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Colorectal Cancer Immunotherapy: State of the Art and Future Directions

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Abstract

Cancer immunotherapy has become an indispensable mode of treatment for a multitude of solid tumor cancers. Colorectal cancer (CRC) has been one of the many cancer types to benefit from immunotherapy, especially in advanced disease where standard treatment fails to prevent recurrence or results in poor survival. The efficacy of immunotherapy in CRC has not been without challenge, as early clinical trials observed dismal responses in unselected CRC patients treated with checkpoint inhibitors. Many studies and clinical trials have since refined

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immunotherapies available for CRC, solidifying immunotherapy as a powerful asset for CRC treatment. This review article examines CRC immunotherapies, from their foundation, through emerging avenues for improvement, to future directions.

Keywords

Colorectal Cancer; Immunotherapy; Microbiome; Immune Checkpoints; Cancer Genomics

Introduction

Colorectal cancer (CRC) is the third most prevalent cancer worldwide and second in cancer-related mortality in both men and women.[1] Fortunately, early screening and advances in treatment have helped to decrease overall CRC-related incidence and mortality. However, a recent study has found shifts in CRC incidence, with more frequent diagnoses in younger patients (ie, under 50 years of age) and those with more advanced disease stages.[2] CRC is associated with a highly disparate 5-year relative survival rate, from 90% in localized CRC to about 15% in distant or metastatic CRC (mCRC).[3] For the last 2 decades, the mainstay of CRC treatment has been chemotherapy with 5-fluorouracil together with oxaliplatin and/or irinotecan.[4] Subsets of CRC patients may also benefit from monoclonal antibodies (mAbs) against vascular endothelial growth factor or epidermal growth factor receptor.[5] The emergence of cancer immunotherapy in the last decade has expanded the available and effective treatment options for select individuals with CRC—beyond the standard of care: surgery, chemotherapy, and radiation. CRC immunotherapies consist of neoantigen-based or cell-based vaccines, checkpoint immunotherapy, microbiome-based therapeutics, and multimodal therapy with cancer immunotherapy. Despite the proven advantage of immunotherapy in CRC, only specific subsets of patients with certain tumor profiles can benefit from current immunotherapies; thus, the need for more inclusive immune-based treatments to accommodate all CRC patients is of high interest. Tumor biomarkers have been an important tool for identifying patients likely to benefit from CRC immunotherapy by predicting an individual patient's responsiveness and even acting as prognostic markers. Significant CRC biomarkers include tumor sidedness (the origin of primary tumor formation, either the right- or left-side of the colon), DNA mismatch repair (MMR) system competency, neoantigen load, microsatellite status, and/or tumor mutation burden (TMB). This review explores the molecular-, microenvironmental-, and clinical-based rationales behind CRC immunotherapy and provides a comprehensive summary of the current methods and future directions of this important cutting-edge treatment (Figure 1).

The Impact of Genomics on CRC Immunotherapy

A key feature differentiating CRCs that respond well to immunotherapy from those that respond poorly is the integrity of their genomes.[6–8] This is determined by measuring the capacity of different CRC subtypes to carry out DNA repair which, in turn, governs their level and type of genomic instability.[9–12] Four subtypes have been identified using the consensus molecular subtype (CMS) classification[10] (Figure 2). CMS 1, or the immune subtype, comprises 14% of CRCs, with 76% of CMS 1 tumors harboring microsatellite

instability (MSI). CMS 2, or the canonical subtype, encompasses 37% of CRCs, but only 2% of CMS 2 tumors display MSI. CMS 3, the metabolic subtype, is 13% of CRCs, while CMS 4, the mesenchymal subtype, accounts for 23% of CRCs. Both CMS 3 and 4 display a microsatellite stable (MSS) phenotype, representing 84% and 94% MSS-based tumors, respectively. High MSI is a consequence of deficient MMR (dMMR), leading to a hypermutable phenotype generally associated with effective immunotherapy responses. MMR proteins such as mutL homolog 1, mutS homolog 2, PMS1 homolog 2, and mutS homolog 6 function to repair single-base nucleotide errors, such as insertions or deletions that arise during DNA replication. dMMR is found in Lynch syndrome (caused by germline inactivating mutations in MMR genes) or in approximately 15% of sporadic CRCs (typically caused by mutL homolog 1 promoter hypermethylation).[13] Tumors harboring a dMMR phenotype exhibit DNA MSI-H and consequently high mutational burden (ie, >12 mutations per 10^6 DNA bases) compared to proficient MMR (pMMR)- MSS tumors (ie, <8.24 mutations per 10^6 DNA bases).[14]

CMS 1 CRCs contain abundant point mutations in repetitive nucleotide tracts throughout the genome. These hypermutable cancers inherently contain abundant $CD8^+$ tumor-infiltrating lymphocytes (TILs), provide patients with a better survival rate than other CRC subtypes, and are the only CRC subtype that reliably responds to checkpoint immunotherapy.[7] In contrast, most other CRCs possess chromosomal instability, leading to large but relatively few chromosome rearrangements. The prevailing explanation for the improved antitumor immune response in dMMR-MSI-H CRCs is that their large number of mutations give rise to many aberrant proteins that can act as neoantigens, thereby increasing the chance of TIL recognition. Ample evidence supports the link between a high TMB and tumor immunogenicity.[15–20] However, evidence also exists that a high TMB and abundant neoantigens are often insufficient to activate antitumor immunity.[21] For example, late stage MSI CRCs are refractory to immunotherapy, and MSI cancers in many other tissue sites do not provide patients with a better prognosis and remain unresponsive to checkpoint inhibitors.[22–27] These findings suggest that cancer response to immunotherapy depends on both their level of neoantigen production as well as on other factors inherent to the tumor, some of which are controlled genetically.

One important genetic determinant of CRC immunogenicity is the tumor's capacity for DNA repair.[7,11,28] In MSI CRCs, hypermutability leads to fragments of DNA leaking into the cytosol of the tumor cells (Figure 3). This initially results in the activation of antiviral sensing immune pathways, especially the cyclic guanosine monophosphate–adenosine monophosphate synthase (cGAS)/stimulator of interferon genes (STING) pathway, and subsequently leads to the production of type I interferons (IFN) by the cancer cells.[29–33] The type I IFN then acts in an autocrine manner to induce the expression of a group of interferon-stimulated genes ISGs() that promote antiviral immunity. Among these are genes for numerous chemokines, such as chemokine ligand 5 (CCL5) and C-X-C motif chemokine ligand 10 (CXCL10), which then facilitate recruitment and infiltration of $CD8^+$ T cells into the tumor microenvironment (TME).[31] This mechanism explains the high levels of $CD8^+$ TILs inherent in MSI CRCs and has been shown to be essential to the improved prognosis of these cancers. Furthermore, given that the infiltration of tumors by TILs is a prerequisite for effective checkpoint inhibitor therapy, this mechanism explains the high

response rates of MSI CRC patients to such treatments. Notably, many non-MSI CRCs have defects in other DNA repair pathways.[11] While loss of different repair pathways appears to have different consequences on CRC immunogenicity, they all result in increased genetic instability and a generally higher level of antitumor immune activation than in CRCs with intact DNA repair.[34] A further implication of the impact of genomic instability on antitumor immunity in CRC is that many chemotherapeutic treatments currently in clinical use to treat CRC, such as the FOLFOX or FOLFIRI regimens, induce DNA damage that can activate the cGAS/STING pathway.[35–38] This implies that combinatorial treatments of chemotherapeutic regimens or radiotherapy with immune checkpoint inhibitors (ICIs) could potentially improve the prognosis of many CRC patients.[39]

A second genetic determinant of CRC immunogenicity is the constellation of oncogene and tumor suppressor gene (TSG) mutations that frequently occur in CRCs.[40–43] This is highly relevant because it has become clear that some cancer driver mutations exert powerful immunoregulatory effects on the TME over and above their direct growth-promoting roles in cancer cells. Among these driver mutations are the 3 most common mutations found in CRCs. Over 80% of CRCs, mostly of the chromosomal instability subtype, are initiated by mutations in the adenomatous polyposis coli gene that leads to constitutive activation of WNT/β-catenin signaling.[7,44–46] Additional drivers are mutations frequently occurring in the *TP53* TSG and the *KRAS* oncogene.[46–48] Each of these mutations suppresses specific aspects of the antitumor immune response. Activation of WNT/β-catenin signaling suppresses the production of important T cell-recruiting chemokines CCL4 and CCL5, which compromises both T cell recruitment and stimulation of the dendritic cells (DCs) needed for T cell activation.[40,41,49–51] In addition, active WNT signaling upregulates the CD47 “don’t-eat-me” molecule on CRC cells that limits uptake of tumor material by DCs and other antigen-presenting cells, thereby limiting cross-presentation of tumor neoantigens. Finally, active WNT signaling upregulates programmed death-ligand 1 (PD-L1) on the surface of CRC cells, limiting the ability of any T cell that does manage to infiltrate the tumor to kill the CRC cells. Activating mutations in *KRAS*, such as *KRAS-G12D*, lead to upregulation of the pro-inflammatory genes interleukin (IL)6, IL8, and granulocyte-macrophage colony-stimulating factor, which facilitate recruitment of immunosuppressive regulatory T cells and myeloid-derived suppressor cells.[40,41,52–54] In addition, hyperactive KRAS decreases the expression of major histocompatibility complex (MHC)-I on the CRC surface, thereby preventing $CD8^+$ T cell-mediated recognition. Loss-of-function mutations in TP53 similarly alter cytokine production by the tumor cells, while also promoting secretion of CXCL2 and recruitment of suppressive neutrophil populations.[40,41,55,56] Perhaps more significantly, mutant *TP53* directly inhibits cGAS/STING signaling and decreases type I IFN signaling in the tumor cells, further compromising antitumor immunity. Interestingly, neoantigens from some of the frequently occurring hotspot mutations in *TP53* can be recognized by circulating T cells in some patients with epithelial cancers, though this has not been shown directly for CRC.[47]

Epigenetic factors such as DNA methylation, histone modification, noncoding RNA regulation, and chromatin remodeling play a pivotal role in the pathogenesis of CRC.[57,58] In particular, DNA methylation has been shown as a favorable target for CRC treatment, especially in the development of CRC immunotherapies. The DNA demethylating agent,

5-aza-2-deoxycytidine, displayed antitumor proliferative effects on CRC-initiating cells.[59] The antitumor effects of 5-aza-2-deoxycytidine were ascribed to the induction of an antiviral response regulated by the MDA5/MAVS/IRF7 viral RNA recognition pathway, resulting in reduced self-renewing proliferation of CRC-initiating cells. Preclinical studies showed promise for another interesting epigenetic target, cyclin-dependent kinase 9. Inhibition of cyclin-dependent kinase 9 reactivates hypermethylated TSGs and increases sensitization to immunotherapy.[60] In a phase II study, a combination of epigenetic therapy consisting of the DNA demethylating agent, 5-azacitidine, and the histone deacetylase inhibitor, entinostat, was tested in mCRC patients.[61] Both cohorts of the study exhibited treatment tolerability to both epigenetic therapies based on hematological and non-hematological toxicities presentation. However, this epigenetic combination therapy did not result in significant clinical activity under the RECIST 1.0 criteria. Nevertheless, since the combination of epigenetic regulators together with immune checkpoints reveals promise in different cancer types, it remains an interesting avenue for CRC treatment.[62–64]

In sum, these recent observations indicate that each CRC has an inherent immunophenotype that is dictated to a large extent by its genotype. Thus, successful treatment of many CRC patients with immunotherapy may require an understanding of their tumors' genetic underpinnings to guide selection of the most appropriate treatment combinations.

Neoantigens in CRC Immunotherapy

Tumor cells accumulate genetic changes, some of which lead to the expression of antigens that are considered foreign by the immune system, that is, neoantigens derived from mutations, frameshifts, gene fusions, and noncoding genomic regions.[65] When presented by the human leukocyte antigen (HLA) proteins, these neoantigens can be bound by high avidity T cell receptors of tumor-specific T cells, enabling tumor cell elimination.[66] High-throughput genome and peptidome analyses combined with computational algorithms have facilitated the identification of neoantigens and prediction of their HLA affinity and immunogenicity.[67] These technologies have allowed for the quantification of the number of changes found in the DNA, called the TMB.

The highest TMB has been reported in MSI/dMMR tumors, with a median of 52 mutations per Mb.[68] The high TMB in these tumors has been correlated to their infiltration with neoantigen-specific T cells[69] and their response to chemotherapy[70] or immune checkpoint immunotherapy[71,72]: both therapies with immune-stimulating capacity.[73,74] Although pMMR-MSS tumors are generally considered TMB low, it has been shown that stratification of patients according to TMB predicts their overall survival following chemotherapy plus cetuximab (anti-epidermal growth factor receptor mAb) or bevacizumab (anti-vascular endothelial growth factor mAb).[68] For these tumors, the TMB can be considered a proxy for the presence of neoantigens and T cell abundance in the tumor.[75]

Though the TMB predicts a tumor's foreignness, it does not predict the quality of neoantigens, that is, their affinity for HLA proteins or immunogenicity. These 2 parameters determine their value as a T cell target. Another parameter that likely predicts the value of a neoantigen as a T cell target, and therefore a therapeutic target, is its oncogenic

role in tumor cells.[76] Several neoantigens derived from frequently occurring mutations that are oncogenic drivers have been identified and proven to be immunogenic, including mutations in *KRAS*,[70,77] *TP53*,[78] and DNA repair genes, such as *POLE*.[79,80] These represent interesting targets for developing neoantigen-based immunotherapies, as evidenced by several preclinical and clinical reports on the adoptive transfer of neoantigen-specific T cells[77,80] and therapeutic vaccination.[81–83]

Cell-based Vaccines

The discovery of antigens that allow cytotoxic T lymphocytes (CTLs) to recognize and kill cancer cells has led to the pursuit of therapeutic cancer vaccines.[84] Their development has mainly been based on the knowledge that mononuclear phagocytes, such as DCs, can activate T cells. Although various functionally specialized DC subsets have been described,[85] they share the ability to acquire antigens and sense danger in their surroundings, resulting in a maturation program. Maturation of DCs results in their migration from the periphery to the T cell zones of lymph nodes by CCR7-CCL19/21 chemotaxis while upregulating HLA-peptide complexes, costimulatory surface molecules (e.g., CD80 and CD86), and cytokines (e.g., IL12) that are key in T cell activation.[86]

Generation of *ex vivo* DCs capable of activating antitumor CTLs mostly involves the isolation of $CD14^+$ monocytes from peripheral blood, followed by their differentiation and manipulation into antigen-presenting mature monocyte-derived DCs (moDCs). Though similar in principle, many protocols exist with variations in methods for peripheral blood collection (eg, whole blood, leukapheresis), monocyte isolation (eg, elutriation, adherence or antibody selection), DC differentiation (eg, cytokine cocktails such as granulocyte-macrophage colony-stimulating factor and IL4, or IFN-β and IL3), antigen delivery (eg, nonviral vs viral delivery), DC maturation (eg, cytokine cocktail, genetic engineering), and timing of each step.[87,88] Several clinical trials in CRC patients have been performed with moDCs pulsed with autologous tumor lysates,[89–92] ensuring presentation of neoantigens and other tumor antigen classes, such as cancer-testis (eg, melanoma antigen) and differentiation antigens (eg, carcinoembryonic antigen [CEA]).[93,94] These trials have provided evidence for the safety of moDC vaccines and their ability to stimulate tumor-specific T cell responses, which has been associated with improved survival.[90] However, a limitation of this approach is the availability of tumor material, which has been circumvented in other trials by pulsing moDCs with allogeneic cell line lysates[95]; peptides (or mRNA) of tumor-associated antigens[96–99] and established neoantigens,[100] such as frameshifted caspase 5[101] and transforming growth factor β receptor II. These studies confirmed the feasibility and tolerability of moDC vaccines and provided evidence of T cell responses and benefit in some patients. Furthermore, peptide-pulsed moDCs are being studied in phase I/II clinical trials as a prophylactic vaccine in Lynch syndrome patients (ie, carriers of germline MMR mutations) at high risk of developing CRC.[100] The rationale for these trials is that moDC vaccination in CRC patients following tumor resection showed fewer and later relapses in the vaccine arm,[92] making a case for immune surveillance, in preventing cancer (re-)occurrence. Despite these encouraging responses, clinical benefit has not been achieved in a considerable cohort of patients. Arguably, moDCs might have a suboptimal intrinsic capacity to induce T cell responses, and conventional DCs might be better

suited to stimulate CTLs.[85] One study on vaccination of CRC patients with blood-derived DCs, isolated following mobilization with FMS-like tyrosine kinase 3 ligand, and pulsed with optimized CEA peptides, resulted in unprecedented objective clinical responses,[102] encouraging continued investment in improved CRC vaccines. In this regard, the use of $CD34^+$ hematopoietic stem cells[103] or inducible pluripotent stem cells[104] paves the way to culture conditions allowing *ex vivo* generation of specific DC subsets. Combined with cell engineering technologies such as mRNA, these culture conditions might lay the foundation for optimal *ex vivo* DC vaccines.[67,105]

Since generating *ex vivo* DCs is time-consuming and expensive, various vaccine platforms have been studied in CRC to deliver tumor antigens to DCs *in situ*. These platforms mirror the strategies used for delivery of antigens and maturation stimuli to *ex vivo* DCs and include the use of autologous or allogeneic irradiated CRC cells,[106] peptides,[107,108] mRNA,[109] viral,[110,111] and bacterial[112,113] vectors often combined with adjuvants.[114] In general, these vaccines have been well-tolerated in clinical trials. However, as with *ex vivo* moDC vaccines, clinical benefit is not always evident, even though T cell responses have been frequently reported. Further improvement of vaccine delivery to specific DC subsets will potentially improve the outcome of the vaccines, although it is generally accepted that the full potency of cancer vaccines will only be achieved when combined with strategies that support the functionality of the activated T cells in the suppressive TME.[115]

A strategy that gained momentum for treatment of CRC is adoptive cell therapy, including transfer of TILs and T cells genetically engineered to express tumor-specific receptors: T cell receptors (TCRs) or chimeric antigen receptors (CARs). These cells can persist in vivo when unhampered by tolerance mechanisms and the TME.[116] The use of TCRs for T cell engineering requires prior knowledge of HLA-mediated tumor antigen presentation by CRC cells as well as the matching TCR. Therefore, CARs have been designed that can bind antigens using antibody fragments, while still conveying signals via CD3ζ, essential for T cell activation.[117] To date, CARs have been generated against a variety of antigens that are highly expressed on the surface of CRC cells (e.g., CEA, EpCAM).[118,119] Several clinical trials are evaluating CAR-T cells in CRC, showing encouraging results.[120] Following optimization, CAR-T cells usually have strong target-specific activity, which entails the risk of on-/off-target toxicity as many of the targeted antigens are also lowly expressed on healthy tissue. Moreover, as with T cells activated via vaccination, the TME can exclude or dysregulate CAR-T cells, even induce irreversible exhaustion.[121] With these limitations in mind, natural killer (NK) cells have been engineered with CARs, as NK cells, like CTLs, exert cytotoxic activity. CAR-NK cells further offer the advantage that they can be used in an allogeneic setting.[122,123] Though these cells have limitations in view of proliferative capacity and in vivo persistence, local injection of CAR-NK cells demonstrated clinical benefit in mCRC patients.[124] Clinical data encourage further exploration of CAR-engineered immune cells for the treatment of CRC. Clinical data encourage further exploration of CAR-engineered immune cells for the treatment of CRC even though the success as observed in hematological malignancies has not yet been achieved. Solutions to obtain similar levels of success encompass altering the route of delivery, the dose, the infusion interval, or combining adoptive cell therapy with other immunotherapies purposed to expand[125] and support[120,126] the adoptively transferred immune cells.

Checkpoint Immunotherapy

Checkpoint immunotherapy modulates the immune system using ICIs by inhibiting negative regulators (immune checkpoints, or “brakes” of the immune system) found on the surface of immune cells. ICIs target coinhibitory receptors, including programmed cell death protein 1 (PD-1) and cytotoxic T lymphocyte-associated protein 4 (CTLA-4), or their ligands, and thus enhance T cell activation and promote cancer cell killing. In early studies, ICIs exhibited limited efficacy in unselected CRC patients whose MSI status was not known.[127–129] A critical turning point for CRC immunotherapy stems from early clinical trials assessing the clinical activity of PD-1 blockade.[6,130] Only 1 of 33 patients responded, a rather disappointing result. In retrospect, that clinical trial provided a key discovery of the DNA MMR system correlating with those who responded well to the blockade therapy vs those who did not; that is, those with dMMR tumors responded to the PD-1 blockade therapy, unlike those with pMMR tumors.[6] This finding led to another clinical trial in phase 2 to evaluate the clinical response of pembrolizumab (anti-PD-1 mAb) in 41 patients with MSI-H CRC, MSS CRC, or MSI-H nonCRCs.[131] This study reported a remarkable pembrolizumab response rate in dMMR CRC, with a 40% (4 of 10 patients) objective response rate (ORR) and 78% (7 of 9 patients) progression-free survival, whereas pMMR CRC patients were observed to have little to no response, with ORR and progression-free survival rate of 0% (0 of 18 patients) and 11% (2 of 18 patients), respectively. Based on these results, in May 2017, the Food and Drug Administration (FDA) granted accelerated approval for pembrolizumab for patients with advanced MSI-H CRC that has progressed after treatment with standard chemotherapy (Table 1). A follow-up study across 12 tumor types demonstrated that pembrolizumab is effective in MSI-H cancers regardless of tissue of origin and that somatic mutational burden significantly correlates with response to treatment.[138]

Another phase 2 study, CheckMate 142, evaluated the efficacy of nivolumab (anti-PD1 mAb) either with ipilimumab (anti-CTLA-4 mAb) or alone as a monotherapy in advanced MSI-H CRC patients.[132,139] The investigators reported an ORR of 31% and a disease control rate of 68% with nivolumab alone, which led to FDA approval for nivolumab to treat metastatic, progressive MSI-H CRC in August 2017. The investigators later reported a disease control rate of > 80% for dual therapy with nivolumab and ipilimumab; however, despite FDA approval of combination therapy, treatment with nivolumab and ipilimumab has been associated with increased toxicity and adverse events related to treatment,[140] which has decreased enthusiasm among clinicians. Treatment-related adverse events from ICIs have been reviewed elsewhere.[141–143]

The success of ICI for treatment of advanced, mCRC led to the intriguing hypothesis ICI could be as effective, or more effective, in the neoadjuvant setting (ie, prior to chemotherapy, radiation, and/or surgery) for treatment of nonmetastatic disease. The NICHE-1 study[135] evaluated the efficacy of combination ipilimumab and nivolumab treatment of 21 patients with dMMR-MSI-H nonmetastatic CRC and found an impressive major pathological response of 95% and complete response (PCR) of 60%; the follow-up NICHE-2 study in 112 patients with dMMR-MSI-H nonmetastatic CRC confirmed these findings with a major pathological response of 95% and a PCR of 67%. A recent phase 2 study tested the novel

hypothesis that dostarlimab (anti-PD1 mAb) alone would be beneficial as a neoadjuvant therapy for MSI-H stage II or III rectal cancer.[137] This treatment was followed by standard chemotherapy, radiation, and surgery if clinically indicated. Remarkably, after 6 months of follow-up, all 12 subjects had a complete clinical response based on magnetic resonance imaging imaging, positron emission tomography scanning, colonoscopy, rectal exam, and biopsy, which demonstrates that locally advanced MSI-H rectal cancer is highly sensitive to PD-1 blockade. Together, these studies suggest that neoadjuvant treatment of dMMR-MSI-H nonmetastatic CRC with ICI may be even more effective than treatment of pretreated, metastatic dMMR-MSI-H CRC.

Similarly, a phase 1 study tested 2 ICIs, botensilimab (anti-CTLA-4 mAb) and balstilimab (anti-PD-1 mAb), in refractory, MSS, mCRC patients without liver metastasis (NCT05608044). Their results demonstrated complete or partial response to both ICIs in 76% of the patients, a 63% 12-month survival rate, and only 12% of patients discontinuing both treatments due to adverse side effects. A recent longitudinal study on melanoma patients determined that the time-of-day infusion resulted on improved overall survival.[144] Their data, despite not being done in CRC, would suggest scheduling ICI infusions before mid-afternoon.

Thus, we can state that the current status of CRC checkpoint immunotherapy divides patients into 2 groups: pMMR/MSS or MSS and dMMR/MSI-H, with the latter group comprising only 15% of CRC patients.[145] In addition, only 3%–6% of advanced-stage CRCs are MSI-H. Furthermore, within this 5%, the ORR ranges from 30% to 70%.[131,138,146,134] The considerable remainder, who do not respond to checkpoint immunotherapy, are indicated as resistant.[147] Although ICIs are generally successful in evoking antitumor immunity in CRC patients, there is still a majority of patients that present with primary (ie, no response at all) or acquired (ie, initial response but response has stopped, or relapse occurred) resistance.[148] Regardless of which type, checkpoint immunotherapy resistance stems from tumor intrinsic and extrinsic factors.

Studies regarding tumor-intrinsic factors, specifically neoantigen presentation and tumor recognition, have been revealed potential approaches to improve ICI efficacy for CRC. One study showed the significance of low neoantigen presentation in MSS CRC since it results in poor T cell priming, and consequently, an overall tolerogenic phenotype of T cells which contributes to tumor immune evasion.[149] This study also evaluated the efficacy of a potential therapeutic approach to rescue these dysfunctional T cells by utilizing agonistic antibodies against the CD40 receptor (anti-CD40). Anti-CD40 therapy in combination with ICIs (e.g., PD-1 and CTLA-4) enhanced T cell priming through strengthening the costimulatory function in antigen-presenting cells. The results showed decreased tumor size and reduced rates of metastasis in an MSS CRC mouse model. Impaired MHC-I antigen presentation following truncation mutations of the beta 2 microglobulin gene is another intrinsic factor that has been posited to lead to tumor immune escape, and thus, ICI resistance[150]; although further studies are required to definitively comment on defective beta 2 microglobulin-associated ICI resistance in CRC.

Tumor-extrinsic factors contributing to CRC-related ICI resistance include the host microbiota (discussed later in this review), and the TME. The TME is composed of cellular components (ie, immune cells, cancer-associated fibroblasts or CAFs, and endothelial cells) and noncellular components (ie, the extracellular matrix).[151] The interactions among these different components generally contribute to the immunosuppressive nature of the TME. For instance, CAFs are a major cellular component within the TME and lead to the promotion of tumor angiogenesis, cell proliferation, and migration through multiple pathways.[151] CAFs secrete activin A, resulting in increased stiffness of the extracellular matrix and promotion to mCRC.[152] Therefore, further observations into the components of the TME are warranted to prevent, or even overturn, ICI resistance in CRC.

Overcoming these types of ICI resistance in CRC requires different approaches. Identifying unique neoantigens is an example of one such approach. Cell migration-inducing and hyaluronan-binding protein expressed by tumors was found as a main driver for immune escape in CRC through the clathrin-mediated endocytosis, and consequently degradation of MHC-I.[153] This shows promise in enhancing ICI response, as MHC-I is a key first signal to induce the $CD8^+$ T cell activation that can kill tumors. We previously aimed at improving current immune checkpoints in CRC and found that atractylenolide I (ATT-I), a small molecule compound, strengthens T cell-mediated cytotoxicity in CRC.[154] ATT-I interacts with the proteasome 26S subunit non-ATPase 4 (PSMD4), an essential component of the immunoproteasome complex, to enhance the antigen processing activity of the immunoproteasome, leading to enhanced MHC-I-mediated antigen presentation on cancer cells. ATT-I treatment ultimately results in enhanced ICI treatment for CRC. Thus, the identification of drug targets allowing for an increased tumor antigen presentation could improve ICI and is a valid avenue worth pursuing.

For those CRC patients who are unreceptive to current ICIs, there is a need to identify novel immune checkpoint targets. Several such alternative targets were identified recently for CRC. Targeting these alternative immune checkpoints serves to bolster the antitumor effects of ICIs by alleviating the immunosuppressive nature of the TME and its constituents. A recent study showed that sialic acid-binding immunoglobulin-like lectin 15 (Siglec-15) is a macrophage-associated immune suppressor of antigen-specific T cell responses.[155] Importantly, Siglec-15 has limited surface expression on normal tissue, in contrast to tumor-associated macrophages and tumor cells which highly express Siglec-15. The same study discerned that a Siglec-15 antibody blockade greatly improves antitumor immunity in the TME, and a separate study also corroborates Siglec-15 as a mAb treatment.[156] Siglec-15 targeting is being tested in the clinic in solid tumors, including in CRC, using NC318, a humanized mAb (NCT03665285). We previously identified stimulation 2 (ST2, IL33 receptor) as a potential checkpoint target for CRC immunotherapy.[157] ST2 (encoded by the IL1RL1 gene) was found to be highly expressed on both murine and human macrophages. Using ST2 KO animals, we showed that the antitumor effects are mainly mediated by $CD8^+$ T cells. Moreover, anti-PD-1 treatment in $Il1rl1^{-/-}$ mice results in a significant decrease in tumor size compared to wild type animals or control animals treated with isotype antibodies. These data suggest that combining current ICI with ICI targeting different cell types (ie, non-T cells) could be a promising approach. Another potential alternate checkpoint target is C-type lectin domain family 1 (CLEC-1), a necrotic cell

sensor expressed by myeloid cells. Inhibiting CLEC-1 precludes an immunosuppressive TME propagated by suppressive myeloid cells, like myeloid-derived suppressor cells, to allow tumor immune escape.[158] Following this evidence, combination therapeutic treatment in the form of anti-CLEC-1 mAb and chemotherapeutic cyclophosphamide reduces tumor growth in mice subcutaneously injected with MC38 tumor cells. Next to these, several other myeloid checkpoint targets are being investigated in CRC including triggering receptor expressed on myeloid cells 2[159] and leukocyte immunoglobulin-like receptor subfamily B member 4.[160]

The Microbiome and Their Immunotherapy Implications

Mounting evidence suggests that the gastrointestinal microbiota and its metabolites can modulate both local and systemic immune responses and, as such, should be considered important variables in both cancer progression and response to immunotherapy. Indeed, it has recently been shown that primary resistance to ICI is associated with gut microbial dysbiosis in clinical trials of patients with advanced cancers.[161] Similarly, reports also show that the gut microbiota composition is predictive of ICI response and that antibiotic treatment can reduce the clinical benefits of ICI[162–164] in patients with solid tumors. Various studies using gnotobiotic mice mirror clinical reports that support the role of the microbiome in ICI response, and they demonstrate that the absence of the microbiome confers resistance to ICI therapy in epithelial and solid tumors.[165–167] Nevertheless, the mechanisms by which the microbiota and metabolites can influence ICI therapy are not well elucidated. Here, we outline the latest studies aimed at unraveling the mechanisms used by the microbiota and metabolites to modulate the efficacy of CRC immunotherapies and highlight clinical trials underway to test the efficacy of microbiota-modulating therapeutic approaches in CRC.

Gut microbiota modulate both adaptive and innate immune cells to enhance ICI efficacy in solid intestinal tumors. Mice engrafted with a defined 11-strain bacterial mix isolated from healthy human donors demonstrated enhanced antitumor immunity attributed to an increase in IFNγ^+ CD8$^+$ T cells in both a spontaneous manner and in response to treatment.[167] In a separate study, mice humanized with stool from patients with CRC showed impaired anti-PD1 efficacy but were rescued upon supplementation with the soluble fiber pectin.[168] In the same study, depletion of CD8$^+$ T-cells reversed the positive antitumor effects of pectin supplementation, hinting at a CD8$^+$ T cell-based mechanism.[168]

A recently published study proposed a potential mechanism that can be targeted to improve PD-1 blockade therapy in microbiome-related immunotherapy resistance: inhibition of programmed cell death 1 ligand 2 (PD-L2) and repulsive guideline molecule b on immune cells that promote antitumor immunity.[169] Additionally, this study defined the type of bacteria (Gram-positive anaerobes) and even pinpointed a bacterium species (*Coprobacillus cateniformis*) from the human microbiome that was able to promote antitumor immunity in combination with ICI treatment. Such an approach could be an appealing strategy toward improving CRC-related immunotherapy resistance.

Interestingly, specific bacterial species have also been shown to have effects similar to that of bulk stool engraftment in preclinical models. In 4 different cancer models, including

colitis-associated cancer, *Bifidobacterium pseudo-longum, Lactobacillus johnsonii,* and *Olsenella* species were shown to enhance ICI response.[170] In several other studies, oral administration of *Bifidobacterium*[166] and *Bacteroides fragilis*[171] augmented DC function and enhanced T cell-mediated antitumor response. Furthermore, an overall reduction in the relative abundance of *Akkermansia muci-niphila* has been described in solid tumor patients who have failed to respond to ICI therapy.[164] In the same study, engraftment of germ-free mice with nonresponder microbiota decreased ICI response, which was rescued upon oral supplementation of *A muciniphilia* in a mechanism dependent on increased tumor infiltration of $CXCR3^+$, $CCR9^+$, and $CD4^+$ T cells.[164] Similarly, systemic administration of *Bifidobacterium* rescued the response to anti-CD47 immunotherapy in nonresponding mice and enhanced the antigen-presenting capacity of DCs.[172]

Another way the gut microbiota can influence host responses is through secreted metabolites. The most abundant microbial metabolites in the colon are short-chain fatty acids (SCFAs).[173] SCFAs are produced by the anaerobic intestinal flora as a by-product of fiber fermentation and play important roles in gut homeostasis. The SCFA butyrate inhibits histone deacetylases and, as such, has been shown to block tumor cell proliferation through inhibition of calcineurin-mediated nuclear factor of activated T cells C3.[174] Similarly, the SCFA propionate was shown to improve the antitumor effects of ICIs in a mechanism dependent on p21 activation and downregulation of inhibitor of apoptosis protein.[164] The purine metabolite inosine has recently been highlighted for its role in enhancing ICI response in T cell-mediated mechanisms,[170] manipulating the TME, increasing tumor-antigen presentation to cytotoxic cells,[175] and providing an alternative energy source to enhance effector T cell function.[176,177]

L-arginine (L-Arg) is another metabolite that has been shown to mediate antitumor immunity, specifically through T cells.[178] For this reason, a nonpathogenic strain of bacteria, *E coli* Nissle 1917, was developed that can colonize tumors and convert ammonia into L-Arg.[179] The combination of the L-Arg-producing bacteria and anti-PD-L1 ICI demonstrated an effective antitumor response as well as increased TILs.

Strengthening our mechanistic understanding of the interplay between the microbiome, metabolome, and anti-tumor immunity is crucial for therapeutic development. Today, several microbiome-modulating therapeutic strategies are being explored in CRC patients. Two phase-II clinical trials are underway to evaluate the efficacy of fecal microbiota transfer for the treatment of mCRC in anti-PD-1 nonresponding patients (NCT04729322) and in conjunction with sintilimab and fruquintinib in advanced-stage CRC patients (NCT05279677). Probiotics, which have been shown to have anticarcinogenic effects,[180] are also being explored in CRC. Results from a clinical trial in CRC patients (NCT03072641) showed that a probiotic mixture of *Bifidobacterium lactis* and *Lactobacillus acidophilus* results in an increased abundance of butyrate-producing bacteria in the tumor, mucosa, and feces of patients while another (NCT03782428) showed that probiotic of *Lactobacillus* and *Bifidobacteria* strains lead to a reduction in pro-inflammatory cytokines in CRC patients following surgery.[181] Another trial is currently underway to assess the safety and efficacy of using *Bifidobacterium trifidum* live powder in conjunction with chemotherapy and targeted therapy in patients with mCRC (NCT04131803). Additionally, a phase-I/II trial in advanced

or metastatic cancers (NCT04208958), including CRC, is assessing the efficacy of combined use of nivolumab and VE800, which is an 11-strain probiotic.

A similar strategy to probiotics is the use of Microbial Ecosystem Therapeutics, where instead of using single or limited strains (probiotics), microbial communities are derived directly from healthy human donors.[182] A phase-II/III trial (NCT03686202) is assessing the safety, tolerability, and engraftment of a defined set of Microbial Ecosystem Therapeutics strains given in combination with ICIs to patients with solid tumors. Beyond whole organisms and their products, genetic engineering approaches allow for more specific targeting and have shown some success in preclinical models.[183] In advanced solid tumors, a phase-1 clinical trial (NCT04167137) is evaluating the safety and possible efficacy of using atezolizumab in combination with SYNB1891, a dual innate immune agonist designed based on the biology of *E coli* that can stimulate the expression of IFNs and achieve antitumor effects.[184] Other trials explore the effects of microbiota-derived SCFAs (NCT02446431, NCT01106872, NCT02624128) or polypeptides with homologous structures to tumor-associated antigens (NCT04187404, NCT04116658) in solid tumors.

In conclusion, the recent interrogation of the microbiome-metabolome-immune axis has provided substantial insights and opportunities for the development of novel microbiome-based therapeutic strategies to be used in conjunction with traditional immunotherapies. However, a deeper understanding of the mechanisms underlying the multifaceted and bidirectional relationship between the gut microbiota and antitumor immunity is needed for the successful advancement of precision-based microbiota adjuvant strategies.

Improving CRC Immunotherapy Through Combination With Radiotherapy

Radiotherapy is a conventional cancer therapy that directly targets tumor cells and can be used as monotherapy or combination therapy. In locally advanced rectal cancer, treatment with chemoradiation followed by total mesorectal surgical excision is the standard of care, although longcourse radiotherapy was recently challenged by the results of the Rectal cancer And Preoperative Induction therapy followed by Dedicated Operation trial.[185] In addition to its established value, preclinical research has demonstrated that radiotherapy has the potential to sensitize tumors to ICIs. Particularly, high-dose radiotherapeutic regimens induce immunogenic cell death,[186,187] have the potential to convert a cold into a hot tumor,[188] and increase PD-L1 expression in the TME.[189,190] In addition, early clinical trials have demonstrated that high-dose radiation cooperates with immunotherapy by activation of systemic immune responses in multiple tumor types, as reviewed elsewhere.[191] These observations have spurred clinical efforts into reinvestigating tumors that are currently considered “nonresponders” to immunotherapy, such as MSS CRC. Unfortunately, most trials investigating combination therapies of radiotherapy and immunotherapy have produced suboptimal results, possibly due to issues such as radiotherapy dosing, fractionation, timing, or radiation of single lesion or multiple lesions, which all differ substantially between trials. No consensus has yet been reached regarding an optimal schedule to combine ICI and radiation.[191] Nevertheless, clinical trials reporting an improvement in overall response rate in MSS CRC serve as an encouragement for future research.[190,192] Clinical trials in this area are still sparse; nevertheless, 23 active studies in CRC are investigating a specific

combination therapy including both radiotherapy and immunotherapy. Of these 23 ongoing trials, 3 are exclusively investigating the combination treatment in an MSS phenotype. Novel combinations, including the addition of radiotherapy, are anticipated to deliver promising results when expanding the use of ICI to a larger array of solid tumors; the results of these ongoing clinical trials are eagerly awaited.

Conclusion

As the diagnoses of CRC shifts to a younger demographic and prevalence of more advanced disease, the need to expand CRC therapies becomes ever more critical. CRC immunotherapy has been a promising avenue when traditional methods are not enough for patients, whether it is in the durability of the response, recurrence, side effects, overall survival, and quality of life after treatment. CRC immunotherapy has been largely implemented as an additional therapy next to first-line methods, like surgery, and is limited to the minority: dMMR/MSI-H and/or mCRC patients. Multiple studies are being conducted to enable the expansion of CRC immunotherapy to more patients, identifying other markers or microenvironments that modulate the antitumor immune response and evaluating immunotherapy, beginning with ICIs, as a primary as well as sole mode of CRC treatment.

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Abbreviations used in this paper:

ADCC	antibody-dependent cellular cytotoxicity
ADCP	antibody-dependent cellular phagocytosis
ATT-I	atractylenolide I
CARs	chimeric antigen receptors
CCR	clinical complete response
CCL4	C-C motif chemokine ligand 4
CEA	carcinoembryonic antigen
CIMP	CpG island methylator phenotype
CKIα	casein kinase I alpha
CRC	colorectal cancer
CMS	consensus molecular subtype
CTLs	cytotoxic T lymphocytes
CTLA-4	cytotoxic T lymphocyte-associated protein 4

DCs	dendritic cells
DCR	disease control rate
ECM	extracellular matrix
EGFR	epidermal growth factor receptor
GSK-3β	glycogen synthase kinase 3 beta
HLA	human leukocyte antigen
ICIs	immune checkpoint inhibitors
IFN	interferon
IRF3	interferon regulatory 3
mAbs	monoclonal antibodies
L-Arg	L-arginine
LRP	lipoprotein receptor-related protein
mCRC	metastatic CRC
MHC	major histocompatibility complex
MMR	mismatch repair
dMMR	deficient MMR
pMMR	proficient MMR
MPR	major pathologic response
MSI	microsatellite instability
MSS	microsatellite stable
moDCs	monocyte-derived DCs
NF*k*B	nuclear factor kappa-light-chain-enhancer of activated B cells
NK	natural killer
ORR	objective response rate
PD-1	programmed cell death protein 1
PD-L1	programmed death-ligand 1
PFS	progression-free survival
SCFAs	short-chain fatty acids
SCNA	somatic copy number alteration

SIRPα	signal regulatory protein alpha
TGFβ	transforming growth factor beta
TILs	tumor-infiltrating lymphocytes
TMB	tumor mutation burden
TCRs	T cell receptors
TCF/LEF	T cell factor/lymphoid enhancer factor
TME	tumor microenvironment
TSG	tumor suppressor gene
VEGF	vascular endothelial growth factor

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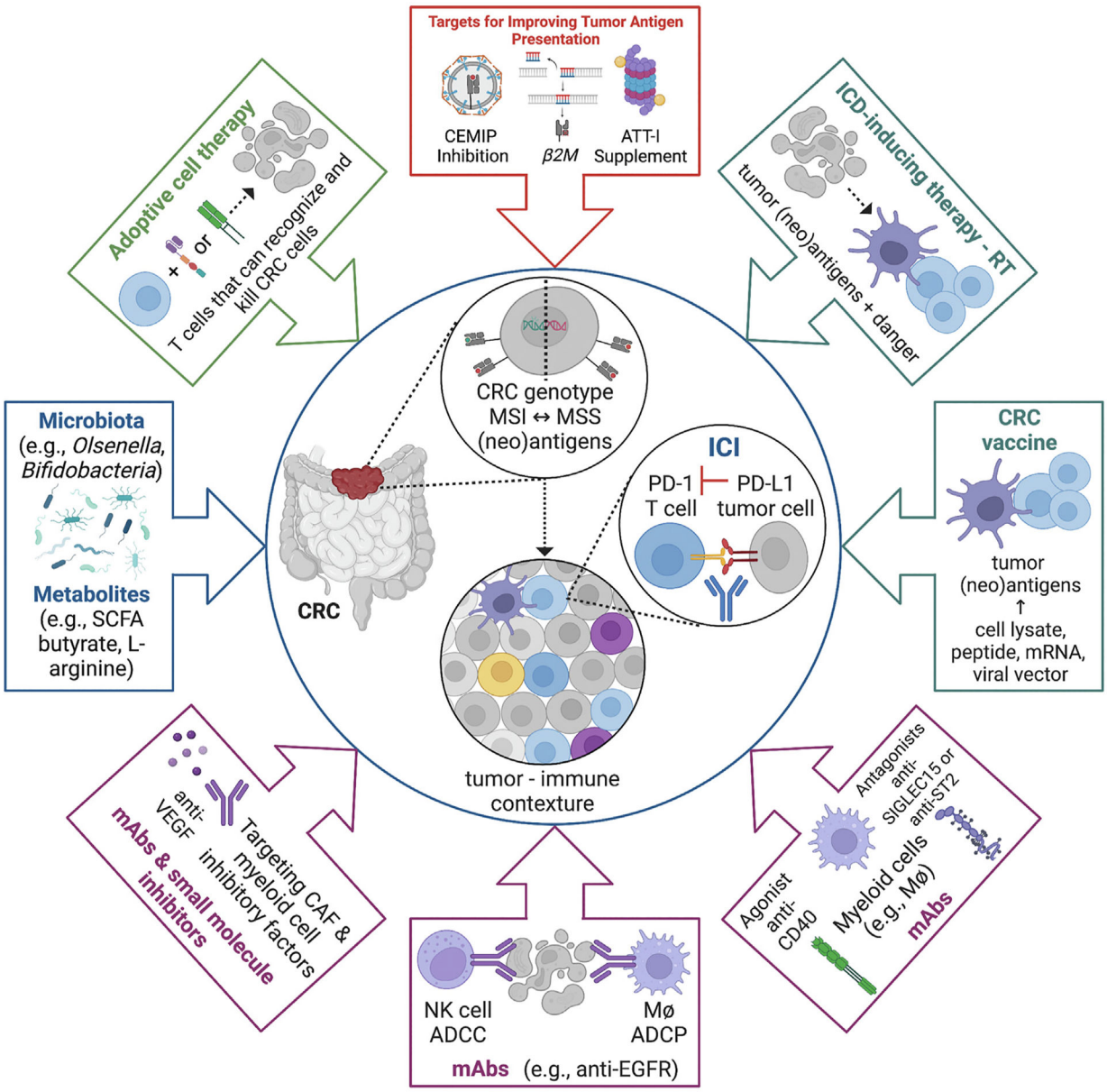

Figure 1.

Schematic representation of explored CRC immunotherapy avenues. Immune checkpoint inhibitors (ICIs) are at the center of CRC immunotherapy, while several strategies are combined with ICIs to improve therapy outcome. ADCC, antibody-dependent cellular cytotoxicity; ADCP, antibody-dependent cellular phagocytosis; EGFR, epidermal growth factor receptor; Mφ, macrophage; VEGF, vascular endothelial growth factor. Created with BioRender.com.

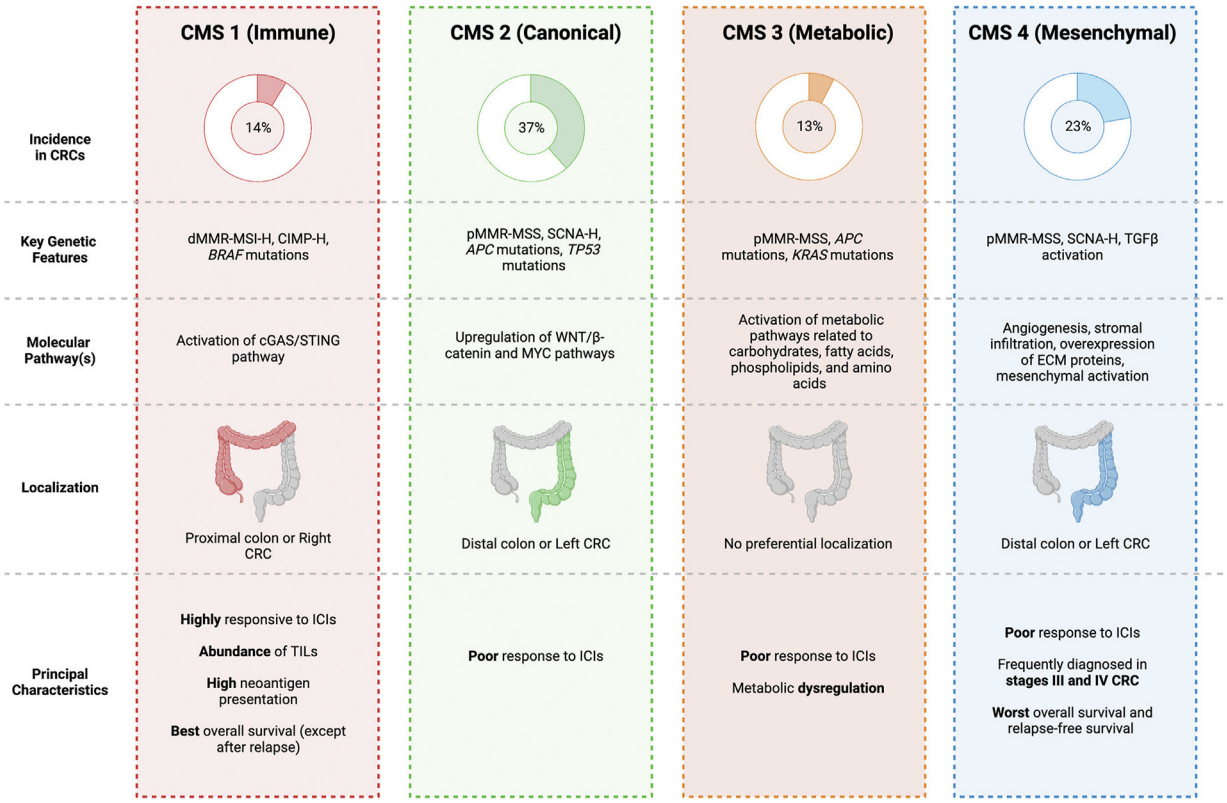

Figure 2.

Consensus molecular subtype classification in CRC. Consensus molecular subtype (CMS) groups highlight important hallmarks observed in CRC. CMS grouping for CRC has been instrumental as a prognostic marker and predictive indicator for treatment efficacy. CIMP, CpG island methylator phenotype; ECM, extracellular matrix; SCNA, somatic copy number alteration; TGFβ, transforming growth factor beta. Created with BioRender.com.

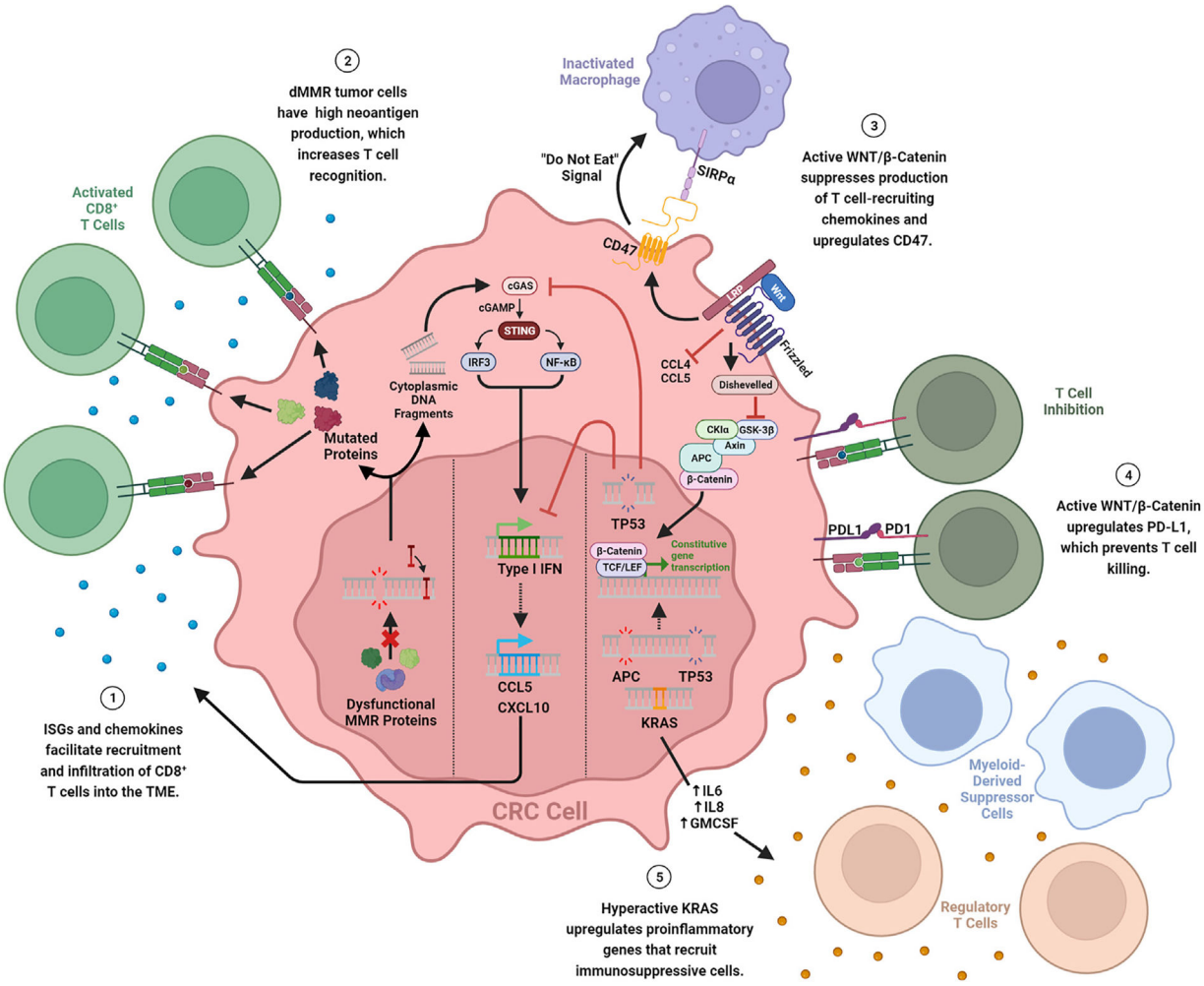

Figure 3.

Impact of tumor genomics on immune responses. Dysfunctional mismatch repair proteins are not able to correct indel mutations, resulting in the production of mutated proteins. These proteins can act as neoantigens for T cell recognition. DNA mutations from dysfunctional mismatch repair proteins can also produce cytoplasmic DNA fragments that can activate the cGAS/STING pathway. The ISGs and chemokines produced then facilitate $CD8^+$ T cell recruitment to destroy the tumor. Conversely, the 3 most common mutations in CRC (*APC, TP53, KRAS*) lead to tumor protection. Mutations derived from these 3 genes lead to the constitutive activation of the WNT/β-catenin pathway. Activation of this pathway upregulates the "do not eat" signal (CD47) and PD-L1. *KRAS* activation upregulates pro-inflammatory genes to recruit immunosuppressive cells, such as myeloid-derived suppressor cells and regulatory T cells. CCL4, C-C motif chemokine ligand 4; SIRPα, signal regulatory protein alpha; LRP, lipoprotein receptor-related protein; CKIα, casein kinase I alpha; GSK-3β, glycogen synthase kinase 3 beta; TCF/LEF, T cell factor/lymphoid enhancer factor; IRF3, interferon regulatory 3; NFκB, nuclear factor kappa-light-chain-enhancer of activated B cells. Created with BioRender.com.

Table 1.

Landmark Clinical Trials of Immune Checkpoint Blockade for Colon and Rectal Cancer

Name	Phase	Drug and dose	Patient population	Results	FDA approval date
KEYNOTE 028 Le et al.[6]	II	Pembrolizumab 10 mg/kg every 14 d	dMMR MSI-H mCRC	ORR 40%; DCR > 12 wk 90%	May 2017
CheckMate 142 Overman et al.[132]	II	Nivolumab 3 mg/kg every 14 d	dMMR-MSI-H mCRC	ORR 31 %; DCR > 12 wk 61%	August 2017
CheckMate 142 (further analysis of subgroup) André et al.[133]	II	Nivolumab 3 mg/kg + Ipilimumab 1 mg/kg every 21 d	dMMR-MSI-H mCRC	ORR 55%; DCR > 12 wk 80%	July 2018
CheckMate 177 André et al.[134]	III	Pembrolizumab 200 mg every 3 wk or chemotherapy	307 patients dMMR-MSI-H mCRC	PFS 16.5 vs 8.2 mo; hazard ratio, 0.60	N/A
NICHE-1 Chalabi et al.[135]	II	Ipilimumab (1mg/kg) + nivolumab (3mg/kg)	21 patients with dMMR-MSI-H and 20 patients with pMMR-MSS nonmetastatic CRC	dMMR-MSI-H: MPR 95%, PCR 60% pMMR-MSS: MPR 20%	N/A
NICHE-2 Chalabi et al.[136]	II	Ipilimumab (1mg/kg) + nivolumab (3mg/kg) for 4 wk followed by surgery within 6 wk	112 patients with T3, dMMR-MSI-H, nonmetastatic CRC	MPR 95% PCR 67%	N/A
Cercek et al.[137]	II	Dostarlimab (500 mg) every 3 wk for 6 mo, followed by standard chemotherapy, radiation, and surgery if clinically indicated	12 patients with dMMR-MSI-H locally advanced stage II or III rectal cancer	CCR 100%	N/A

DCR, disease control rate; PFS, progression-free survival; MPR, major pathologic response; PCR, complete response; CCR, clinical complete response.