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REVIEW

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ABSTRACT

Introduction: In the last few years, significant advances in molecular biology have provided new therapeutic options for colorectal cancer (CRC). The development of new drugs that target the immune response to cancer cells seems very promising and has already been established for other tumor types. In particular, the use of immune checkpoint inhibitors seems to be an encouraging immunotherapeutic strategy.
Areas covered: In this review, the authors provide an update of the current evidence related to this topic, though most immunotherapies are still in early-phase clinical trials for CRC. To understand the key role of immunotherapy in CRC, the authors discuss the delicate balance between immune-stimulating and immune-suppressive networks that occur in the tumor microenvironment.
Expert opinion: Modulation of the immune system through checkpoint inhibition is an emerging approach in CRC therapy. Nevertheless, selection criteria that could enable the identification of patients who may benefit from these agents are necessary. Furthermore, potential prognostic and predictive immune biomarkers based on immune and molecular classifications have been proposed. As expected, additional studies are required to develop biomarkers, effective therapeutic strategies and novel combinations to overcome immune escape resistance and enhance effector response.

1. Introduction

Colorectal cancer (CRC) is the third most common cancer and the fourth leading cause of cancer-related deaths worldwide in older adults, with a 5-year survival rate that largely depends on disease stage [1,2].

In the last 15 years, it has been shown that specific gene alterations have both prognostic and predictive value in CRC [3,4], with important implications for clinical practice. However, patients with the same TNM stage often show different clinical outcomes, reflecting the molecular heterogeneity of this cancer. More recently, other molecular features have been elucidated and, as it has for other solid tumors [5,6], molecular heterogeneity has also been studied in CRC [7,8].

Interestingly, CRC is currently classified into four consensus molecular subtypes (CMS) and a fifth unclassified group [9]. CMS1 includes tumors with microsatellite instability (MSI); CMS2 consists of chromosomal instable (CIN) tumors; CMS3 comprises tumors with KRAS mutations and metabolic dysregulation; and CMS4 includes tumors with a mesenchymal phenotype. Among these subtypes, the most immunogenic are CMS1 and, to a lesser extent, CMS4. Indeed, MSI tumors have a significantly higher mutational load than other tumor types,

primarily due to a deficient mismatch repair (dMMR) mechanism [10]. This latter feature causes the presentation of many non-self-antigens and triggers potent immune-response [11].

Despite the immunogenicity of these subtypes, the tumor is known to establish several mechanisms to escape immune surveillance [12]. Therefore, different solutions may restore the immune response against these easily targetable cells. To restore patient immunity against cancer cells, diverse strategies may be pursued, including an *active immunotherapy* (cytokines, immune checkpoint inhibitors, co-stimulatory pathways and cancer vaccines) or a *passive immunotherapy* (adoptive cellular therapy and monoclonal antibodies) approach [11,13].

Among these strategies, checkpoint inhibition seems promising, especially for MSI tumors, due to the significant mutational load and high expression of immune checkpoint molecules, which cause substantial immunogenicity. In addition to assessing CRC subtypes and the tumor microenvironment (TME), an immune score that can predict the outcome of CRC in patients with tumor infiltrating lymphocytes (TILs) has been proposed [13,14].

The aims of this review are to present the available knowledge on the underlying molecular features and immunogenicity of CRC, discuss the role of novel possible
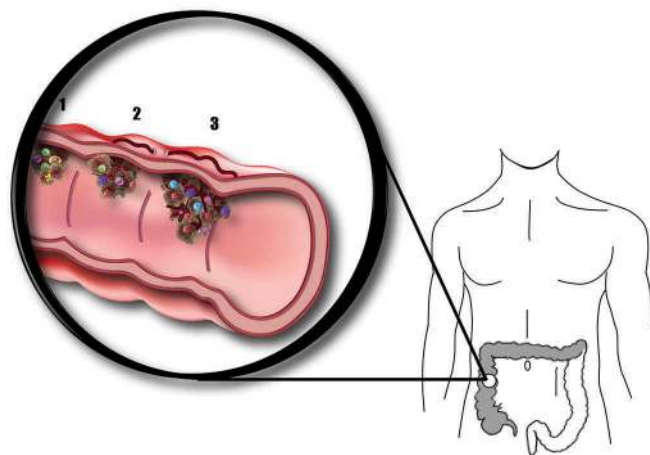
predictive biomarkers, illustrate the modern immunotherapeutic approaches and introduce the most relevant ongoing clinical trials. Although more work is required to understand the complex interactions between tumor cells and the immune system, we are at the very beginning of an exciting revolution. If the promise of these developments is fulfilled, it could guide clinicians toward a more 'personalized' treatment for advanced CRC patients.

2. The role of the immune system in CRC: the tumor microenvironment and the local immune system

Traditionally, the aggressiveness of a tumor has been defined by its clinical-pathological characteristics. More recently, advances in immunology and molecular biology have enabled us to understand the mechanisms underlying the metastatic potential of tumors. Several studies have increased the knowledge in this field and clarified the role of the immune system in regulating cancer growth. Innate immune system cells (macrophages, neutrophils, myeloid derived suppressor cells [MSDCs], mast cells, eosinophils and antigen-presenting cells [APCs]) and adaptive immune cells (T and B lymphocytes and to a lesser extent NK cells) [15,16] are among the main 'characters'. *In vitro* studies as well as experimental animal models have provided insight into the complex machinery that functions at the TME level. Preclinical evidence suggests that abnormal cells without specific antigen recognition first recruit the innate immune system, and the subsequent inflammatory response is able to promote angiogenesis and tumor cell growth. Subversion of immune surveillance, orchestrated by the tumor, involves precise mechanisms developed by the neoplasm during clonal selection. Notably, the adaptive immune response requires the recognition of non-self-antigens by interactions between peptides and the major histocompatibility complexes (MHCs) of APCs and T cell receptors (TCRs) of CD8+ and CD4 + T cells during antigen presentation [17]. Loss of tumor antigenicity may be due to antigens recognized as self as well as to acquired defects in their presentation. Systematically, a tumor progresses through three well-defined phases: *elimination, equilibrium and escape* (Figure 1). In the first phase (*elimination*), immune cells manage to remove high-immunogenicity cells that express surface proteins that can easily trigger the elimination of proliferating cells. In the *equilibrium* phase, some cells clonally survive due to their ability to hide surface molecules or by inhibiting T cells and macrophages in their 'tumor clearance process' via the expression of co-inhibitory molecules such as PD-1/2 that bind B7-H1 on APCs. Similarly, in peripheral lymphoid organs, B7 binds CTLA-4 on T lymphocytes, subsequently inhibiting them [18,19]. During this phase, a minority population of tumor cells can develop a high mutational load and stimulate the immune response, while the majority acquires CIN, which results in abnormal activation of intracellular proliferation, making these cells

Figure 1. Immunoediting in colorectal cancer.
The figure summarizes the three phases of immunoediting in colorectal cancer, underling the roles of the main effectors in the response of immune system. Immune cells are able to promote tumor cells killing but have also a series of protumorigenic effects, mainly through host immune-response. Elimination phase. Immune cells (NK, T-cells and macrophages) manage to remove the neoplastic cells which are so 'naïve' to express surface proteins. Equilibrium phase. Specific subclones are able to survive thanks to their ability to hide antigenic surface molecules or direct ihinibition of t-cells and macrophagesEscape phase. Several cancer variants escape from the killing process with subsequent evasion and proliferation of resistant clones.

more likely to escape the immune system [20]. Moreover, extracellular matrix degradation by metalloproteinases and neovasculature produced by abnormal angiogenesis helps *circulating tumor cells* move to metastatic sites. The 'seed and soil' theory posits that these cells migrate to favorable environments (metastatic sites), where they enter the *extravasation and retention* phase, during which they exert their damaging effects on the metastatic site [12].

2.1 Immune-stimulating network

Immune cells can either destroy or sustain cancer cells. Indeed, immune cells participate in the physiological repression of tumor proliferation through a complex network, leading to tumor eradication or resulting in cancer promotion [21].

Below, we summarize the different cell types involved in the immune-stimulating network.

2.1.1 CD4+ T cells
The CD3+/CD4+ group of cells represents a family of different T cells, generally referred as T-helper (Th) cells, which act as regulators of the inflammatory response directed against foreign cells. Among these, CD4+Th1 and CD4+Th17 cells stimulate the production and activity of cytotoxic T lymphocytes (CTLs) by secreting cytokines such as interferon γ (IFN-γ), tumor necrosis factor α (TNF-α), interleukin (IL)-2 and IL-17 [22–24].

2.1.2 CD8+ T cells
This population of cells is responsible for destroying tumor cells via direct lysis (CTLs) or producing cytokines that in turn cause a cytotoxic response. It has been shown in the literature that a high number of TILs (consisting of activated CD69+ and cytotoxic CD107a+ cells) characterize earlier stage CRCs [25,26].

2.1.3 NKT cells
These cells express both a functional T cell receptor (TCR) αβ and the NK receptor NK1.1. Furthermore, they are able to interact with glycolipid antigens presented by 'MHC class-I like' CD1d. After stimulation, NKT type I cells promote CTL and NK cell activation but also have NK-like direct cytolytic activity. By contrast – as Cardell described in 1995 – the NKT type II subset plays a role in suppressing CTL- and NK-mediated tumor lysis as well as in cross-regulating NKT type I cells. In addition, regulatory T cells (Treg cells or Tregs) have been demonstrated to reduce the number of NKT type I cells and thereby down-regulate tumor immunity [27].

2.1.4 NK cells
Classical fully mature NK cells (CD16+ CD57+ KIR+ and LIR+) as well as memory/adaptive NK cells (CD16+ CD57+ KIR+ LIR+ and NKG2C+) show antibody-dependent cell-mediated cytotoxicity (ADCC) and natural cytolytic activity against tumor cells. In addition, another small subset of NK cells has been demonstrated to express the inhibitory PD-1 receptor, display less effective killing activity and favor cancer escape [28].

Dendritic cells (DCs). After internalizing and processing antigens, DCs present functional MHC I- and II-peptide complexes to naive CD8+ and CD4+ T cells, respectively, to activate a specifically directed immune response. DC density has also been shown to be a positive prognostic factor in CRC [29].

2.2 Immune-suppressive network

Immune-suppressive cells in the TME can promote cancer growth. Below, we describe the cells involved in this network.

2.2.1 CD4+ T cells
CD3+/CD4+ T lymphocytes, the so-called Th2 cells, may secrete cytokines such as IL-4, IL-5 and IL-10 and inhibit CTL proliferation. The resulting TME is then enriched by B cells and Treg cells [30].

2.2.2 CD4+ Treg cells
CD4+/CD25+ Treg cells usually suppress chronic inflammation and contribute to downregulating the immune response by producing IL-10 and transforming growth factor β (TGF-β) [22,31]. In many cancers, pronounced infiltration of FoxP3+ CD4+ Tregs is associated with a worse prognosis, based on the observation that they can suppress the host immune response [32,33]. On the other hand, their role in CRC has not been fully elucidated. Treg tumor infiltration can correlate with a positive prognosis due to the central role of inflammation in CRC tumor progression. This process could be slowed down by the suppressive action of Tregs [34,35].

2.2.3 MSCs (mesenchymal stem cells)
MSCs are non-hematopoietic stromal cells with an extensive proliferative potential and the ability to differentiate into various cell types [36]. Notably, MSCs have broad immunosuppressive properties with a remarkable role in the TME. Their immunosuppressive function is elicited by the release of pro-inflammatory factors. In turn, MSC recruitment through TGF-β and prostaglandin E2 secretion inhibits lymphocyte proliferation and DC maturation by the downregulation of MHC and co-stimulatory molecules expression [37]. They promote the production of tolerogenic DCs, M2 macrophages and Treg cells. The main factors involved in this signaling are plasminogen activator inhibitor 1, IL-6, neuregulin 1, human epidermal growth factor receptor 2/3, phosphatidylinositol-4,5-bisphosphate 3-kinase and the AKT signaling pathway [38]. MSCs function in tumor development and immune surveillance evasion by encouraging the emergence of resistant clones through selective pressure and subsequent evasion and rapid proliferation. The stromal cell compartment has an important prognostic relevance to CRC patient outcomes. MSCs in the colon TME employ many pathways that result in tumor initiation, angiogenesis, resistance to chemotherapy, invasion and metastasis [36,39].

2.2.4 CAFs (cancer-associated fibroblasts)
Among the stroma players, CAFs have a critical role in CRC immunosuppression. As a result, they may strongly promote tumor progression, epithelial mesenchymal transition, and metastasis through TGF-β/SMAD signaling [40]. High levels of CAFs are correlated with poor prognosis in CRC [41].

2.2.5 MDSCs

These cells arise from different myeloid-derived cells at various stages of differentiation. They are able to enhance immune suppression by acting both on the innate and adaptive immune system. They are involved in tumor development and progression through the release of immunosuppressive molecular mediators such as NOS, TGF-β, IL-10, and PGE2 [38]. A study of 64 patients with CRC indicated that these patients had a high absolute blood count of MDSCs compared with negative control individuals; similarly, patients with high levels of MDSCs had a more advanced stage of the disease [42].

2.2.6 Mast cells

Mast cells also play an important role in the immunosuppressive cross talk: they interact with MDSCs via the CD40/CD40L axis, releasing cytokines and chemotactic factors and enhancing both the number and activity of MDSCs in the TME [43].

2.2.7 Macrophages

Macrophages are cells that clear neoplastic cells through phagocytosis. They also release matrix-degrading substances called metalloproteinases and cysteine cathepsin proteases with the same function. This mechanism facilitates tumor invasion of the stromal compartment, which allows the neoplasm to spread through the organism [44].

Pro-inflammatory or anti-inflammatory signals may activate macrophages. M1 macrophages act as effector cells in Th1 responses through the release of IL-1β, TNF-α, IL-12, IL-23, reactive oxygen species (ROS) and nitric oxide (NO), thus killing tumor cells. Conversely, M2 macrophages are involved in tumor growth and metastasis [45,46]. They inhibit the generation of M1 macrophages and block immune surveillance by expressing arginase 1 and secreting cytokines such as IL-1, IL-6, TGF-β, and IL-10. Notably, tumor associated macrophages (TAMs) display an M2-like phenotype. In turn, they recruit immunosuppressive cells by secreting cytokines that differentiate lymphocytes into Th17 cells (IL-23, IL-6, IL-1β, TGF-β) or Tregs (TGF-β, IL-10) [38,47,48]. High levels of metalloproteinase-9 and M2 cells have been found to be independent predictors of metastasis and of poor outcome in CRC [41,44]. Furthermore, TAMs can induce resistance to 5-fluorouracil (5-FU)-based chemotherapy. This fluoropyrimidine triggers the p-JNK/caspase-3 pathway, inducing cell death, but also stimulates the production of putrescine in macrophages through ornithine decarboxylase (ODC), inducing chemoresistance [49].

Fusobacterium nucleatum: Evidence has recently shown that this bacterium contributes to CRC development. Particularly, some studies have demonstrated that fusobacterium could accelerate tumorigenesis in mice. This is primarily due to MDSC recruitment and expansion, resulting in immunosuppression and immune escape. Moreover, a correlation between MDSCs, TAMs, DCs and *Fusobacterium* was reported. Remarkably, an up-regulation of PTGS2 (COX-2), IL-1β, Il-6, IL-8, TNF (TNF-α) and MMP3 was noted, suggesting an NF-κB-driven pro-inflammatory response [50]. In a recent study, *F. nucleatum* was detected in 13% of CRC tissues. Patients without *F. nucleatum* showed a higher density of CD3+ T cells compared to patients with *F. nucleatum* (multivariable odds ratio, .47; 95% CI .26–.87; P_{trend} = .006). However, a significant association between the presence of *F. nucleatum* and CD8+, CD45RO+, or FOXP3+ T cells (P_{trend} > .013) was not observed [51].

3. How to select patients who are likely to benefit from immunotherapy

Increasing insights have established the pivotal role of the immune system in cancer growth control. More specifically, immunological manipulation has led to the development of new agents that unleash the immune system against cancer. Indeed, in this era, immunotherapy represents a promising option for the treatment of an increasing number of malignancies, including CRC. Unfortunately, only some CRC patients seem to benefit from immunotherapy. By investigating potential prognostic and predictive immune biomarkers, we may be able to identify patients who would benefit from these new strategies [52].

Though this may be difficult at first, it is essential to clarify the role of both the immune and molecular classifications of CRC.

3.1 Immune classification: immunoscore

For more than 10 years, TILs, defined as CD3+ and CD45RO+ cells within the tumor [53], have been known to have a critical impact on patient outcome [54]. In particular, the observation that CRC patients with a high infiltration of memory T cells and CD8+ T cells experienced a longer progression free survival (PFS) and overall survival (OS) [55] led to the proposal of an immunoscore classification [54].

The immunoscore is obtained by counting two lymphocyte populations identified by CD3/CD45RO, CD3/CD8 or CD8/CD45RO positivity, in both the tumor core (TC) and invasive margin (IM). Evaluating both tumor regions (TC and IM) increases the accuracy of survival prediction. The immunoscore, which ranges from a score of 0 (a low density of both cells in both cancer regions) to a score of 4 (high density), may predict disease free survival (DFS) and OS in CRC [56] and may help identify patients with early-stage disease who might potentially benefit from adjuvant chemotherapy [57].

3.2 Molecular subtype classifications

CRC is classified as four CMS subgroups and a fifth residual unclassified group (13%) [9]. CMS1 (MSI-like, 14%) includes MSI tumors; CMS2 (canonical, 37%) consists of CIN tumors with epithelial differentiation and a strong upregulation of WNT and MYC downstream targets; CMS3 (metabolic, 13%) encompasses tumors with KRAS mutations and metabolic dysregulation; and CMS4 (mesenchymal, 23%) includes tumors with a mesenchymal phenotype. Only two of these subgroups showed high expression of immune signatures: CMS1 and CMS4. More specifically, CMS1 encompasses tumors with deviations (expansion or contraction) in microsatellite regions, defined as MSI. The cause of this alteration is dMMR enzymes, with an increased mutational rate (genomic instability) resulting from mutations in DNA MMR genes (i.e. *MLH1, MSH2, MSH6 and PMS2*) [58,59]. A high frequency of MSI in CRC has

been revealed to be an independent prognostic factor for favorable outcome and reduced metastatic spread in early stages of the disease [60]. More recently, some studies, evaluating the polymerase genes *POLE* and *POLD1* in MSI-high tumors, have identified a hypermutated phenotype with up to more than 1,000,000 base substitutions per tumor when these genes are mutated. Notably, MSI-high tumors have a mutational rate 20 times higher than MSS tumors, reflecting the tendency to express a higher load of neo-antigens, thus improving the response to immunotherapy [61].

CMS1 also includes tumors with methylated CpG islands (CpG island methylator phenotype, CIMP-H), which often results in gene silencing, and tumors with mutations in the BRAF oncogene [9,61,62]. Interestingly, this subgroup displays a diffuse immune infiltrate. Moreover, this subtype exhibits high expression of T cell-recruiting chemokines as well as the expression of Th1 cytokines that have been shown to correlate with good prognosis in CRC [55]. Indeed, dMMR causes a high mutational oncogenic load, such as frameshift mutations and neo-antigen expression, which can induce an active immune microenvironment reaction characterized by a high density of TILs [63]. Further investigations have explored the association between neo-antigens and immune infiltrate in CRC. A higher neo-antigen load was shown to be associated with a high lymphocyte score (Spearman's rank correlation coefficient = .29, p value = 2.6×10^{-11}) and with increased CRC-specific survival (log rank test, p value = .004; multivariate HR = .57 [95% CI, .35–.93], p value = .03) [64]. Therefore, tumors with a high neo-antigen load would seem to benefit more from immunotherapy [61].

Conversely, CMS4 includes tumors with a mesenchymal phenotype characterized by TGF-β activation, stromal invasion, and angiogenesis. This subgroup displays high expression of myeloid chemokines, angiogenic factors, immunosuppressive factors and complement components, which correlate with worse prognosis in CRC.

Interestingly, the genes (CD274 and PDCD1LG2) encoding the PD-1 ligands are highly expressed in CSM1 tumors and in some tumors of CSM4 [55].

3.3 Integrating immune and molecular classification: individualization of potential prognostic and predictive factors

A recent analysis of 270 patients from The Cancer Genome Atlas (TCGA) found that MSI cancers have higher intratumoral immune gene expression in all immune subpopulations and higher immunoscores than MSS tumors [14,65]. These data support the treatment of MSI-like CRC patients with anti PD-1/PD-L1. Indeed, a recent clinical trial showed that MSI patients responded to PD-1 blockade [66]. Le's study, testing pembrolizumab (anti-PD-1), as well as data recently presented at ASCO 2016 regarding the combination of nivolumab (anti-PD-1) and ipilimumab (anti-CTLA-4) in CRC patients with either MSI or MSS tumors confirmed that the optimal candidates for anti-PD-1 or anti-PD-L1 therapy are those with MSI tumors [67]. The response to PD-1/PDL-1 blockade might not be the same in all MSI CRC patients. Indeed, only one-third of MSI

CRC patients can benefit from immunotherapy, and the duration of response is not homogeneous [68]. Although this study provides novel insights into the pathological and molecular bases of PD-L1 expression in CRC, important questions remain with respect to genetic or epigenetic alterations that could critically affect PD-L1 expression in CRC. The different responses to anti-PD-1/PD-L1 therapy of MSI CRCs with PD-L1 positivity in tumor cells or in immune cells should also be considered.

Moreover, some evidence has shown that a high density of MDSCs is associated with a poor prognosis in many tumors. Interestingly, granulocyte MDSCs that expressed higher levels of PD-L1 induce robust immunosuppressive activity in metastatic CRC (mCRC). MDSC levels are apparently higher in the blood of patients with mCRC rather than in healthy subjects. Moreover, a high level of granulocyte MDSCs is associated with worse prognosis, and reduced blood levels of granulocyte MDSCs after treatment with 5-FU, oxaliplatin and bevacizumab seemed to be associated with a longer median PFS [42]. Certainly, further studies are needed to confirm if granulocyte MSDC density is an independent prognostic factor in mCRC.

In conclusion, the combination of different biomarkers (i.e. properly defined and documented PD-L1 expression, the presence of TILs, and molecular classification) may help identify responders to immunotherapy among MSS patients and non-responders among MSI patients (Figure 2).

Notably, current evidence has suggested that patients with CRC tumors that exhibit the presence of TILs, MSI-H and high expression of neo-antigens are good candidates for checkpoint immunotherapy [61,69]. Like CMS1 tumors, CMS4 tumors also exhibit immune, inflammatory and immunosuppressive cells, indicating that immunotherapy could also be applied to cohorts of patients with a mesenchymal CRC phenotype [55].

However, there is no universal consensus regarding the use of PD-1 and PD-L1 expression in CRC treatment decisions. This lack of uniformity is due to heterogeneity in the testing methodology and to the variable expression of the latter; therefore, more studies are required to validate the use of PD-1 and PD-L1 as predictive biomarkers. Furthermore, some studies have demonstrated that PD-L1 expression is inversely associated with MSI-H status as well as FOXP3+ cell density [70].

4. Clinical trials of mCRC immunotherapies

While immunotherapies are being developed for different cancer types, many trials are currently recruiting patients to explore the safety, activity and efficacy of these new agents for mCRC.

Ipilimumab is one of the first drugs designed to interfere with an immune checkpoint. Ipilimumab is a fully human monoclonal antibody (IgG1) that blocks CTLA-4 to promote antitumor immunity, and it has extended survival in patients with advanced melanoma [71]. Novel promising monoclonal antibodies that target other checkpoints such as PD-1 (e.g. pembrolizumab, nivolumab) or PD-L1 (e.g. atezolizumab) can boost the immune response against cancer cells. These drugs are now being tested in clinical trials either alone or in
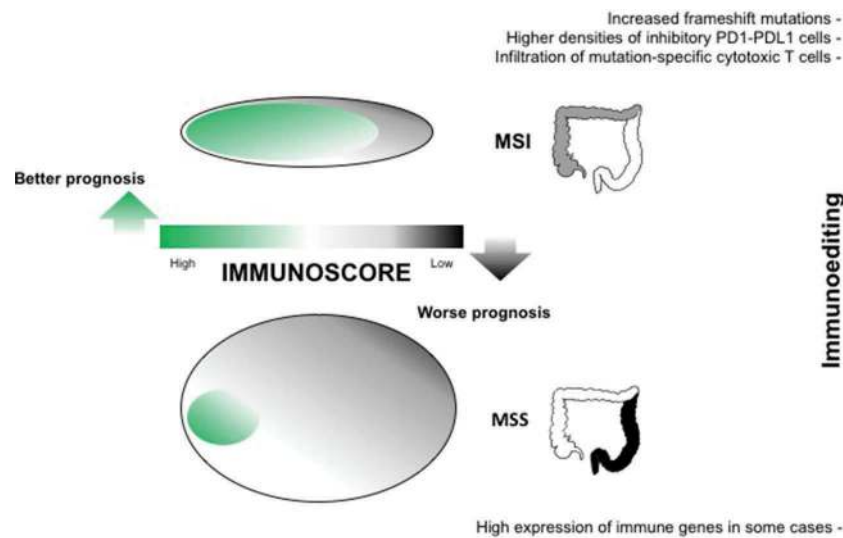
Figure 2. Immunoscore, gene profile, tumor location and prognosis in colorectal cancer.
The figure summarizes the overlapping of immune and molecular classification and differences in clinical presentation and prognosis. A high immunoscore is more frequent in right side MSI tumors and is correlated with good prognosis. On the other hand, left side MSS tumors commonly show a low immunoscore and worse prognosis.

combination. However, many of these mCRC trials are ongoing, and only a few mature results have been obtained.

The ESMO consensus guidelines [72] recommend MSI testing because of its strong predictive value for the use of checkpoint inhibitors in the treatment of CRC patients, suggesting the potential use of pembrolizumab in patients with dMMR tumors. According to reports from a small phase II trial, the immune-related objective response rate (ORR) and immune-related 6-month PFS rate were 40% and 78%, respectively, for the 11 CRC patients with dMMR and 0% and 11% for the 21 CRC patients with proficient MMR (pMMR). This result supports the hypothesis that MMR status could predict the efficacy of immunotherapy [73].

Based on promising results published by Le [73], in the phase III KEYNOTE-177 trial [74], patients with MSI-high (MSI-H) or dMMR advanced CRC will be randomly assigned to receive either pembrolizumab or an investigator's choice chemotherapy regimen among six different choices for the upfront treatment of advanced CRC. The primary trial endpoint is PFS, and the results are expected in 2019. Another phase III trial [75] is comparing regorafenib [76] to cobimetinib (MEK inhibitor) plus atezolizumab or to atezolizumab monotherapy in pretreated CRC patients. Cobimetinib promotes MHC I expression, inducing the accumulation of intratumoral CD8 T cells and thereby sensitizing tumors to atezolizumab. The rationale for the combination derives from preclinical models showing that cobimetinib favors PD-L1 upregulation and accordingly enhances anti-PD-L1 activity in serial biopsies taken from patients included in a phase Ib trial. The results of the phase Ib trial [77] were presented at the 2016 ASCO Meeting and then at ESMO 2016. The study included three patients with mCRC (two KRAS mutant, one KRAS WT) in the dose escalation cohort and 20 (all KRAS mutant) in the dose expansion cohort. The combination was well tolerated, and only 9% of patients had treatment-related serious adverse events (nausea/vomiting and cerebrovascular accident). The ORR was 17% (four partial response, five stable disease), with the duration ranging from 5.4 to 11.1 months. Activity did not correlate with PD-L1 expression. Interestingly, three of the responders were pMMR. These results showed that even patients with MSS CRC are likely to respond to the combination of cobimetinib and atezolizumab, paving the way for the ongoing phase III trial. This trial is recruiting patients with histologically confirmed CRC who have experienced disease progression on at least two systemic chemotherapy regimens for mCRC. Only MSI-stable patients will be eligible, and the primary endpoint is OS.

Finally, the IMPALA trial is testing MGN1703 as switch maintenance therapy in patients with mCRC who achieved a partial response after first-line standard chemotherapy. MGN1703 is a DNA-based Toll-like receptor (TLR) agonist that acts as an immunomodulator and showed promising activity in the IMPACT phase II trial when compared to placebo [78]. The study requires no molecular selection.

The results of an interim analysis of CheckMate-142 [67], an international phase II, open-label, noncomparative trial enrolling patients regardless of their MSI status, were recently presented [67]. MSI-H CRC patients received nivolumab (n = 70) or 3 mg/kg nivolumab plus 1 mg/kg ipilimumab (n = 30) for four doses followed by 3 mg/kg nivolumab every 2 weeks until unacceptable toxicity or disease progression. The primary endpoint was investigator-assessed ORR in MSI-H patients. The investigator-assessed ORR for MSI-H patients receiving 3 mg/kg nivolumab with at least 12 weeks of follow up was 25.5% and 33.3% for the combination arm. The six-month PFS rate was 45.9% (95%CI: 29.8–60.7) and 66.6% (95% CI: 45.5–81.1) for nivolumab and nivolumab plus ipilimumab, respectively. The six-month OS rate was 75% (95% CI: 58.5–85.7) and 85.1% (95% CI: 65.0–94.2) respectively.

Therapeutic vaccines, adoptive cell therapy, oncolytic virus therapy and cytokines are also under investigation. Most of these immunotherapies are still in early-phase clinical testing,

but their activity results in other cancer types suggest testing them in CRC as well. Table 1 summarizes key clinical trials that are now evaluating the activity/efficacy of different immunotherapeutic agents in mCRC.

5. Looking ahead

Immunotherapy is a promising treatment option for many cancer patients. Specifically, the advent of ipilimumab, nivolumab and pembrolizumab in clinical practice has markedly improved the outcomes of these patients with durable responses and significant survival benefits. Unfortunately, patients do not all equally benefit from this new strategy. To understand the reasons for the heterogeneity of the responses, we should first consider the potential mechanisms of primary and acquired resistance to immunotherapy and then attempt to understand how to enhance the benefit of this therapeutic approach.

5.1 Immunotherapy: how do tumor cells acquire resistance?

Tumor cells may escape immune surveillance by acquiring different genetic alterations. Indeed, some patients exhibit an innate resistance to immunotherapy. A higher expression of mesenchymal transition genes (AXL, ROR2, WNT5A, LOXL2, TWIST2, TAGLN, and FAP), immunosuppressive genes (IL10, VEGFA, and VEGFC) and chemokines that recruit immunosuppressive cells (CCL2, CCL7, CCL8, and CCL13) may be associated with innate anti-PD-1 resistance.

A recent study [80] used an innate anti-PD-1 resistance signature (IPRES) to evaluate mesenchymal transition, angiogenesis, hypoxia and wound healing in metastatic melanoma. IPRES-enriched tumors were associated with anti-PD-1 nonresponding cancer (OR = 4.6; p = .013), while IPRES-low tumors were associated with anti-PD-1 responding cancer (OR = .15; p = .04). Conversely, the IPRES signature showed no similar association in the context of anti-CTLA-4.

By contrast, some patients quickly develop resistance, even after an initial benefit with a significant reduction in tumor burden, suggesting that a rapidly proliferating resistant clone may cause the progression of resistance. Interestingly, Ribas et al. identified acquired mutations in four patients treated with pembrolizumab at disease progression through wholeexome sequencing. They showed that two out of the four patients presented a loss-of-function mutation in the IFN receptor pathway, specifically involving Janus kinase-1 (JAK-1) and JAK-2. Moreover, in a third patient, the resistance was due to a truncating mutation of beta-2-microglobulin (B2M), an essential component of the MHC-I structure, which is necessary for antigen binding and presentation. As a result, these mutations cause decreased antigen presentation and immune escape [81].

Likewise, high tumor PGE2 expression represents a key mediator of immune resistance, mainly due to the secretion of suppressive chemokines and the recruitment of gMDSCs, which results in immunogenic loss [82].

5.2 Immunotherapy effect: how can it be enhanced?

Following the reports of positive data obtained in melanoma, renal tumors and lung cancer, the use of immunotherapy to treat many other cancer types, including gastrointestinal malignancies, has attracted interest. Ongoing research efforts are aimed at identifying new targets and developing novel approaches to enhance immunotherapy [83]. More specifically, immunotherapy alone appears to have modest success, likely due to the complexity of the TME. Therefore, recent trials have been evaluating novel combined approaches, such as immune-chemotherapy or combo immunotherapy, that could be more effective than chemotherapy or immunotherapy alone [67,84,85].

Moreover, some authors observed that VEGF-A blockade could help sensitize T cells to anti-PD-1 treatment and that high VEGF-A levels may be involved in resistance to this treatment [86]. Therefore, these data suggest a potential rationale for the association between anti-angiogenic molecules and checkpoint inhibitors, with particular interest for VEGF-A-producing tumors. It will be interesting to verify if combining immunotherapy with chemotherapy and/or biological therapies (anti-EGFR or anti-VEGF) could produce a synergistic effect in CRC (Table 1). Obviously, many clinical trials are required to evaluate the efficacy and safety of these novel approaches.

It would also be useful to understand how to enhance immunotherapy, increasing the effector response and reducing the inflammatory component. Indeed, tumor cells can exploit inflammation for cancer promotion. COX-2 deregulation plays a pivotal role in tumor cells. Unlike COX-1, which is expressed constitutively in most cells, COX-2 is produced in response to growth factors and cytokines [87,88]. Once synthesized, prostaglandin-2 (PGE-2) acts in an autocrine and paracrine manner though four receptors to direct epithelial-mesenchymal transition, angiogenesis, HIF-1 transcription, acid oxidation production, chemo-resistance, M2 polarization, and Treg and MDSC recruitment. Furthermore, a cross talk between the immunosuppressive microenvironment and the EGFR pathway activates several signal transduction cascades, including the MAPK, AKT, and PI3K pathways, and subsequent tumor growth and immunosuppression [89].

Preclinical studies [82,90,91] found that COX inhibition could enhance the efficacy of anti-PD-1 blockade. Zelenay and colleagues [91] inoculated Ptgs2-deficient and BRAFV600E mutated cells in WT mice and found that the loss of COX-2 expression leads to a significant decrease in immunosuppressive cytokine (IL-6) and chemokine (CXCL1) expression and a simultaneous marked increase in immune-stimulating factors (IFN-g, T-bet, CXCL10, IL-12 and IFN-I) and co-stimulatory molecules. Unlike in COX-deficient tumors, DCs are absent in COX-competent tumors. More interestingly, in the same study, mice were randomly assigned to receive aspirin, celecoxib, or anti-PD-1 in monotherapy or the combination of a COX inhibitor plus anti-PD-1. As expected, the combination promoted a much more rapid tumor regression, with the eradication of BRAFV600E melanoma cells. This suggests that the association of COX inhibitors and immune checkpoint blockers could enhance

Table 1. Key clinical trials testing immune checkpoint modulators in colorectal cancer (according to https://www.clinicaltrial.gov/, 30th of December 2016).

	Experimental arm (In combination with)	Control arm (In comparison to)	Phase	Lines	Conditions	Target population	Primary end point	Sample	Status	NCT number
Ipilimumab	Nivolumab (sin)	Nivolumab	I/II	≥2	CRC	MSI	ORR	240	Recruiting	02060188
Nivolumab	Enoblituzumab	-	I	>2	B7H3 tumors	All	Safety	84	Recruiting	02381314
	-	-	1		Solid tumors	All			Completed [79]	00729664[1]
	TAS-102	-	II	≥3	CRC	MSS	irORR	35	Recruiting	02860546
	Epacadostat	-	I/II	All	Solid tumors	All	DLTs/ORR	291	Recruiting	02327078
	Varlilumab (anti-CD27)	-	1I/II	All	Solid tumors	All	DLTs/ORR	190	Recruiting	02335918
	Chemotherapy	-	I/II	//	Solid tumors	All	RP2D	49	Recruiting	02423954
	Arginase Inhibitor CB-1158	Arginase Inhibitor CB-1158	I	All	Solid tumors	All	Safety	236	Recruiting	02903914
	Enadenotucirev	-	I	All	Solid tumors	All	MTD+Mfd	30	Recruiting	02636036
	TSR-022 (anti-TIM-3)	-	I	All	Solid tumors	All	Safety/ORR	402	Recruiting	02817633
	DS-8273a	-								
Pembrolizumab	-	Standard therapy	IIIR	≥2	CRC	All	irORR	20	Recruiting	02991196
	Modified FOLFOX6	-	II	1	CRC	MSI-H/dMMR	PFS	270	Recruiting	02563002
	-	-	II	1	CRC	All	PFS	30	Active, not recruiting	02375672
	-	-	II	≥2	CRC	MSI-H/dMMR	ORR	120	Active, not recruiting	02460198
	-	-	II		Solid tumors	MSI			Completed [73]	01876511[2]
	Azacitidin	-	II	All	CRC	All	ORR	40	Active, not recruiting	02260440
	Radiotherapy	Ablation	II	All	CRC	All	ORR	48	Recruiting	02437071
	Cetuximab	-	I/II	All	Solid tumors	All wt	RP2D	90	Recruiting	02318901
	lymphocyte depleting regimen → autologous TIL	-	II	All	Solid tumors	All	ORR	290	Recruiting	01174121
	BBI608 (Napabuca	-	I/II	≥2	Solid tumors	MSI-H	DLTs/ORR	403	Recruiting	02178722
	Cetuximab	-	I/II	≥2	CRC	MSI	irORR	56	Not yet recruiting	02851004
	Cetuximab	-	I/II	≥2	CRC	All	Safety/ORR/PFS	42	Recruiting	02713373
	Romidepsin, oral CC	-	I	≥3	CRC	MSS	Change in TIL	30	Recruiting	02512172
	SBRT liver	-	I	≥2	CRC	Liver mts	RR at 1 year	15	Not yet recruiting	02837263
	AMG820	-	I/II	All	Solid tumors	All	Safety/irORR	197	Recruiting	02713529
	CM-24 (anti-CEACAM1)	-	I	All	Solid tumors	All	Safety	196	Recruiting	02346955
	Jak inhibitor/ PI3 K-delta inhibitor	-	I	All	Solid tumors	MSI	Safety	78	Recruiting	02646748
	Ziv-aflibercept	-	I	≥2	Solid tumors	All	Safety/DLT	36	Recruiting	02298959
	AM0010 (PEGylated recombinant human Interleukin-10) + CT	-	I		Solid tumors	All	Safety	300	Recruiting	02009449
	mFOLFOX+ celecoxib	-	I/II	//	GI tumor	All	Safety	39	Not Recruiting	02268825
	Selinexor	-	I	All	Solid tumors	All	MTD	470	Recruiting	02419495
	Poly-ICLC	-		≥3	CRC	All	MTD	42	No yet recruiting	02834052
	Nintedanib	-		All	Solid tumors	All	MTD	18	Recruiting	02856425
	CY/GVAX	-	II	>2	CRC	pMMR	ORR	30	No yet recruiting	02981524
	Enoblituzumab	-		>2	B7H3 tumors	All	Safety	75	Recruiting	02475213
	p53MVA	-		//	Solid tumors with p53 mutation	P53 mutated tumors	Safety	12	Recruiting	02432963

(Continued)

Table 1. (Continued).

	Experimental arm In combination with	Control arm In comparison to	Phase	Lines	Conditions	Target population	Primary end point	Sample	Status	NCT number
Atezolizumab	± Cobimetinib	Regorafenib	III	≥3	CRC	All	OS	360	Recruiting	02788279
	Cape + bev	Cape + bev	II	All	CRC	All	PFS	135	Not yet recruiting	02873195
	5FU+bev	5FU+/-bev +/-vemurafenib	IIR	Biomarker-driven maintenance	CRC	All	PFS/ORR	610	Recruiting	02291289
	Cobimetinib+bev	-	I	≥2	CRC	All	Safety	33	Not yet recruiting	02876224
	CPI-444	CPI-444	I	<5	Solid tumors	MSI	DLTs/ORR	534	Recruiting	02655822
	-	-	I	//	Solid tumors	All	Safety/DLT	604	Recruiting	01375842
	Bev +/- CT	-	I	All	Solid tumors	MSI	MTD/DLT/ Safety	225	Recruiting	01633970
MGN1703	-	Standard	IIIR	maintenance	CRC	RP after first line	OS	540	Recruiting	02077868
Durvalumab (MEDI4736)	-	-	II	AD	CRC	All	ORR	48	Recruiting	02227667
	-	-	II	AD	Epithelial tumor with SNC mts	-	Brain ORR	136	Recruiting	02669914
	Tremelimumab	-	I	AD	Solid tumors	All	Safety	105	Recruiting	01975831
	ONCOS 102	-	I/II	//	Solid tumors with peritoneal disease	All	Safety	78	No yet recruiting	02963831

TIL: tumor infiltrating lymphocytes; CC: oral azacitidine; SBRT: stereotactic body radiation therapy for liver metastases; Cape: capecitabina; bev: bevacizumab; CT: chemotherapy; R: randomized; AD: advance disease; CRC: colorectal cancer; GI: gastrointestinal; SNC: central nervous system; mts: metastases; MSI: microsatellite instability; MSS: microsatellite stable; MSI-H: high; dMMR: DNA mismatch repair status; RP: partial response; ORR: objective response rate; irORR: immune-related objective response rate; DLTs: dose-limiting toxicities; RP2D: recommended phase 2 dose; MTD+MFD²: maximum tolerated dose /maximum feasible dose; PFS: progression free survival; OS: overall survival; //: line of therapy not specified.

the efficacy of immunotherapy and prevent resistance development.

6. Conclusion

In recent years, several therapeutic approaches have reshaped the overall strategy of treating CRC patients and have markedly improved patient survival. Significantly, emerging novel immunotherapeutic approaches could change the CRC landscape. Moreover, selection criteria are necessary to identify patients who may benefit from immune checkpoint inhibitors. To this end, the presence of TILs is one of the most important predictors. Through the immunoscore determined by the quantification of two lymphocyte populations in both the core and the invasive margin of the tumor, CRC has been classified into four different subgroups. Two of these, the first consisting of MSI tumors and the other one including tumors with a mesenchymal phenotype, seem to be appropriate subgroups for PD-1 inhibitor immunotherapy. DNA MMR and MSI status is now clinically significant to determining whether patients may be eligible for immunotherapy in clinical trials, but we ignore the potential predictive factors in MSS patients. To date, the predictive role of the differential expression of PD-1 and PD-L1 has not been completely clarified, although some evidence suggests that high expression correlates with a better immunotherapy efficacy. Currently, most immunotherapies are still in early-phase clinical testing for CRC, but their successful use in other types of cancers suggests that they may ultimately prove useful for CRC as well. As the field of immunotherapy treatment continues to evolve, a more comprehensive knowledge of resistance mechanisms will be mandatory, which will lead to the development of novel strategies to overcome both primary and acquired resistance to anti-PD/PDL-1 antibodies.

7. Expert opinion

Since the first presentation in 2010 of ipilimumab data in melanoma, checkpoint immunotherapy has *revolutionized* the treatment of cancer. This strategy later succeeded in other diseases, such as renal cell carcinoma and non-small cell lung cancer. Despite a strong rationale for adopting the same strategy in CRC, first clinical data only became available in 2015, and the clinical development of checkpoint inhibitors in this field is still in an early phase. This did not occur by chance. In fact, CRC has received less attention than other cancers in the field of humoral immunotherapy primarily because of the lack of CRC patients who respond to this treatment. As far as we know, only MSI-H CRC tumors respond to checkpoint inhibition. In this review, we explained the basis for this and the results obtained thus so far. This apparent limitation could be seen as a point of strength. Each time a new drug is added to the list of approved agents in oncology, the scientific community claims to have powerful and ready-

to-use predictive markers, which is what we have with microsatellite status and checkpoint inhibitors in CRC.

Though the only available data have come from phase II trials, as phase III trials are currently ongoing, the results achieved so far are exciting [67,73]. Based on these results, the Food and Drug Administration (FDA) has granted Breakthrough Therapy Designation to pembrolizumab for the treatment of MSI-H CRC. This paves the way for new therapeutic possibilities but also raises new doubts and questions, some of which concern very practical matters.

The first is 'positioning'. Impressive data regarding immune checkpoint inhibitors in CRC have been reported, but stage IV CRC is not curable in 90% of cases by definition. Should we use checkpoint inhibitors in all mCRC patients as a first approach, or is it more prudent to wait for head-to-head trials comparing immunotherapy to the standard of care? Using these agents in the treatment of stage IV radically resected patients or as adjuvant therapies is even more challenging. Certainly, drug labels will help determine how we use these agents, but we can easily anticipate that medical oncologists will have to balance economic limitations and the best interests of their patients.

The second is 'response assessment'. Traditionally, tumor response assessment is based on the RECIST criteria. These criteria are applicable to chemotherapeutic agents, due to their cytotoxic action. Drugs with different mechanisms of action require different response evaluation criteria. This problem has already been encountered in CRC treatment with anti-angiogenic agents, and a definitive solution has not yet been found. The different mode of antitumor action of immunotherapy requires modified tumor response criteria (irRC) that consider new phenomena such as pseudoprogression [92]. Much work needs to be done in this field in the very near future.

The third is 'tolerability'. Immune checkpoint inhibitors have been shown to have a different toxicity profile compared with traditional chemotherapy and targeted agents [93]. The first results have been reassuring and have not noted specific concerns for CRC patients. Nevertheless, the number of patients that have been treated up to this point is quite limited, and the follow-up times are rather short. We cannot forget that preliminary efficacy results suggest that many patients are candidates to receive the treatment for years. What about immune-related colitis and intestinal resections? What about the coexistence of inflammatory bowel disease?

Other important questions concern the direction of future clinical research:

- Is MSI the only biomarker? Are there other potential candidate molecular predictors of response? Beyond dMMR tumors, another subset of hypermutated CRCs, such as those bearing somatic or germ line mutations in the DNA polymerase E (POLE) gene, has been described [94]. This is a small subgroup of patients, approximately 0.5–2% of patients with mCRC, but these patients may have a strong biological basis for checkpoint inhibitor sensitivity [94].

- Is there any difference between different checkpoint inhibitors (i.e. nivolumab vs pembrolizumab)? Is there a real benefit in combining the anti-CTLA-4 ipilimumab with nivolumab? What

about the treatment duration in patients undergoing a complete response or post-progression treatment for those who acquire resistance to immune checkpoint inhibitors?

- What about the other tumor types? Is there anything we can do to 'ignite a cold tumor', to sensitize the vast majority of CRC patients, who are affected by MSS cancers [95], to checkpoint inhibitors? Combination therapies to increase tumor sensitivity to immunotherapy are under investigation. The rationale for combining a MEK inhibitor such as cobimetinib with an antibody directed against PD-L1 such as atezolizumab is described above [77]. A more 'dirty' option may be combining immune checkpoint inhibitors with systemic therapies (i.e. chemotherapy or targeted therapy) or local treatments (i.e. radiation therapy) to exploit the immunogenic release of antigens from cancer cells. Innovative approaches under discussion include pretreating the tumor with alkylating mutagenic agents, such as temozolomide [96,97], followed by immune checkpoint inhibitors alone or in combination.

- Is there any 'alternative' immunotherapy? Immune checkpoint inhibition is not the only immunotherapy option, although this is the first successful approach. Alternative strategies currently under investigation include vaccines, cytokine therapy, TLR agonists, and adaptive cell therapy. In CRC, some of these efforts have failed in the past. However, the present level of knowledge and technology makes success more likely. Most of the studies are still in early phase I and II trials, but results from phase III studies, such as the IMPALA (testing the TLR9 agonist MGN1703) are awaited in the near future [96].

In conclusion, even though novelty always brings concerns and new challenges, we are excited for the potential to treat some CRC patients with checkpoint inhibitors and for the opportunities that immunotherapy will likely provide.

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Declaration of interest

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