributions**

Conceptualization: Andriy Trailin, Kari Hemminki and Václav Liška. Supervision: Kari Hemminki; project administration: Václav Liška; funding acquisition: Kari Hemminki and Václav Liška. Data curation: Sergii Pavlov, Filip Ambrozkiewicz, Esraa Ali, Wenjing Ye, Rajtmajerová Marie. Sergii Pavlov wrote the first draft of the manuscript and performed data analysis. All authors performed interpretation of the data, revising drafts of manuscript and approval of final version.

### **Findings**

Supported by the grants AZV NU21-03-00506; SALVAGE project (OP JAK; reg. no. CZ.02.01.01/00/22\_008/0004644) - co-financed by the European Union and the state budget of the Czech Republic.

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### **Ethics approval and consent to participate**

Not Applicable.

### **Consent for publication**

Not applicable.

### **Competing interests**

The authors have no financial and non-financial competing interests.

Table 1

**The main markers of macrophages, their biological properties, and their association with CRC**

<b>Marker, Localization</b>	<b>polarization state</b>	<b>Biological effects</b>	<b>Associations with CRC</b>	<b>References</b>
F4/80 (adhesion G protein-coupled receptor E, EMR1), cell membrane	M0	induction of efferent CD8+ regulatory T cells necessary for peripheral tolerance	<p>EMR1 is abnormally expressed in CRC and is a risk factor for LNM and poor RFS in patients with CRC. Inducing EMR1 in macrophages may promote LNM and CRC progression via JAK2/STAT1,3 signaling upregulation.</p> <p>EMR1 is significantly associated with CD68+ CD163+ macrophages, and CRC with a high combined EMR1+CD68+CD163+ score showed worse RFS.</p>	[1, 2, 3]
CCR2 (C-C chemokine receptor type 2 for the chemokine CCL2), cell membrane	M0	involved in the recruitment of monocytes to sites of inflammation	Aberrant expression of CCR2 is associated with negative outcomes in IBD, and colon-related metastasis. CCR2 inhibition can reduce the recruitment of myeloid-derived suppressor cells and decrease lung metastasis in breast cancer models Endothelial CCR2 expression has been linked to promoting tumor cell extravasation and pulmonary metastasis, highlighting its significance in cancer progression .	[4, 5, 6]
CD14, cell membrane	M0	co-receptor for bacterial LPS	High CD14 expression in CRC is associated with microsatellite instability, BRAF mutations. High CD14 expression predicts worse outcomes in CRC. High density of CD14+HLA-DR- cells (immature monocytic phenotype) in intraepithelial regions is	[7, 8, 9]  <a href="https://www.proteinatlas.org/ENS/G00000170458-CD14">https://www.proteinatlas.org/ENS/G00000170458-CD14</a>

			associated with higher CRC-specific mortality, however, high density of CD14+HLA-DR+ cells (mature monocytic phenotype) in both intraepithelial and stromal regions is associated with lower CRC-specific mortality.	
CD68 (SCARD1-Scavenger Receptor Class D Member 1 GP110, LAMP4), lysosomes, endosomes, cell membrane	M0	A transmembrane glycoprotein that binds to tissue- and organ-specific lectins or selectins, clear cellular debris, promote phagocytosis, and mediate the recruitment and activation of macrophages	CD68+ cells are predominantly found at the invasive front of the tumor compared to the intratumoral area or adjacent normal mucosa. There is a moderate correlation between CD68 and CD163 staining, suggesting that tumors with higher CD68+ infiltration also tend to have higher CD163+ cell infiltration. Prognostic associations in CRC are contradictory. High infiltration of CD68+ TAMs, especially in the tumor stroma, correlates with worse RFS and OS in late-stage CRC patients receiving bevacizumab combined with chemotherapy. Specifically, in stage III CRC, higher infiltration of CD68+ cells in the intratumoral area was associated with reduced OS. A high CD206+/CD68+ ratio is associated with improved survival in adjuvant chemotherapy. High CD68+/CRC cell ratio was linked to better survival in 205 CRC patients.	[10-15]  <a href="https://www.proteinatlas.org/ENSG00000129226-CD68">https://www.proteinatlas.org/ENSG00000129226-CD68</a> <a href="https://doi.org/10.1158/1538-7445.AM2022-2530">https://doi.org/10.1158/1538-7445.AM2022-2530</a>
CSF1R (receptor for colony-stimulating factor 1, CD115), cell membrane	M0	CSF1R promotes the release of pro-inflammatory chemokines in response to IL-34 and CSF-1, contributing to homeostasis in the colon. CSF1R-expressing macrophages are involved in	The CSF1/CSF1R axis is essential for the survival and differentiation of M2 TAM in CRC. CSF1R expression is enriched in TAMs within CRC tumors, and its expression in macrophages is associated with poor prognosis in CRC patients.	[16]  <a href="https://www.ncbi.nlm.nih.gov/gen/1436proteinatlas.org/ENSG00000182578-CSF1R">https://www.ncbi.nlm.nih.gov/gen/1436proteinatlas.org/ENSG00000182578-CSF1R</a>

		tissue repair processes in the colon.		
Ly6C1 (lymphocyte antigen 6 family member C1), cell membrane	M0	Ly6C1, a member of the Ly-6 superfamily, plays a crucial role in immune responses and cell signaling. Ly6C1 is anchored to the cell membrane through a glycosyl-phosphatidylinositol (GPI) lipid anchor, allowing them to localize to specific membrane domains called lipid rafts. Cross-linking of Ly-6 proteins like Ly-6A/Sca-1 can trigger simultaneous stimulatory and inhibitory responses in cells, leading to cytokine production, growth inhibition, and apoptosis. Additionally, Ly6C1 may be involved in regulating complement activation and pathogen clearance, highlighting its importance in host defense mechanisms.	Ly6C-high macrophages contribute to tumor initiation and malignant progression in CRC. Participate in the creation of a pre-metastatic niche and subsequent colonization of metastatic sites by tumor cells.	[17, 18] Klikněte nebo klepněte sem a zadejte text.
PPARG (Peroxisome Proliferator-Activated Receptor Gamma), nucleus, cytoplasm	M0	a type II nuclear receptor, a transcription factor. PPARG is an important regulator of macrophage polarization, with PPARG activation driving the M2 phenotype through upregulation of Arg1 and Mgl1 genes.	High PPARG expression in tumors was not significantly associated with worse prognosis in CRC patients, as indicated by a study analyzing PPARG gene expression in CRC tumors and adjacent normal tissues . However, in lung adenocarcinoma (LA), low PPARG expression was linked to poor prognosis. The study on hypopharyngeal squamous cell	[19-23] <a href="https://www.proteinatlas.org/ENS/G00000132170-PPARG">https://www.proteinatlas.org/ENS/G00000132170-PPARG</a>

			carcinoma (HSCC) revealed that PPARG expression variations were significantly associated with the Tumor-Node-Metastasis (TNM) stage, impacting chemosensitivity in HSCC patients. Role of PPARG expression in cancer prognosis and metastasis can vary depending on the specific cancer type.	
CX3CR1 (C-X3-C Motif Chemokine Receptor 1 for CX3CL1), cell membrane	M0	Involved in the adhesion and migration of monocytes, macrophages, and other immune cells	CX3CR1 expression is significantly elevated in poorly differentiated CRC compared to moderate-well-differentiated tumors. Higher CX3CR1 expression is associated with advanced clinical stages, metastasis, and recurrence within 3 years.	[24, 25]
ITGAM (Integrin alpha M subunit, CD11b), cell membrane	M0	Cell adhesion, migration, and phagocytosis	The concentration of ITGAM-positive exosomes is lower in both primary CRC and metastatic CRC groups compared to the healthy control group. ITGAM expression is highest in healthy controls (HC), followed by colonic adenomas, and lowest in primary CRC and CRC with hepatic metastases.	[26, 27] <a href="https://www.proteinatlas.org/ENS/G00000169896-ITGAM">https://www.proteinatlas.org/ENS/G00000169896-ITGAM</a>
FCGRIA (Fc Gamma Receptor Ia, CD64), cell membrane	M1	antibody-dependent cell-mediated cytotoxicity (ADCC), antibody-dependent cellular phagocytosis (ADCP)	High infiltration of CD64+ macrophages in CRC, particularly at the tumor front, is associated with improved patient survival. Mediates ADCC by macrophages against tumor cells.	[28, 29] Klikněte nebo klepněte sem a zadejte text.
CD80, cell membrane	M1	CD80 is an inducible co-stimulatory molecule on APC. Interacts with CD28 and CTLA-4 on T cells to regulate T cell activation and tolerance.	CD80 macrophages are more prevalent in less invasive T1 tumors compared to more advanced stages. CD80+ macrophages, although present at low numbers, are associated with better prognosis in CRC.	[30-31] <a href="https://www.proteinatlas.org/ENS/G00000121594-CD80">https://www.proteinatlas.org/ENS/G00000121594-CD80</a>

			In stage III colorectal tumors, a lower CD80/CD163 ratio is associated with decreased overall survival.	
CD86, cell membrane	M1, M2b	co-stimulatory molecule related to CD80	Lower infiltration of CD86+ macrophages is associated with more advanced tumor stages and higher rates of tumor recurrence and mortality. Stage II-III CRC patients with a low CD86/CD163 ratio had shorter RFS and OS.	[32-34] <a href="https://www.proteinatlas.org/ENSG00000114013-CD86">https://www.proteinatlas.org/ENSG00000114013-CD86</a>
CD40, cell membrane, secreted	M1	type I transmembrane protein on antigen-presenting cells and is required for their activation. Promotes pro-inflammatory and anti-tumor responses when activated on macrophages	High CD40 expression in CRC tissues is associated with better OS and DFS.	[30, 35] <a href="https://www.proteinatlas.org/ENSG00000101017-CD40">proteinatlas.org/ENSG00000101017-CD40</a>
iNOS, cytoplasm	M1, M2d	Produces nitric oxide (NO) with a cytotoxic activities against pathogenes and tumor cells	M1 macrophages, which express iNOS, have anti-tumor effects. In a study of 205 CRC patients, iNOS+ macrophages did not demonstrate any significant benefit to patient outcomes. Also, infiltration of CD68+/iNOS- TAMs in the tumor stroma is a negative prognostic factor.	[10, 36-39] Klikněte nebo klepněte sem a zadejte text.
MHC-II, cell membrane	M1, M2a M2b	antigen presentation to CD4+ T cells	In primary CRC, increased MHC-II expression is associated with increased tumor-infiltrating lymphocytes and improved prognosis. Low MHC-II expression may reflect poor interactions between APC and helper T-cell and reduced cytotoxic T lymphocytes mediated anti-tumor activity.	[40-43] Klikněte nebo klepněte sem a zadejte text.
TLR2	M1, M2c	Recognize a wide range of pathogen-associated molecular	Among stage III patients a strong TLR2 expression associates with a better prognosis.	[30, 44-46]

TLR4 (Toll-Like Receptor 2 and 4), cell membrane, cytoplasm		patterns (PAMPs) from gram-positive bacteria, mycobacteria, fungi, and viruses	Among patients with stage II CRC, a strong TLR4 expression associate with a worse DSS.	<a href="https://www.proteinatlas.org/search/TLR2">https://www.proteinatlas.org/search/TLR2</a>
IL1R1 (Interleukin-1 Receptor Type 1), cell membrane	M1 M2b	receptor for IL-1 $\alpha$ and IL-1 $\beta$ , pro-inflammatory cytokines that initiate inflammatory responses and mediate innate immunity against pathogens	Patients with progressive CRC present higher levels of IL-1R1 in the pCRC tissue than patients responsive to the therapy or with a stable disease. IL-1R1 this is a maker of poor prognosis in CRC	[47, 48]
IL-10R (Interleukin-10 Receptor), cell membrane	M1 M2b	IL-10R signaling helps maintain immune homeostasis by suppressing excessive inflammatory responses, as IL-10 is a crucial immunosuppressive agent. Also, IL-10R signaling can modulate the expression of MHC class II and co-stimulatory molecules, affecting antigen presentation by macrophage	The absence of IL-10R in colorectal tissue can cause severe spontaneous colitis, creating a risk for CRC. The expression level of IL-10 in the serum is lower in patients 7 days after CRC surgery compared to earlier levels, while patients with CRC recurrence after surgery had significantly higher IL-10 levels in the serum indicating that IL-10 may serve as a prognostic biomarker for recurrence CRC.	[49-52] <a href="https://www.proteinatlas.org/search/IL-10R+">https://www.proteinatlas.org/search/IL-10R+</a>
CD163, cell membrane	M2a, M2c	Scavenger receptor involved in clearance of hemoglobin-haptoglobin complexes and anti-inflammatory functions	High levels of CD163 expression in serum and tumor tissues have been associated with a worse prognosis. High expression levels correlate with lower overall survival rates.	[53-55] <a href="https://www.proteinatlas.org/ENS/G00000177575-CD163">https://www.proteinatlas.org/ENS/G00000177575-CD163</a>
CD206 (Mannose Receptor), cell membrane	M2a, M2c	a C-type lectin, Involved in pathogen recognition and tissue remodeling	Higher density of CD206+ macrophages is associated with poorer prognosis in CRC. A high ratio of CD206+/CD68+ macrophages is	[10, 33, 56] <a href="https://www.proteinatlas.org/ENS/G00000260314-MRC1/pathology">https://www.proteinatlas.org/ENS/G00000260314-MRC1/pathology</a>

			<p>significantly associated with poor survival in stage II CRC patients.</p> <p>Adjuvant chemotherapy significantly improved RFS and OS for patients with a high CD206+/CD68+ ratio of TAMs.</p>	
<p>CLEC7A (CD301) (Dectin-1), cell membrane</p>	M2a	<p>C-type lectin receptor, is a key innate immune receptor involved in coordinating host defense against fungi. It recognizes <math>\beta</math>-1,3-glucan, a major structural component of fungal cell walls, induces anti-fungal responses.</p>	<p>CLEC7A promotes pro-tumor functions of macrophages</p> <p>CLEC7A promotes tumor progression by regulating the immune microenvironment.</p> <p>Depletion of Clec7a in macrophages in vivo increases the infiltration of tumor tissue by CD4+ and CD8+ T-cells.</p> <p>An analysis of the literature did not demonstrate the role of CLEC7A as a prognostic marker in CRC.</p>	<p>[57, 58]</p> <p><a href="https://www.proteinatlas.org/ENSG00000172243-CLEC7A">https://www.proteinatlas.org/ENSG00000172243-CLEC7A</a></p>
<p>CD36, cell membrane</p>	M2a	<p>scavenger receptor that mediates the uptake of oxidized lipids and apoptotic cells. Promotes inflammatory responses and phagocytosis. CD36 may interact with other receptors, such as integrins, TLRs, or tetraspanins.</p>	<p>High CD36 mRNA levels are associated with reduced 5-year survival in CRC patients (<a href="https://www.proteinatlas.org/ENSG00000135218-CD36/pathology">https://www.proteinatlas.org/ENSG00000135218-CD36/pathology</a>).</p> <p>CD36 expression is highest in macrophages in the liver, particularly in MAMs within metastatic liver tumors</p>	<p>[59-61]</p> <p><a href="https://www.proteinatlas.org/ENSG00000135218-CD36/pathology/colorectal+cancer">https://www.proteinatlas.org/ENSG00000135218-CD36/pathology/colorectal+cancer</a></p>
<p>CD209 (DC-SIGN)(Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin),</p>	M2a, 2b, 2c, 2d	<p>a C-type lectin receptor present on the surface of both macrophages and dendritic cells. Involved in antigen uptake and presentation.</p>	<p>Expression is increased in metastatic CRC cell lines and patient tissues.</p> <p>Higher DC-SIGN expression is associated with reduced OS in CRC patients.</p> <p>DC-SIGN facilitates CRC metastasis both in vitro and in vivo. It forms a complex with Lyn and p85, promoting metastasis by increasing PI3K/Akt/<math>\beta</math>-catenin signaling.</p>	<p>[62-64]</p>

cell membrane			Soluble DC-SIGN (sDC-SIGN) levels in serum are significantly higher in CRC patients with distant metastasis compared to non-metastatic patients.	
FCGR3A (Fc Gamma Receptor IIIa, low affinity Fc receptor, CD16), cell membrane, secreted	M2a, 2c	mediates antibody-dependent cell-mediated cytotoxicity (ADCC) and antibody-dependent phagocytosis (ADP)	Genetic polymorphisms in FcγRIIIa have been associated with response to anti-EGFR antibody therapy in metastatic CRC patients. Patients carrying the FcγRIIIa-158F/F genotype tend to have a less favorable prognosis compared to those with V/V or V/F genotypes. Survival analysis indicated that FCGR3A serves as a prognostic risk factor in most types of cancer.	[65-66] Klikněte nebo klepněte sem a zadejte text.
MSR1 (CD204) cell membrane	M2a, 2b, 2c, 2d	a scavenger receptor that recognizes and clears modified lipoproteins and bacterial products.	Higher infiltration of CD204-positive macrophages into colorectal tumors is associated with shorter OS and RFS in patients with stage II and III CRC. Also, in vitro studies have shown that M2-polarized macrophages with high CD204 expression enhance the proliferation and invasion of CRC cell lines.	[67-69]
TIMD4 (T-cell immunoglobulin and mucin	M2a	a phosphatidylserine receptor involved in recognition and clearance of apoptotic cells.	TIMD4 expression on macrophages may serve as a marker for a subset of tissue-resident macrophages with immunosuppressive functions in CRC. The presence of TIMD4-positive macrophages in CRC is associated	[70, 71]

domain containing 4), cell membrane			with worse clinical outcomes, including increased tumor growth and metastasis.	
CLEC10A (C-type lectin domain family 10 member A receptor, CD301), cell membrane	M2a	recognizes and binds to various glycan structures.	CLEC10A expression has been correlated with clinical outcomes in several cancers, including CRC. Higher expression levels of CLEC10A ligands on tumor cells have been associated with poorer disease-free survival in stage III CRC patients. The presence of CLEC10A-positive macrophages in the tumor microenvironment may indicate a more aggressive tumor phenotype and poorer prognosis.	[72-74]
FIZZ1 (RELM- $\alpha$ ) (RELM- $\alpha$ ) (Resistin-Like Molecule Alpha or RELM- $\alpha$ ), cell membrane	M2a	FIZZ1 is a resistin-like molecule involved in wound healing and tissue repair processes.	Some studies have explored the presence of FIZZ1 in stool samples from American patients with CRC, suggesting its potential as a biomarker for CRC detection. No prognostic associations in CRC have been established yet.	[75-78]
Arg1 (Arginase-1), cytoplasm	M2a	An enzyme involved in the urea cycle, catalyzing the conversion of L-arginine to L-ornithine and urea.	The expression levels of Arg-1 is significantly higher in CRC compared to the corresponding normal colon tissues. Increased Arg-1 expression is associated with stage III-IV tumors. Arg-1 overexpression was associated with shorter OS and DFS in advanced CRC stages (III + IV) , but not at early stages (I + II) in multivariate analysis. The activation of ARG1 is also associated with the migration ability and metastatic colonization of colon cancer cells, and blocking this process may be a novel strategy for controlling malignancies.	[79-81] <a href="https://www.proteinatlas.org/ENS/G00000118520-ARG1">https://www.proteinatlas.org/ENS/G00000118520-ARG1</a>

<p>CD155 (poliovirus receptor (PVR), cell membrane, secreted</p>	<p>M2a</p>	<p>-</p>	<p>Macrophages in the CRC tissue express high levels of CD155 compared to those from adjacent normal tissues. The expression level of macrophage CD155 was higher in stage III/IV CRC compared to stage I/II and was negatively associated with the survival of CRC patients. Additionally, CD155+ macrophages promote the migration, invasion, and growth of CRC cells.</p>	<p>[82, 83]  <a href="https://www.proteinatlas.org/ENS/G00000073008-PVR">https://www.proteinatlas.org/ENS/G00000073008-PVR</a></p>
<p>VEGF (Vascular Endothelial Growth Factor), secreted  what about VEGFR1 (VEGF receptor), cell membrane</p>	<p>M2d</p>	<p>VEGF stimulates endothelial cell proliferation, migration, and survival, facilitating the formation of new blood vessels. The VEGF receptor (VEGFR1) is a family of three closely related, membrane-spanning peptides containing seven extracellular immunoglobulin-like domains and two intracellular tyrosine kinases. Binding of VEGF to VEGFR1 stimulates endothelial cell migration, and may mediate vascular organization.</p>	<p>VEGF overexpression in CRC is associated with poor OS. While VEGFR1 expression in primary CRC tumor patients did not predict prognosis; high percentage of VEGFR1+ cells in liver metastasis was associated with worse patient outcome. VEGFR1+ metastasis-associated macrophages contribute to metastasis in CRC and were identified as a potential new prognostic marker for disease recurrence.</p>	<p>[84-86]  <a href="https://www.proteinatlas.org/ENS/G00000112715-VEGFA/pathology">https://www.proteinatlas.org/ENS/G00000112715-VEGFA/pathology</a></p>
<p>SIGLEC1 (CD169), Sialoadhesin, Plasmatic membrane, intracellular</p>	<p>Non M1/M2</p>	<p>Cell adhesion molecule. Antigen presentation and the modulation of T-cell responses</p>	<p>A high density of CD169+ macrophages in RLNs is significantly associated with longer OS in CRC patients. CD169+ cells to CD68+ cells ratio in RLNs was an independent prognostic factor for CRC. The number of CD169+ sinus macrophages in RLNs decreased in CRC patients with LN metastasis. Also, the density of CD169+ macrophages in</p>	<p>[87-90]  <a href="https://www.proteinatlas.org/ENS/G00000088827-SIGLEC1/pathology">https://www.proteinatlas.org/ENS/G00000088827-SIGLEC1/pathology</a></p>

			<p>RLNs positively correlates with the number of CD8+ cytotoxic T cells infiltrating tumor tissues. CD169+ macrophages in RLNs are thought to promote CD8+ T-cell-mediated antitumor immunity, contributing to a better prognosis for CRC patients.</p> <p>In case primary CRC, CD169 macrophages can exhibit protumor effects.</p>	
<p>CD63, membranes of intracellular vesicles (constitutive), cell membrane (inducible)</p>	<p>Non M1/M2</p>	<p>CD63 is a member of the tetraspanin superfamily of activation-linked cell surface antigens. Regulates phagocytosis, antigen presentation, and secretion of inflammatory mediators</p>	<p>High CD63 expression in CRC is associated with advanced stages of the disease, poor differentiation, mucinous histology and EMT-associated secretory phenotype. It predicts an unfavorable prognosis in CRC patients, including those with metastatic disease in pCRC. CD63 immunohistochemistry can be used to identify patients with an increased risk of recurrence who might benefit from adjuvant therapy.</p>	<p>[91-93]  <a href="https://www.proteinatlas.org/ENS/G00000135404-CD63/pathology">https://www.proteinatlas.org/ENS/G00000135404-CD63/pathology</a></p>

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Table 4.

**Key differences between KCs in Normal liver and LM**

<b>Sign, Function, Role</b>	<b>Normal Liver</b>	<b>reference</b>	<b>TME of Metastasis</b>	<b>reference</b>
Morphology	<p>Kupffer cells are amoeboid-shaped and are attached to the sinusoidal endothelial cells.</p> <p>spindle or stellate-shaped cytoplasm, components of liver vascular walls</p> <p>Kupffer cells maintain a normal, non-activated state.</p>	[1, 2]	<p>Kupffer cells may become activated in response to tumor cells, leading to hypertrophy (enlargement) of the cells. Activated Kupffer cells may show increased cytoplasmic vacuoles</p> <p>Kupffer cells and other macrophages were found to leave the sinusoids and migrate to sites of potential tumor development where they interacted with tumor cells and intimately wrapped their processes around fat storing cells</p>	[2-4]
Phenotypic polarization	KCs typically exhibit an M1-like phenotype (CD80, CD86, Ly6C1) and Pan macrophages (CD68, CD14, CCR2, CD163)	[5-6]	KCs often undergo polarization towards an M2-like phenotype (CD36, LCD206, CD209, CD163)	[5-7]
Recruitment and polarization	KCs are maintained through self-renewal and local proliferation, with minimal recruitment from circulating monocytes	[5]	In CRC LM, there is increased recruitment of monocyte-derived macrophages, which differentiate into M2-like KCs.	[6, 8] 8
Phagocytic Activity	KCs exhibit robust phagocytic activity, clearing pathogens, cellular debris, and potentially	[9]	The phagocytic activity of KCs can be impaired or altered, potentially contributing to tumor	[4, 10]

	tumor cells in the early stages of metastasis.		cell survival and metastatic progression.	
Interactions with other cells	KCs interact with resident liver cells, such as hepatocytes and stellate cells	[9]	KCs interact with cancer cells, cancer-associated fibroblasts (CAFs), and other immune cells	[4, 10, 11]
Cytokine and Chemokine Production:	KCs produce a balanced array of cytokines and chemokines to maintain immune homeostasis and regulate inflammatory responses.	[4]	KCs often produce higher levels of immunosuppressive cytokines (e.g., IL-10, TGF- $\beta$ ) and pro-angiogenic factors (e.g., VEGF), promoting tumor growth and metastasis.	[4]
Functional roles	Crucial role in clearing pathogens, removing cellular debris, and maintaining liver homeostasis through their phagocytic and immunomodulatory functions	[4, 12]	Formation of premetastatic niches.  KCs can exhibit both pro-tumor and anti-tumor functions, depending on the stage of metastasis and the specific microenvironmental cues.  contribute to an immunosuppressive microenvironment	[4]

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