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**Is the combination of immunotherapy with conventional chemotherapy the key to increase the efficacy of colorectal cancer treatment?**

Immunotherapy on colorectal cancer

**Abstract**

Colorectal cancer (CRC) is among the most prevalent and deadly neoplasias worldwide. According to GLOBOCAN predictions, its incidence will increase from 1.15 million CRC cases in 2020 to 1.92 million in 2040. Therefore, a better understanding of the mechanisms involved in CRC development is necessary to improve strategies focused on reducing this oncological pathology's incidence, prevalence, and mortality. Surgery, chemotherapy, and radiotherapy are the main strategies for treating CRC. The conventional chemotherapeutic agent utilized throughout the last four decades is 5-fluorouracil (5-FU), notwithstanding its low efficiency as a single therapy. In contrast, combining 5-FU therapy with leucovorin and oxaliplatin (FOLFOX) or irinotecan (FOLFIRI) increases its efficiency. However, these treatments have limited and temporary solutions and aggressive side effects. Additionally, most patients treated with these regimens develop drug resistance, which leads to disease progression. The immune response is considered a hallmark of cancer; thus, the use of new strategies and methodologies involving immune molecules, cells, and transcription factors has been suggested for CRC patients diagnosed in stages III and IV. Despite the critical advances in immunotherapy, we are still on the way to generating the expected impact of "immune checkpoint inhibitors" (ICIs) on CRC because less than 25% of CRC patients display increased 5-year survival. The causes of CRC are diverse and include modifiable environmental factors (smoke, diet, obesity, and alcoholism), individual

genetic mutations in each patient, and inflammation-associated bowel diseases. Due to these diverse causes, the solutions likely cannot be generalized. Interestingly, new strategies, such as single-cell multiomics, proteomics, genomics, flow cytometry, and massive sequencing for tumor microenvironment analysis, are beginning to clarify the way forward. Thus, the individual mechanisms involved in developing the CRC microenvironment, their causes, and their consequences need to be understood from a genetic and immunological perspective. This review aims to underline the importance of altering the immune response in CRC. It focuses on drugs that may modulate the immune response and show specific efficacy, contrasting with evidence that immunosuppression or the promotion of the immune response is the answer to generating effective treatments with combined chemotherapeutic drugs.

**Key Words:** Colorectal cancer; Immunotherapy checkpoint inhibitors; chemotherapy; Immunotherapy; Immune response

Olguin JE, Mendoza-Rodriguez MG, Sanchez-Barrera CA, Terrazas LI. Is the combination of immunotherapy with conventional chemotherapy the key to increase the efficacy of colorectal cancer treatment? . *World J Gastrointest Oncol* 2022; In press

**Core Tip:** This review focuses on those drugs that may modulate the immune response and show specific efficacy, contrasting with evidence that immunosuppression or promotion of the immune response is the answer to generating effective treatments with combined chemotherapeutic drugs.

## **INTRODUCTION**

The origin of CRC is heterogeneous. The general classification of CRC is divided into inherited, sporadic, and intestinal bowel diseases. Inherited CRC, which represents ~5% of all CRC cases, includes either the presence or absence of colonic polyps, such as Lynch syndrome and serrated polyposis syndrome<sup>[1]</sup>. Sporadic CRC (~70% of CRC

cases) is sustained by environmental and modifiable risk factors, including stress, diet, and age. Sporadic CRC has a monoclonal origin and is characterized by mutation accumulation in oncogenes and tumor suppressor genes. This second pathway of colorectal cancer includes the traditional *APC-KRAS* pathway and the microsatellite instability group, both having an essential role in clinical studies [2]. The third pathway includes intestinal chronic inflammatory diseases, such as Crohn's disease and ulcerative colitis, which could result in colitis-associated colon cancer (CAC) [3]. From a biological perspective, this evidence demonstrates that the origin of CRC is diverse. The response to therapies is not always homogeneous in patients. The best treatment should be based on the tumor's unique characteristics. Effective treatments need to be broad and involve, chemical and immunological molecules. The context of the broad causes of CRC development is highly involved in the low effectiveness of either single chemotherapeutics or classical Immunotherapy by Checkpoints Inhibitors (ICI) administration during this oncological pathology. For a further and more precise description of the CRC origin and development, including a role for both immune response and inflammation, please see [4,5].

### **Conventional treatments for colon cancer and mechanisms of action from a genetic perspective**

Colorectal cancer (CRC) is one of the deadliest diseases in the world. Despite advances in diagnosis, treatment strategies remain an essential bottleneck affecting survival, in which the pathological stage represents the most important prognostic factor for patients for this neoplasia. The accurate classification of lesions is the primary tool to decide the most appropriate treatment and therapy [6]. The treatment for early-stage CRC (stage I and stage II) currently consists of resectioning the tumor area with regional lymph nodes, with a 5-year disease-free survival rate of 95% [7]. In the advanced stage of the disease (stages III and above), the rate of disease-free survival drops from 90 to 50% for surgery alone, requiring the administration of chemotherapeutics, and only 17%-20% of these patients ultimately survive [8,9].

5-Fluorouracil (5-FU) has been central in treating advanced colorectal cancer since 1957. Unfortunately, the response rate to 5-FU as the first-line chemotherapy in advanced CRC is still only 10–15%. In contrast, 5-FU combined with other anticancer drugs as adjuvants, such as leucovorin and oxaliplatin (FOLFOX), and 5-FU leucovorin calcium together with irinotecan (FOLFIRI) increases the effectiveness of 5-FU by 50% [10,11].

5-FU is the third most commonly used chemotherapeutic agent for the treatment of solid malignancies worldwide [12]. Heidelberger synthesized it in the early 1950s as a derivate of fluoropyrimidines. This drug was one of the first chemotherapeutics reported to have anticancer activity. It was tested in diverse tumors in rats and mice, where it significantly reduced tumor burden. Additionally, tumoral tissues incorporated this compound more rapidly than normal tissues, which pointed to its potential use as a chemotherapeutic drug [13,14]. In 1962, the Food and Drug Administration (FDA) approved the use of 5-FU for treating colorectal cancer.

In intravenous administration, 5-FU is incorporated rapidly into the cells through facilitated transport as uracil [15]. Subsequently, its metabolism can be driven in two ways, i.e., *via* anabolic and catabolic routes, which compete with each other. In sensitive cancerous cells, the anabolic pathway leads to the conversion of this drug into several active metabolites, such as fluorodeoxyuridine monophosphate (FdUMP), fluorodeoxyuridine triphosphate (FTP), and fluorouridine triphosphate (FUTP) [16]. The active metabolites interfere with nucleoside metabolism and can be incorporated into RNA and DNA, leading to cytotoxicity and cell death [17,18]. This mechanism is due to its similar structure to pyrimidine, molecules of DNA and RNA, an analog of uracil with a fluorine atom at the C-5 position in place of hydrogen [19]. FdUMP disrupts the function of thymidylate synthase (TS), a key enzyme responsible for providing deoxynucleotides triphosphates (dNTPs), which are necessary for DNA replication and repair, catalyzing the reaction of deoxyuridine monophosphate (dUMP) to dTMP synthesis [14,15]. An insufficiency in dTMP leads to the depletion of dTTP, which perturbs the levels of the other dNTPs [16,20] (Figure 1).

5-FU has primarily been used in the treatment of solid cancers of digestive origin, such as colorectal, anal, pancreatic, esophageal, gastric, and ampullary tumors, and less frequently, in breast, cervix, and head and neck cancers [21–23]. CRC treatment includes various chemotherapeutic drugs to manage this neoplasia. As the backbone of treatments, 5-FU has been used for more than five decades, and more recently, it has been combined with other chemotherapeutic drugs to potentiate its anticarcinogenic effect [22,24].

The use of alternative broad-spectrum chemotherapeutics in addition to 5-FU has been proposed for colon cancer treatment. Doxorubicin treatment, combined with other drugs, such as metformin and sodium oxamate, reduces the proliferation rate of colon cancer cell lines *in vitro* [25]. However, the use of doxorubicin in patients is limited in the first instance by the side effects frequently associated with this drug, such as hepatotoxicity, nephrotoxicity, pulmototoxicity, and cardiotoxicity [26,27]. Additionally, doxorubicin can lead to chemoresistance in tumoral cells through nuclear factor kappa B (NFkB) translocation to the nucleus and DNA binding because of the damage induced by this drug, triggering the expression of antiapoptotic genes [28].

In CRC, NF-κB nuclear translocation is a characteristic in more than 70% of patients, limiting the use of doxorubicin [29]. Another disadvantage of this drug is its anthracycline nature since it is extracted from *Streptomyces* spp. Cancer cells frequently show rapid resistance to naturally occurring cancer drugs, diminishing their effectiveness, whereas they are more sensitive to antimetabolites, such as 5-FU and cisplatin, among others [30]. Outstanding the use of other chemotherapeutics, such as tamoxifen, which is highly effective and frequently used in breast cancer treatment, has an adverse effect in the treatment of CRC [31]. Due to the molecular characteristics of each type of cancer, the successful use of tamoxifen in breast cancer lies in its mechanism of action. Approximately 80% of all breast cancers are positive for the estrogen receptor (ER), and tamoxifen inhibits the expression of estrogen-regulated genes by the competitive inhibition of this receptor. Different reports indicate that

tamoxifen has the opposite effect on CRC, increasing the risk of developing this type of cancer [31,32].

### **Molecular perspective for the use of chemotherapeutic strategies in colorectal cancer treatment; which is the right drug?**

CRC is a molecularly heterogeneous disease in which genetics and cellular events accumulate to endow tumor cells with aggressive characteristics, including chemotherapy resistance. Chromosomal instability, mismatch repair, and methylator phenotype are the three major pathways involved in acquiring tumorigenesis and a malignant phenotype and could be present in sporadic and inherited neoplasia [33,34]. The choice of better therapy is based on cancer-related features and patient-related factors, such as the <sup>8</sup> number and localization of metastases, tumor progression, presence or absence of biochemical markers, and comorbidity<sup>[35–37]</sup>. Despite all these characteristics, treatment based on the antineoplastic effects of 5-FU is the cornerstone of therapy in advanced CRC stages.

Treatment with 5-FU in combination with other drugs, such as oxaliplatin, irinotecan, capecitabine, bevacizumab, cetuximab, panitumumab, ziv-aflibercept, regorafenib, and ramucirumab, increases its effectiveness and has been approved by the FDA for the management of CRC [38]. Thus, during stages III or IV in resected CRC patients, the use of combination treatment, such as 5-FU plus folinic acid, oxaliplatin (FOLFOX), or irinotecan (FOLFIRI), is common as a first-line treatment. This strategy significantly increases the survival rate of these patients [39]. The initial chemotherapy scheme and the decision on better combinatory drugs depends on multiple conditions in the patients: (1) In metastatic CRC limited to the liver or lung, surgery and the rapid initiation of chemotherapy appears to be the best option, hoping for a good response from the patients; when CRC cure is not possible, three additional scenarios can arise: (2) patients with advanced tumors and symptoms require rapid tumor shrinkage to provide palliation, which begins with chemotherapy; (3) asymptomatic patients with bulky tumor and possible rapid progression are likely to become symptomatic in a short period; (4) patients without symptoms but disseminated disease who never had



resectable disease but whose tumors remain nonbulky are likely to remain asymptomatic for an extended period. In the last two scenarios, the initiation of chemotherapy can be discussed<sup>[22,40]</sup>. Previous work in the Nordic population demonstrated that early treatment with 5-FU plus leucovorin in asymptomatic patients with advanced CRC prolonged survival and delayed both disease progression and the onset of symptoms<sup>[41]</sup>. In another study in Australasian and Canadian populations of asymptomatic patients using the same chemotherapy regimen, no difference was reported between early or delayed chemotherapy use until symptoms appeared<sup>[42]</sup>. Thus, clinical treatment requires medical discretion and the patients' preference when cure is not possible. The spectrum of molecular alterations that offer alternative management for this disease could be explored.

Alterations in genes related to survival, angiogenesis, proliferation, and apoptosis incorporate additional strategies into CRC treatment. The RAS, KRAS, and NRAS genes play an essential role as prognostic and predictive indicators in CRC treatment<sup>[43–45]</sup>. Mutations in the DNA at position 12 in the KRAS protein are significantly associated with a poor prognosis: a 5-year survival rate of ~3%<sup>[46]</sup>. Patients with this mutation are not candidates to receive treatment with monoclonal antibodies, such as cetuximab or panitumumab, which target the epidermal growth factor receptor (EGFR)<sup>[47–50]</sup>. Blocking EGFR represents the second line of treatment in patients with RAS without mutation, together with the backbone FOLFIRI therapy. Therefore, the patient's genetic and tumor-specific factors need to be considered when choosing chemotherapeutic and combination schemes to avoid resistance and undesired responses to these therapies. Drug resistance and consequent therapy failure are the main problems clinicians face in treating different neoplasias, limiting the quality of life and long-term remission rates as a consequence of tumor growth and spreading, leading to 90% of patients dying<sup>[51,52]</sup>. Drug resistance is a highly complex process that is commonly classified into two types: intrinsic and acquired resistance. Both types of drug resistance lead to the regulation of molecular mechanisms of chemoresistance, such as the activation of transporter pumps,



oncogenes, tumor suppressor genes, mitochondrial alteration, DNA repair, autophagy, epithelial-mesenchymal transition (EMT), cancer stemness, and exosomes [53,54].

In the intrinsic phenotype, diverse alterations existing before drug administration in the patient complicate the selection of chemotherapy. The inherent genetic mutations in tumors, such as a KRAS mutation in exon 2 in codon 12 or 13, are the most frequent mutations associated with poor prognosis and drug resistance in CRC [55,56]. Therapies based on the first line of treatment using FOLFOX or FORFIRI plus cetuximab or panitumumab (anti-EGFR) are ineffective in patients with KRAS mutations [57]. Recent studies indicate that mutations in genes related to the pathways that regulate tumor cell survival and proliferation and inhibit apoptosis in tumoral cells, such as AKT1 and CTNNB1, and contribute to 5-FU chemotherapeutic resistance in CRC. The CTNNB1 gene encodes the b-catenin protein, which plays a crucial role in cancer by activating the Wnt/b-catenin signaling pathway. This pathway is associated with tumorigenesis and CRC resistance, and it upregulates genes and proteins, such as multidrug resistance gene (MDR1) and inhibitor of apoptosis (Bcl2), to induce epithelial-mesenchymal transition (EMT) and regulate the tumor microenvironment [58–60].

### **Is chemotherapy an inducer of immunosuppression in colorectal cancer?**

The heterogeneity of tumors, including CRC, consists of heterogeneity in cancer and infiltrated resident host cells, extracellular matrix, and immune and inflammatory cells, such as <sup>1</sup>macrophages, dendritic cells (DCs), myeloid-derived suppressor cells (MDSCs), T cells, mast cells, and natural killer (NK) cells. These components comprise tumor microenvironment (TME), which has a dynamic composition [61]. <sup>1</sup>It is well known that one of the main functions of the TME is to provide a protective function for tumor cells, inducing crosstalk between immune and nonimmune cells that leads to tumor-mediated immunosuppression, supporting tumor growth and survival [62]. Recent reports indicate that the TME in CRC contributes to cancer progression and drug resistance through high interstitial pressure, fibrosis, and the degradation of the

therapeutic agent by enzymatic activity and inducing immunosuppression [61,63,64]. These findings indicate that the regulation of immune cells surrounding the tumor has a critical role in the response to therapies for CRC. Thus, chemotherapy and immunotherapies targeting the recovery activity of immune cells are likely necessary to fight CRC.

The study of the effect of chemotherapeutic drugs on immune cells is controversial. The central concept here is that chemotherapy reduces the capacity of the immune system to function, but how could a drug affect the capacity and efficiency of the immune response to induce an efficient posttreatment response? Perhaps the "original" concept has a flawed approach. Evidence suggests that after 5-FU treatment in a mouse model, bone marrow cellularity decreases, but platelets and thrombopoietin, which are close to the immune response, rebound [64,65]. Similarly, the serum of patients diagnosed with stage III/IV CRC who had received FOLFOX chemotherapy showed increased levels of heat shock protein 70 (HSP70), which belongs to the Damage-Associated Molecular Patterns (DAMPs) recognized by innate receptors [66]. Later, in vitro studies showed that the supernatants of dying CRC cells treated with oxaliplatin (OXA) and 5-FU induced a mature phenotype in dendritic cells coexpressing HLA-DR, CD80, and CD86 and producing IL-1 $\beta$ , TNF- $\alpha$ , and MIP-1 $\alpha$  in a TLR-4-dependent manner [66]. These results strongly suggest that OXA/5-FU treatment induces the activation of the innate immune response during CRC. Additionally, increased numbers of myeloid-derived suppressor cells (MDSCs) have been reported in a mouse model of thymoma, and treatment with 5-FU combined with gemcitabine selectively induced apoptosis in MDSCs. Consequently, increased antigen-specific T-CD8<sup>+</sup> cells produced more IFN- $\gamma$ , generating a T-cell antitumor response (Figure 3) [66].

Conversely, high levels of the chemokine CCL20 recruit Treg cells in CRC patients resistant to FOLFOX [67]. However, in blood samples from metastatic FOLFOX-sensitive CRC patients, a reduced percentage of Foxp3<sup>+</sup> Treg cells was recorded after treatment [68]. Therefore, the increase in Treg cells is associated with 5-FU chemoresistance. Thus, evidence of 5-FU chemotherapy suggests a role in the specific and direct reduction of

suppressive immune cells during CRC. Additionally, the increased apoptosis-induced death of tumor cells by 5-FU could increase the ability of immune cells to recognize DAMPs released by these dying tumor cells, inducing protective inflammation. Evidence needs to be accumulated in this field to clarify whether chemotherapy may be an inducer of immune cell activation (Figure 2).

### **Classical immune checkpoint inhibitors (ICIs) used as monotherapy during CRC.**

The effectiveness of ICI as an immunotherapy treatment has been evaluated in the last decade. The efficacy of these agents is evident in liquid tumors, such as melanoma, leukemia, and solid non-small cell lung carcinoma (NSCLC). Classical ICIs used to treat these oncological pathologies are anti-PD1, anti-PDL1, and anti-CTLA4 monoclonal antibodies, where anti-CTLA4 has a lower clinical efficacy [68,69] (Table 1).

Diverse reports suggest single or combined therapy using different antibodies targeted against the same or another molecule. In melanoma therapy, nivolumab, a human IgG4 anti-death 1 pathway (PD1) monoclonal antibody, results in an overall survival rate of 72.9%, whereas the antineoplastic chemotherapeutic dacarbazine resulted in a survival rate of 42.1% [69]. In metastatic melanoma refractory to chemotherapy, treatment with ipilimumab, an anti-CTLA4 antibody, shows high efficacy when combined with anti-PD1 antibodies, either pembrolizumab or nivolumab [70]. Additionally, nivolumab and ipilimumab combination treatment prolongs progression-free survival, mainly in patients with tumors testing positive for PDL1 expression [71]. Although *in vitro* studies suggested that atezolizumab, avelumab, and durvalumab, all anti-PDL1 antibodies, more effectively block PD1/PDL1 signaling (Figure 3) [72], evidence of the use of anti-PDL1 antibody as a single approved treatment without immunotherapy or chemotherapy combination is insufficient to conclude their role in inducing protection in metastatic melanoma [73]. Immunotherapy with the anti-PDL1 antibody atezolizumab has been approved to treat NSCLC combined with chemotherapy, and anti-PDL1 has been approved to treat triple-negative breast cancer [74,75]. Therefore, increased PDL1 expression in the tumor microenvironment of melanoma patients is an efficient marker

to predict response to anti-PD1 treatments, which must be applied in all types of cancer when this immunotherapy is suggested [76].

Notwithstanding the successful use of immunotherapy in the neoplasias mentioned above, few reports show an influential role for classical monoclonal ICI using anti-CTLA4 and anti-PD1/PDL1 axis antibodies in CRC. Most clinical assays were disappointing. For example, tremelimumab, a human fully IgG2 anti-CTLA4 antibody, did not produce clinically meaningful results when it was used as a monotherapy in patients with refractory metastatic CRC [77] (Table 1).

Another monotherapy treatment using nivolumab in CRC patients with microsatellite instability and FOLFOX chemoresistance showed excellent control of the disease, and patients tolerated this treatment well [78]. CRC patients treated with nivolumab combined with an oral HSP90 inhibitor showed safety profiles and antitumor activity associated with reduced activity of Treg cells and better response of tumor-infiltrating lymphocytes [79]. Interestingly, a report of a woman treated with nivolumab for melanoma with no hereditary CRC background developed colon carcinoma after seven years of anti-melanoma treatment. The medical service then decided to switch the treatment to ipilimumab, and after four cycles of monotherapy, the colon tumor was in complete remission [80]. Pembrolizumab used as monotherapy in either microsatellite instability-high or mismatch repair-deficient CRC patients produced improvements in health-related quality of life compared to patients treated with leucovorin, fluorouracil, and oxaliplatin (mFOLFOX6) [81]. In one study of refractory mismatch repair, CRC patients treated with pembrolizumab as monotherapy plus maraviroc, an agonist of CCR5 that promotes the activation and recruitment of macrophages inducing immune cell infiltrate in tumors, showed a beneficial toxicity pattern. The overall survival (OS) was higher than expected [82].

ICI with avelumab monotherapy in unresectable metastatic CRC patients who failed FOLFOX chemotherapy showed an overall survival of 72.2% at 8.1 mo, which was similar to the effect of ICI monotherapy using either pembrolizumab or nivolumab. However, some patients showed treatment-related adverse events [83]. Durvalumab

used as monotherapy in microsatellite-instability high/mismatch repair-deficient metastatic CRC patients whose disease had progressed after chemotherapy showed efficiency and a satisfactory progression-free survival of 58.2%; however, side effects were found in 36.4% of patients [84].

Taken together, these results suggest that contrary to anti-CTLA4, ICI monotherapy blocking the PD1/PDL1 axis has a better effect in high microsatellite instability/mismatch repair-deficient metastatic CRC patients who previously displayed chemoresistance. Additionally, combining ICI PD1/PDL1 monotherapy with either antibodies or immune cell stimulators improves treatment efficacy. However, only a small number of clinical trials show increased OS (Table 1). Most likely, the tumor microenvironment reduces the access of ICI antibodies to the target molecules expressed in either immune or epithelial cells (Figure 2).

#### **Does combined immunotherapy increase the effectiveness of treatment for CRC?**

Little evidence of the apparent effect of ICI monotherapy on CRC development is available. Conversely, increasing evidence suggests a better outcome using combined ICI, i.e., the use of two monoclonal antibodies targeting CTLA4 or the PD1/PDL1 axis. To improve the immune response, combination treatment with tremelimumab and durvalumab was used in patients for the preoperative management of resectable CRC and liver metastases. These patients improved their OS to 24.5 mo; interestingly, their CD4<sup>+</sup>, CD8<sup>+</sup>, and B cells displayed an activated profile [85]. Additionally, first-line treatment consisting of nivolumab plus low-dose ipilimumab treatment in patients with microsatellite instability and metastatic CRC without previous chemotherapy showed that this combination was well tolerated at the primary endpoint, with robust and durable clinical benefit. However, the study is ongoing, and OS data are not yet available [86]. Previous ICI studies using similar inclusion criteria and antibody doses showed that follow-up over 12 mo of combined treatment resulted in 85% OS [87]. Recently, the combined use of ipilimumab plus nivolumab before surgery in either mismatch repair-deficient or mismatch repair-proficient CRC patients induced an antitumoral response associated with a lack of signs of cancer after surgery with

increased infiltration of CD8<sup>+</sup>PD1<sup>+</sup> cells; the authors suggested that this combined ICI therapy may be the standard treatment for mismatch repair-deficient CRC patients [21].

Additionally, circulating tumor DNA detection in the blood of patients with durable and ongoing responses to ipilimumab plus nivolumab could be used as a monitoring response and dynamic marker of this combined ICI treatment [88,89]. Finally, a study attempted to analyze whether pseudo progression is observed in mismatch repair-deficient CRC patients treated with nivolumab plus ipilimumab, showing that this treatment rarely induces

and confirming a high disease control rate of 86% [90]. Taken together, this evidence strongly suggests that the combined ICI blockade of CTLA4-PD1/PDL1 in mismatch repair-deficient CRC patients is highly successful. However, these patients represent only a tiny fraction of the all CRC-diagnosed patients. Therefore, more clinical trials must be developed to obtain sufficient evidence to conclude the positive effects of the classic ICI combination.

#### **Use of either monotherapy or combined immunotherapy plus chemotherapy to increase the effectiveness of treatments for CRC**

The combined use of atezolizumab plus aobimetinib, a MAPK/ERK kinase (MEK) 1 and 2 inhibitor that increases CD8<sup>+</sup> cell infiltration in tumors of patients with microsatellite-stable metastatic CRC, showed no improvement in OS and was consistent with the safety of using both drugs [91] (Table 1). A similar effect showing only acceptable tolerance to this treatment was observed with durmalumab plus MEKi, an inhibitor of MEK, in microsatellite stable metastatic CRC patients [89]. No improved OS was observed using atezolizumab combined with cobimetinib in metastatic CRC patients [92]. A multicenter phase I/II study in June 2017 aimed to analyze the role of durmalumab plus tremelimumab combined with FOLFOX chemotherapy in patients with metastatic CRC, expecting a 6-month progression-free survival of over 70.7%; however, the authors do not have the final results to date [93]. Recently, the combination of atezolizumab with FOLFOX chemotherapy plus bevacizumab (monoclonal anti-VEGF antibody) in mismatch repair metastatic CRC patients induced a median

progression-free survival of 13 mo, while FOLFOX plus bevacizumab alone resulted in 11 mo of progression-free survival, suggesting that the addition of atezolizumab improves progression-free survival. However, 42% of patients showed neutropenia, and 27% displayed severe adverse events [94]. The same treatment consisting of atezolizumab combined with FOLFOX plus bevacizumab was used in patients with untreated unresectable metastatic CRC, showing no improvement in OS and safety signals [95]. The use of the anti-epidermal growth factor receptor (EGFR) antibody cetuximab plus avelumab for treating RAS WT metastatic CRC patients was safe; the authors suggested that an analysis of circulating DNA in plasma could be an indicator of the positive effects of this treatment. However, the data are insufficient to show the impact on the OS rate [96]. Treatment with cetuximab plus the ICI avelumab in microsatellite stable metastatic CRC patients showed that subclones of tumors expressing PDL1 mutations mediate the resistance to direct avelumab antitumor effects but also increase T-cell killing [97]. An analysis of the neutrophil-to-lymphocyte ratio (NLR) in the blood of chemo-refractory metastatic CRC patients treated with cetuximab plus showed that a high NLR is a poor prognostic factor. Thus, the NLR could also be a predictor for the effectiveness of the combined ICI cetuximab plus avelumab [98]. Regorafenib, an inhibitor of protein kinases in tumoral angiogenesis used in combination with avelumab in microsatellite stable CRC patients, showed increased infiltration of T-CD8<sup>+</sup> cells associated with better outcomes, with an OS of 10.8 mo [99]. Metastatic CRC patients who previously received two radiotherapies and who were treated with durvalumab plus tremelimumab before the third round of radiotherapy showed increased circulating, differentiated, and proliferating T-CD8<sup>+</sup> cells, but the authors concluded that this finding does not meet the prespecified endpoint criteria to consider this combined ICI plus radiotherapy worthwhile for further study; specifically, the authors suggest an objective response rate at least of 25%, but they only obtained a response rate of 8.3% [100]. The addition of FOLFOX-based chemotherapy to avelumab plus an adenovirus vector vaccine capable of inducing a CD4<sup>+</sup>/CD8<sup>+</sup> T-cell response in mismatch repair-deficient microsatellite instability-high metastatic CRC patients



showed no improvement in progression-free survival <sup>[101]</sup>. However, The Canadian Cancer Trials Group suggests that combining tremelimumab and durvalumab to treat patients with high microsatellite instability who had previously received chemotherapy (fluoropyrimidines, oxaliplatin, irinotecan, and others) may prolong OS. They correlated the increased effectiveness of this immunotherapy combination with the tumor mutation burden elevated in plasma <sup>[102]</sup>.

It is essential to mention that some research about the combination of chemotherapy and ICI is under development <sup>[93]</sup>. For example, a phase II trial in 2020 will show whether atezolizumab combined with oxaliplatin, radiotherapy, and bevacizumab may increase progression-free survival in microsatellite instability CRC patients <sup>[103]</sup>. Additionally, in microsatellite instability-high metastatic CRC patients with deficient mismatch repair, a study is currently underway to prove the improvement of disease-free survival by ICI with avelumab plus fluoropyrimidine; the authors suggest that this ICI plus chemotherapy treatment will improve the expected 3-year disease-free survival rate by 12% <sup>[104]</sup>. Evidence showing that combining ICI with chemotherapy improves treatment efficacy continues to accumulate. Most clinical trials reported here are recent, and perhaps the evidence is insufficient to conclude that a treatment criterion has already been established, but the bases are under construction. Consequently, evidence supports the hypothesis that the use of classical ICIs improves chemotherapy treatment, mainly in CRC patients with high microsatellite instability. It is crucial for patients who do not have a good prognosis with chemotherapy alone to have a better response with the combination of classical ICI antibodies.

### **Multomics, individualized immunotherapy, adoptive transfer of "trained" immune cells, and nonclassical ICIs are a new hope for CRC**

In recent years, mechanisms have begun to be developed to understand and explain why, in some cases, classical immunotherapy is sufficient to generate benefits in some patients. Multiple factors participate in the development of any pathology, such as the patient's clinical history, genetics, and, excitingly and prominently, the ability of their immune cells to act during cancer. We emphasize the importance of the recruitment of

immune cells to the tumor microenvironment; in the case of melanoma, non-small cell lung cancer, and leukemia, the capacity of the immune cells to access is greater, which, together with ICI, increases the bioavailability of monoclonal antibodies to find the antigens expressed in the required enclosures to be detected and removed or blocked [105]. In contrast, other types of oncological pathologies are available when access to the tumor site is more difficult for both immune cells and classical ICI antibodies, such as CRC [106]. In addition, the causes of CRC are multiple, and we cannot attempt to generalize a unique treatment for all varieties of CRC to reduce the statistics of this oncological pathology on the rise.

The single-cell multiomics technique has begun to shed light on the complexity of the individual immune response elicited against any agent. This approach facilitates the individual characterization of groups of cells by identifying the gene transcripts at a specific time [107]. This technique depends on the efficiency of flow cytometers to distinguish and separate individual cells, the equipment used to amplify mRNA transcripts and synthesize complementary strand DNA (cDNA), and sequencing equipment, allowing for robust data with high precision and certainty [108]. The advancement of these latest-generation technologies allows not only the expression of the classic ICI (CTLA4, PD1/PDL1) to be distinguished but also the characterization of mRNA transcripts in specific immune or epithelial cell populations at a particular time. These transcripts, already expressed as proteins, can individually be proposed as new nonclassical ICIs in patients [109], generating a wide range of therapeutic targets that, as in the case of CRC, increase the efficiency of previous treatments.

Some surface molecules involved in suppressive functions for T-activated cells, regulatory T cells, macrophages, neutrophils, and epithelial cells have been proposed as immunotherapy targets. GITR, TIM3, LAG3, CD39, CD73, CD47, and SIRP-1 $\alpha$  (a do not eat me signal) [110] warrant evaluation as a monotherapy or combined therapy in CRC.

T-cell immunoglobulin and mucin domain 3 (Tim-3) is an overexpressed inhibitory receptor in active immune cells, including myeloid and lymphoid cells, with suppressive and modulatory characteristics. It is relevant in reducing IFN- $\gamma$  production

by Th1 cells after binding to its ligand, galectin-9 [111]. Tim-3 is also overexpressed by Treg cells in a colitis-associated colon cancer mouse model [112]. The combination of Tim-3 with anti-PD1 ICI antibodies is a good prospect in a murine breast cancer model [113]. Sabatolimab, an anti-Tim-3 antibody, has already been used as a treatment in ovarian, CRC, and non-small cell lung cancer combined with anti-PD1 antibody, being well tolerated and improving antitumor activity [114]. Glucocorticoid-induced TNFR-related protein (GITR) has a role in the immunomodulation of effector T cells and increases tumor resistance [113,115]. Treatment with anti-GITR antibody combined with pembrolizumab improves the disease control rate compared with anti-GITR used as monotherapy for treating CRC, melanoma, and adrenal carcinoma [116].

Lymphocyte activation gene-3 (LAG3 or CD223) has a structure similar to that of the CD4 molecule, joining MHC-II in antigen-presenting cells. However, two of their immunoglobulin-like domains can bind receptors in tumoral cells [81]. Blockage of LAG3 induces increased IL-2 production and enhances T-cell proliferation [117]. Therefore, LAG3 has been proposed as an ICI; the genes LAG3 and IDO1 were shown to be overexpressed in a phase II study of pembrolizumab use as ICI monotherapy in patients with esophageal squamous cell carcinoma; the authors suggested that a combination of ICIs is needed to induce immunity against this tumor [118]. CD73 is an extracellular adenosine receptor expressed in immunosuppressant cells (such as Treg cells), favoring tumor progression [119]. Recently, single-cell RNA sequencing in a colitis-associated CRC murine model showed that an anti-CD73 antibody has a significant role in improving the anticancer functions of Treg cells, and exhausted T-CD8<sup>+</sup> cells became activated T-CD8<sup>+</sup> cells. In contrast, anti-PD1 antibodies in the same model deplete Treg cells and M2 macrophages [120], suggesting a synergistic role for the new ICI anti-CD73 that may improve the positive effects of anti-PD1 monotherapy. *Ex vivo* samples of blood and tumors from microsatellite instable CRC patients showed that atezolizumab alone could reactivate T cells. Furthermore, adding tiragolumab, an anti-TIGIT antibody, restored intraepithelial T-CD4 and T-CD8 cell function by favoring IFN- $\gamma$  and TNF- $\alpha$  production [120,121]. TIGIT is a receptor upregulated in NK and activated T cells when the

modulation of their effector abilities is necessary for the microenvironment, such as cancer. It is also overexpressed in Treg cells [122]. The use of avelumab plus the adoptive transfer of autologous dendritic cell vaccine in chemotherapy-treated mismatch repair-proficient metastatic CRC patients had a successful result because this treatment was well tolerated. Furthermore, treatment was terminated early because 11% of patients were disease free at six months, and progression-free survival was increased by 40% [123]. This evidence also suggests that new ICI research could open other possibilities for specific and beneficial treatment for CRC patients, probably because either immune or epithelial cells may express nonclassical ICI molecules (Figure 2).

## **CONCLUSION**

Chemotherapy using 5-FU remains the primary treatment for CRC, despite its high toxicity and low efficacy. New strategies targeting ICI have been useful in some oncological pathologies; however, evidence showing the effectiveness of classical ICI monotherapy in CRC is scarce. A combination of classical ICI antibodies targeting CTLA4 and PD1/PDL1 molecules showed stronger efficacy for CRC treatment. Finally, classical ICI plus conventional chemotherapy is effective, as evidenced by increased OS, but these strategies are not yet well established, and some clinical studies are ongoing. Evidence suggests that chemotherapy produces neoantigens, increasing tumor immunogenicity that may activate immune responses [124]. This increased immunogenicity is likely the reason for a better response when classical ICI plus chemotherapy is used and may represent a pathway to design new therapeutic strategies aimed at improving the response in CRC patients based on immunological reactivation combined with conventional chemotherapy. Knowledge of the tumor microenvironment in CRC is essential to understand immunosuppression. New options for nonclassical ICIs obtained by single-cell sequencing are shedding light on this area and will probably improve the effectiveness of many treatments.

We are possibly on the verge of major findings in the study of CRC, where the immune response will continue playing a leading role and where new proposals with

nonclassical ICIs may reduce the current statistics and poor prognoses for this oncological pathology.

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