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Abstract

Colorectal tumors have been classified based on histologic factors, genetic factors, and consensus molecular subtypes, which all affect the tumor microenvironment (TME). Elements of the TME serve as therapeutic targets and might be used as prognostic factors. For example, immune checkpoint inhibitors are used to treat tumors with microsatellite instability, and anti-angiogenic agents may be used in combination with other drugs to slow or inhibit tumor growth. We review the features of the colorectal tumor stroma that associate with outcomes of patients and discuss potential therapeutic agents that target these features.

Keywords : CRC, MSI, microenvironment, matrix

In patients with colorectal cancer (CRC), distinct molecular features of tumor cells alter the tumor's microenvironment (TME) to affect its growth and metastasis[1,2]. The TME contains an extracellular matrix (ECM) made of collagen fibers, layers of fibroblasts, blood and lymphatic vessels, nerves, and cells of hematopoietic origin[1,3]. Among the hematopoietic cells, lymphocytes and myeloid cells influence tumor development directly or via the mediators they produce. In general, colorectal tumors are most heavily infiltrated by macrophages, followed by T and B cells [4]. These immune cells interact with tumor cells and other stromal cells. The tumor stroma determines interactions among lymphocytes, myeloid cells, fibroblasts, endothelial cells, lymphatics, and tumor cells. Different components of the TME can affect clinical outcome.

There are different types of colorectal tumors, which each have different features of the TME. For example, colorectal tumors with microsatellite instability (MSI) have defects in DNA repair enzymes and are highly infiltrated by lymphocytes[5]. Most colorectal tumors, however, have low levels of infiltration by lymphocytes and varying densities of myeloid cells, endothelial and lymphatic cells, and fibroblasts. [6,7]. However, a subgroup of colorectal tumors is characterized by medium levels of infiltration by lymphocytes and high densities of endothelial cells and fibroblasts. This group, called mesenchymal colorectal tumors, has high metastatic potential and patients have poor prognoses[8]. These highly aggressive tumors have a complex stroma. Colorectal tumors with specific mutations in RAS are an intriguing subgroup that are resistant to inhibitors of epidermal growth factor receptor (EGFR) [9–11]. The efficacy of antibodies against EGFR might depend on their interactions with immune cells[12], so learning more about the TME can lead to strategies to improve therapies. We review the major components of the colorectal tumor stroma and their potential for therapeutic targeting. We discuss new therapeutic strategies to alter the colorectal tumor stroma.

The Microenvironment in Tumorigenesis

CRC develops via a multistep process that involves the sequential accumulation of mutations in colonic epithelial cells[13]. However, colorectal tumor development also involves interactions between these cancer cells and their microenvironment. This environment includes immune cells, neurons, fibroblasts, blood vessels, and lymph tissues (see Table 1).

Inflammation

Inflammatory diseases of the colon, such as ulcerative colitis and Crohn's disease,[14,15, 16] promote tumorigenesis via alterations to immune cells, blood vessels, and the colon mucosa[17]. Mice given dextran sulfate sodium (to induce colitis) followed by azoxymethane (an mutagenic alkylating agent) develop colitis-associated cancer[17] similar to that in patients. Studies of these mice have indicated the roles of toll-like receptors and inflammatory cytokines, as well as colonic microbiome, in the generation of CRC [18].

Patients with inflammatory bowel diseases have a significant increase in risk of CRC, due to the neoplastic effects of chronic intestinal inflammation. Chronic inflammation can lead to chromosome abnormalities, MSI, and epigenetic changes such as DNA hypermethylation. Inflammation also involves changes in expression of cytokines, chemokines, cyclooxygenase enzymes, and transcription factors, as well as in production of reactive oxygen species and the composition of the intestinal microbiome. Loss of p53 from colorectal tumors is associated with increased intestinal permeability, causing formation of an NF-κB-dependent inflammatory microenvironment and the induction of epithelial–mesenchymal transition (EMT)[19].

T cells, myeloid cells, and blood and lymphatic vessels are found in the center of the tumor and its invasive margin. Natural killer (NK) cells are often anergic[35] and relegated to the invasive margin. B cells are primarily incorporated in lymphoid aggregates, called tertiary

lymphoid structures (TLS), within the invasive margin[36]. Macrophages are the most abundant hematopoietic cells in the colorectal TME and are distributed between the tumor center and the invasive margin, whereas T-helper 17 (Th17) cells, mast cells, and neutrophils are mostly present in the invasive margin[37]. Although most mature dendritic cells are present in TLS, which are in close contact with T cells, immature dendric cells are also detected throughout the center of the tumor. Intestinal microbes can also induce production of inflammatory cytokines such as interleukin 17 (IL17)[20]. Increased levels of IL17 in colorectal tumors have been associated with shorter survival times of patients[21].

Neural cells

The colon contains millions of neurons, which interact with lymphoid tissue in the intestine. Neural cells located in the tumor stroma facilitate migration of metastatic cells—neural invasion of a tumor is an early sign of its invasiveness[24]. In prostate tumors, neurogenesis is initiated from neural progenitors from the central nervous system and newly formed nerve fibers sustain tumor initiation and progression[25]. Signaling by the chemokine CXCL13 via its receptor CXCR5 mediates interactions between neural cells, cancer cells, and the TME. High levels of CXCL13 and CXCR5 correlate with neural invasion of the TME and shorter survival times for patients with advanced CRC[26]. CXCL13 can induce the migration of CXCR5-positive neural precursor cells across the endothelium in humans[27]. Moreover, CXCR5 expression is required for differentiation of neural precursor cells into neurons in adult zebrafish[28]. CXCR5 is expressed by many colon cancer cell lines[29], by B cells, and a subset of T cells in the TME[30,31]. More studies are needed to investigate interactions among nerves, immune cells, and tumor cells.

Fibroblasts

Fibroblasts are major constituents of the invasive margin, where they provide a physical barrier and remodel the ECM [3]. The densities, the location, and the functional orientation of these different cell types, as well as the presence or absence of TLS are prognostic factors for patients with CRC[1].

Cancer-associated fibroblasts (CAFs) originate from several cell types, including epithelial and endothelial cells, local fibroblasts, and mesenchymal cells from the bone marrow (reviewed in ref 3). CAFs are a heterogeneous population of cells that undergo epigenetic modifications during cancer development. CAFs that resemble quiescent fibroblasts facilitate tissue regeneration during the early steps of carcinogenesis. Activation of CAFs during tumor progression is regulated by factors including transforming growth factor beta (TGFB), platelet derived growth factor (PDGF), hedgehog, bone morphogenic protein, IL1, IL6, TNF, and reactive oxygen species[32]. Activated CAFs acquire a highly contractile and proliferative phenotype and produce ECM proteins (collagen, fibronectin, proteoglycan, periostin and tenascin C). They support tumor growth indirectly, via collagen fibers that form the stiff ECM that prevents the entry of lymphocytes and drugs in the tumor center, and through the production of immunosuppressive, angiogenic, and inflammatory factors (Table 1). For a review on CAFs in colorectal tumor development, see Kabayashi et al[32]. Better markers are needed to detect CAF subtypes and determine their prognostic value.

Vasculature

Blood and lymphatic vessels infiltrate the tumor core and the invasive margin[24]. Endothelial cells regulate angiogenesis, but pericytes, (peri-endothelial smooth muscle cells that express α smooth muscle actin) support endothelial cell function and are required for development of a tumor vascular network[33] (Table 1). Eberhard et al have shown that the percentage of

endothelial cells covered with pericytes varies among tumor types (such as 65% pericyte coverage in colorectal tumors vs 13% in glioblastomas)[34].

Endothelial cells within the tumor form new blood vessels. However, these cells are highly proliferative and prone to apoptosis, unlike the endothelium of normal tissue[120]. Tumor vessels are disorganized, tortuous, and dysfunctional, whereas the normal vasculature has a hierarchical branching pattern of arteries, veins, and capillaries[19]. In the normal vasculature, expression of VEGF and angiogenic factors is tightly regulated, and levels decrease rapidly upon new vessel formation. However, during tumor growth, the balance in expression of angiogenic vs anti-angiogenic factors is shifted toward continuous neoangiogenesis[121].

VEGF signaling is complex[122], with VEGF gene expression upregulated by hypoxia, through activation of the HIF1 transcription factor, and by integrin or oncogene signaling[123] (such as EGFR signaling). VEGF receptors are expressed not only by vascular endothelial cells but also by other cells, including macrophages and monocytes[125], providing evidence for a role in immune modulation, angiogenesis and metastatic progression . Other signaling pathways interact with VEGF signaling, such as the angiogenin, TIE1, and Notch signaling pathways[126].

In a meta-analysis, Wang et al associated higher levels of VEGF with tumor metastasis to lymph nodes and blood vessels.[127] Tumor level of VEGF might therefore be a prognostic marker for patients with CRC. Similarly, the incidence of metastases was higher in patients whose tumors expressed high levels of VEGF, which might be used in prognosis. Sustained levels of angiogenesis, revealed by the tumor endothelial cell signature, correlate with reduced patient survival times [51]. Mohamed et al showed that patients whose colorectal tumors expressed high levels of VEGF, CD105 (endoglin; glycoprotein involved in TGF receptor complex), and CD31 (endothelial cell marker) had poor outcomes [128].

Lymphangiogenesis

Lymphatic vessels maintain fluid balance by draining interstitial fluid to regional lymph nodes. During metastasis, they provide a pathway for tumor cell dissemination.[145,146] Lymphangiogenesis (the process where new lymphatic vessels are formed) occurs in and around tumors [147]. In colorectal tumors, there is a correlation between lymphatic microvessel density and risk of metastasis[146].

Lymphangiogenesis is mediated by VEGFC and VEGFD[148],[149,150]. These factors bind to the receptor tyrosine kinase VEGFR3 expressed on lymphatic cells resulting in neo-lymphangiogenesis. Other factors such as HGF, PGDF, FGF2, IGF1, and IGF2 stimulate lymph vessel outgrowth[148]. Lymphangiogenesis is inhibited by TGFB1, which also regulates tumor development. In mice undergoing wound repair, addition of exogenous TGFB1 inhibited assembly of lymphatic vessels, reduced lymphatic endothelial cell proliferation, and inhibited lymphatic endothelial cell migration[151].

Tumor Cell Mutations and the TME

Among patients with colorectal tumors with high levels of MSI, 16% were found to have Lynch syndrome[40]—an inherited cancer syndrome caused by mutations in genes that encode DNA repair enzymes. These tumors have a high mutation burden and are infiltrated by a large number of lymphocytes[38]. Patients with these tumors have better outcomes than tumors without MSI, because of the adaptive immune response mediated by T cells that recognize the tumor neo-antigens created by the high-frequency mutations[41]. This immune response slows tumor growth and metastasis. Microsatellite-stable (MSS) tumors[4,88], alternatively, have less infiltration of by lymphocytes. MSS tumors often have mutations in oncogenes such as *APC, KRAS, TP53,* or *PIK3CA.* These tumors can acquire additional mutations due to

mutations in the DNA polymerase epsilon gene (*POLE*)[43,44], which increases their activation of the anti-tumor immune response. Patients with these colorectal tumors have longer than average survival times [45].

COLOSSUS (www.colossusproject.eu), a multi-disciplinary European Commission-funded research network, is studying the development, stromal composition, and resistance mechanisms of colorectal tumors with RAS mutations. Tumors with *RAS* mutations are resistant to treatment with antibodies against EGFR[9,10]. Most mutations in *KRAS* occur in exon 2 (codon 12 and 3)[46]; patients whose tumors have these mutations do not benefit from anti-EGFR therapy[47], with the possible exception of patients with tumors with the KRAS.G13D mutation[48,49]. Other mutations in RAS (KRAS exons 3 and 4; NRAS exons 2, 3, and 4) are also associated with poor response to anti-EGFR treatments[50]. BRAF is downstream of Ras in the EGFR signaling pathway and the BRAF V600E mutation is associated with resistance to EGFR therapy[51]. It is not clear why antibodies that bind and activate effector cells in the TME do not induce tumor cell killing by macrophages or NK cells. Tumors with RAS mutations might become resistant to NK cell killing downregulate the anti-tumor immune response by unknown mechanisms [52,53]. For a review of tumor mechanisms of resistance to EGFR inhibitors, see ref [54].

Molecular Classifications and the TME

Transcriptome-based classifications of colorectal tumors have been proposed [55–62] . A Consensus Molecular Classification system[63] has been developed an provides a useful tool to classify tumors and study their corresponding TMEs. There are 4 consensus molecular subtypes (CMSs). CMS1 (14% of colorectal tumors) contains most, although not all, hypermutated MSI tumors with BRAF mutations and the high CpG island methylator phenotype, resulting in the methylation and subsequent inhibition of transcription of the

mismatch repair gene *MLH1* [64] and few somatic copy number alterations. CMS2 (37% of colorectal tumors) is characterized by mutations in *APC* and activation of WNT and MYC. CMS3 tumors (13% of colorectal tumors) have metabolic deregulation and have many tumor cells with KRAS mutations. CMS4 tumors (23% of colorectal tumors) upregulate genes involved in the EMT, TGFB signaling, angiogenesis, and ECM remodeling[63].

An in-depth analysis of the composition and activation states of the stromal components associated with each CMS[42] revealed that CMS1 tumors had a high expression of genes that regulate T-cell trafficking and activation as well as differentiation of Th1 and cytotoxic T cells, and high expression of CXCL13. CMS1 tumors have a high density of infiltrating $CD8^+$ T cells[42]. Patients with CMS1 tumors have longer survival times than patients with other CMSs, supporting the concept that hypermutated tumors of this subtype induce specific T- and B-cell responses that control tumor dissemination and metastasis. However, CMS1 tumors express high levels of immune check point molecules such as PD1 and cytotoxic T-lymphocyte associated protein 4 (CTLA4).

The TME of CMS2 tumors is characterized by low numbers of lymphocytes, macrophages endothelial, and fibroblastic cells. CMS3 tumors are heterogeneous but are characterized by low levels of immune cell infiltration[42]. CMS4 tumors are the most aggressive subtype with the worst outcomes. CMS4 tumors express immune checkpoint molecules, and are highly infiltrated by macrophages, myeloid-derived suppressor cells (MDSCs), MDSCs, and fibroblasts. CMS4 tumors express high levels of the chemokines CCL2 and CXCL12, which recruit myeloid cells and promote neural migration. CMS4 tumors have low levels of CXCL13, which regulates formation of TLS, indicating a disorganized anti-tumor immune response and lack of T and B cells that recognize tumor antigens[31]. CMS4 tumors are characterized by an inflammatory gene expression signature, with high expression of genes encoding components of complement system. They also

express high levels of genes that encode TGFB and LGALS1, which are immune-suppressive, and the angiogenic factors VEGF and PDGFC [42,63,65]. The abundance of fibroblasts found in CMS4 tumors correlates with myeloid and endothelial cell abundance, indicating that fibroblasts might promote angiogenesis and recruitment of inflammatory cells [42].

In the stroma of CMS4 tumors, fibroblasts express high levels of VEGFB, VEGFC, PDGFC, LGALS1, CXCL12, PTGS1, and TGFB to promote angiogenesis, lymphangiogenesis, and immune suppression. Endothelial cells in these tumors express high levels of CCL2, PDGFB, and TGFB1 and TGFB2. Finally, monocytes in CMS4 tumors express complement components (C1QA, C1QC, C3, C3AR1, and C5AR1) and chemokines that attract macrophages (CCL19 and CCL23). These cell populations contribute to progression of CMS4 colorectal tumors by promoting inflammation, angiogenesis, and immunosuppression.

Although practical, the CMS classification system faces many hurdles. Integration of cancer cell and stromal gene expression signatures depends on the purity of the tumor sample analyzed. CMS1 and CMS4 are over-represented in samples containing a large proportion of stromal tissue[66]. In addition, the CMS system is based on average characteristics, and does not take into account tumor heterogeneity[66]. The cancer cell intrinsic subtype classification system, established from tumor patient-derived xenografts[67] appears to be more robust, but does not integrate the TME. Other immune classification systems overlap imperfectly with the CMS system, so further molecular classifications are needed to guide TME targeted therapies.

Chromosome Instability and the TME

There have been many studies of gene copy number changes in colorectal tumors;[68] these have also been used to create a CRC classification system, based on chromosome instability. [69]. Copy number load was initially studied as a potential biomarker of response to bevacizumab in patients with metastatic CRC. Specifically, 472 primary tumors that metastasized were classified into 3 subgroups (clusters 1–3), each characterized by different degrees of chromosome instability. Tumors with increasing cluster numbers (cluster 1–3) had an increasing number of chromosomal breakpoints and a higher proportion of the genome with copy number alterations (CNAs).

Researchers used publicly available TCGA datasets to correlate clusters of CNAs with CMSs [77–84]. Gene set enrichment analysis of 50 cancer-associated pathways applied to differentially expressed genes between clusters of CNAs revealed that cluster 1 tumors were characterized by a strong immune-activated microenvironment, whereas cluster 2 and 3 tumors were characterized by angiogenesis, EMT, and inflammatory response pathways. Tumors from cluster 1 overlapped with CMS1 or CMS3 tumors, whereas cluster 2 and 3 tumors overlapped with CMS2 or CMS4 tumors, respectively.

Colon Side and the TME

Left-sided and right-sided colon tumors have distinct histologic and molecular characteristics. Right-side colon tumors arise from the ascending colon and proximal two thirds of the transverse colon whereas left-side colon tumors arise from the descending or sigmoid colon, and distal third of the transverse colon[70]. Right-side stage III or IV colon tumors are generally associated with short survival times. These tumors are more commonly MSI, have *BRAF* mutations, and are hypermutators[71], compared with left-side tumors, which have chromosome instability. High numbers of PD1+ $CD8^+$T cells, $FOXP3^+$ T cells, $CD20^+$ B cells, and $CD138^+$ $IGKC^+$ plasma cells in tumor tissues has been associated with increased OS survival

times of patients with right-side colon tumors[72]. Differences in immune cell features of the right vs left colon might account for the different outcomes of patients with right-side vs left-side colon tumors[70].

The human colon contains complex and diverse microbial colonies of approximately 10^{13} to 10^{14} bacteria,[70] with colony numbers increasing from right to left. Therefore, the left colon, with the highest concentration of microbes, has a more tolerant immune environment. Tumors that develop in the right colon face a more active immune environment than tumors in the left colon,[70] and are infiltrated by higher numbers of lymphocytes. Tumors in the left colon have a higher level of immune-suppressive cells than tumors of the right colon[73].

Clinical Effects of TME Composition

Primary tumors with no perineural infiltration and no vascular or lymphatic invasion have a higher density of memory T cells than tumors with early signs of metastasis[74]. High densities of memory and effector T cells, particularly $CD8^+$ T cells, in the center and the invasive margins of tumors correlated with longer progression-free survival (PFS) and overall survival (OS) times of patients [75]. Analysis of hepatic[76] and lung[77] metastases also associated higher densities of $CD8^+$ T cell with better outcome. Although infiltration by $CD8^+$ T cells appears to have positive effects for tumors of all stages, more advanced tumors (stages III and IV) have lower densities of these cells than early-stage tumors[78]. A reduced adaptive immune response might therefore promote tumor progression[78,79].

Analyses of primary tumors and metastases from the same patients provided evidence for immune selection of malignant cells[80]. This mechanism is prevalent in MSI tumor cells, which often lose membrane HLA molecules,[81–83] so they escape T-cell cytotoxicity but not NK cell cytotoxicity. This reduces their metastatic potential[52]. Analyses of tumor transcriptomes revealed that high expression of genes that regulate T-cell chemotaxis

(CXCL9, CXCL10, CXCL11), T- and NK-cell activation (IL15), and Th1 cell development (IFNG) associated with longer survival times of patients [1,84]. Tumors with mutations resulting in loss of expression of IL15[85] or CXCL13[4], which attracts B cells and is involved in TLS formation, resulted in shorter survival times of patients. Infiltration of colorectal tumors by Th2[21] and Th17[4] cells has been associated with shorter survival times, whereas T follicular helper cells were associated with longer survival times, as were high levels of tumor infiltration by B cells[2,4,86] and the presence of TLS[2,87]. The overall positive effects of high T-cell density in colorectal tumors led to the establishment of an Immunoscore, based on quantification of $CD3^+$ and $CD8^+$ T cells in the center and the invasive margin, which was associated with increased survival times of patients with MSI or microsatellite stable (MSS) tumors[4,88].

CRC tissues are enriched in commensal bacteria such as *Bacteroides fragilis* and *Escherichia coli*[89]. These bacteria produce stimuli that upregulate expression of genes encoding chemokines that attract T cells to tumors, associated with longer survival times of patients. The abundance of these bacteria in colorectal tumors also correlates with expression of chemokines that recruit T cells[30]. The mechanisms of bacterial species such as *Fusobacterium nucleatum*, which are associated with lower densities of T cells[90], lymph node metastasis[89], and poor outcomes, [91] require further study [92].

Tumor-associated macrophages (TAMs) form a heterogeneous and versatile population of cells, most of which are located in the stroma along the invasive front. The presence of $CD68^+$ macrophages has been associated with increased survival times of patients with CRC[2] whereas $CD163^+$ macrophages have a negative effect [2,93]. Whereas colorectal tumor cells express low levels of the immune checkpoint ligand CD274 molecule (also called PDL1), TAMs located in the invasive margin express high levels of PDL1 and are more abundant in MSI than MSS tumors, indicating a role in the CRC adaptive resistance

phenomenon [94]. Neutrophils are also present in the TME and correlate with improved outcomes and response to 5 FU-based chemotherapy[95]. Macrophages and neutrophils might derive from the local differentiation of MDSCs, a heterogenous population of immature myeloid cells that lack robust cell surface markers for detection by immunohistochemistry. However, the prognostic value of MDSCs requires evaluation in large cohorts of patients. Mast cells are associated with poor outcomes in 1 study[96].

A gene expression pattern characteristic of fibroblasts associated with reduced survival time survival and decreases the positive effects of a cytotoxic cell signature[86] This finding has been attributed mostly to the fact that CAFs produce immunosuppressive TGFB[97,98] and VEGF,[99] which impair immune responses even when lymphocytes are able to cross the fibroblastic barrier[3].

Immune-based Therapies

CRC was once considered to be resistant to immunotherapy. This changed in 2015, when the striking response of patients with metastatic MSI tumors to anti-PD-1 therapy[100] has opened new avenues and raised questions about therapies to target the tumor stroma. These agents re-activate T-cell anti-tumor responses by blocking checkpoint molecules such as programmed cell death 1 (PDCD1, also called PD1) [100]. Immunotherapy for CRC has become the paradigm for all types of tumors with MSI, and for tumors with a high mutation burden[101]. Trials are underway to determine whether the combination of anti-PD1 and anti-CTLA4 increases the response rate and survival times of patients with MSI tumors[102,103] (see Table 2).

MSI tumors have all characteristics required to respond to immune checkpoint inhibitors [104], are surrounded by PD1+ CD8+ T cells, and express high levels of PDL1 [94]. MSI tumors account for only 3%–5% of all metastatic colorectal tumors[23]. However,

extension of immune checkpoint inhibitor therapy to treatment for primary colorectal tumors might increase the number of patients who benefit from these therapies. Computational strategies (deep residual learning) diminish the price to identify tumors with MSI based on histologic features that might result in immune checkpoint inhibitor therapy for a larger number of patients with CRC [105].

Interestingly, the efficacy of PD1 inhibitors against MSI tumors raises questions that, if answered, might reveal new treatment options for MSS tumors. For example, due to immune-cell selection, many MSI tumors express few or no HLA molecules, which are required for antigen presentation to $CD8^+$ T cells[38]. This might explain the growth of primary MSI tumors despite their infiltration by T cells. However, HLA defects are rare in liver metastases, in contrast to metastases in other organs[81], which could account for the response of patients with metastatic CRC, which usually spreads to the liver, to PD1 inhibitors [100]. Studies of responses in patients with primary MSI tumors that have lost HLA expression[82] should help answer this important question and provide additional information about mechanisms of immune checkpoint inhibitor therapy. In MSI tumors that have lost HLA expression, responses to PD1 inhibitors could resemble the sensitivity of Hodgkin disease, which despite a loss of HLA expression responds to PD1 inhibitors.[106] Other T-cell subsets or NK cells might act as effectors.

It is also important to learn why MSS colorectal tumors that are highly infiltrated by T cells do not respond to immune checkpoint inhibitors. It has been proposed that the Immunoscore (the density of $CD3^+$ and $CD8^+$ T cells) more accurately predicts survival times of patients with CRC than MSI[107]. In MSS tumors, $CD8^+$ T cells might control tumor growth but still cannot promote tumor regression—other elements of the TME might continue to support tumor development. CMS4 tumors are characterized by high levels of myeloid cell infiltration, high levels of angiogenesis, and fibroblastic contents.[42] These tumors might be a

paradigmatic stroma-rich colorectal tumor sub-class that contains many different cell types and would be a good candidate for testing TME-targeted therapies. Studies are needed to determine whether immune checkpoint inhibitors can be used in combination with other strategies for treatment of MSS tumors. This question is further under consideration by the COLOSSUS CRC research network.

Most TAMs have an M2 phenotype, produce complement components, and have inflammatory and angiogenic activities[108] such as production of VEGF[109] and immunosuppressive cytokines (IL10) cytokines,[110] resulting in T-cell exhaustion [111] and angiogenesis[112]. Therefore, the combination of anti-angiogenic and immune checkpoint inhibitor therapy might result in reactivation of the anti-tumor immune response [112]. This combination is currently being tested in patients with MSI colorectal tumors (TECENTRIC, NCT02982694). Agents that block colony stimulating factor 1 receptor, or the CCL2 receptors CCR2 and CCR5, which are expressed by macrophages and MDSCs and induce macrophage repolarization, [113] are being tested in MSS in combination with anti-PD1 and anti-PDL1 in patients with advanced CRC (NCT02713529 with AMG 820, and MARACON, NCT03184870).

Neutralization of inflammatory complement components is a new strategy for treatment of CRC [108]. Agents that block C5aR reduced tumor growth in mice, alone[6] or in combination with a checkpoint inhibitor.[29, 109, 110] A phase 1 study is underway to test the combination of a C5aR inhibitor and anti-PDL1 in patients (STELLAR-001, NCT03665129 (see Table 2). Agents that target different steps of the complement cascade might be adapted for cancer therapy [112].

Fibroblasts produce TGFB and VEGF and mechanically prevent entry of therapeutic cells and agents into the tumor core[3]. It is a challenge to target fibroblasts therapeutically, given their heterogeneity, plasticity, and the role of ECM in maintenance of tissue stiffness.

Of the immune-suppressive cytokines present in CMS4 tumors, TGFB is a challenge to target, given its multiple functions [114] . It may be similarly challenging to target IL10, due to its dual effects on the immune response[115].

Indoleamine deoxygenase (IDO) is expressed by mesenchymal cells, myeloid dendritic cells, T-regulatory cells, and tumor cells. Trials of the IDO inhibitor, epadadostat, in combination with anti-PD1 blockade (ECHO-204, NCT02327078) are underway in patients with CRC. Oncolytic viruses replicate specifically in tumor cells and promote tumor infiltration by lymphocytes and induction of specific anti-tumor immune responses. These might be used to increase the response of MSS colorectal tumors to anti-PD1 therapy (NCT02963831 with ONCOS102). Tumor development results in epigenetic alterations that reduce antigen presentation and responses of T cells to tumor cells, allowing tumors to evade immune surveillance. Demethylating agents, which increase expression of genes including HLA genes, might increase antigen presentation[116]. These types of agents reduced tumor growth in mice and are being tested in combination with immune checkpoint inhibitors in patients with melanomas (Di Giacomo et al, Clin Cancer Res, in press).The histone deacetylase inhibitor entinostat is being tested in combination with anti-PD1 in patients with MSS colorectal tumors (NCT02437136).

Chemotherapies that include oxaliplatin have been reported increase immune cell cytotoxicity toward cancer cells and activation of the adaptive immune response[117]. Also, oncolytic viruses not only induce immune-cell killing of tumor cells, but also remodel the TME[118]. Inhibitors of beta-catenin or PAX4 might increase tumor infiltration by immune cells, and strategies are being developed to increase the immune response against MSS tumors (see Figure 3).

Other ways to increase the anti-tumor immune response would be to deliver T cells directly to the tumor core, using chemokines such as CXCL9 and CXCL10. It might be

possible to increase interaction of tumor cells with immune cells using bi-specific antibodies, or by infusing effector T cells, tumor-infiltrating lymphocytes, or T cells with chimeric antigen receptors against tumor antigens (reviewed in[103]). Stem cell features of cancer cells have been associated with a suppressed immune response, higher intra-tumor heterogeneity, and reduced survival times of patients[119]. Inhibitors of stem cell markers such as CD133 or the polycomb group protein BMI1, or agents that induce tumor cell differentiation, might slow tumor growth or progression and reduce immunosuppression.

Targeting the Tumor Vasculature

In tumor tissues, the most common method to evaluate angiogenesis is to measure microvessel density (MVD), based on endothelial markers such as CD31, CD34, or endoglin. Nevertheless, studies have produced conflicting results on the prognostic value of MVD for patients with CRC. MVD was correlated with depth of invasion, metastasis to lymph nodes and distant sites, and tumor node metastasis stage; there was an inverse correlation between MVD and OS. [127,129–131] In other studies, researchers found no correlation between MVD and PFS or OS [132–135]. Specifically, Prall et al [134] reported that patients with tumors with high MVD had longer times of cancer-specific survival. The conflicting results might be due to differences in the methods used to determine MVD[136]. For markers that are detected by immunohistochemistry (CD31, CD34, and Von Willebrand factor) and size of area examined varied among studies. MVD has also been studied in patients treated with bevacizumab, a humanized monoclonal antibody that binds VEGF and inhibits its binding to its receptor. However Jubb et al[137] did not associate MVD with efficacy of bevacizumab in a post-hoc analysis of data from a trial of bevacizumab in addition to the standard of care (irinotecan, 5-fluorouracil, and leucovorin) in patients with previously untreated metastatic CRC[138].

Angiogenesis inhibitors (antibodies or small molecules) are included in standard treatments for patients with CRC (for review, see ref [139]). Bevacizumab was the first angiogenesis inhibitor approved by the Food and Drug Administration for treatment of renal cancer [140]. It was subsequently approved as a first-line treatment agent for metastatic CRC, in combination with irinotecan, 5-fluorouracil, and leucovorin [138]. Inclusion of bevacizumab in this combination increased the mean OS time of patients from 15.6 months to 20.3 months. A retrospective analysis found no association between tumor mutations in *KRAS*, *BRAF*, or *TP53* and survival time after bevacizumab therapy[141]. Bevacizumab alone is approved for first-line therapy for CRC, whereas other anti-angiogenic agents have only been approved for treatment of patients with tumor progression. The efficacy of bevacizumab as a second-line agent has been evaluated in patients whose metastatic CRC progressed after they were given the standard bevacizumab-containing regimen as their first-line therapy.[142] This study found that that continued bevacizumab therapy prolonged OS (by 1.4 months) and PFS (by 1.6 months) (Table 2).

Drugs with a broader scope of inhibition have approved for treatment of CRC, such as aflibercept (VEGF Trap). Aflibercept is a high-affinity soluble decoy receptor for VEGF that was approved (in combination with FOLFIRI) for treatment of patients with metastatic CRC that progressed or is resistant to oxaliplatin-based therapies[143]. Ramucirumab is a monoclonal human IgG1 against the extracellular domain of VEGFR2 that prevents binding of VEGF A–E, and consequently, VEGFR2 activation[144]. The small molecule angiogenesis inhibitor regorafenib has been approved for treatment of metastatic CRC. It is an orally administrated inhibitor of the tyrosine kinases VEGFR1–3, TIE2, FGFR1, PDGFR beta, KIT, and RET, RAF, RAF1, BRAF, and BRAFV600E. Regorafenib has been approved for salvage monotherapy in patients with refractory metastatic CRC, but it has a significant toxicity

profile and questionable efficacy. Nevertheless, the drug is being tested in combination with FOLFIRINOX (NCT03828799) (See Table 3).

Li et al found an inverse correlation between levels of SMAD4 and TGFB1 with lymphatic microvessel density in a study of 147 patients colorectal tumors.[152] Additionally, patients with SMAD4-positive tumors had significantly longer overall and tumor-free survival times than patients with SMAD4-negative tumors, indicating that TGFB1 signaling inhibits lymphatic outgrowth and reduces metastasis. Nevertheless, TGFB1 is a complex pleiotropic growth factor with paradoxical effects—it inhibits proliferation of normal epithelial cells and cells in early-stage tumors, but promotes proliferation of malignant and stroma cells in late-stage tumors. For a review of TGFB1 signaling in metastatic colorectal tumors and therapeutic strategies, see ref [153]

Agents designed to block lymphangiogenesis are being tested for their ability to prevent colorectal tumor metastasis. Unfortunately, lymphangiogenesis has proven a difficult process to specifically target in patients with CRC[148]. Sorafenib, an inhibitor of multiple tyrosine kinases, blocks VEGFR3, which regulates lymph vessel outgrowth.[154] First-line treatment of patients with CRC with sorafenib in combination with FOLFOX did not increase patient survival time (RESPECT trial, NCT00865709)[155]. There is a large amount of redundancy in lymphangiogensis signaling, so if 1 pathway is blocked, another will compensate. No agent that interferes with lymphangiogenesis is being used in treatment of CRC[148].

Biomarkers of Response to Treatment

Resistance of tumor cells to drugs (initial or acquired during treatment) poses an constant challenge[156], and strategies are needed to determine which tumors are most likely to respond to which therapies. Genomic[50,69,157–163] and other classes of biomarkers of response have been

proposed,[164] but there are no markers that can be used to predict response to anti-angiogenic agents.

Chromosome instability was reported to be a biomarker of response to bevacizumab in patients with metastatic CRC [69]. Tumors with intermediate to high levels of chromosome instability (clusters 2 and 3) had better responses to chemotherapy with bevacizumab than to chemotherapy alone (prolonging PFS by 149 days for cluster 2 and 85 days for cluster 3). Colorectal tumors with low levels of chromosome instability (cluster 1), which include those with mutations in POLE and MSI, did not have an increased to chemotherapy that included bevacizumab, nor did metastatic colorectal tumors in phase 2 MoMa study (NCT02271464). A chromosome instability threshold in which ≥25% of chromosomal regions contained CNAs has been proposed for identification of tumors most likely to respond to bevacizumab. Patients whose tumors were above this threshold who received bevacizumab therapy had significantly longer times of PFS than those given the standard of care chemotherapy (REF132). This difference was not observed in when patients with tumors with high levels of chromosome instability were compared with patients with tumors with low levels of chromosome instability given chemotherapy alone. These require confirmation in a prospective trial, but CNA might be a biomarker of response to certain therapies.

Tebbutt et al investigated the association between CMS and response of patients with unresectable metastatic CRC to capecitabine; capecitabine and bevacizumab; and capecitabine, bevacizumab, and mitomycin (NCT00294359). [165] Patients with CMS2 tumors (and possibly CMS3 tumors) given the combination of capecitabine and bevacizumab or capecitabine with bevacizumab and mitomycin had longer PFS than patients given capecitabine alone, but this association was not observed in patients with CMS1 or CMS4 tumors[166]. A retrospective analysis of patients with colorectal tumors without mutations in RAS treated with either mFOLFOX6 or FOLFIRI, combined with

bevacizumab or cetuximab as first-line therapy, reported equal survival times (CALGB/SWOG 80405 trial).[167] Interestingly, patients with CMS1 tumors had longer survival times after bevacizumab-based treatment than cetuximab-based treatment. This study compared the effects bevacizumab with those of different control groups (patients treated with cetuximab vs standard of care chemotherapy) than those included in the analyses of Smeets et al.[69] Nevertheless, findings from all 3 studies indicate a need for additional analyses, using large and diverse patient cohorts to confirm the association between CMS and outcomes of patients treated with bevacizumab.

The side of the colon in which a tumor develops associates with response to therapy. In the FIRE3 trial (NCT00433927) of patients with metastatic colorectal tumors without mutations in RAS, those with left-side colon tumors had a significantly better outcomes after first-line therapy with FOLFIRI and cetuximab (an antibody against EGFR) than with FOLFIRI and bevacizumab (OS time, 38.3 months vs 28 months)[168]. In contrast, in patients with right-side colon tumors, there was no significant difference in survival between patients given either combination (OS 18.3 months vs 23.0 months respectively). Yoshimo et al showed that addition of ramucirumab to FOLFIRI (in the RAISE trial, NCT01183780) as a second-line therapy increased survival times of patients with metastatic colon cancer, regardless of tumor side (or mutational status)[169]. These findings indicate that the side of the colon on which the tumor develops affects to some treatment regimens, but not all.

Future Directions

Colorectal tumors develop via many different pathways that result in many different TMEs. Mutations in DNA repair genes, the DNA polymerase E gene, and BRAF, as well as the CpG island methylator phenotype, result in high mutation burden and tumor infiltration by lymphocytes. Conversely, APC mutations are associated with lack of lymphocyte infiltration,

due to activation of beta-catenin. Tumors with mutations in RAS (and probably BRAF) are resistant to anti-EGFR therapies whereas MSI colorectal tumors often respond to immune checkpoint inhibitors.

Different colorectal tumors subtypes therefore respond differently to therapies that target the TME. Integration of data on TMEs with genome and transcriptome profiles might identify the best therapeutic combinations for each patient's tumor type, comprising chemotherapies, immunotherapies, anti-angiogenic therapies, and anti-stromal agents. Immunogenic chemo- and radiotherapies, and oncolytic virus-based therapies, are in development. Cell-based therapies such as autologous tumor-infiltrating lymphocytes or T cells with chimeric antigen receptors are also being developed and might be effective against tumors that do not induce and immune response. These types of therapies will be selected based on specific features of each patient's tumor and TME.

Table 1. Cells the Tumor Microenvironment and Functions

Cell Type	**STRUCTURES**	**FUNCTIONS**
Lymphocytes		
NK cells	MHC class I-negative cells IgG bound to target cells	killing MHC class I-negative cells antibody-dependent cellular cytotoxicity of ar
B cells	native soluble or membrane antigens	antibody production antigenic peptide presentation via MHC class
CD8+ T CELLS	peptides presented by MHC class I	killing MHC class I-positive cells regulate responses of T and B cells
CD4+ T CELLS		produce IFNG
Th2 cells	peptides presented by MHC class II	produce IFNG IL2 activate CD8+ T cells to become cytotoxic
T-follicular helper cells		produce IL4, IL13 activate B cells to become antibody-producin
Th17 cells		produce CXCL13 recruit and activate B cells in TLS
T-regulatory cells		produce IL17 activate macrophages to produce IL6 and IL8
		produce IL10, TGFB suppress responses of T and B cells
NK T Cells		killing target cells in an MHC-unrestricted ma
Myeloid Cells		
Dendritic cells	danger signals (DAMP, PAMP	produce IL12, IL18 present antigen to T cells via MHC I and II
Macrophages M1	danger signals (DAMP, PAMP) IgG-coated target cells	produce IL1, IL6 phagocytosis of target cells antibody-dependent cytotoxicity of antibody-
M2		produce VEGF, IL10, TGFB, and complemen promote angiogenesis, fibroblast activation in
Mast cells		produce inflammatory mediators (serotonin, l
Polymorphonuclear cells		phagocytosis of target cells or inflammatory
MDSC	danger signals (DAMP, PAMP)	immature cells of heterogeneous population fr suppress immune responses
Stromal cells		
Follicular dendritic cells		present antigen to B cells via immune comple
CAF		produce angiogenic factors (VEGF, CXCL12 produce immunosuppressive TGFB and M2 p CXCL18

Table 3. Angiogenic Agents Approved for Treatment of CRC

		originate from tumor cells through the EMT transdifferentiate from endothelial cells, peric form a mechanical barrier preventing entry of
Endothelial Cells		produce VEGFA–E, CXCL12, FGF2, and cor support tumor growth through nutrients and o
Pericytes		produce PDGF-BB and regulate vascular func

Table 2. Clinical Trials of Agents Designed to Target Tumor Stroma

Trial	Drug combination	Patient population	Status
anti-angiogenics			
bevacizumab (avastin) solstice	1st line tas 102 + bevacizumab vs capcitabine + bevacizumab	854 untreated patients with MCRC who were not candidates for irinotecan or oxaliplatin therapy	recruiting. due for completion September 2022
vitality	1st line oxaliplatin, 5-fluorouracil, leucovorin +/-bevacizumab and vitamin C vs line oxaliplatin, 5-fluorouracil, leucovorin +/- bevacizumab	428 previously untreated patients with tumors with mutations in RAS. given	recruiting, due for completion December 2020
bevacizumab (avastin)	bevacizumab + binimetinib (MEK inhibitor) + prembrolizumab	40 patients with MCRC without a response to prior therapy	Recruiting, due for completion August 2019
bevacizumab (avastin)	2nd line bevacizumab + 5-fluorouracil, leucovorin + irinotecan + onvansertib (an inhibitor of polo-like kinase 1)	44 patients with metastatic colorectal tumors with a mutation in KRAS failed by previous treatment, or patients who are intolerant to, oxaliplatin	recruiting. due for completion May 2021
aflibercept (zaltrap)	aflibercept + prembrolizumab	78 patients with advanced solid tumors	recruiting. due for completion December 2021
ramucirumab (cyramza) ramtas	tas 102 +/- ramucirumab	144 patients with advanced MCRC that progressed on or after, or patients who did not tolerate, fluoropyrimidines, oxaliplatin, irinotecan, or anti-angiogenic therapies	Recruiting, due for completion June2021
donafenib (multi tyrosine kinase inhibitor)	2nd line donafenib vs best supported care	510, patients with MCRC that progressed during or within 3 months of final dose of therapy	active not recruiting, due for completion April 2020
regorafenib (stivarga)	salvage regorafenib + irinotecan vs regorafenib only	78 previously treated patients with MCRC that progressed during or within 3 months of last treatment of standard therapy	recruiting, due for completion June 2023
remety	2nd line tas 102 + regorafenib	18 patients with MCRC that progressed after standard therapy	Recruiting, due for completion October 2019
folfirinox-r	regorafenib + 5-fluorouracil, leucovorin + irinotecan + oxaliplatin (folfirinox)	87 patients with metastatic colorectal tumors with mutant RAS	recruiting. due for completion March 2022
regorafenib (stivarga)	regorafenib + pembrolizumab	75 patients with MCRC failed by, or patients who are intolerant to, oxaliplatin, irinotecan, or fluorouracil	not yet recruiting, due for completion in July 2022
checkpoint	BRAF, $BRAF_{v600E}$, FGFR1, RET	full-length RAS[b]	in PFS and CMS2 and CMS4 in OS[c,d]

Aflibercept (Zaltrap) — VEGFA, VEGFB, placental growth factor — Decoy receptor — 2nd line in combination with FOLFIRI — phase 1: MCRC that progressed on or after, or patients who did not tolerate, fluoropyrimidines, oxaliplatin, irinotecan, or anti-angiogenic therapies — ziv-aflibercept in treating patients with advanced solid tumors (NCT02298959) — CMS4 tumors, based on bevacizumab

Ramucirumab (Cyramza) — VEGFR2 — Fully human anti-VEGFR2 — 2nd Line in combination with — RAMTAS: Ramucirumab in combination with — bearing genotype a/a of *GGND1* — predicted to have efficacy against

Regorafenib — PDGFRB, — Multi — Salvage — phase 1b. —

inhibitors			
atezolizumab (Tecentriq)	1st line folfox6 + bevacizumab + atezolizumab or atezolizumab alone or FOLFOX6 and bevacizumab	347 patients with MSI tumors	active, currently recruiting, due for completion April 2022
pembrolizumab (Keytruda) keynote-177	1st line folfox6 or folfiri + bevacizumab or cetuximab +/- pembrolizumab	308 patients with MSI tumors or DNA mismatch repair-deficient tumors	active, not recruiting, due for completion February 2021
pembrolizumab (Keytruda)	amg820 (anti csf1r monoclonal antibody) + pembrolizumab	116 patients with advanced solid tumors	active, currently recruiting, due for completion May 2020
pembrolizumab (Keytruda)	pembroluzimab + entinostat (a histone deacetylase inhibitor)	50 patients with DNA mismatch repair-proficient colorectal tumors who have not been treated with anti-PD1 or anti-PDL1	active, not recruiting, due for completion August 2019
nivolumab (Opdivo) checkmate 9x8	first-line FOLFOX + bevacizumab +/- nivolumab	180 patients with MMS tumors or DNA mistach repair proficient tumors that cannot be treated by curative resection	active and recruiting, due for completion August 2022
nivolumab (Opdivo) echo204	epacadostat (indoleamine 2, 3-dioxygenase 1 inhibitor) + nivolumab +/- standard of care	307 patients with advanced solid tumors including colorectal tumors	active not recruiting. due for completion in august 2022
durvalumab (imfinzi) stellar001	durvalumab+ IPH5401 (human monoclonal antibody against C5AR)	100 patients with advanced solid tumors	active and recruiting, due for completion June 2021
durvalumab (imfinzi)	durvalumab + oncos102 (an adenovirus that encodes GMCSF)	78 patients with advanced peritoneal disease failed by chemotherapy	active not recruiting, due for completion October 2022
other			
c-kit/mast cell inhibitor	third- or fourth-line masitinib + FOLFIRI vs best supportive care	219 patients with MCRC failed by second- or third-line therapy	active not recruiting, due for completion December 2020
CCR1 and CCR5 antagonist	BMS813160 +/- folfiri/nab-paclitaxel/gemcitabine or nivolumab	348 patients with advanced colorectal or pancreatic tumors	active, not recruiting due for completion December 2021

MCRC, metastatic colorectal cancer; MMRP, DNA mismatch repair proficient; PLK1, polo-like kinase1;

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Figure legends

Figure 1. The Microenvironment of Colorectal Tumors
Dispersed immune cells are in the tumor center, mostly in the invasive margin that juxtaposes the non-tumor area of the colon, with some forming TLS. Blood vessels, high endothelial venules, and lymphatic vessels allow entry and/or egress of immune cells.

Figure 2. The Consensus Molecular Subtypes
CMS1 and CMS4 tumors are highly infiltrated by immune cells, whereas CMS1 tumors are characterized by a Th1-cell response and activated and inflamed TME. These tumors can be treated with immune checkpoint inhibitors. CMS4 tumors have an inflamed, complement-rich, suppressive and highly angiogenic TME that can be targeted with combination therapies. CMS2 do not activate an anti-tumor immune response, due to activation of the beta-catenin pathway, and CMS3 considered to be metabolic tumors.

Figure 3. Treatment of MSI and MSS Tumors
MSI tumors should be treated with immune checkpoint inhibitors, specifically with inhibitors of PD1, potentially combined with inhibitors of CTLA4. Among MSS tumors, patients with tumors without mutations in RAS respond to cetuximab or panitumumab in combination with chemotherapy. Tumors with mutations in *APC* and activation of WNT signaling to beta-catenin might be treated with inhibitors of beta-catenin or PAX4. Mesenchymal-type tumors might be treated with a combination of immune checkpoint inhibitors and anti-angiogenic, anti-inflammatory, anti-complement, or anti-TGFB agents, in combination with chemotherapy. RAS mutant tumors might respond to anti-angiogenic therapies, T-cell–based, or T-cell activating immunotherapies.