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# Checkpoint molecules on infiltrating immune cells in colorectal tumor microenvironment

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Colorectal cancer (CRC) is one of the most prevalent cancer types worldwide, with a high mortality rate due to metastasis. The tumor microenvironment (TME) contains multiple interactions between the tumor and the host, thus determining CRC initiation and progression. Various immune cells exist within the TME, such as tumor-infiltrating lymphocytes (TILs), tumor-associated macrophages (TAMs), and tumor-associated neutrophils (TANs). The immunotherapy approach provides novel opportunities to treat solid tumors, especially toward immune checkpoints. Despite the advances in the immunotherapy of CRC, there are still obstacles to successful treatment. In this review, we highlighted the role of these immune cells in CRC, with a particular emphasis on immune checkpoint molecules involved in CRC pathogenesis.

## KEYWORDS

tumor microenvironment, macrophages, neutrophils, lymphocytes, colorectal cancer, immune checkpoint

## Introduction

Colorectal cancer (CRC) is the third most commonly diagnosed cancer in 2020, affecting 10% of the global population (1). The increasing mortality rate in patients with advanced CRC is of concern and reflects the limited range of treatment options. This could be attributed to the diagnosis of CRC at a late stage when the tumor has already metastasized. Furthermore, in most CRC patients, surgical resections are not the ultimate cure as there is a high possibility of recurrence of the disease in a more aggressive form; thus, using additional therapeutic modalities is mandatory (2). CRC is not a single disease and every patient has a unique illness due to distinctive genetic/epigenetic causes (3). The molecular classification of CRC is changing over

time. Global genomic status [microsatellite instability (MSI) status and chromosomal instability (CIN) status] and epigenomic status [CpG island methylator phenotype (CIMP) status] contribute significantly to the clinical, pathological and biological properties of CRC. CIN tumors are mostly microsatellite stable (MSS) and have been associated with an aggressive clinical picture (4–6). Such tumors usually have large genomic abnormalities that lead to higher average DNA copy number compared with MSI tumors (7). MSI is typically diagnosed by the variable lengths of DNA microsatellites (mononucleotide and dinucleotide repeats) (8), which are caused by epigenetic silencing (9, 10) or mutation of DNA mismatch repair (MMR) genes, leading to accumulated mutations at 10–100 times the normal rate promoting cancer progression (8). CRC tumorigenesis has been reported to be triggered by gene mutations associated with multiple signaling pathways such as KRAS, BRAF, and PIK3CA (11). Several studies have confirmed that association between BRAF and KRAS mutations, in addition to BRAF mutations being more linked to MSI status (3, 12–14).

The tumor microenvironment (TME) is a dynamic and ever-changing phenomenon that has a pivotal role in determining CRC initiation and progression. The TME is a unique environment that develops during tumor progression due to its interactions with the host. It comprises several components, such as immune cells, stromal cells, myofibroblasts, vessels, and extracellular matrix (ECM), which differ according to tumor type (15). The tumor growth occurs in a multi-step process, where the neoplastic cells recruit stromal and immune cells to establish the TME. Then, within the tumor site, the deranged production of inflammatory cytokines and growth factors by cellular components in the TME leads to further recruitment of various immune cells (16). Finally, angiogenesis and ECM degradation occur during the tumor growth, eventually leading to invasion and metastasis. Several multiplexed technologies, such as single-cell RNA sequencing and mass cytometry, explore the functional diversities of tumor-infiltrating immune cells and the recent progress in the cancer immunotherapy (17). Furthermore, multiplex immunohistochemistry/immunofluorescence (mIHC/IF) provides throughput staining and standardized quantitative analysis that could be a proficient approach to detect specific proteins or molecular aberrations as well as explore the immune evasion (18). Thus, it could have a great potential to discover novel prognostic and predictive biomarkers in cancer immunotherapy and contribute in translational research and clinical practice (19). During multiplex IHC, more than three markers can be analyzed simultaneously in a single cut of formalin fixed paraffin embedded tissue (FFPE) with good cell discrimination and spatial information due to recent developments in multiple immunolabeling and multispectral imaging (20–23). A valuable method for assessing the expression of numerous markers simultaneously in a single tissue section

was a multiplex IHC with tyramide signal amplification (TSA) (20–24). This is a more sensitive method than standard chromogenic IHC and may be able to identify proteins that are expressed at lower quantities (20, 25). In this review, we aim to discuss the various cellular immune components, focusing on the impact of immune checkpoint molecules on the CRC TME.

## Immune checkpoint molecules

The therapeutic use of antibodies that disrupt immune checkpoints was a critical turning point in the cancer immunotherapy (26). Blocking inhibitory coreceptors and pathways, which constrain immune cell activities in normal physiologic contexts, might “loosen the brakes” on immunological response, thus eliminating tumors. Immune cell activities are known to be exploited in malignancies (27). In addition, multiple immune checkpoint molecules have been identified in CRC pathogenesis and on various cell types, including lymphocytes, macrophages and neutrophils (28).

The co-inhibitory receptor programmed death-1 (PD-1), also known as CD279, is expressed inducibly on CD4<sup>+</sup> T cells, CD8<sup>+</sup> T cells, B cells, natural killer T cells, and macrophages (29). PD-L1 (B7-H1) and PD-L2 (B7-DC) are two known PD-1 ligands. PD-L1 is constitutively expressed on various immune and non-immune cells. However, PD-L2 expression can be induced in response to microenvironmental stimuli (30). The upregulation of PD-1 on tumor-infiltrating lymphocytes (TILs) and the increased expression of its ligands on tumor cells have been linked to tumor immune evasion, resulting in the suppression of tumor-specific CD8<sup>+</sup> T cells. This receptor upregulation has also been linked to T cell exhaustion in malignant tumors, defined as a reduction in the proliferation and cytokine production (31). Thus, blocking PD-1 and PD-L1 using monoclonal antibodies (mAbs) might be effective in stage IV solid tumors by overcoming this immune suppression (32, 33).

A well-known immune checkpoint molecule is cytotoxic T lymphocyte antigen-4 (CTLA-4), expressed on T lymphocytes' surfaces. CTLA-4 binds to B7-1 (CD80) and B7-2 (CD86) costimulatory receptors present on antigen-presenting cells (APCs), leading to inhibition of T cell activity by competitive blocking of CD28 (29). Therefore, CTLA-4 has been a hot target for mAbs cancer immunotherapy such as Ipilimumab (28). A remarkable target for immune checkpoint blockade (ICB) is lymphocyte activation gene-3 (LAG-3), a surface molecule of the immunoglobulin superfamily. LAG-3 interacts with MHC class II markers, thus leading to negative regulation of T cells, natural killer (NK) cells, B cells, and plasmacytoid dendritic cells (DCs) (34, 35). T cell immunoglobulin and mucin-containing protein-3 (TIM-3) is another immune checkpoint marker expressed on T helper 1 (Th1) and CD8<sup>+</sup> cytotoxic T cells (CTLs). TIM-3 plays a critical role in inhibiting Th1 responses by causing cell

death and is also known as hepatitis A virus cellular receptor 2 (HAVCR2) (36). Hence, blocking TIM-3 boosted the anti-tumor activity, with a greater efficiency upon combinatorial effect with PD-1 blockade (36). On the other hand, blockage of the inducible T-cell co-stimulator (ICOS), belonging to the B7-CD28 immunoglobulin superfamily, gained promising results in the treatment of different malignancies. Its expression is linked to a better prognosis in CRC patients, as the percentage of ICOS<sup>+</sup> CD4<sup>+</sup> cells operating as Th1 cells in either primary tumor tissue or peripheral blood could be a clinical predictive marker for a favorable prognosis (37).

CD40, a member of the tumor necrosis factor (TNF) family, was characterized on immune cells such as DCs, B cells and macrophages, as well as non-immune cells. The ligand of CD40 (CD40L) is expressed by activated B and T cells as well as platelets (38). CD40/CD40L interactions regulate T cell activity, cytokine production and antigen presentation (38, 39). In some cases, this interaction could inhibit tumor growth (40). On the other hand, tumors could utilize the CD40/CD40L to manipulate both T-cell and antigen-presenting compartments, thus contributing to the establishment of the immunosuppressive TME (38, 41). For instance, this immunosuppression could be achieved by inducing their proliferative capacity, growth, and survival (42).

Sialic acid-binding immunoglobulin-type lectins (Siglecs) are expressed on most white blood cells of the immune system, as well as TILs, DCs, and macrophages. Hypersialylation of neoplastic cells was identified as a hallmark of poor clinical outcomes and contributes to tumor escape from immune surveillance (43). Therefore, they are considered potential immune checkpoint targets for anticancer therapy (44, 45). Another promising target for cancer immunotherapy is the T cell immunoreceptor with immunoglobulin and ITIM domain (TIGIT). Its expression was known to be upregulated by various immune cells such as activated T cells, regulatory T (Treg) cells and NK cells. In addition, it can bind to two known ligands, CD155 and CD112, expressed by tumor and antigen-presenting cells in the TME (46).

## Therapies targeting immune checkpoint molecules in colorectal cancer

Several immunotherapeutic strategies are under clinical trials, especially in metastatic CRC; however, the results in MSS-CRC are generally modest. The ongoing studies investigate the outcome and potential biomarkers of metastatic CRC using various immunotherapy-based modalities, including immune checkpoint blockers (ICB) such as PD-1 blockers (e.g., nivolumab, pembrolizumab, atezolizumab, avelumab, durvalumab) and CTLA-4 blockers (e.g., ipilimumab, tremelimumab). This is besides the use of other approaches

such as cancer vaccines (autologous, peptide, viral vector, and dendritic cell-based) that aim to stimulate an immune response against tumor cells, as well as adoptive cell transfer using chimeric antigen receptor T-cell therapy to kill the tumor cells directly, and oncolytic virus therapy (e.g., herpes simplex virus and NV 1020) where the viruses selectively replicate in cancer cells to destroy them with no harm to normal cells. Also, among immunotherapies under clinical trials are indoleamine 2,3-dioxygenase 1 (IDO-1) inhibitors, OX40 antagonists (e.g., epacadostat, indoximod) that enhance the immune response, and biphasic antibody targeting carcinoembryonic antigen (e.g., RO6958688) on T cells (47, 48).

Multiple clinical trials in this research area are at different phases, and some of which have been completed and the results are expected to be published soon. To mention a few examples, a phase II clinical trial investigated a combination of pembrolizumab and azacytidine in metastatic CRC refractory to chemotherapy. The findings demonstrated the safety and tolerability of this regimen, however, the clinical effect was modest in the investigated cohort, likely due to DNA methylation and immunomodulation of the tumor as an effect of azacytidine therapy (NCT02260440) (49). Another remarkable study was IMblaze370, which did not meet its primary endpoint of improved overall survival with atezolizumab plus cobimetinib or monotherapy using atezolizumab vs. regorafenib in previously treated metastatic CRC (NCT02788279). The study findings highlighted the challenge of using immunotherapy in tumors with low baseline levels of immune inflammation, such as that observed in the MSS metastatic CRC (50). Results from ongoing comparative clinical trials, such as Morpheus-CRC, are likely to thoroughly evaluate the role of immunotherapy in CRC. Morpheus-CRC is an ongoing study to evaluate the efficacy and safety of multiple immunotherapy combinations in metastatic CRC (NCT03555149) (48).

There are several challenging factors in using immunotherapeutic agents in CRC. In contrast to melanoma, which represents a successful example of immunotherapy, patients with metastatic CRC responded modestly to immunotherapy treatment, with many trials with high failure rates. Several mechanisms may explain the discrepancy in immunotherapy outcomes in different types of cancer. The tumor mutational burden (TMB) has been early identified as a potential predictor for effective response to immunotherapy. For example, MSI in CRC, where there is deficient DNA repair, gives rise to high TMB. In addition, appropriate immune response in the intestine could be preserved by ameliorating the host immune system that must tolerate commensal bacteria while maintaining the ability to face infections, otherwise, severe chronic inflammatory reactions might occur (51). Another important aspect of the poor outcome of CRC to immunotherapy is the fact that most tumors are associated with activated WNT/ $\beta$ -catenin signaling which can promote dendritic cell and T-cell exhaustion (52). This is

similar to metastatic melanoma, where the activation of the WNT/ $\beta$ -catenin signaling pathway resulted in T-cell exclusion and resistance to anti-PD-L1/anti-CTLA-4 monoclonal antibody immunotherapy (53). Similarly, in a mouse model of hepatocellular carcinoma, the  $\beta$ -catenin pathway enhanced immune escape and suppressed the recruitment of DCs, and consequently led to impaired T-cell activity (54). Apart from the MSI status of the tumor, at the moment, no predictive biomarkers of immunotherapy response in CRC are available.

## Immune components of the colon cancer microenvironment

The cellular landscape of the TME includes various immune cells, namely, TILs such as T, B, and NK cells, as well as tumor-associated macrophages (TAMs) and tumor-associated neutrophils (TANs). Various immune checkpoint molecules are expressed on these immune cells, thus modulating the colon cancer microenvironment and regulating the pathogenesis and response to therapy (Figure 1). The anti-tumor and pro-tumor roles of these immune cells on the TME have been previously discussed in CRC context [reviewed in (55)].

## Tumor-infiltrating lymphocytes

TILs mainly include CD8<sup>+</sup> T cytotoxic and CD4<sup>+</sup> T helper lymphocytes, in addition to B and NK cells. They are usually considered the host protecting element against tumor formation, as they induce the recruitment, maturation, and stimulation of immune cells that repress tumor growth (56).

### T cells

In conventional terms, TILs represent the heterogeneous population of  $\alpha\beta$  T cells, both CD4<sup>+</sup> and CD8<sup>+</sup> subsets, present within the TME (57). CD8<sup>+</sup> T cells (CTLs) recognize tumor-associated antigens (TAAs) along with proteins of HLA class I. These cells become differentiated into killer cells, release perforins, and express the apoptotic inducer FasL after expansion. Perforins disrupt the cell membrane, aiding the entry of granzymes inside the cells, causing cleavage of caspases' precursors, thus directing the neoplastic cells toward apoptosis. Additionally, CD4<sup>+</sup> T helper cells proved to have an essential role in the anti-tumor immunity by responding to antigens presented by antigen-presenting cells (APCs) such as macrophages (58).

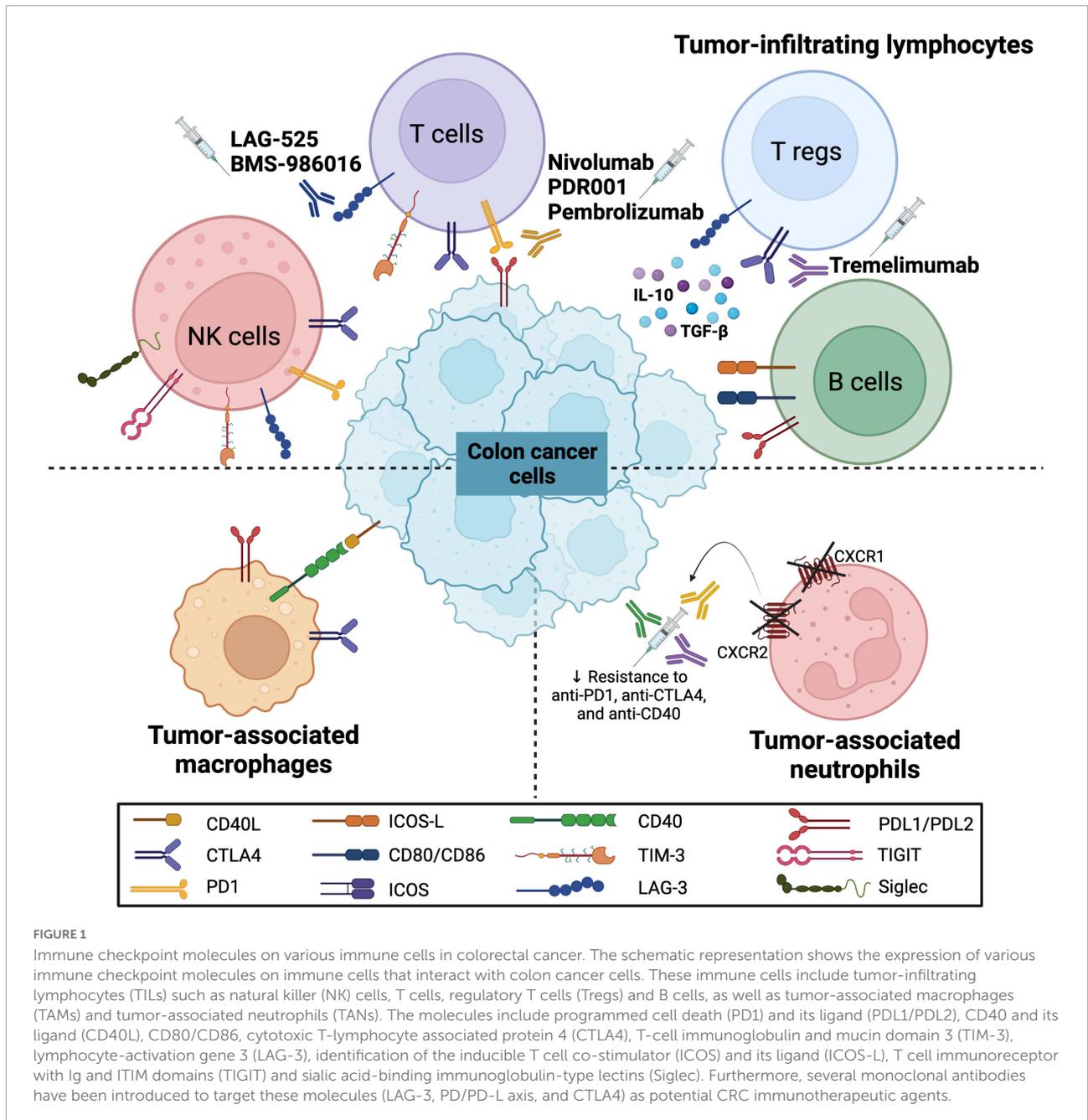
Increased TILs is a favorable prognostic factor in many malignancies, including CRC (59). In addition, the quantification of lymphocyte infiltration has prognostic significance, suggesting that lymphocyte infiltration is not passive but may actively modulate tumor growth (60). This was supported by a large multicenter study spanning more than

10 years, which demonstrated levels of lymphocyte infiltration into primary tumors to be a strong independent predictor of relapse and overall survival (61). Using expression profiling of CRC, they further defined the relevance of specific immune signatures, demonstrating that Th1 type interferon- $\gamma$  (IFN- $\gamma$ ) dominant immune profiles signified an improved prognosis. In contrast, Th17 type IL-17 dominant immune profiles signified a poor prognosis (61).

A recent study of most tumor-infiltrating immune cell subtypes revealed that CD8<sup>+</sup> T cells had the most significant impact on patients' survival (62). CD8<sup>+</sup> CTLs mediate tumor rejection by recognizing TAAs and directly killing transformed cells. Effector CD8<sup>+</sup> T cells in the TME generate IL-2, IL-12, and IFN- $\gamma$ , which enhance the cytotoxic potential of CD8<sup>+</sup> CTLs, leading to a targeted tumor cell killing (63, 64). On the other hand, CD4<sup>+</sup> helper T cells present in the TME are involved in activating CTLs against tumor cells (65). Exhaustion of CTLs could be caused by long-term interaction between CTLs and antigens, leading to loss of their efficiency and function.

Similarly, tumor cells suppress the immune response by inducing the exhaustion of CTLs in the TME through the expression of inhibitory immune checkpoint receptors such as PD-1, CTLA-4, and LAG-3 (66, 67). In CRC pathogenesis, PD-1 was shown to be upregulated on CD8<sup>+</sup> T cells in the TME, and its ligand was associated with cytokines and perforin impairment (30). Furthermore, a study by Hua et al. reported an inverse relationship between T cell density in the TME and the expression of PD-L1 on CRC cells (68). This was accompanied by an expansion of Treg cells, further linking the presence of PD-L1<sup>+</sup> tumor cells and poor prognosis (68).

CTLA-4 was found to be expressed on TILs within the epithelial component of the tumor, the surrounding tumor stroma and the invasive front of the tumor. Further, CTLA-4 was identified on subsets of Treg cells, where high expression of CTLA-4 was revealed along with a significant increase of activated Tregs (CD45R Foxp3<sup>+</sup> T cells) in the blood and tissues of CRC patients (69). Also, a highly suppressive subset of the CD4<sup>+</sup> Foxp3<sup>-</sup> T cell population was described in CRC patients to express multiple immune checkpoints (such as LAG-3, PD-1, and CTLA-4) and produce immunosuppressive cytokines such as IL-10 and transforming growth factor (TGF)- $\beta$  (70). Therefore, CTLA-4 expression on Treg cells highlighted its potential role as a therapeutic target in CRC, such as in the case of Tremelimumab, which has been investigated in a phase II study for CRC patients with refractory metastatic adenocarcinoma who failed standard chemotherapy (70). Additionally, LAG-3 was reported to regulate the function of Treg cells, and its expression on CD4<sup>+</sup> CD25<sup>+</sup> cells was associated with potent inhibitory activity (71). Exhausted CD8<sup>+</sup> T cells were observed to express LAG-3 along with other inhibitory receptors, such as PD-1, and thus inhibition of both PD-1



and LAG-3 could boost T cell activity (72). There are several clinical trials with LAG-3 inhibitors (LAG-525 and BMS-986016) with or without the combination of PD-1 inhibitors (Nivolumab and PDR001) in patients with advanced solid malignancies (28).

Xu et al. found considerably greater levels of circulating TIM-3<sup>+</sup> PD-1<sup>+</sup> CD8<sup>+</sup> T cells in CRC patients' peripheral blood samples than in healthy subjects' blood (73). The expression of TIM-3 and PD-1 on CD8<sup>+</sup> and CD4<sup>+</sup> T cells was also revealed in peripheral blood collected after surgery. Furthermore, both TIM-3 and PD-1 expression appeared to

be linked to decreased T cell activity (74). In comparison to adjacent colonic tissues, tumor tissue had a higher number of TIM-3<sup>+</sup> PD-1<sup>+</sup> CD8<sup>+</sup> T cells. Together with the lack of quantifiable responses to PD-1 blockage in a large group of CRC patients, these findings point to TIM-3 as a more prominent inhibitory receptor in CRC patients, thus limiting T cell responses. Furthermore, inhibiting this route may help to restore damaged cell-mediated immunity following surgical resection. These findings support the development of TIM-3 inhibitors and show considerable promise in CRC patients as single or combined treatments (34).

Immunoregulatory cells such as Treg cells, mesenchymal derived stem cells (MDSCs), and M2 macrophages possess the ability to control and modulate T cell function by releasing cytokines such as IL-10 and TGF- $\beta$  that can activate specific inhibitory immune checkpoints (75–77). Likewise, tumor cells and other cells in the TME can express these inhibitory ligands and activate their receptors, thus impairing T cells' activity (78). This was reported to disrupt the proliferation of CTLs and reduce the immune response against CRC (79).

A known prognostic approach for immune checkpoint inhibitor therapy is MSI. Furthermore, MSI is linked with an MMR system that recognizes and repairs DNA damage. Several clinical trial data highlight that deficient MMR (dMMR) or MSI were able to predict treatment response across different solid tumor types, including CRC (80). In particular, MSI is known to be a good predictor of CRC prognosis, as it is closely associated with the abundance of tumor-infiltrating T cells. Several immunohistochemical studies have revealed high infiltration of intraepithelial activated CD8<sup>+</sup> T cells within MSI colorectal tumors (81–83). Furthermore, Dolcetti et al. found that cytotoxic infiltrating structures were highly abundant in tumor epithelial cells of MSI-high (MSI-H) patients. The exact pathophysiology of TILs accumulation in MSI-H CRC has not been elucidated. However, an early proposal was that MSI-H tumors produce many abnormal proteins that trigger a host immune response. This was supported in a study by Smyrk et al. which reported an active immune microenvironment in MSI/dMMR tumors that are characterized by a more favorable prognosis compared to MSS/MMR-proficient (pMMR) tumors (8). In the MSI/dMMR subset of CRC, the high accumulated mutation creates many tumor-specific neoantigens, typically 10–50 times that of MSS/MMR-proficient subset (84), which might be the reason for the high level of TILs and active Th1/CTL immune microenvironment in MSI/MMR-tumors observed in many previous studies (8).

Moreover, granzyme B expression and other cytotoxic effects were more active in MSI-H tumors (85). Additionally, pMMR-microsatellite instability-low (MSI-L)/MSS have low tumor mutational burden, poor infiltration by TILs and often have a worse prognosis than dMMR-MSI-H as well as a poor response to immune checkpoint inhibitors (86). In the TME, the PD/PD-L1 pathway leads to the escape of tumor cells from the immune response via the inhibition of CTLs (87, 88). Additionally, the expression of PD-L1 on tumor cells is related to the exhaustion of T cells, therefore blocking this pathway has been demonstrated to be a successful approach for the treatment of different types of cancers, including non-small cell lung cancer, melanoma, breast, renal cell carcinoma, and CRC (87–92). In particular, higher expression of PD-1 and PD-L1 has been associated with a better prognosis in CRC patients. Furthermore, PD-1 expression in TILs has been found to be an independent prognostic factor for overall survival and disease-free survival of CRC patients, especially for MMR-proficient

tumors (93). Therefore, the upregulation of the PD-1/PD-L1 axis in CRC is correlated with a favorable clinical outcome. Such a pattern could be a compensatory upregulatory mechanism in the TME in order to identify the tumor and trigger an immune response. Furthermore, an association between PD-L1 on tumor cells and a high TILs density could further support this hypothesis, similar to that observed in breast cancer (94, 95). Moreover, there is a remarkable high expression of checkpoint molecules such as PD-1, PD-L1, CTLA-4, and LAG-3 in MSI CRC in comparison to MSS CRC, which could contribute to the immunosuppressive microenvironment that aids MSI tumors evade immune destruction by the infiltrating immune cells. Therefore, this explains why the MSI subset of CRC could be a potentially good candidate for the checkpoint immunotherapy (9). ICB was described as more effective in MSI CRC in a phase 2 trial of Pembrolizumab, a fully human mAb targeting PD-1. In addition, another PD-1 mAb, Nivolumab, showed efficacy in CRC, where a patient showed complete response with no disease recurrence and demonstrated MSI (27, 96). Therefore, MMR status is a critical key for response to therapy, as shown by different clinical trials with anti-PD-1 and anti-PD-L1 therapy. Moreover, it was also demonstrated that CTLA-4 expression is increased in MSI tumors compared to MSS cancers (84).

## B cells

Tumor-infiltrating B cells constitute a significant proportion of the immune infiltrates in CRC. Until recently, B cells have not been considered an important population of TILs, despite that they compose around 40% of TILs (97, 98). They are considered positive regulators of immunity, often collaborating with T cells to generate potent, unrelenting immune responses (98).

B cells can exert anti-tumor effects by activating antibody-dependent cell cytotoxicity (ADCC) and the complement cascade (99). In tumor tissues, B cells can be found in lymphoid aggregates, known as tertiary lymphoid structures (TLSs) or could be sparsely distributed in the TME. B cells present in the immature TLSs were reported to possess immunoregulatory functions by the secretion of anti-inflammatory cytokines and thus leading to the inhibition of anti-tumor immunity (100). Also, B cells can act as APCs besides their main function as antibody producers. Furthermore, B cells possess the unique capability of concentrating antigens through membrane immunoglobulin mediated uptake, which might also facilitate T cell activation above certain thresholds for TAAs (98, 101). Autoantibodies were shown to react primarily with autologous tumor targets or allogeneic tumors of the same tissue type, suggesting recognition of TAAs (102). Antibodies were believed to play a negligible role in the TME, so their relevance in tumor biology has been overlooked. However, studies revealed that B cell markers such as CD20 and CD138 correlated significantly with a lower CRC stage (103).

A study by Maletzki et al. observed that tumor-infiltrating B cells in primary CRC were of a mature immunophenotype, suggesting activation and antigen-induced maturation (104). This was supported by other studies where most tumor-infiltrating B cells reside in follicular aggregates in CRC. Likewise, peritumoral follicular aggregates of lymphocytes have been previously reported as a “Crohn’s-like reaction” and interpreted as an immune-mediated anti-tumor effect in CRC (105, 106). Similar to T cells, B cells express checkpoint ligands on their surface, such as PD-L1, CD80/CD86, and ICOS-L (107–109). Furthermore, a study by Helmink et al. observed significantly higher levels of B-cell-related gene expression, increased B cell receptor diversity, and clonal expansion in tumor samples from melanoma patients who responded to ICB treatment compared to other patients (110).

### Natural killer cells

Being members of the innate immunity, NK cells can lyse tumor cells without prior sensitization or clonal expansion, unlike T cells. NK cells can be classified into two major groups, where the CD56<sup>bright</sup> CD16<sup>−</sup> subset represents 10–15% of circulating NK cells and are more immunoregulatory by releasing cytokines such as IFN- $\gamma$ . They mainly reside in the secondary lymphoid organs, such as lymph nodes and tonsils (111). In contrast, CD56<sup>dim</sup> CD16<sup>+</sup> cells represent the significant population (90% of circulating NK cells) and predominantly mediate cytotoxicity (112, 113). NK cells play a fundamental role in cancer immunosurveillance through their anti-tumor activity (114). This has been supported by studies where the elimination of NK cells led to increased malignancy occurrence (115). NK cells perform their anti-tumor activity mainly when the expression of MHC class I molecules is downregulated. Moreover, upregulation of stress-induced molecules such as ligands of the activating receptor C type lectin receptor D (NKG2D) on cancerous cells makes them prone to NK-cell killing (116).

Most neoplastic cells and tumor-associated cells in the TME secrete factors that block the activation of NK cells, such as IL-6, IL-10, IDO, TGF- $\beta$ , and prostaglandin E2 (PGE2), through downregulating NK cells activating receptors including NKG2D (117). Thus, NK cells, which infiltrate the tumor stroma, might proficiently lose their tumor-killing function due to these immunosuppressive mediators (118). For instance, IDO causes tryptophan depletion and kynurenine accumulation leading to immunosuppression of T and NK cell functions as well as the stimulation of Treg cells (119). Additionally, PGE2 suppresses IFN- $\gamma$  production and responsiveness to IL-12 and IL-15 (120). Moreover, there is a reduction in the cytokine production of intra-tumoral NK cells (121). TGF- $\beta$  affects the IL-15 signaling pathway, thus dampening NK cell proliferation and cytotoxicity (122). Furthermore, hypoxia and poor nutrient levels in the TME suppress NK cell activity (116). On another note, NK

cell migration and penetration into the tumor growth site might be halted by ECM accumulation and increased interstitial fluid (123).

Furthermore, the recruitment of immunosuppressive cells such as MDSCs and the emergence of NK cell-resistant tumor variants result in primary tumor overgrowth. On the other hand, other tumor cells try to increase the expression of MHC class I molecules, such as human leukocyte antigen (HLA)-E, which engages the inhibitory receptor NKG2A on NK cells. This has been supported by studies where high expression of HLA-E and NKG2A led to a high inhibitory signal, potentially leading to poor outcomes and tumor growth (124–126).

NK cells have the potential to regulate the function of the adaptive immune system. For example, NK cells have been found to enhance T cell infiltration, thus triggering immune responses through their cytokine and chemokine secretion turning tumors immunologically “hot.” In contrast, the absence of these immune cells leaves the tumors immunologically “cold” (127). Consequently, CD8<sup>+</sup> T cell recruitment in the TME and their interaction with NK cells elicit tumor regression. In addition, NK cells possess anti-metastatic activity by possible elimination of circulating tumor cells, “i.e., metastatic clones” (118, 127). However, tumors could escape NK cell activity through several mechanisms, including immune checkpoints expression by NK cells: PD-1, CTLA-4, LAG-3, and TIM-3. Upon binding to their receptors, NK cell activity is dampened (128), which can be surpassed by ICB, thus restoring NK and CD8<sup>+</sup> T cell anti-tumor immunity. Nevertheless, many tumors still develop resistance to ICB therapy, representing a potential therapeutic target (129).

Another major obstacle in solid tumors is the homing of immune cells such as NK cells to tumor growth sites. This could be attributed to a dysregulation in the chemokine gradient in the TME, thus preventing NK cells from reaching the tumor growth sites (130). This has been reported in several studies where aberrant signaling pathways led to alterations in chemokines, including CCL27, CCL2, and CXCL11, hence impairing leukocyte migration (131–133). In CRC, loss of MHC class I expression is quite common, allowing NK cell recognition and killing of tumor cells (134, 135). However, like other types of cancer, a decreased number of NK cells in CRC patients was reported, which was associated with an increased frequency of CRC tumor recurrence (136, 137). This has been further supported where a negative correlation between peripheral NK cells and the CRC staging was reported, especially at early (I) and late (IV) stages of the disease (138). Phenotypically, CRC patients exhibited a reduction in the expression of the natural cytotoxicity receptors, NKp44 and NKp46 (139).

Furthermore, other activating receptors such as NKG2D, NKp30, NKp46, and DNAX accessory molecule-1 (DNAM-1) were reduced in the peripheral blood of patients with CRC (140–142). Upon tumor progression, the percentages of NKG2D<sup>+</sup> NK

cells were decreased, indicating a role in the metastasis of CRC (143). It has been shown that reduced expression of NKG2D on NK cells was correlated with high soluble serum levels of its ligand MHC-class I related molecule A (MICA) (144). The pathway of NKG2D and its ligands has been reported to be affected by TGF- $\beta$ , which is highly expressed by colorectal cells (145). Hence, ligands of the activating receptor NKG2D were detected in the early stages of CRC, but as an immune evasion strategy, their expression decreased upon disease progression (146). Additionally, dysregulated NK cells displayed impaired function in CRC, including IFN- $\gamma$  secretion and degranulation (140). Moreover, phenotypic alteration has been observed in the circulating CD56<sup>dim</sup> population of NK cells in CRC patients (139). Interestingly, a subpopulation of NK cells that is positive for CD16 and CD56 was studied and correlated negatively with the occurrence of CRC and the staging of CRC (147). The inhibitory receptor, NKG2A, has been reported to be an interesting target as a checkpoint molecule in cancer (148). Thus, blocking the inhibitory NKG2A receptor enhances tumor immunity by promoting both NK and CD8<sup>+</sup> T cell effector functions. Monalizumab, a humanized anti-NKG2A antibody, was reported to induce NK cell activity against various tumor cells, especially in combination with PD axis blockade (149). This is under investigation in multiple clinical trials in solid tumors such as CRC (149).

Differentiated CRC cells were found to be more resistant to NK cells compared to cancer-initiating cells that were more susceptible to NK cell killing (150). It has been established by both *in vitro* and *in vivo* studies, where NK cells were shown to mediate the direct killing of human tumor cells in colon cancer (151–153). This has been implemented in clinical settings, where autologous NK cells were utilized in patients with advanced gastric or colorectal cancers combined with trastuzumab or cetuximab chemotherapy (154, 155). Colon adenocarcinomas exhibited low NK cell infiltration rates, thus causing the NK cell population to remain in the outer stroma and halting them from performing their anti-tumor activity (60, 134, 156, 157). Additionally, infiltration of NK cells was proposed to be a potential predictive marker of therapy. The homing and migration of NK cells are dependent on selectins, adhesion molecules and chemokines. Hence, future clinical trials should target the trafficking of NK cells into tumor sites rather than focusing on the simple administration of a single cytokine/chemokine as a therapeutic approach (157).

Another interesting aspect that is critical for immunotherapy for CRC is the expression of immune checkpoint molecules on NK cells (158). These include CTLA-4 and PD-1 receptors as well as TIGIT, CD96, LAG-3, and TIM-3. In CRC animal models and human patients, NK cell exhaustion was reported to be associated with the expression of TIGIT. Furthermore, the presence of NK cells was critical for the efficacy of TIGIT and PD-L1 checkpoint inhibitors, as they regulate the frequency of effector CD8<sup>+</sup> T cells secreting

IFN- $\gamma$  and TNF- $\alpha$  (159). The combination of these checkpoint inhibitors showed a synergistic effect in their anti-tumor potential that was accompanied by prevention of NK cell exhaustion in both animal models and CRC patients (159, 160). In addition, PD-1 was found to be upregulated on tumor-infiltrating and peripheral NK cells in digestive cancers such as esophageal, gastric, biliary, and CRCs (161).

Other recently reported immune checkpoints are the Siglec family receptors, such as Siglec-7 and -9, CD47, and CD200. On another note, NK cells express Siglec-7 and Siglec-9 receptors, with a further upregulation on the cytotoxic CD56<sup>dim</sup> NK cell subset (162, 163). In addition, Siglec-9 was found to be upregulated on tumor-infiltrated CD8<sup>+</sup> cytotoxic T cells in various solid tumors, including CRC (164, 165). An interesting fact about the Siglec immune checkpoint molecules is that they are expressed on various immune cells and are usually expressed on T cells that concomitantly express PD-1, further enhancing the co-inhibitory signal (165). Furthermore, they were known to play an inhibitory effect on NK cell function against tumor cells, particularly cytotoxicity.

On the other hand, blocking these immune checkpoint molecules such as Siglec-9 antibodies improved the anti-tumor cytotoxic potential of NK cells. This was due to the blockage of Siglec markers on tumor cells as well as the NKG2A receptor on NK cells (164). Also, sialidase treatment was found to enhance NK cell killing against various cell lines, including the colon cell lines. Therefore, anti-Siglec-7 and anti-Siglec-9 blocking antibodies could be developed to be used for cancer immunotherapy, along with other immune checkpoint inhibitors.

## Tumor-associated macrophages

TAMs are the dominant inflammatory constituent in the TME and are ample in all stages of carcinogenesis. Activated infiltrating TAMs secrete a plethora of proteolytic enzymes as well as growth and inflammatory mediators, known to modulate different molecular pathways involved in tumor progression and metastasis (166).

Macrophages can be classified into two well-defined subtypes: M1 macrophages “classically activated” and M2 macrophages “alternatively activated.” M1 macrophages have a pivotal role in eradicating different organisms and cancerous cells, as they have an inflammatory function by secreting pro-inflammatory cytokines like TNF- $\alpha$ , IL-6, and IL-1 $\beta$ . On the contrary, M2 macrophages release anti-inflammatory cytokines, such as TGF- $\beta$ , IL-10, and IL-13, and have been implicated in tissue healing and tumor progression. M1 and M2 are distinguished with certain markers in the tumor samples, where M1 macrophages are characterized by the expression of HLA-DR, CD11c, CD86, inducible nitric oxide synthetase (iNOS), and phosphorylated signal transducer and

activator of transcription 1 (pSTAT1), while M2 macrophages express CD163, CD204, and CD206 (167). In the TME, TAMs are mostly pro-tumorigenic/anti-inflammatory “M2 phenotype form.” Their significance in tumor evolution and progression is accentuated by the fact that they may comprise up to 80% of the tumor mass (168). The suppression of an immune response, activation of angiogenesis, and remodeling of ECM are important functional characteristics of TAMs. Furthermore, TAMs produce proteolytic enzymes such as matrix metalloproteinases (MMPs) and cathepsins that cause ECM breakdown, leading to the intravasation of tumor cells into the bloodstream, thus enhancing metastases (169). Additionally, TAMs release angiogenic factors, allowing tumor cells to spread beyond the primary tumor site and contributing to metastasis (170). They also provide a favorable environment for metastatic tumor cells by releasing inflammatory mediators like IL-1 $\beta$ . Furthermore, reactive oxygen species (ROS) produced by TAMs are implicated in malignant cell instability, a hallmark of cancer (168). On another note, TAMs could promote cancer cell proliferation by releasing growth factors such as epidermal growth factor (EGF) (170).

Recently, the effect of colon cancer ECM on macrophage polarization was investigated, where it was discovered that tumor ECM-educated macrophages could develop into M2 macrophages. The anti-inflammatory markers (IL-10, CCL18, and TGF- $\beta$ ) were upregulated, and the pro-inflammatory markers (TNF- $\alpha$  and IL-6) were downregulated by the macrophages that are differentiated within the tumor matrices. It was also found that MMP1, the MMP responsible for M2 polarization, was upregulated in tumor matrices. These results indicated that tumor-derived matrices caused an anti-inflammatory M2-like macrophage polarization significantly (171). Additionally, clinical staging and lymph node metastases were found to be associated with macrophage infiltration and vascular density in CRC (172). Moreover, blocking the colony-stimulating factor 1 receptor (CSF1R), required for TAMs' recruitment, differentiation, and survival, is one of the most effective ways to target TAMs (173). Small molecule inhibitors or mAbs against CSF1R diminish the number and/or affect the behavior of TAMs in mice models of solid tumors such as CRC, breast cancer, and glioblastoma, thus impairing tumor formation and progression (174–176).

TAMs were reported to express molecular triggers of checkpoint proteins that regulate T-cell activation. Such proteins are the site of action of checkpoint-blockade immunotherapies (177). On another note, TAMs are key players in immunological resistance and their manipulation could improve the efficiency of immunotherapies, possibly through the NF- $\kappa$ B pathway. Such a pathway could be inhibited to increase the efficacy of immunotherapies by repolarizing M2 TAMs and to decrease the expression of PD-L1 on them (178). A recent study in

CRC by Fiegle et al. showed that the combined blockade of CTLA-4 and PD-L1 increased the levels of the pro-inflammatory Th1/M1-related cytokines, increased NOS<sup>+</sup> macrophages in the tumor tissue and reduced PD-L1<sup>+</sup> macrophages (179). The role of TAMs as therapeutic targets was reviewed by Malfitano et al. (177). Also, CD40<sup>+</sup> TAMs and plasma sCD40 in CRC tissues have been identified as favorable prognostic markers (180). Apoptotic susceptibility is dependent on the “quality” of the signal, as death occurs when the CD40 signal is delivered in membrane-bound form (mCD40L), whereas the soluble CD40 agonists are non-apoptotic (181). Blocking of CD40 using membrane-bound CD40L showed pro-apoptotic signal and pro-inflammatory cytokine production in CRC cells, thus suggesting CD40 as a promising therapeutic in CRC (182).

## Tumor-associated neutrophils

Neutrophils play an intricate and complex role in cancer (183). Many reports support the dual function of neutrophils, including anti-tumoral and pro-tumoral roles, and thus TANs are segregated into anti-tumor (N1) and pro-tumor (N2) phenotypes (184). However, these cells do not have specific cell surface markers to discriminate N1 and N2 neutrophils. Some studies indicate that N1 neutrophils have a higher expression of CD54, CD95, TNF- $\alpha$ , CXCL10, and low production of IL-8, while N2 neutrophils have high expression of CD182 and IL-8 production (185). In addition, neutrophils play a role in the immunosuppression of tumors (186), through the release of different mediators, including IL-4, TGF- $\beta$ , immune checkpoint ligands, ROS, and reactive nitrogen intermediates (187). On the other hand, releasing nitric oxide by neutrophils could enhance cancer cell killing and suppress CRC growth and metastasis (188).

Under the effect of TGF- $\beta$  present in the TME, neutrophils polarize into pro-tumor N2 neutrophils, which produce proangiogenic factors and exert immunosuppressive activity through the secretion of arginase-1 (Arg1) (184, 189, 190). TANs mediate direct suppression of Th1 and CTL in tumors (191). On the other hand, upon blockade of TGF- $\beta$  or administration of type 1 IFN, neutrophils could polarize into anti-tumor N1 neutrophils, which activate CD8<sup>+</sup> T cells, thus exerting anticancer cytotoxic activity, by reducing the expression of the proangiogenic factors (e.g., VEGF and MMP-9), and increasing the expression of T cell-attracting chemokines (e.g., CCL3, CXCL9, and CXCL10) (184, 189, 192).

Neutrophils are recruited to the tumor site through inflammatory molecules such as granulocyte-colony stimulating factor (G-CSF), tumor-derived cholesterol derivatives (oxysterols) (193) and anaphylatoxin C5a (complement component) (99, 194). In CRC, neutrophils play an anti-tumoral role through the secretion of IFN- $\beta$ , IFN- $\gamma$  and

Granulocyte macrophage-colony stimulating factor (GM-CSF), and are known to express CD66b, CD11b, CD101, and CD177 (187). Neutrophils may promote tumor metastasis by accumulating in the metastatic niche. Tumor and stromal cells expressing G-CSF, CXCL1, and CXCL2 enhance neutrophil recruitment in the metastatic sites (195).

In solid tumors, neutrophils' accumulation is a poor prognostic marker associated with tumor progression and metastases (196–198). However, in CRC, high infiltration of TANs was reported to be associated with a better response to 5-FU-based chemotherapy (199). In this regard, CRC represents an exception from other solid tumors in which a high number of TANs is associated with poor response to chemotherapy and radiotherapy (200). Different key players in tumor immunobiology among different cancers may explain the discrepancy of TANs function in CRC compared to other tumors (e.g., ovarian and gastric).

Noteworthy, neutrophils interact with TILs. Using an inducible colon tumor mouse model, Germann et al. reported that the most potent inhibitor of T-cell activity in the TME was the TANs. The suppression is exerted through matrix metalloproteinase-mediated activation of TGF- $\beta$  (201). Interestingly, MMP-9 secreted by TANs, converts TGF- $\beta$  precursor into an active form. Thus, inhibiting the MMP-9/TGF- $\beta$  axis eliminates the immunosuppressive effect of neutrophils and suppresses their tumor-promoting functions (201). On the other hand, a recent study reported that the pre-operative and post-operative neutrophil to lymphocyte ratio was associated with histological markers of CRC progression. Also, there was a trend of association between post-operative neutrophil count and disease-free survival (202). Different factors affect neutrophil polarization and may, at least in part, explain the apparent paradoxical impact of TME neutrophil count.

The link between TANs infiltration and tumor angiogenesis determines to a great extent the response to ICBs. It has been reported that neutrophil infiltration in the TME is associated with significant resistance elements to ICBs and their adjuvant anti-angiogenic agents. More than 100 clinical trials investigate the combination of bevacizumab (Avastin; anti-VEGF-A antibody) with ICBs (203). In addition, inhibition of CXCL1 or CXCL5/CXCR2 signaling in tumors with low TILs causes a reduction in TANs infiltration, with an increase in the number of PD-1<sup>+</sup> CD8<sup>+</sup> T cells. Furthermore, this enhances the sensitization of cancer cells to the anti-CD40, anti-CTLA-4, and anti-PD-1 combination immunotherapy (204). Moreover, the use of CXCR2 inhibitors might overcome the resistance to anti-PD-1 immunotherapy in KRAS<sup>G12D</sup>-expressing CRC (205). Such findings, together with similar ones in other cancers, promoted the development of phase I and II clinical trials, using CXCR1 and CXCR2 inhibitors in combination with anti-PD-1 in patients with metastatic CRC with MSI-L and Ras-mutation (195). Furthermore, the

“neutrophil extracellular trap” or “NET” is considered an important element of the TME that leads to resistance to ICB therapy (206, 207). Accordingly, DNase I, an inhibitor of NETs, was reported to significantly enhance the therapeutic effects of anti-PD-1 in an MC38-bearing mouse model of CRC (208).

## Conclusion

Blocking immune checkpoints has ushered in a new era of cancer treatment. Targeting immunological checkpoints in CRC TME is an intriguing novel cancer therapeutic approach via altering the immune cells' function. Increasing evidence suggests that patients' responses are linked to different pro-tumor and anti-tumor immune cells in the TME, such as TILs, TAMs, and TANs. Anti-PD-1, anti-PD-L1 and anti-CTLA-4 are well-known ICBs showing promising results in CRC patients. In addition, other intriguing immunological checkpoints that can suppress T or NK cell activity have emerged in recent years, such as TIM-3 and LAG-3. As a result, combining ICBs with other therapeutic modalities has shown encouraging results and could be a successful step forward in CRC treatment.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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