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REVIEW

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Cellular mechanisms of combining innate immunity activation with PD-1/PD-L1 blockade in treatment of colorectal cancer

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Abstract

PD-1/PD-L1 blockade therapies have displayed extraordinary clinical efficacy for melanoma, renal, bladder and lung cancer; however, only a minority of colorectal cancer (CRC) patients benefit from these treatments. The efficacy of PD-1/PD-L1 blockade in CRC is limited by the complexities of tumor microenvironment. PD-1/PD-L1 blockade immunotherapy is based on T cell-centered view of tumor immunity. However, the onset and maintenance of T cell responses and the development of long-lasting memory T cells depend on innate immune responses. Acknowledging the pivotal role of innate immunity in anti-tumor immune response, this review encapsulates the employment of combinational therapies those involve PD-1/PD-L1 blockade alongside the activation of innate immunity and explores the underlying cellular mechanisms, aiming to harnessing innate immune responses to induce long-lasting tumor control for CRC patients who received PD-1/PD-L1 blockade therapy.

Keywords Colorectal cancer, Innate immunity, PD-1/PD-L1 blockade, Combinational therapy, Cellular mechanism

Introduction

Colorectal cancer (CRC) stands as the third most common malignancy and ranks as the second leading cause of cancer death worldwide [1]. Nearly half of patients with CRC had liver metastasis, thus lost the chance to undergo surgery. The current first-line treatment of metastatic CRC is the use of single targeted agents plus chemotherapy regimens. Recently developed immunotherapy, especially PD-1/PD-L1 blockade, have changed the treatment paradigm in many types of cancer, including CRC [2].

PD-1 (programmed cell death-1, also known as CD279), is a receptor belongs to the family of immune checkpoint proteins and is expressed on the cell surface of activated T cells and pro-B cells. Its ligands programmed death ligand1 (PD-L1) and programmed death ligand2 (PD-L2) are expressed on macrophage or dendric cells (DCs). In normal condition, binding of PD-1 and PD-L1 induces protective signals to inhibit over-activation of immune system, protecting host from immune attack. However, evolved tumor cells overexpressed PD-L1 and evaded immunosurveillance in PD-1/PD-L1 interaction dependent manner. PD-1/PD-L1 interaction hampers function of cytotoxic T lymphocytes in response to cancer and induces T cell exhaustion, thus PD-1/PD-L1 inhibitors have been designed to block this inhibitory immune checkpoint [3].

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Although PD-1/PD-L1 blockade therapies have displayed extraordinary clinical efficacy for melanoma, renal, bladder and lung cancer, only 15% of CRC patients (Mismatch repair-deficient and microsatellite instability-high) benefit from these treatments, demonstrating the need to improve the efficacy of PD-1 blockade for the majority of patients with CRC [4]. Moreover, PD-1/PD-L1 blockade immunotherapy is based on T cell-centric perspective of tumor immunity. However, T cells were not dissociated with whole anti-tumor immune system, especially the initiation and maintenance of T cell responses as well as the establishment of enduring adaptive immune memory depend on innate immune responses.

Innate immunity is composed of myeloid lineage and lymphoid cells. The former includes monocytes, macrophages, DCs, granulocytes and mast cells, and the latter is mainly natural killer (NK) cells. The innate immune system is the first line to defend viruses, bacteria, parasites, or to detect wounds [5]. Upon infection or wound happens, innate immune system rapidly activates immune cells to destroy invader, or to initiate repair. After the innate immune system activation, phagocytosis by professional phagocytes (macrophages, neutrophils, monocytes and DCs) and natural cytotoxicity by NK cells were prominent processes via antibody-dependent cellular phagocytosis (ADCP) and antibody-dependent cell cytotoxicity (ADCC) [6].

Given the crucial role of innate immune responses in anti-tumor immunity, harnessing innate immune responses most likely enhanced the efficacy of PD-1/PD-L1 blockade for CRC patients with MSS phonotype (MSS, Microsatellite stability). Here we reviewed the tumor immune environments of CRC, the mechanism of resistance to PD-1/PD-L1 immunotherapy, and the pre-clinical and clinical studies on anti-tumor efficacy of combinational therapy in CRC, pointing out new strategy for improvement of the efficacy of PD-1/PD-L1 blockade for CRC patients.

Tumor immune microenvironment in CRC

Tumor microenvironment (TME) in CRC is complex as it includes different cell components, such as tumor cells, immune cells, fibroblasts and endothelial cells. All components in TME affect the prognosis of CRC patients in a directly or indirectly manner.

Cell components in tumor microenvironment

Tumor infiltrating lymphocytes

Cytotoxic T lymphocytes CRC patients with higher cytolytic immune cell infiltration showed beneficial overall survival (OS) and disease-free survival (DFS). CD8 + T cells accounts for the majority of cytolytic cells in TME, which kills tumor cells by secreting GZMB, perforin, IFN-γ, TNF-α or via Fas ligand (FasL) pathway [7]. Besides, cytotoxic CD4 + T cells has been observed in tumor which recognize tumor antigens presented by major histocompatibility complex (MHC) class II, and kill tumor cells in a similar way with cytotoxic CD8 + T cells [8]. However, the role of cytotoxic CD4 + T cells in CRC is still not fully explored and needs to be further investigated.

T help cells (th cells) *Th1/Th2 cells*

Th1 cells are subsets of CD4 + T cells which affect CRC prognosis by producing cytokines IFN-γ, TNF-α and IL-2. CRC patients with high expression of Th1-associated gene in tumor, such as *T-bet*, *IRF1*, *IL12Rb2* and *STAT4*, exhibit a favorable prognosis. In contrast, there is no significant correlation between frequency of GATA3 + Th2 cells in CD3 + T cells and clinic-pathologic features in advanced CRC patients [9].

Th9

Th9 is a subset of IL-9 producing T help cells, and IL-9 production from Th9 cells can be induced by IL-4, IL-21 and TGF-β but inhibited by IFN-γ. Th9 cells have been proved to be involved in the development of inflammatory bowel disease (IBD) and CRC, and Th9/IL-9 displayed both anti-tumor and pro-tumor role in CRC development. It is reported that IL-9 conversed suppressive regulatory T cells (Tregs) to Th9 cells to inhibit CT26 tumor growth. While overexpression of IL-9 in colon cancer cell lines induced cell proliferation via upregulation of c-Myc and cyclin D1 [10].

Th17

Th17 cells, the IL-17 A/F-expressing Th cell subtype, can be induced by several cytokines, including IL-1β, IL-6, IL-21, IL-23 and TGF-β. Th17 cells is related to colorectal inflammation and tumorigenesis and IL-17 play a critical role in metastasis and prognosis of CRC [11]. Although studies showed an antitumor role of IL-17, most studies demonstrated that Th17/IL-17 trigger and amplify the inflammatory immune response and tumorigenesis in the colorectum. IL-17/IL-17 receptor interaction promoted tumor angiogenesis by stimulating VEGF production from endothelial cells or CRC tumor cells. Furthermore, IL-17 induced chemoresistance in CRC via activating the mTOR pathway [10].

Th22

Th22 cell is a novel subset of T helper cells. Th22 cell differentiation is dependent on transcription factor

RORγt, but not T-bet. Th22 cells produce IL-22, IL-26 and IL-33, and stimulates intestinal epithelial cells to produce immunosuppressive IL-10, thus it promotes the development of CRC. Moreover, higher IL-22 level in serum was related to chemoresistance in CRC patients [10].

Regulatory T cells (Tregs) Tregs, the specialized subset of T cells, act to suppress immune response. Tregs consume IL-2 by highly expressed CD25, release adenosine produced by CD39 and CD73 ectoenzymes, induce immunosuppressive factor (IDO, IL-10, TGF-β and IL-35), inhibit dendritic cells maturation and promote angiogenesis in tumor. High FOXP3 + expressing T cells infiltration is associated with poor outcome of CRC patients.

Natural killer cells (NK cells) NK cells are one of the prominent lymphocytes of the innate immune system. It plays anti-tumor roles via producing granzyme B and perforin or through expression of FasL and TRAIL (TNF-related apoptosis-inducing ligand). Nevertheless, cancer cells potentially escape the recognition of NK cells by regulating MHC class I molecule expression, inducing secretion of immunosuppressive factors, including IL-10, TGF-β and IDO. Besides, NK cell exhaustion, characterized with PD-1 expressing NK cells, can be induced when CRC occurred [12].

B lymphocytes B cells play a major role in humoral immunity. Gut plasma cells produced sIgA and sIgM to protect epithelial barrier from intestinal bacterial dysbiosis. In CRC, infiltrated B cells in TME are characterized by terminally differentiated memory B cells or plasma cells [13]. The high frequency of B and plasma cell in TME is positively correlated with the favorable prognosis of patients with CRC; however, regulatory B cells (Bregs), another subset of B cells, express PD-L1 and exhibit immune-suppressive function in advanced tumors and metastases, can be recruited by upregulated CXCL9/10 in CRC tumors [12].

Myeloid cells

Dendritic cells (DCs) DCs serve as specialized antigen-presenting cells (APCs) which are essential in induction and maintenance of the anti-tumor immune responses by bridging innate immunity and adaptive immunity. DC population can be classified into plasmacytoid DC (pDC) and myeloid DC, which are also referred to conventional DC (cDC1 and cDC2). pDCs could support the tumoricidal processes but also induce Tregs generation. cDC1, such as CD103 + cDC1, are critical in inducing CD8 + T cell-mediated immune responses to tumor, and cDC2 play an important role in inducing CD4 + T cell responses against tumors. It has reported that higher pDCs infiltration in CRC correlated with poor prognosis, while mature cDCs infiltration within the tumor correlated with favorable prognosis in CRC [14], but tumor cells and stromal cells in TME hamper DC differentiation and maturation by secreted cytokines, such as VEGF, prostaglandin E2 (PGE2), TGF-β, IL-1β, IL-10 and IL-13. Immature DCs in TME induced T cell exhaustion and T cells expressing PD-L1, Tim-3, LAG-3, IL-10, IDO and TGF-β further enhanced immunosuppression in TME [12].

Myeloid-derived suppressor cells (MDSCs) MDSCs has been identified as immunosuppressive cell which assist tumor cell to escape the immune surveillance and promote tumor development [15]. MDSCs population can been divided into two subgroups: monocyte origin (Mo-MDSCs) and polymorphonuclear origin (PMN-MDSCs) in human and mice. MDSCs activated inducible NO synthase (iNOS) and arginase-1 (ARG1), promoted production of several immunosuppressive factors, such as NO and ROS, and lead to inactivation and proliferation inhibition of T cells. MDSCs population in blood circulation was both increased in premalignant states and late stage of CRC. CRC tumor growth could be supported by MDSCs. Inflammatory and soluble mediators such as histamine, prostaglandins, miRNA, mRNA, Hsp72 and local hypoxic and low pH microenvironments also promoted suppressive function of MDSCs by regulating proliferation and ARG1, iNOS, PD-L1 and VISTA expressions in CRC [16].

Tumor-associated macrophages (TAMs) TAMs are most abundant immune cells in tumors. Generally, TAMs were distinguished into two subtypes: M1- and M2-like TAMs. M1-like TAMs play critical role in inhibiting tumor progression, while the role of M2-like TAMs were not. The phenotypes of TAMs are plastic and the anti-tumor or oncogenic activities of TAMs was depended on various factors within the TME. In CRC, the presence of CD68 + macrophages within the invasive margin of tumors is associated with a favorable prognosis for patients, however, it has widely recognized that TAMs in CRC promote angiogenesis and metastasis with production of VEGF in TME, moreover, in the advanced CRC, the predominant polarization of macrophages shifts towards pro-tumorigenic M2 macrophages [12].

Granulocytes Granulocytes are a type of leukocytes which containing large numbers of cytoplasmic granules

including neutrophils, eosinophils, and basophils. Single-cell transcriptome analysis revealed that CRC metastases harbored a relatively higher granulocytes compared with normal samples, suggesting this subset play a prominent role in CRC prognosis [17]. Neutrophil granulocytes were regarded as the first defender of the innate immune system to fight against extracellular pathogens. Recently, the role of neutrophils in tumor attracts more attention. Tumor associated neutrophils (TANs) exhibit plasticity and can be polarized into anti-tumorigenic N1 neutrophils or a pro-tumorigenic N2 neutrophils in response to environmental stimulation [18]. N1 TANs inhibit angiogenesis and eliminate tumor cells by the production of TNF-α, ROS and Fas or downregulating arginase expression, while N2 TANs promote tumor invasion and angiogenesis by producing MMP-9, VEGF, and NETs formation. Besides, N2 TANs is capable in T cell proliferation inhibition and T cell apoptosis induction. Neutrophils to lymphocytes ratio (NLR) in blood has shown prognostic benefit to CRC. Lower level of basophil granulocytes in blood from CRC patients is associated with poor survival [19], while for eosinophil granulocytes, increasing studies suggested a positive correlation with good prognosis in CRC patients. Human eosinophils induce apoptosis in CRC cell lines by release of ROS, EPO, ECP, EDN, TNF, and granzyme A [20].

Mast cells Mast cells (MCs) play a vital role in anti-tumor immunity. In response to danger signal, MCs trigger rapid and longer-term inflammatory responses by releasing a variety of immune mediators, such as histamine, serotonin, cytokines (IL-6, IL-9, IL-13 and TNF), chemokines (CXCL8, CCL2 and CCL5), and proteases (chymase, tryptase and carboxypeptidase). Prognostic role of MCs varies in different cancer types, and the role of MCs in CRC progression remains a topic of debate. Mao et al. reported lower density of tumor-infiltrating MCs is associated with prognostic benefits in CRC, while Malfettone et al. found that MCs infiltration is linked to survival advantage [21].

Cancer-associated fibroblasts (CAFs)

During colon tumorigenesis, fibroblasts, the major stromal population, was reeducated to cancer-associated fibroblasts by cytokines in TME, such as platelet-derived growth factor (PDGF), TGF-β, IL-4, IL-6, insulin-like growth factor II (IGF-II), fibroblast growth factor 2 (FGF2) and prostaglandin E (PGE). Modified CAFs secrets immune-related factors, including epidermal growth factor (EGF), FGF-1, FGF-3, hepatocyte growth factor (HGF), IGF-1, IGF-2, VEGF, CXCL12, macrophage migration inhibitory factor (MIF), various vitronectin and miRNAs [13]. MIF and EGF derived from CAFs contributes to the development of chemoresistance in CRC [22, 23]. CAFs also secrete HGF to enhance the migration of colon cancer cell via the HGF-MET signaling pathway [24]. FGF-1/-3 and VEGF secreted from CAFs promote colon cancer cell growth and angiogenesis [25, 26]. CAFs also produce CXCL12 which enhance immune escape by through inhibiting degradation of PD-L1 [27]. Thus, CAFs regulate the development of CRC and contribute to prognosis of patients with CRC.

Tumor-associated endothelial cells

Endothelial cells (ECs) are instrumental in the development and functionality of blood and lymph vessels, and dysregulated angiogenesis and lymph angiogenesis is one of the hallmarks of CRC. Lymph (angiogenesis) was regulated by pro-(lymph) angiogenic factors, such as VEGF family (VEGF-A/B/C/D, PIGF) and their receptors (VRGFR1-3). ECs in TME show an irregular shape, size and function, and they regulate immune response in TME. Tumor-derived cytokines act on tumor endothelial cell directly or indirectly, regulating adhesion molecules expression on endothelial cells (s-endoglin, ICAM1, VCAM1, E-selection, CLECER1, MadCAM1), and then affects T cell extravasation within tumor. Moreover, ECs regulate T cell activation by inducing co-stimulatory/inhibitory molecules expression (PD-L1, FasL, TRAIL, CD137 and OX40L) and affect T cell metabolism by upregulating enzymes (IDO1, eNOS and Arginase) [28].

Collectively, these cell components in TME work together in immune regulation within CRC tumor via direct cell-to-cell contact and/or indirectly cytokine/chemokine productions (Fig. 1). Thus far, extensive investigation into the tumor microenvironment of CRC has been conducted with the deployment of cutting-edge single-cell sequencing technology to elucidate a diverse array of immune cell types. Nevertheless, the pivotal role of a particular immune cell subtype throughout initiation and progression of CRC remains undefined.

Consensus molecular subtypes of CRC

CRC is characterized with genetic heterogeneity, and several molecular pathways has been reported to be implicated in its initiation and development. Consensus molecular subtype (CMS) classification has been proposed based on differential gene expression of tumor and infiltrating cells, and four major groups have been identified according to the CMS classification (Fig. 2).

CMS1 tumors (the MSI immune subtype, MSI, Microsatellite instability), accounting for approximately 14% of all CRC cases, exhibit MSI type, hypermutation, higher CIMP (CpG island methylator phenotype) and enrichment of *BRAF* mutations and immune cells in

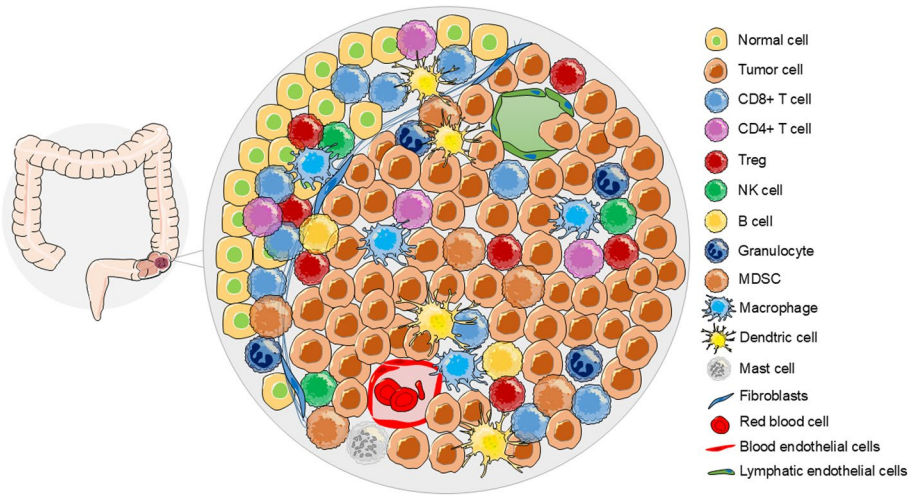

Fig. 1 Tumor immune microenvironment in colorectal cancer. The cellular composition in TME of CRC is notably heterogeneous, predominantly consisting of great majority of cancer cells, alongside stromal cells and infiltrating immune cells. Accumulating experimental and clinical evidence indicates that immune cells exert diverse and essential roles in the development and progression of CRC, manifesting both pro- and anti-tumor functions. In CRC, intratumoral immune cells could be classified into lymphoid or myeloid lineages based on the progenitor cells. Lymphoid cells in CRC encompass tumor infiltrating T lymphocytes (TILs), natural killer cells (NK cells), B lymphocytes. TILs can be further delineated into cytotoxic T lymphocytes, T help cells (Th1, Th2, Th9, Th17 and Th22) and regulatory T cells (Tregs). Myeloid cells found in CRC include dendritic cells (DCs), myeloid-derived suppressor cells (MDSCs), tumor-associated macrophages (TAMs), granulocyte and mast cells. Additionally, non-immune cells within tumor regulated the prognosis of CRC patients, such as cancer-associated fibroblasts (CAFs) and tumor-associated endothelial cells. It is important to note that each component of intratumoral immune cell network does not function in isolation; rather they interact and regulate the growth or death of tumor cells. The function of immune cells could be influenced by same or other type of immune cells as most of immune cells share unspecific protein or molecules in activating innate or adaptive immune responses. Ultimately, the progression of CRC was largely determined by the complexity of TME. Underscoring the intricate interplay among various cell types will provide promising therapeutic opportunities for CRC patients

TME. CMS2 tumors (the canonical subtype), accounting for about 37% of CRC cases, are characterized with MSS type (MSS, Microsatellite stability), higher SCNA (somatic copy number alterations), activation of Wnt and Myc pathways, but with lower CIMP. Besides, CMS2 tumor had high expression of oncogenes, including EGFR, ERBB2, insulin-like growth factor 2 (IGF2) and insulin receptor substrate 2 (IRS2) and transcription factor hepatocyte nuclear factor 4α (HNF4A). CMS3 tumors (the metabolic subtype), accounting for about 13% of CRC cases, display MSS type, frequent *KRAS* mutations and exhibited dysregulation of metabolic pathways, including glutaminolysis and lipidogenesis activation. CMS4 tumors (the mesenchymal subtype), accounting for about 23% of all the cases, are characterized with MSS type, high SCNA, low gene hypermethylation, activated pathway related to EMT (epithelial-mesenchymal transition) and stemness including TGF-β pathway and stromal activation, increased angiogenesis and immunosuppression, and remarkable stromal cell infiltration, particularly cancer-associated fibroblasts [29].

CMS2 and CMS3 are regarded as "cold" tumors in term of immunogenic and immune infiltration condition. CMS1 tumors are considered as immune activated subtypes while CMS4 subtype is referred to as immune-inflamed. CMS1 CRC particularly have strong infiltration of CD8 + CTLs, CD4 + Th1 cells and NK cells. However, increased immune checkpoint molecules CTLA-4, PD-1 and PD-L1 in tumor might lead to immune evasion in CMS1 CRC, thus CMS1 is also regarded as immune activated subtype [30]. CMS4 tumors also displayed more immune cells infiltration than CMS2 or CMS3, while the majority of immune cells are Tregs, MDSCs, monocyte-derived cells and Th17 cells [31]. In this immune inflamed subtype, interaction with stromal cells and tumor cells released immunosuppressive chemokines and cytokines (CXCL12, CCL2, TGF-β, IL-17 and IL-23), inhibited cytotoxic immune cells and promoted the migration and proliferation of MDSCs, B cells and Tregs [32]. Unlike CMS1 or CMS4 tumor, CMS2 and CMS3 tumor were defined as "immune desert" type as they are generally PD-L1 negative and lack of tumor-infiltrating lymphocytes and immunoregulatory cytokines in TME [31]. Poorly immunogenity of CMS2 or CMS3 might be explained by oncogenic-driven cancer cell pathways, lack of MHC I (major histocompatibility complex class 1) molecules and increase of non-classical human leukocyte antigens (HLA) [33]. "Cold" tumors are not static as they

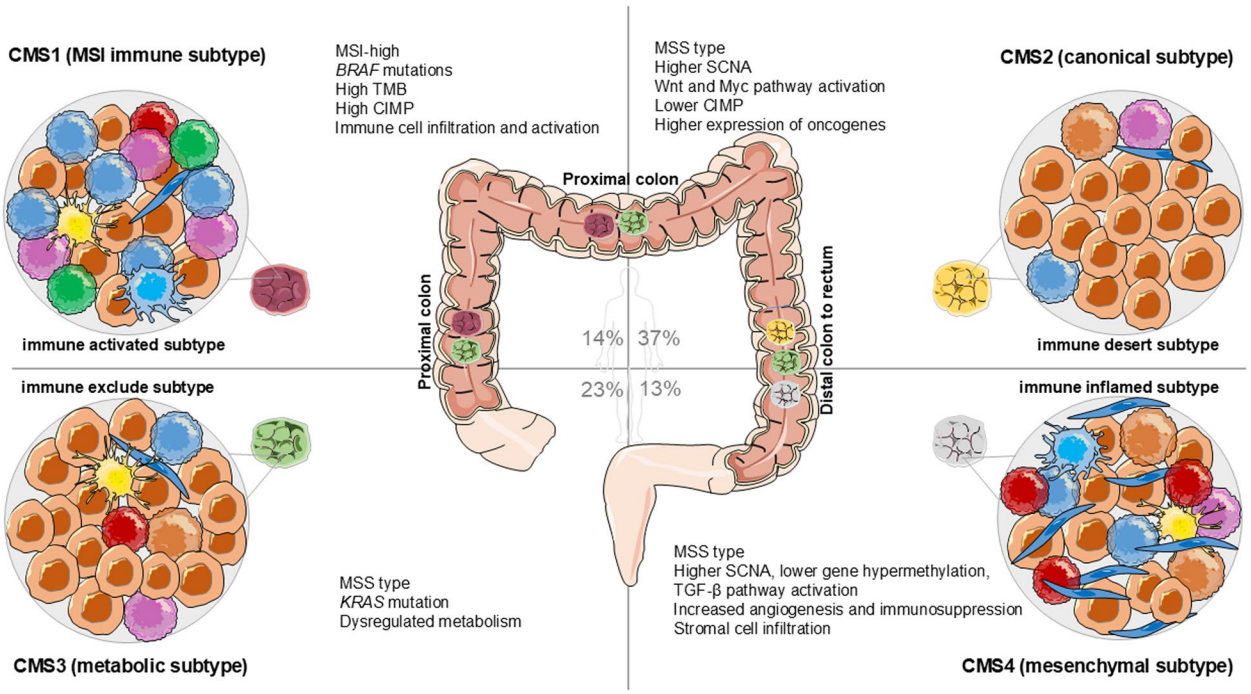

Fig. 2 Consenses molecular subtype of CRC. CRC is characterized with genetic heterogeneity, and several molecular pathways have been reported implicated in tumor initiation and development. Consensus Molecular Subtype (CMS) classification has been developed based on differential gene expression of tumor and infiltrating cells. Four major groups have been identified according to the CMS classification. CMS1 (MSI immune subtype, 14%) was defined by high MSI status, high TMB and CIMP (CpG island methylator phenotype), *BRAF* mutations, and immune infiltration and activation. CMS2 (Canonical subtype, 37%) had a high SCNA (somatic copy number alterations) level as well as WNT and MYC activation, lower CIMP and higher oncogenes expression. MSS status, *KRAS* mutations, and metabolic dysregulation were found in CMS3 (Metabolic subtype, 23%). CMS4 (Mesenchymal subtype, 13%) was characterized by MSS status, a high level of SCNA, stromal cells infiltration, TGF-β pathway activation, angiogenesis and immunosuppression. In term of immune infiltration condition, CMS2 and CMS3 are regarded as "cold" tumors. CMS1 CRC, also called as immune activated subtype, particularly have strong infiltration of CD8+ CTLs, CD4+ Th1 cells and NK cells. However, increased immune checkpoint molecules CTLA-4, PD-1 and PD-L1 in tumor might lead to immune evasion. CMS4 tumors also displayed more immune cells infiltration than CMS2 or CMS3, while the majority of immune cells are Tregs, MDSCs, monocyte-derived cells and Th17 cells. In this immune inflamed type, interaction with stromal cells and tumor cells released immunosuppressive chemokines and cytokines, inhibited cytotoxic immune cells and promoted the migration and proliferation of MDSCs, B cells and Tregs. Unlike CMS1 or CMS4 tumor, CMS2 and CMS3 tumor were defined as "immune desert" type as they are generally PD-L1 negative and lack of TILs and immunoregulatory cytokines in TME

can be transformed from cold to hot through clinical treatments such as chemotherapy, radiotherapy, microwave ablation, and cryotherapy.

Resistance to PD-1/PD-L1 inhibitors in CRC

Researchers have recently uncovered multiple tumor-intrinsic mechanisms to immunotherapy. Intrinsic factors inducing primary or adaptive resistance are listed as follow: lack of tumor antigen expression and mutations, decreased or lack of HLA expression, absence of antigen processing machinery (deletion in TAP or B2M or silence of HLA) or in MAPK, PI3K, WNT, IFN-γ signaling pathways, and constitutive PD-L1 expression in tumor. Besides, intrinsic factors are involved in the acquired resistance to immunotherapy, and these factors include loss of tumor antigen and HLA, alteration in interferon signaling, and decreased function of T cells [34]. Tumor cell-extrinsic factors, such as lack of T cells or antigen-specific TCRs, also contribute to the resistance mechanisms. Inhibitory immune cells, such as Tregs, MDSCs and M2 TAMs, have shown ability to inhibit CTL function, besides, other inhibitory immune checkpoints or suppressive factor, such as VISTA, LAG-3, TIM-3, TGF-β, adenosine, G-CSF, CD39, CD73 and IDO in TME induced T anergy [34] (Fig. 3).

Mechanisms of resistance to PD-1 blockade in CRC

Tumor-based resistance

Absence of antigenic proteins and antigen presentation MSS CRC presents low mutation load when compared with MSI CRC, non-small cell lung cancer or melanoma which are responsive to PD-1 blockade [35], thus neo-antigen from MSS CRC is quite low to induce CD8 + T cells recognition. Atkins et al. found that MHC class I antigen processing machinery (APM) component

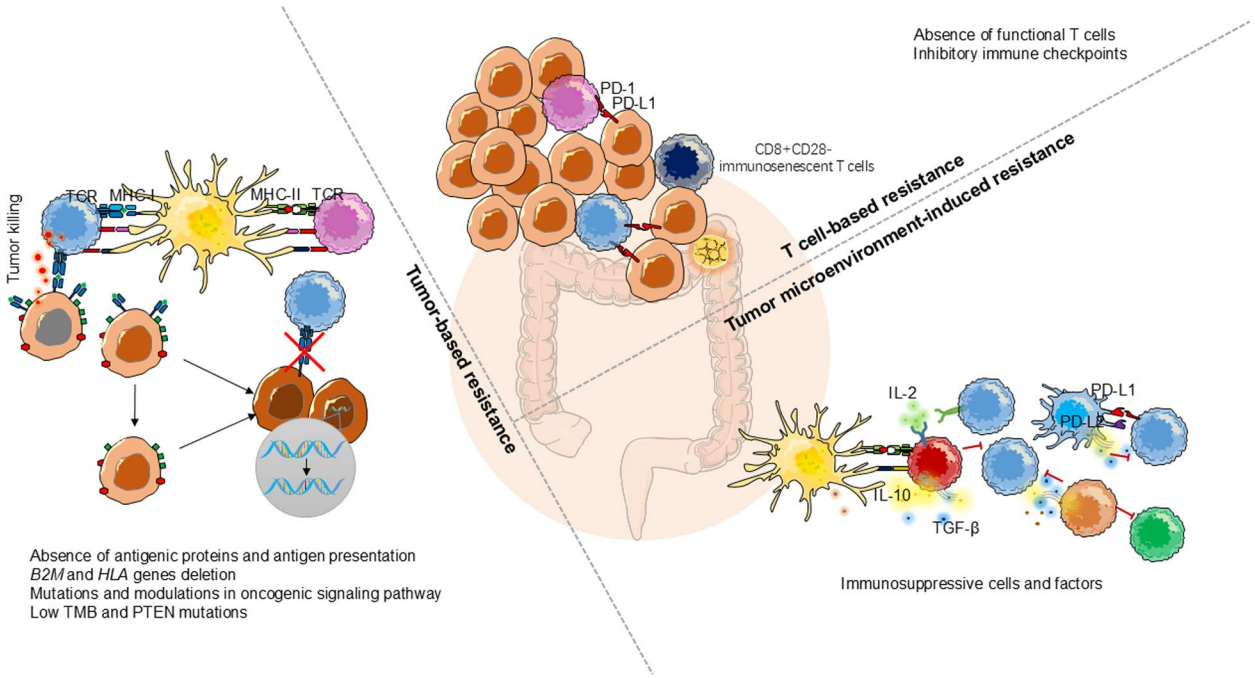

Fig. 3 Mechanisms of resistance to PD-1 blockade in CRC. Multiple tumor-intrinsic and extrinsic resistance mechanisms to immunotherapy have been explored. Herein, mechanisms of resistance to PD-1 blockade in CRC are listed as follow: tumor-based resistance, T cell-based resistance, and tumor microenvironment-induced resistance. Tumor-based resistance is characterized with absence of antigenic proteins and antigen presentation, the *B2M* and*HLA* genes deletion, mutations and modulations in oncogenic signaling pathway, low TMB and PTEN mutations. T cell based resistance includes absence of functional T cells and increased expression of inhibitory immune checkpoints. Resistance to PD-1 blockade was partly ascribed to the absence of CD8+T cells in MSS CRC. Moreover, CD8+CD28- immunosenescent T cells with impaired proliferation capacity account for the majority of intratumoral CD8 + T cells. Exhausted CD4+ and CD8 + T cells expressing PD-1 was observed in MSS CRC tumor, and CD8+ T cells also overexpressed other inhibitory immune checkpoints, such as CTLA-4, LAG-3, TIGIT, TIM-3 and VISTA. Tumor microenvironment-induced resistance is characterized with immunosuppressive cells and factors. Immunosuppressive Tregs, TAMs and MDSCs in colon from CRC is significantly higher than healthy control. Besides, Immunosuppressive molecular, such as TIM-3, LAG-3, CTLA-4, TGF-β, IL-10 and IL-17A was overexpressed in TME

(TAP1, LMP2 and tapasin) deficiencies in *KRAS*-mutated CRC, suggesting downregulated expression of MHC class I APM component mediates immune escape in *KRAS* mutations in CRC [36] and Grasso et al. have found MSI-high CRC displayed B2M and HLA genes deletion [37]. More PD-1 + T cells infiltration was found in *B2M*-mutation CRC tumor than the *B2M*-wild type [38].

Mutations and modulations in oncogenic signaling pathway Coelho et al. found that MAPK signal (ERK) was activated in *RAS* mutated CRC and demonstrated that RAS-MEK signaling increased PD-L1 expression in CRC by modulating PD-L1 mRNA stability [39]. Ebert et al. found that inhibition of MAPK (ERK) enhanced antitumor efficacy of PD-L1 blockade in murine CT26 CRC models, and MEK inhibition protected tumor-infiltrating CD8 + T cells from apoptosis driven by chronic TCR stimulation but keeping cytotoxic activity [40]. Grasso et al. confirmed all types of CRC present genetic mutation in WNT/β-catenin signaling, leading to decreased T cell infiltration in CRC. Xiao et al. found that inhibition of Dickkopf-related protein 2 (DKK2) enhanced the efficacy of PD-1 blockade therapy to MC38 tumor cell via activation of NK cells and CD8 + T cells in tumors [41]. Chida et al. confirmed that low TMB and *PTEN* mutations in CRC both compromised the efficacy of PD-1 blockade therapy, with the increase of CD204 + tumor-associated macrophages and the decrease of intratumoral CD8 + T cells. Besides, mutations in STK11, FBXW7, JAK1, B2M and HLA genes were found in non-respond patients [42]. Loss of JAK1 mutations in MSI CRC patients increased transcriptional signatures related to resistance to PD-1 blockade, showing lower IFN-γ gene expression than wild-type [43].

T cell-based resistance

Absence of functional T cells Di et al. analyzed T cell phenotypes in tumor samples from 18 patients with MSS

CRC by single cell mass cytometry, and found that percentage of CD8 + T cells in tumor is significantly lower than that in non-tumorous adjacent tissues [44], suggesting resistance to PD-1 blockade was partly ascribed to the absence of CD8 + T cells in MSS CRC. Moreover, CD8 + CD28- immunosenescent T cells with impaired proliferation capacity account for the majority of CD8 + T cells in tumor, suggesting T functionality was limited in MSS CRC tumor [44].

Inhibitory immune checkpoints The increase of immunosuppressive or exhausted T cell phenotypes in tumor, especially CD4 + and CD8 + T cells expressing PD-1 was observed in MSS CRC tumors, and CTLA-4 was expressed in CD8 + PD-1 + T cells [44], suggesting other inhibitory immune checkpoint, such as CTLA-4, LAG-3, TIGIT, TIM-3 and VISTA, were involved in resistance to PD-1 blockade in MSS CRC. It was reported that LAG-3 + FoxP3 + Treg was expanded in tumors from CRC patients [45], and IL-10 and TGF-β-producing Treg leads to poor prognosis of CRC patients [46]. Besides, other immune checkpoint molecules (IDO1, TIGIT, VISTA and PD-L1) was expressed in MSI + and MSS CRCs tumors [47].

Tumor microenvironment-induced resistance

Immunosuppressive cells and factors The proportion of Tregs in colon from CRC is higher than that in healthy colon tissue [48]. Moreover, Tregs in CRC patients exhibited higher immunosuppressive molecules, such as TIM-3, LAG-3, TGF-β, IL-10, CD25 and CTLA-4 [49]. MDSCs were found in the late stage of CRC; however, MDSCs in circulation was increased in colon polysis, the premalignant states of CRC [50]. TAMs is another important component in TME, and PD-L1 + TAMs exits in both CRC liver metastatic lesions and primary tumor [51]. IL-17 A has been reported to induce high PD-L1 expression in CRC cells. Blocking IL-17 A enhanced efficacy of PD-1 blockade in CT26 or MC38 tumors, and mice in combinational therapy exhibited more CTLs and less MDSCs in tumors [52].

Cellular mechanisms underlying combinational innate immunity activation and PD-1/PD-L1 blockade in CRC treatment

Facilitating tumor cells recognition by innate immune cells

Anti-microbial immune response to CRC

Antimicrobial immunity not only contributes to the recognition of bacterial or viral infections but also involved in tumor cell recognition. For instance, nucleic acids released by killed tumor cells elicit anti-tumor immune response via nucleic-acid sensing receptors, such as endosomal Toll-like receptors (TLRs), RIG-I-like receptors (RLRs) and STING (stimulator of interferon gene) (Fig. 4).

Antimicrobial immune can be activated by synthetic immunostimulatory products which resemble immune factors after bacterial or viral infection. Certain products are capable of directly eliminating tumors while simultaneously releasing tumor antigens whereby inducing DCs maturation through direct or indirect mechanisms. Released tumor antigens was presented to matured DCs and amplified bioprocess was further induced which includes the proliferation of tumor specific CD8 + T cells, production of pro-inflammatory cytokines and chemokines, the increase of phagocytosis and cytotoxicity. Preclinical advances have found that monotherapy targeting of TLRs, STING or RLRs by intratumoral injection or systemic administration has yielded promising findings, and combination of immune agonist and PD-1/PD-L1 blockade in CRC further enhanced the anti-tumor efficacy (Table 1).

Toll-like receptors (TLRs) TLRs, a group of pattern recognition receptors (PRRs), trigger the innate immune response by recognizing damage associated molecular patterns (DAMPs) after infection. Ten TLRs (ranging from TLR1 to TLR10) in human and 13 TLRs (ranging from TLR1 to TLR13) in mice have been identified in which TLR1, TLR2, TLR4, TLR5, TLR6 and TLR10 were expressed on cell surface, whilst TLR3, TLR7, TLR8, TLR9, TLR11 and TLR13 localized in the endosome [118].

TLR3

BO-112 (nanoplexed poly I: C targeting TLR3) induced the cytotoxicity of MC38 and human colon cancer line in vitro. Intratumoral injection of BO-112 to transplanted MC38 tumors suppressed tumor growth and induced T cell infiltration, and anti-tumor efficacy is dependent on tumor specific CD8 + T cells but not CD4 + T cells. Besides, BO-112 induced PD-1 expression on CD8 + T cells in MC38-derived tumors, thus combinational BO-112 and αPD-L1 strategy was further investigated [53]. In their study, BO-112 enhanced anti-tumor efficacy of αPD-L1 in mice bearing B16-OVA tumor, with more CD8 + T cells and higher CD8/Treg ratio in tumor-draining lymph nodes (tdLNs) and tumor. Moreover, therapeutic effect was dependent on IFN-α signaling and *Batf3*-dependent DCs. Type-I IFN-related transcriptomic changes was significantly promoted by BO-112 [53]. Lee et al. developed the L-Pampo, a dual TLR2/3 agonist, and also confirmed that combinational therapy with L-Pampo further boosted the efficacy of αPD-1 monotherapy,

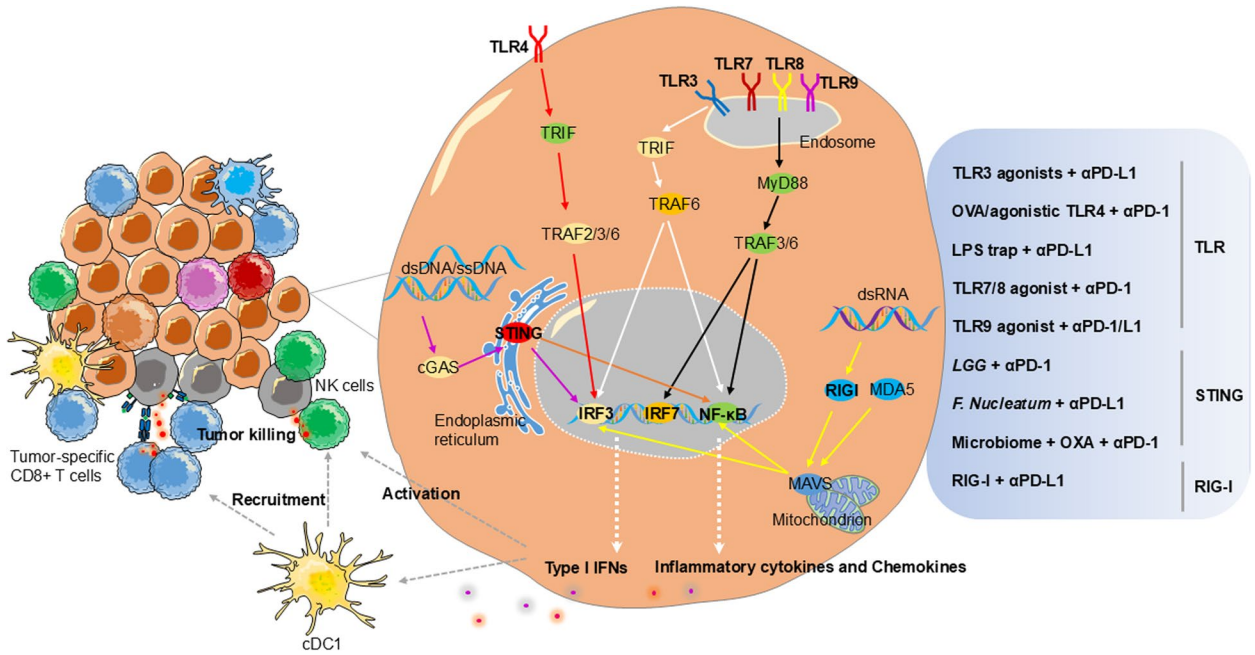

Fig. 4 Facilitating tumor cells recognition by innate immune cells. Antimicrobial immunity not only contribute to the recognition of bacterial or viral infections but also is involved in tumor cells recognition. For instance, nucleic acids released by killed tumor cells elicit anti-tumor immune response via nucleic-acid sensing receptors, such as endosomal Toll-like receptors (TLRs), RIG-I-like receptors (RLRs) and STING (stimulator of interferon gene). TLR3, TLR7, TLR8 and TLR9 are endosomally expressed while TLR4 is expressed on cell surface. TLR3 and TLR4 signal induces the production of type I IFNs via TRIF-TRAF6-IRF3 pathway, besides, TLR3 signal induce inflammatory cytokines and chemokines production via TRIF-TRAF6-NF-κB pathway. TLR7, TLR 8 and TLR9 active IRF7 and NF-κB pathways via MyD88 and downstream TARF3/6. dsRNA stimulates IRF3 and NF-κB pathways via RIG-I and MDA5 signal and downstream MAVS, while dsDNA/ssDNA activates NF-κB and IRF3 pathway through cGAS-STING signal. Antimicrobial immune can be activated by synthetic immunostimulatory products which resemble immune factors after bacterial or viral infection. Various products were aimed to increase type I IFNs and inflammatory mediators which activate tumor killing-medicated by NK cells and cDC1 recruitment to tumor site. Besides, these products could kill tumor directly whereby releasing tumor antigens, or inducing DCs maturation directly or indirectly. Released tumor antigens was presented to matured DCs and amplified bioprocess was further induced which includes generation of tumor-specific CD8 T cells, production of pro-inflammatory cytokines and chemokines, the increase of and phagocytosis and cytotoxicity. Preclinical advances have found that monotherapy targeting of TLRs, STING or RLRs by intratumoral injection or systemic administration has yielded promising findings, and combination of immune agonist and PD-1/PD-L1 blockade in CRC further enhanced anti-tumor efficacy in CRC, such as TLR3 agonist, OVA/agonistic TLR4, LPS trap, TLR7/8 agonist, TLR9 agonist, LGG, *Fusobacterium nucleatum*, RIG-1 and microbiome plus OVA

inducing complete tumor regression and extended overall survival in MC38 tumor bearing mice. Tumor specific immune response was activated and characterized with increased tumor-specific CD8 + T cells and M1 macrophages within TME, and the anti-tumor efficacy was dependent on CD8 + T cells and IFN-γ [54].

TLR4

CT26-FL3 tumors (MMR-proficient CT26 cells with liver metastasis potential) exhibit no responsive to αPD-L1 therapy. However, the combination therapy (LPS trap and αPD-L1) suppressed tumor growth, promoted survival of model mice and inhibited tumor metastasis to liver. LPS trap is lipid-protamine-DNA (LPD) nanoparticle gene delivery system to yield LPS trap protein and selectively blocks LPS (TLR4 agonist) in tumor. LPS trap reduced MDSCs and induced the increase of CD86 + and MHC II + DCs, CD8 + and CD4 + T cells; however, CD8 + T cells instead of CD4 + T cells depletion abrogated anti-tumor efficacy. LPS trap also regulated the production of cytokine and chemokines, with decreased IL-1β, IL-6, Ptgs2, and increased CXCL9 and CXCL10 which both play critical roles in CTL recruitment [55]. However, Tsukamoto et al. have demonstrated that OVA/agonistic TLR4 mAb combined with αPD-1 inhibited MC38-OVA tumor growth in comparison with monotherapy regimen. Tumor suppression was dependent on CD8 + T cells not CD4 + T cells. At a mechanistic level, OVA/anti-TLR4 mAb not only induced the proliferation of antigen-specific T cells but also activated splenic CD4 + and CD8 + T cells, displaying high CD44 expression and the increase of OVA-specific CD8 + IFN-γ + T cells. The interesting question is that why TLR4 blockade or activation both enhanced the efficacy of PD-1 blockade in CRC treatment and the mechanism should be

Table 1 Cellular mechanisms underlying combination innate immunity activation strategies for improving PD-1/PD-L1 blockade efficacy in colorectal cancer

Target	Treatments	Mechanism	Ref.
TLR3	BO-112 αPD-L1	Induced cytotoxicity and immunogenic cell death Dependent on tumor specific CD8 + T cells not CD4 + T cells More CD8 + T cells and higher CD8/Treg ratio in tdLNs and tumor Dependent on IFN-α signaling and *Batf3*-dependent DCs Increased type-I IFN-related transcriptomic changes	[53]
	L-Pampo αPD-1	Increased tumor-specific CD8 + T cells and M1 macrophages within TME Dependent on CD8 + T cells and IFN-γ	[54]
TLR4	LPS trap αPD-L1	Increased CD8 + and CD4 + T cells, CD86 + MHCII + DCs and reduced MDSCs Decreased IL-1β, IL-6, Ptgs2 and increased CXCL9 and CXCL10	[55]
	OVA/agonistic αTLR4 αPD-1	Proliferation of antigen-specific T cells Splenic CD4 + and CD8 + T cells activation with high CD44 expression and the OVA-Specific IFN-γ-producing CD8 + T cells	[56]
TLR7/8/9	banNVs αPD-1	Dependent on tumor specific CD8 + T cells not CD4 + T cells, NK cells DC secret cytokines (IL-12, IL-6 and TNF-α) and overexpressed CD80 and CD86	[57]
	CaP nanoparticles αPD-L1	Increased Ki67 + CD8 + T cells and CD8 + GzmB + CD43 + cytotoxic T cells in tdLNs Type I IFNs dependent Increased eomesodermin+, CXCR3+, CCR4+, CCR5 + CD8 + T cells	[58]
	CpG-C αPD-1	Increased intratumoral CD3 + CD8 + T cells Increased IFN-γ+, granzyme B + or perforin + T cells and effector memory CD8 + CD44 + CD62L- and CD4 + CD44 + CD62L-T cells	[59]
	Fe_3O_4@IR820@CpG αPD-L1	Increased activated CD8 + T cells (CD8 + CD69 + and CD8 + IFN-γ + T) including effector memory (CD8 + CD44 + CD62L-) and central memory T cells (CD8 + CD44 + CD62L+) Increased M1 macrophages and decreased M2 macrophages and Tregs	[60]
RIG-1	Ad-MAVS αPD-L1	Increased intratumoral CD4 + and CD8 + T cells	[61]
STING	*Lactobacillus rhamnosus* GG αPD-1	Infiltration and activation of cytotoxic CD8 + T cells and DCs Increased secretion of CXCL9, CXCL10, IFN-γ and IFN-β from DC cGAS-STING-TBK1-IRF7-IFN-β cascade signaling activation in DC *Lactobacillus murinus* and *Bacteroides uniformis* enrichment	[62]
	STING agonist (RR-CDA) αPD-1	Increased iNOS2 + M1 like macrophages and reduced CD206 + M2-like macrophages Increased CD8 + T cells and activated CD8 + GzB + T cells or IFN-γ secreting T cells Normalization of the intraperitoneal vascular-immune microenvironment is dependent on both CD8 + T cells and Type I IFN signaling	[63]
	PMM NPs αPD-1	Polarization from suppressive M2 macrophage to anti-tumor M1 subtype	[64]
Microbiome	OXA αPD-1 Microbiome	ileal crypt IECs undergo Casp3/7-dependent cell death Increased serum IgG TFH cell and B cell maturation and of antigen specific CD8 + type 1 Tc cells activation Induced CD103 + CD11b- (Batf3+) DCs migration to mLN Induced an TFH cell immune response dependent on secreted IL-1β and IL-12	[65]
	Fusobacterium nucleatum αPD-L1	Increased CD8 + TILs or CD8 + IFN-γ + TILs Dependent on CD8 + T cells	[66]
	Roseburia intestinalis αPD-1	Production of cytotoxic CD8 + granzyme B+, CD8 + IFN-γ + or CD8 + TNF-α + cells Directly bound to TLR5 on CD8 + T cells and activated NF-κB signaling pathway	[67]
GM-CSF	GM-CSF-BCG loaded gel RFA αPD-1	Reduced Ly6-Gr1 + CD11b + MDSCs infiltration Increased CD8 + T cells infiltration Expanded TNF-α producing CD4 + and CD8 + T cells and IFN-γ producing CD8 + T cells in spleen	[68]

Table 1 (continued)

Target	Treatments	Mechanism	Ref.
FLT3L	FLT3L αPD-1 αCTLA4	Increased intratumoral dendritic cell infiltration (total DC, CD103 + CD11b- DC and CD103-CD11b + DC) and T cell infiltration and activation (CD8 + T cells, CD8 + granzyme B + T cells, CD8 + PD1 + T cells, and CD4 + FOXP3-PD1 + T cells)	[69]
CD40	αCD40 αPD-1	Increased CD8 + Ly6C-PD1 + TILs, CD4 + Foxp3-Ly6C + PD1 + TILs and CD8 + Ly6C + PD1 + TILs and enhanced proliferation of TILs Monocytes was promoted to MoDCs which produced iNOS and supported TIL expansion	[70]
4-1BB	surrogate FS222 αPD-L1	Induced proliferation of CD4+/CD8 + T cells Increased IFN-γ, TNF-α and IL-6 productions	[71]
	αCD137 SFV-αPDL1	Increased tumor specific CD8 + T cells in tumors, tdLN and peripheral blood Upregulated IFN-stimulated genes	[72]
	αOX40 α4-1BB αPD-L1	Increased intratumoral CD8 + T cells and ratio of CD8 + T cells/Tregs Dependent on CD8 + instead of CD4 + T cells or NK cells Expanded a novel stem-like CD8 + T cell subpopulation with PD-1^{low} KLRG-1 + Ki-67 + phenotype in a CXCR3 dependent manner	[73]
	HK010	Immune cell infiltration, activation (NK cells, CD4 + T cells, CD8 + IFN-γ + T cells) and proliferation (CD8 + Ki67 + T cells)	[74]
OX40	MSC-C9×T4a αPD-1	More CD4 + T, CD8 + T, and NK cells infiltration Increased GrB + CD8 + T and GrB + NK cells Tumor cell proliferation inhibition Dependent on tumor specific CD8 + T cells and NK cells	[75]
	Adenovirus-based CEA vaccine N-803 αOX40 α4-1BB αPD-L1	Induced CD4 + and CD8 + T cell proliferation and activation Increased Ki67 + or IFN-γ + T cells	[76]
ICOS	αICOS αPD-L1	Increased infiltrating CD8 + T cells in tumor CD4 + or CD8 + TAI expressed both activating (ICOS) and inhibitory (LAG-3 and PD-1)	[77]
IFN-α	IFN-α-transfected tumor cell vaccine αPD-1	CD4 and CD8 + cells infiltration Dependent on CD4 + and CD8 + T cells instead of NK cells Higher levels of IFN-γ secretion but reduced IL-10 production by splenocytes Enhanced cytotoxicity Inhibited apoptosis of lymphocytes	[78]
IL-2	F8-IL2 αPD-1	Local T-cell was activated and expanded Less mature phenotype and quicker turnover rate Increased NK cells infiltration and proliferation Increased Granzyme B and Ki67 expressions on CD8 + T cells or NK cells in tumors and TdLN	[79]
	αPD-L1 fusing hIL-2	Dependent on CD8 + T cells Splenic CD8 + IFN-γ + T cells induced more significant tumor inhibition	[80]
	CD122-directed IL-2 complexes αPD-1	Expansion of stem-like CD8 + T cells in spleen and blood Dependent on CD8 + T cells and CXCR3 pathway	[81]
IL-12	L19-mIL12 αPD-1	Increased NK and CD8 + T cells and decreased Tregs in tumor Dependent on NK and CD8 + T cells Upregulation of IL-6, TNF-α and IFN-γ	[82]
	Intratumoral IL-12 mRNA therapy αPD-L1	Recruited CD8 + T cell into tumors to induce tumor-specific cell lysis Dependent on CD8 + T cells, not CD4 + T cells, NK or NKT cells IFN-γ and cytotoxic T cell-medicated immune response	[83]

Table 1 (continued)

Target	Treatments	Mechanism	Ref.
IL-15	mIL-15 αPD-L1	Higher percentages of IFN-γ producing CD8 + T cells in spleen Less IL-10 secretions by CD8 + T cells Enhanced specific cell lytic activity of CD8 + T cells	[84]
	N-803 αPD-L1	Anti-tumor efficacy dependent on CD8 + T cells and NK cells Less number of G-MDSCs and Tregs More infiltration and activation of NK cells in spleen and tumor Enhanced cytotoxic function of NK cells CD8 + T cells activation ($CD44^{hi}CD62L^{hi}$ TCM) and proliferation, more GrB + T cells More IFN-γ and TNF-α productions	[85]
	LH01	Increase of CD8 + T cells and NK cells in tumor Reduced Tregs	[86]
IL-21	IL-21-αHSA αPD-1	Increased ratio of CD3+/CD45 + cells, CD8+/CD3 + cells, NK cells/CD45 + cells and the percentage of Ki67 + CD8 + T cells	[87]
TIM-3	αTIM-3 αPD-L1 DC-targeted cancer cell vaccines.	Reduced Tregs and increased TNF-α + IFN-γ + CD8 + T cells Increased CD8 + T cells proliferation and cytolytic activity	[88]
TIGIT	αTIGIT αPD-L1	CD8 + T cell-dependent	[89]
	αTIGIT αPD-L1	Therapeutic efficacy was dependent on NK cells Increased cells expressing CD107a, TNF-α, IFN-γ or CD226 on tumor NK cells Antitumor memory response Infiltrating CD8 + T cells expressing IFN-γ or TNF-α dengpent in NK cells	[90]
	αTIGIT αPD-1	Myeloid cell activation with production of CXCL10, CXCL11, IL-23 and TNF-α Upregulation of MHC class II, CD86, or CD40 expression on APC Persistent granzyme B and perforin production	[91]
	αTIGIT αPD-L1 Fractionated radiotherapy	Increase of total T-cells, CD4 + T cells, CD8 + T cells, CD8+/Treg ratio, CD8+/granzyme cells, TAM1/TAM2 ratio Decrease of myeloid cells, TAM2 and MDSCs GAS-STING pathway, CD8 + T cell activation and differentiation pathway, IFN-γ production and response pathways	[92]
LAG-3	αLAG-3 αPD-L1	Increased infiltrating CD8 + T cells in tumor CD4 + or CD8 + TAI expressed both activating (ICOS) and inhibitory (LAG-3 and PD-1)	[77]
VISTA	CA-170	Induced more proliferating CD4 + or CD8 + T cells in tumor Higher level of co-stimulatory molecule OX-40 on CD8 + T cells in tumor Higher intracellular levels of granzymeB in CD8 + T cells in Blood	[93]
	αVISTA αPD-1/αCTLA-4	Reduced myeloid-mediated suppression Upregulate costimulatory genes Reduced the expression of regulators that maintain T-cell quiescence	[94]
CD47	RT IMD@Hf-DBP/αCD47 αPD-L1	Immunogenic cell death induced by RT Enhanced macrophage phagocytosis M1 macrophages repolarization IFN-γ producing cytotoxic T cells in splenocytes CD4 + and CD8 + T cells, NK cells and B cells infiltration in tumor	[95]
	RT/αCD47/αPD-1 therapy or RT/αSIRPa/αPD-1	Reduced M2 macrophage and did not increase MDSCs Upregulated CD86 expression on DCs and Mo-Macrophages Primed and activated TAA-specific CD8 + T cell Increased T cell clonality and clonal diversity Dendritic cell not macrophage is responsible for CD8 + T cell priming STING activation	[96]

Table 1 (continued)

Target	Treatments	Mechanism	Ref.
Adenosinergic pathway	CPI-444 αPD-1	Reduced PD-1 and LAG-3 expression on CD8 + CD44 + effctor T cells and Tregs at tdLNs Increased population of CD8 + T cells expressing TNF-α and IFN-γ Increased activation marker 41-BB within the TME Increased transcription factors T-bet expression on infiltrating CD8 + T cells	[97]
	CPI-444 αPD-L1	CD8 + T cells dependent Increase of active GITR + IL7Rα + T-cell Promotion of a Th1 gene expression signature Recovery of T cell function	[98]
	AB680 αPD-1	Reduced myeloid cells and Ikzf2^{high} CD4 + Tex cells by αPD-1, not AB680 Increased CD69^{high} CD8 + T cells and reduced Malat1^{high} Treg by αPD-1 or AB680 Increased TCR diversity of Entpd1(CD39 gene)-negative T cells and Pdcd1(PD-1 gene)-positive T cells by αPD-1 Induced Ccr2^{high}Tlr2^{high} M1 macropahge by AB680 not αPD-1 Reduced Cx3cr1^{high}/Csf1^{high}/Nt5e + M2 macrophage by both AB680 and αPD-1 Induced Treg depletion in AOM/DSS induced CRC model by αPD-1 Increased CD8 + T cells activation in vitro by AB680 therapy	[99]
IDO	diABZI 1-MT αPD-1	Increase of CD8 + T cells (CD8 + IFN-γ + TILs) and dendritic cells (CD11b + CD86 + cells) Decreased infiltration of MDSCs	[100]
TGF-β	RT αCD137 αPD-1 αTGF-β	Higher serum IFN-γ level Increased CD8 + not CD4 + T cell population in tumor Increased granzyme-B + CD8 + TILs	[101]
	Galunisertib αPD-L1	More intramural CD8 + T cell infiltration CD11b + myeloid cells were slightly reduced	[102]
	Galunisertib αPD-L1	Increased CD3+, CD4 + and CD8 + cells infiltration in tumor Increased and T-bet and IFN-γ expression in CD4 + Th cells Increased GZMB production in CTLs Activated Th cells and CTLs	[103]
	Bintrafusp alfa	Increased cytotoxic CD8 + T cells infiltration Decreased PD-L1 expressions on CD45^{neg} cells and immune cells (TILs, Tregs, M-MDSCs and G-MDSCs)	[104]
	TRC105 αPD-1	Dependent on FcγR-mediated ADCC and CD8 + T cells Increased intratumoral CD8 + T cells, the ratio of CD8+/Foxp3 + and CD8 + granzyme B + T cells and in blood Reduced intratumoral CD25+/Foxp3 + cells	[105]
EP4	TP-16 αPD-1	Increased cytotoxic CD8 + T cell and reduced MDSCs and M2 macrophages Decreased PD-L1 expression and reduced p-STAT3 and p-AKT expressions Inhibited production of IL-6 and CXCL1 Upregulation of inflammation- and immunity-related pathways	[106]
Arg-2	Arg2 deletion in CD8 + T cells αPD-1	Enhanced CD8 + cell activation and cytokine induction Upregulation of key genes implicated in CD8 + T cell function, involved in cytotoxicity, IFN-γ signaling, cytokines and inflammatory response, and IL-2 signaling pathway	[107]
IL-6/STAT3	αPD-L1 αIL-6	Accumulation of cytotoxic CD8 + T cells and CD11c + I-Ad ^{high} mature dendritic cells	[108]
	αPD-L1 Danvatirsen	Reversed the suppressive macrophage to pro-inflammatory subtype Enhanced functionality and proliferation of cytotoxic CD8 + T cells	[109]
CCR2	RFA αPD-1 Blockade of CCR2	TAMs promote the CCL2 production of tumor cells through TNF-α/TNF-α receptor signaling Tumor-derived CCL2 recruited monocyte and TAMs	[110]

Table 1 (continued)

Target	Treatments	Mechanism	Ref.
CXCR2	αGr-1 αPD-1	Oncogenic KRAS on CRC cells promoted CXCL3 secretion by inhibiting IRF2 CXCL3 recruited MDSCs into tumor through binding CXCR2 MDSCs inhibited T cell proliferation and activation	[111]
	SB265610 αPD-L1	Decreased MDSCs infiltration Increased CD8 + T cells infiltration	[112]
	Ionizing radiation αPD-1 SB225002	The infiltration of CD8 + T cells and NK cells were not affected by CXCR2 blockade CXCR2-recruited immunosuppressive cells hampered the efficacy of αPD-1	[113]
Epigenetic-modulating drugs	5-azacytidine Entinostat αPD-1 αCTLA-4	Decreased Foxp3 + Tregs and G-MDSCs Function of effector T cells was inhibited by suppresser cells Anti-tumor efficacy is dependent on deletion G-MDSCs not Tregs	[114]
CSF-1R	Oncolytic viruses, PLX3397 αPD-1	T cell infiltration CD8 + T cell-dependent anti-tumor immune response Inhibition onhe recruitment of TAMs Reprogrammed TAMs to M1 phenotype Downregulated immunosuppressive genes and increased expression of proinflammatory gene IFN-γ + CD8 + T cells expansion Restrained co-inhibitory molecular expressions but increased co-stimulatory molecular expressions	[115]
	PLX3397 αPD-1 αCTLA-4	Dependent on CD4 and CD8 + T cells Decrease MDSCs and increased CD45 + cells and T cells in tumor Mainly IFNγ + CD8 + and CD4 + effector T cells but not Treg cells in TILs	[116]
	C19 αPD-1	Induction of TAM-derived CXCL9 generation to recruit CD8 + T cells Dependent on CD8 + T cells	[117]

further clarified. One explanation is that LPS not only activates TLR4 but also triggers intracellular inflammatory caspases, leading to NF-κB activation, however proinflammatory TNF-α, IL-6 and IL-1β secretions triggered by anti-TLR4 mAb were less than that to LPS stimulation. LPS/TLR4 pathway exerts as a two-edge sword in combination with PD-1 blockade and antitumor efficacy might be based on inflammation level. Inflammation induced by TLR4 stimulation is essential for the augment of specific anti-tumor adaptive immunity, however immoderate inflammatory response might be harmful to CRC patients [56].

TLR7/8/9

Ni et al. developed a bi-adjuvant nanovaccine, named banNVs, which contains neoantigen (Adpgk), TLR7/8 agonist R848 and TLR9 agonist CpG. The Adpgk-specific MC38 tumor suppression and prolonged survival rate was more significant in mice treated with banNVs plus αPD-1 compared with αPD-1 alone. Depletion of CD8 + T cells, instead of CD4 + T cells or NK cells compromised anti-tumor efficacy against MC38. Moreover, CpG NPs/R848 stimulated DC secret cytokines (IL-12, IL-6 and TNF-α) and overexpressed costimulatory molecular CD80 and CD86 [57]. Heße et al. investigated therapeutic potential of a tumor-peptide based nanoparticle (CaP nanoparticles functionalized with TLR9 agonist CpG and tumor antigens HA) in CT26-HA colorectal cancer model, and they observed that PD-L1 blockade alone depressed tumor growth, while combinational therapy further inhibited tumor growth. Flow cytometric analysis have shown that the percentage of Ki67 + CD8 + T cells and CD8 + GzmB + CD43 + cytotoxic T cells in tdLNs or tumor in combinational therapy was significantly elevated compared to αPD-L1 treatment alone. Type I IFNs play a crucial role in the antitumor response elicited by CaP/CpG/HA nanoparticle, and transcription factors of cytotoxic CD8 + T cells and CXCR3, CCR4, CCR5 expressions on CD8 + T cells were also increased by CaP/CpG/HA nanoparticle [58]. Li et al. found that both CpG-B and CpG-C exhibited synergistic enhancement of antitumor effects with the αPD-1 in CT26 tumor bearing mice. In the combination group, intratumoral CD3 + CD8 + T cells was increased, and the percentages of IFN-γ+, granzyme B + or perforin + T cells and effector memory CD8 + CD44 + CD62L- and CD4 + CD44 + CD62L-T cells were more profound than monotherapy [59]. Wang et al. developed a laser-activatable in situ vaccine, Fe_3O_4@IR820@CpG (TLR-9 agonist), enhanced the anti-tumor efficacy of αPD-L1 in MC38 tumor bearing mice, displaying increased activated

CD8 + T cells (CD8 + CD69 + and CD8 + IFN-γ + T) including effector memory (CD8 + CD44 + CD62L-) and central memory T cells (CD8 + CD44 + CD62L+), increased M1 macrophages and decreased M2 macrophages and Tregs [60].

Retinoic-acid inducible gene I (RIG-I) Tumor-intrinsic MAVS expression in CT26.CL25 or MC38 xenografts bearing mice induced innate and adaptive immune responses which triggered abscopal effect with the help of cytotoxic CD8 + T cells. Overexpression of MAVS using injected Ad-MAVS sensitize MC38 tumor for αPD-L1 therapy and significantly increased intratumoral CD4 + and CD8 + T cells in the TME [61].

Stimulator of interferon genes (STING) STING serve as is an endoplasmic reticulum-associated membrane molecular that recognize pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAPMs), thereby inducing type I interferon production. It is found on innate immune cells such as dendritic cells and macrophages. The STING signaling pathway plays an essential role in innate immune response to pathogen and contributes to anti-tumor immune reactions as well.

Si et al. found that oral intake of *Lactobacillus rhamnosus GG* (*LGG*) promoted the effectiveness of PD-1 blockade therapy in MC38 colon cancer model, and the combinational therapy induced more infiltration and activation of cytotoxic CD8 + T cells than αPD-1 alone. Antitumor effect relies on CD8 + T cells. Besides, *LGG* induced intratumoral DCs infiltration (CD45 + CD11c + MHC II + CD103 + and CD45 + CD11c + MHC II + cells). The productions of CXCL9, CXCL10, IFN-γ and IFN-β from DC after *LGG* stimulation were induced. Blocking of type I IFN signaling impaired the antitumor efficacy of *LGG*. They further confirmed that cGAS-STING-TBK1-IRF7-IFN-β cascade signaling is involved in immune response to *LGG* in DCs. Finally, enhanced antitumor efficacy in the combinational therapy group was severely comprised after deletion of cGAS/STING ($Cd11c^{cre}Sting^{f/f}$ mice or deletion of cGAS/STING signaling) in mice. After combinational treatments, gut microbial community is characterized with *Lactobacillus murinus* and *Bacteroides uniformis* which has been reported to induce DC activation and T cell recruitment [62]. Moreover, Lee et al. found that STING agonist (RR-CDA) improved antitumor efficacy of αPD-1 in MC38 peritoneal carcinomatosis of colon cancer (PCCC) model. Intratumoral immunological alternation in combinational group include increased iNOS2 + M1 like macrophages but reduced CD206 + M2-like macrophages, increased CD8 + T cells and activated CD8 + GzB + T cells or IFN-γ secreting T cells than αPD-1, besides, vascular normalization was achieved by RR-CDA plus αPD-1 and normalization of the intraperitoneal vascular-immune microenvironment is dependent on both CD8 + T cells and Type I IFN signaling [63] Liu et al. also confirmed that TME-responsive nanoparticles (PMM NPs) which induce STING activation augment the efficacy of αPD-1 in colon tumor model with polarization from suppressive M2 macrophage to anti-tumor M1 subtype [64].

Microbiome Robertiet al. found that ileal microbiota regulated anti-tumor efficacy of immunogenic chemotherapy either when used alone or in conjunction with ICIs. In mice model harboring MSI (MC38) or MSS (CT26) tumors, oral delivery of immunogenic or tolerogenic commensals modulated the efficacy of OXA and αPD-1. Immunogenic *B. fragilis* or *E. ramosum* enhanced the therapeutic effects of OXA + αPD-1 with reduced tumor growth and increased serum IgG levels, whereas administration of tolerogenic *P. clara* or *F. nucleatum* comprised the effectiveness of the combinational therapy with uncontrolled tumor growth and lower serum IgG levels. Furthermore, they found that immunogenic commensals (*B. fragilis*, *E. ramosum* and *A. onderdonkii*) induced TFH cell and B cell maturation and of antigen specific CD8 + type 1 Tc cells activation in background of Casp3/7-dependent cell death of ileal crypt IECs, whereas tolerogenic commensals (*P. clara* or *F. nucleatum*) promoted Th17 accumulation in tdLNs. Immunogenic commensals induced CD103 + CD11b- (*Batf3*+) DCs migrate to mLN and induce an TFH cell immune response dependent on the secretion of IL-1β and IL-12 [65]. *Fusobacterium nucleatum* was demonstrated to improve therapeutic response to αPD-L1 in CT26 tumor bearing mouse model, an AOM/DSS-induced CRC model or CRC organoids. The increase of CD8 + TILs or CD8 + IFN-γ + TILs was more profound in combinational therapy than αPD-L1 monotherapy, and removal of CD8 + T cells abolished anti-tumor effect. Recent evidence showed that enhanced therapeutic effect of αPD-L1 by *F. nucleatum* was mediated by activating STING signaling [66]. Kang X. et al. found *Roseburia intestinalis* is a potential adjuvant to enhance the efficacy of αPD-1 against CRC. The administration of either *R. intestinalis* or butyrate inhibited tumor growth by stimulating the production of cytotoxic CD8 + granzyme B+, CD8 + IFN-γ + or CD8 + TNF-α + cells in mouse models bearing MC38 or CT26 tumor cells. The underlying mechanism is involved that butyrate directly bound to TLR5 on CD8 + T cells, which activated NF-κB signaling pathway [67].

Enhancing the effectiveness of PD-1/PD-L1 blockade therapy for CRC patients through modulation of the gut microbiota presents distinct benefits. Initially, the gut microbiota stands apart from conventional chemotherapy and targeted treatments by imparting minimal harm to the body. Moreover, the approach of intervening in the gut microbiota demonstrates a high degree of target specificity, enabling a direct impact on the localized tumor microenvironment within the intestines. Additionally, by selectively screening the gut microbiota of CRC patients who are responsive to αPD-1/αPD-L1 immunotherapy, it becomes feasible to administer tailored supplements of metabolic products or microbial consortia to those who are non-responsive, thereby precisely orchestrating the therapeutic efficacy of PD-1/PD-L1 blockade treatment.

Immunogenic cell death (ICD)

The induction of immunogenic cell death (ICD) of tumor cells has been demonstrated to trigger anti-tumor effect in preclinical research. Dying tumor cells can release immunostimulatory signals into the TME to activate adaptive immune response. These signals are known as damage-associated molecular patterns (DAMPs). DAMPs induce the "eat-me" signal by cell surface exposure of calreticulin (CRT) or heat-shock proteins (HSP70 and HSP90). Besides, extracellular released molecules, including high mobility group box 1 (HMGB1), adenosine triphosphate (ATP), type I IFNs and IL-1 family cytokines contribute to the activation of tumor-specific immune responses [119]. Chemotherapy and radiotherapy have been reported to trigger ICD, which further stimulates phagocytosis of dead tumor cells, promotes tumor antigen presentation and induces tumor-specific T lymphocytes infiltration, thereby reversing the immunosuppressive TME (Fig. 5).

Stewart et al. determined the antitumor effect of αPD-L1 as monotherapy and in combination with oxaliplatin in CT26 xenograft mouse, and found that the rate of complete tumor elimination was increased to 62.5% in the combined therapy group from 25% in monotherapy group. Subsequently, they found HMGB1 expression in tumors was significantly induced by oxaliplatin. The increase of CD69 and ICOS expressions on splenic CD4 + and CD8 + T cells was also found after PD-L1 blockade [120]. Wen et al. successfully developed an MMP2-responsive controlled-release system Pd-Dox@TGMs NP which mediates chemotherapy and photothermal therapy (PTT) to tumor. Combining Pd-Dox@TGMs NPs with αPD-L1 efficiently reduced metastatic tumor nodules in the CT26 lung metastatic model with boosted CD8 + T cells infiltration in tumor, whilst reduced immunosuppressive Foxp3 + T cells accumulation. Pd-Dox@TGMs NP stimulated dead CT26 tumor cells to release ICD-related molecules, such as ATP, HMGB1 and CRT, potentially promoting tumor recognition of immune system [121]. Limagne et al. demonstrated FTD/TPI and oxaliplatin induced ICD of CT26 cells, as well as in various human MSS colorectal cancer cell lines, including SW620, Caco-2 and Colo-320, leading to the increase of CRT exposure, EIF2α activation, HMGB1 release, and the extracellular release of ATP. Intratumoral CD8 + T cells in combinational therapy group (FTD/TPI plus αPD-1) was characterized with increased granzyme B, IFN-γ, and TNF-α expressions than monotherapy. Besides, FTD/TPI and oxaliplatin eliminated M2 type tumor-associated macrophages, consequently reversing immunosuppressive TME [122]. Schaer et al. found that folate pathway inhibitor pemetrexed promoted anti-tumor efficacy of αPD-L1 in CT26 or MC38 tumor model, inducing tumor growth inhibition and longer survival. Combinational therapy induced higher percentage of CD8 + T cells, Ki67 + Foxp3- CD4 + effector T cells. Ratios of CD8+/CD4 + T cells and CD8+/Treg were increased. Combinational treatments also induce more CD11b + DCs and less Ly6G + granulocytic MDSCs infiltration in tumor, macrophage activation and increased MHC II on tumor. Furthermore, pemetrexed induces ICD of both CT26 and MC38 cells, characterized with increased HMGB1 and CRT [123]. Li et al. developed a nanoparticle called SK/siR-NPs which codelivery Shikonin (SK) and PD-L1 siRNA. SK/siR-NPs induced more potent anti-tumor effects than silencing of PD-L1 alone in mice bearing CT26 tumor. SK/siR-NPs showed an ability to induce ICD (more CRT exposure in SK/siR-NPs than control) whereby inducing DC maturation [124]. Recently, new reagents have been reported to improve the anti-tumor effects of αPD-1/αPD-L1 dependent on ICD, such as the cyclodextrin-based nanoformulation delivering ginsenoside Rg3 and quercetin [125], camptothesome nanovesicles comprising sphingomyelin-derived camptothecin bilayers [126], Fluorinated Mitochondria-Disrupting Helical Polypeptid [127], and the nuclear-targeting delivery system TIR@siRNA [128]. Ren et al. found that combinational mitochondria-targetable dynamic supramolecular nanoassemblies (mtDSN-2) and αPD-1 induces more significant inhibition on tumor growth than αPD-1 alone in MC38/R tumor mice model (MC38 αPD-1 resistant tumor). mtDSN-2 was also confirmed to induce endoplasmic reticulum stress, and cause apoptosis/paraptosis-associated ICD [129].

Amplification of immune response

APC activation and differentiation

Innate immunity is involved in promoting T cell effector functions. Numerous efforts to amplify this effect was implemented in preclinical experiments and most

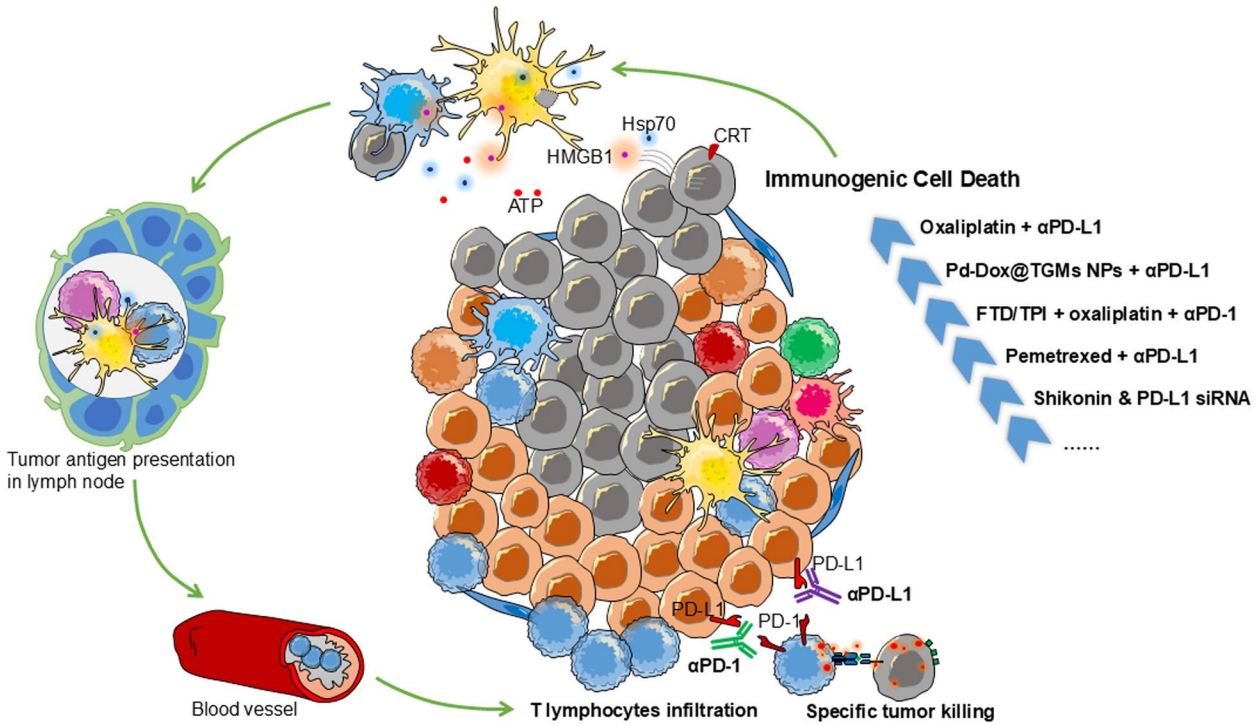

Fig. 5 Induced immunogenic cell death in CRC. Immunogenic cell death (ICD) is a type of cell death that primes the systemic innate and adaptive immune response. ICD has been demonstrated to induce anti-tumor effect in preclinical research, thus induction of ICD remains a popular and active area in cancer therapy. Dying cancer cells can release immunostimulatory signals into TME to activate adaptive immune response, which are called damage-associated molecular patterns (DAMPs). DAMPs induce eat me signal by cell surface exposure of calreticulin (CRT) and heat-shock proteins (HSP70 and HSP90), besides, extracellular released molecules, such as high mobility group box 1 (HMGB1), and adenosine triphosphate (ATP), type I IFNs and IL-1 family cytokines contribute activation of tumor-specific immune responses. Chemotherapy and radiotherapy have been reported to trigger ICD, which further stimulates phagocytosis of dead tumor cells, promotes tumor antigen presentation and induces tumor-specific T lymphocytes infiltration, thereby reversing the immunosuppressive TME. However, PD-1 was induced to express on T cells after activation. After interaction with its ligands PD-L1, TCR proximal signaling pathway was dephosphorylated and the dephosphorylation inhibits the proliferation and activation of T cells, suppresses cytokine secretion, regulates metabolism and functions of cytotoxic CTL, and ultimately leads to death of activated T cells. Besides, tumor cells overexpressed PD-L1 has the capability to escape host immune surveillance. Blockade of PD-1/PD-L1 in CRC reinvigorate the exhausted T cells in TME, and growing preclinical evidence has shown the combinational PD-1/PD-L1 blockade with ICD inducer displayed synergistic anti-tumor role in CRC treatments, such as with oxaliplatin, Pd-Dox@TGMs NP, FTD/TPI plus oxaliplatin, pemetrexed and SK/siR-NPs. Thus, ICD enhance tumor immunogenicity which can improve overall efficacy of anti-PD-1/ PD-L1 checkpoint blockade in CRC

approaches have showed synergistic efficacy with PD-1/PD-L1 blockade, such as GM-CSF, FLT3L, type I IFNs, IL-2, IL-15, stimulation of costimulatory signals (Fig. 6).

GM-CSF Li et al. evaluated the potency of combination GM-CSF-secreting CT26 tumor cell (CT26.GM) immunotherapies with PD-1 blockade in mice, and found that combining PD-1 blockade with CT26.GM significantly inhibited tumor growth and prolonged the survival of model mice compared to αPD-1 monotherapy, with more than 80% of mice survived [130]. Lemdani et al. reported that local immunomodulation in situ with GM-CSF-BCG loaded gel (RFA + Gel-GM-CSF-BCG) promoted antitumor efficacy of PD-1 blockade plus radiofrequency ablation (RFA), resulting in a complete cure of distant colorectal carcinoma and longer survival. Ly6-Gr1 + CD11b + MDSCs infiltration in the distant tumors was significantly reduced in mice from combinational therapy group. However, the proportion of F4/80 + macrophage or CD11c + CD80 + CD86 + DCs was not significantly changed. Additionally, CD8 + T cells, not CD4 + T cells infiltrations were increased in distant tumors from mice after combinational therapy treatment. Further analysis confirmed that CD4 + TNF-α + and CD8 + T cells and CD8 + IFN-γ + T cells in spleen were significantly expanded in the combinational therapy group in comparison with RFA plus αPD-1 group [68].

FMS-like tyrosine kinase 3 ligand (FLT3L) Combination of Flt3L and ICIs therapy (αPD1 plus αCTLA4) is

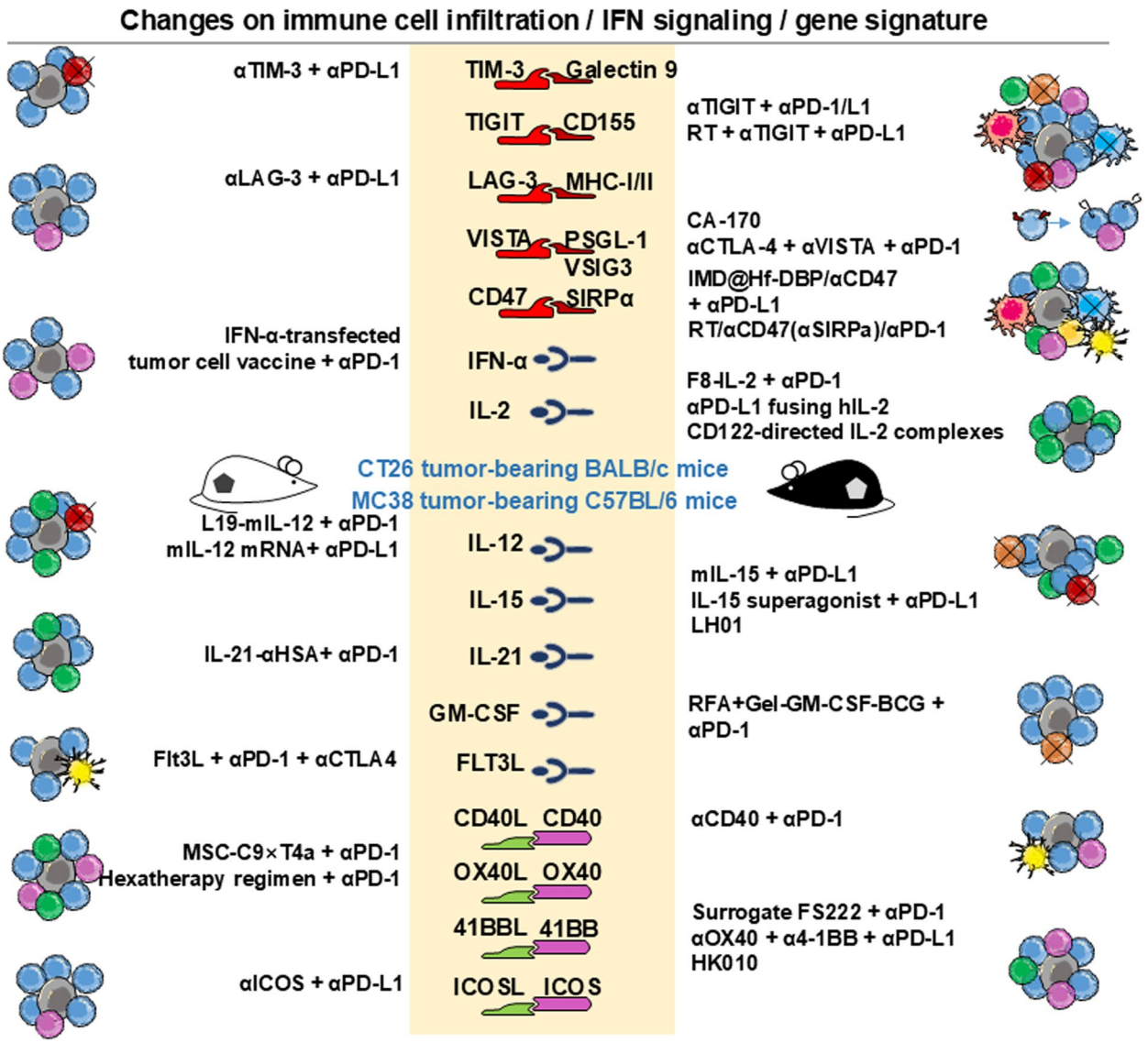

Fig. 6 Boosting of the effector responses of innate immunity. Innate immunity is involved in promoting T cell effector functions. Blockade of immune checkpoint, such as TIM-3, TIGIT, LAG-3, VISTA and CD47 have been developed to boost effector response of innate immunity. Also, attempts such as addition of GM-CSF, type I IFNs, IL-2, IL-12, IL-15, or stimulation of costimulatory signals have yielded synergistic efficacy with PD-1/PD-L1 blockade in CT26 or MC38 tumor bearing mouse model. The cellular mechanism underling combinational therapy was listed as follows: αTIM-3 + PD-1/PD-L1 blockade: characterized with reduced Tregs but increased TNF-α+ IFN-γ+ CD8+ T cells. αTIGIT + PD-1/PD-L1 blockade: increased total T cells, NK cells and TAM1/TAM2 ratio, and decreased TAM2 and MDSCs. αLAG-3 + PD-1/PD-L1 blockade: increased infiltrating CD8+ within tumor. αVISTA + PD-1/PD-L1 blockade: reduced myeloid-mediated suppression and reversion on T cell quiescence. αCD47 + PD-1/PD-L1 blockade: enhanced macrophage phagocytosis, upregulated CD86 expression on DCs and Mo-Macrophages, M1 macrophages repolarization, and CD4+, CD8+ T cells, NK cells and B cells infiltration. IFN-α + PD-1/PD-L1 blockade: dependent on increased CD4+ and CD8+ T cells. IL-2 + PD-1/PD-L1 blockade: activated and expanded CD8+ T cells, and increased NK cells infiltration and proliferation. IL-12 + PD-1/PD-L1 blockade: increased NK and CD8+ T cells and decreased Tregs. IL-15 + PD-1/PD-L1 blockade: reduced G-MDSCs and Tregs, increased NK cells infiltration, activation and cytotoxic function, and CD8+ T cells activation and proliferation. IL-21 + PD-1/PD-L1 blockade: increased ratio of CD8+ and NK cells. GM-CSF + PD-1/PD-L1 blockade: increased CD8+T cells infiltrations and reduced MDSCs. FLT3L + PD-1/PD-L1 blockade: increased intratumoral dendritic cell infiltration and CD8+ T cell infiltration and activation. αCD40 + PD-1/PD-L1 blockade: increased TILs and iNOS+ MoDCs. αOX40 + PD-1/PD-L1 blockade: more CD4+T, CD8+T, and NK cells infiltration, and increased GrB+ CD8+T cells and GrB+ NK cells. α4-1BB + PD-1/PD-L1 blockade: induced proliferation of CD4+/CD8+ T cells, activation of tumor specific CD8+ T cells. αICOS + PD-1/PD-L1 blockade: increased infiltrating CD8+ T cells expressed both activating (ICOS)

more effective than ICIs therapy in treating pMMR CRC liver metastases, with longer survival time of mice. Cellular alternation includes increased intratumoral T cells and dendritic cell infiltration (total DC, CD103 + CD11b- DC and CD103-CD11b + DC), and CD8 + T cells activation (CD8 + T cells, CD8 + Granzyme B + T cells, CD8 + PD-1 + T cells, and CD4 + FOXP3- PD1 + T cells) [69].

Costimulatory signals

CD40 Schetters et al. reported that combinational agonistic αCD40 and αPD-1 displayed more significant tumor inhibition than αPD-1 monotherapy in MC38 tumor bearing mouse model. Combinational therapy increased CD8 + Ly6C-PD1 + TILs, CD4 + Foxp3-Ly6C + PD-1 + TILs and CD8 + Ly6C + PD-1 + TILs. Further evidence showed the proliferation of TILs was induced in the combinational group. Besides, αCD40 plus αPD-1 therapy promoted monocytes to MoDCs which produced iNOS and supported TIL expansion [70].

4-1BB (CD137) Buñuales et al. developed a high-capacity adenoviral vector (HCA-EFZP-αPD-L1) which induced PD-L1 blocking antibody expression and found that MC38 tumor growth was inhibited by HCA-EFZP-αPD-L1 with increased Tet + CD8 + T cells population. However, HCA-EFZP-αPD-L1 did not show significant anti-tumor efficacy in colorectal cancer peritoneal metastases model, and combination with HCA-EFZP-αPD-L1 with agonistic αCD137 did not increase the synergetic therapeutic effect on MC38 tumor than HCA-EFZP-αPD-L1 alone. Interestingly, removal of macrophages plus HCA-EFZP-αPD-L1 induced more significant survival benefit of mice [131]. In contrast, Lakins et al. reported that surrogate FS222, a bispecific antibody targeting CD137/PD-L1, displayed more potent anti-tumor efficacy than αPD-L1 monotherapy in CT26 or MC38 tumor bearing mice. In CT26 tumor bearing mouse, surrogate FS222 increased CD4 + and CD8 + T cells in peripheral and tumor, and induced Ki67 + CD4 + /CD8 + T cells in a dose- and time- dependent manner. PD-L1 receptor occupation rate was increased by surrogate FS222 and serum proinflammatory cytokines IFN-γ, TNF-α and IL-6 were increased [71]. Ballesteros-Briones et al. developed Semliki Forest virus (SFV) vectors expressing anti-PD-L1 mAb. Intratumoral injection into MC38 leads to complete regression and even induces abscopal effects. Tumor specific CD8 + T cells was increased in tumors, tdLNs, and peripheral blood. After SFV-αPD-L1 injection, and IFN-stimulated genes (ISGs), such as *Mx1*, *OAS-2*, *TRIM-21* and *STAT-I*, was significantly upregulated. In addition, the population of CD8 + TILs expressing CD137 was increased in mice treated with SFV-αPD-L1, and combination with systemic αCD137 mAb and SFV-aPD-L1 exhibited potent antitumor efficacy in MC38 tumors bearing mice than monotherapy [72]. Braeckel-Budimir et al. showed that OX40 or 4-1BB co-stimulation enhanced the efficacy of PD-L1 blockade in MC38 tumor bearing mouse model, especially TCT therapy (αOX40 + α4-1BB + αPD-L1) exhibited extraordinary anti-tumor effect than αPD-L1 alone. The combinatorial treatments induced the increase of intratumoral CD8 + T cells and ratio of CD8 + T cells/Tregs. CD8 + T cells depletion, not CD4 + T cell or NK cell, compromised the antitumor effect of TCT. Triple combinatorial treatments specially expanded a novel stem-like CD8 + T cell subpopulation with PD-1^{low} KLRG-1 + Ki-67 + phenotype in a CXCR3 dependent manner [73]. HK010, a Fc-muted bispecific antibody targeting PD-L1 and 4-1BB exhibited stronger antitumor efficacy than αPD-L1 alone in the humanized mouse model bearing MC38/hPD-L1 tumors, with more immune cell infiltration activation (NK cells, CD4 + T cells, CD8 + IFN-γ + T cells) and proliferation (CD8 + Ki67 + T cells) [74].

OX40 Chae et al. observed tumor regression in a patient with MSI-high metastatic colorectal cancer who was treated with combination of OX40 agonist and PD-L1 antagonist. However, there was an pseudo progression, marked by 163% increase in baseline tumor burden before the onset tumor regression was observed [132]. Yin et al. reported MSC-C9×T4a, a delivering system based on mesenchymal stem cell (MSC)-containing CXCL9 and OX40 ligand (OX40L)/tumor necrosis factor superfamily member 4 (TNFSF4), promoted anti-tumor efficacy of PD-1 blockade in CT26 tumor bearing mice, with reduced tumor growth and improved mouse survival. MSC-C9×T4a also elicited anti-tumor immune response in AOM (azoxymethane)/DSS (dextran sulfate sodium)-induced spontaneous colon cancer mouse models, showing less colorectal tumor numbers and Ki67 + cells but more NK cells and CD8 + T cells infiltration. In CT26 xenograft mice MSC-C9×T4a induced more CD4 + T, CD8 + T and NK cells infiltration than controls, especially the frequencies of GrB + CD8 + T, and GrB + NK cells were increased. Besides, MSC-C9×T4a also significantly inhibited the growth of MHC class I-deficient MC38 tumors, and depletion of CD8 and NK cells comprised anti-tumor efficacy [75]. Fabian et al. reported the success of hexatherapy regimen (adenovirus-based CEA vaccine; IL-15 superagonist, N-803; αOX40 and α4-1BB; αPD-L1) with enhanced therapeutic efficacy than αPD-L1 therapy alone in MC38-CEA mouse model. Hexatherapy treatments induced CD4 + and

CD8 + T cell proliferation and activation, characterized with increased Ki67 + or IFN-γ + T cells [76].

Inducible T-cell costimulator (ICOS) Beyrend et al. reported that PD-L1 blockade inhibited MC38 tumor growth with significantly increased infiltrating CD8 + T cells in tumor, while they also observed that CD4 + or CD8 + TAI cells expressing both activating (ICOS) and inhibitory (LAG-3 and PD-1) factors were selectively expanded within tumor after PD-L1 blockade. Subsequently, combinational ICOS antibodies and αPD-L1 therapy was implemented and eventually enhanced survival and tumor growth delay [77].

Cytokines

Cytokines are crucial factors in sustaining immune homeostasis, fostering immune responses to infection or tumor cells and orchestrating the development of immune memory. The preclinical studies on the efficacy of the combinational therapy containing cytokines and PD-1/PD-L1 blockade in CRC tumor bearing mice were summarized previously [133].

IFN-α Omori et al. have revealed the enhanced antitumor efficacy achieved through the combination of IFN-α-transfected tumor cell vaccine and PD-1 blockade in MC38 tumor bearing mice. It significantly suppressed tumor growth via CD4 + and CD8 + T cells, but not NK cells. The combined treatments promoted CD4 and CD8 + T cells infiltration within tumors. Compared with αPD-1 monotherapy, αPD-1 plus IFN-α-overexpressing tumor cells promoted higher levels of IFN-γ secretion but reduced IL-10 production by splenocytes in vitro. A notable enhancement in cytotoxicity against MC38 was found in combinational therapy when compared to treatment alone. In addition, apoptosis of lymphocytes isolated from mice immunized with MC38-IFN-α was suppressed by αPD-1 [78].

IL-2 Hutmacher et al. designed an antibody-IL-2 fusion protein (F8-IL2), which selectively target tumor and be restricted in the tumor site. Then they evaluated the antitumor efficacy of F8-IL2 in combination with αPD-1 or αPD-L1 in CT26 tumor bearing mice. The combination with αPD-1 suppressed tumor growth, but most tumors eventually regrew, whereas the combination with αPD-L1 displayed the lowest therapeutic activity. In tumor, the percentage and the expression of PD-1, TIM-3, and Ki-67 on CD8 + T cells were increased, suggesting local T cells were activated and expanded. The percentage of KLRG1 + CD11b + NK cells was higher in control group than the combined therapy group, indicating NK cells in the controls exhibited more mature phenotype and displayed a slower turnover rate. In tdLNs, the increase of NK cells in mice from all treated groups (F8-IL2 or anti-CTLA-4 antibody or combined therapy) were observed compared with control mice, and F8-IL2 therapy induced the proliferation and activation of NK cells with CD11b and CD27 expression. Similarly, mice treated with F8-IL2 showed increased Ki-67, CD11b, and KLRG1 expression in splenic NK cells. In MC38 tumor bearing mice, the increase of Granzyme B and Ki-67 expressions in CD8 + T cells or NK cells in tumors and tdLNs was observed after F8-IL2 therapy [79]. Chen et al. found that BIPI, a novel αPD-L1 fusing hIL-2 induced longer survival of mouse in CT26 lung metastasis model than αPD-L1 alone. The anti-tumor effect was dependent on CD8 + T cells, and splenic CD8 + IFN-γ + T cells from BIPI group exhibited more significant tumor inhibition than counterparts from αPD-L1 treatment alone [80]. Onyshchenko et al. also demonstrated that CD122-directed IL-2 complexes enhanced the efficacy of radiation plus αPD-1 therapy in C51 colon carcinoma mice model, exhibiting the expansion of stem-like CD8 + T cells in spleen and blood. Depletion of CD8 + T cells or CXCR3 blockade abolished the anti-tumor efficacy [81].

IL-12 Puca et al. developed a novel fusion protein, L19-mIL12, by fusing murine IL-12 to L19 antibody. It displayed a potent anti-tumor activity in CT26 tumor bearing mice, whereas L19-mIL12 plus αPD-1 further enhanced anti-tumor effectiveness, however, L19-mIL12 plus αPD-L1 was not well tolerated. Increased NK and CD8 + T cells and decreased Tregs in tumor was observed after L19-mIL12 treatment. Depletion of NK and CD8 + T cells compromised anti-tumor efficacy of L19-mIL12. The predominant population of CD8 + T cells in CT26 tumors were found to specifically reactive to retroviral AH1 antigen. Protein extracts from tumor indicated that L19-mIL12 induced IFN-γ expression. And injection of L19-mIL12 into BALB/c mice induced upregulation of IL-6, TNF-α and IFN-γ [82]. Hewitt et al. developed a novel intratumoral IL-12 mRNA therapy, and the efficacy of mIL-12 mRNA in a MC38-resisitant model (less responsive to αPD-L1 or αPD-1) was enhanced by αPD-L1. The tumor regression was observed in both local and distant tumor and cellular mechanism exploration revealed that mIL-12 mRNA recruited CD8 + T cell into tumors to induce tumor-specific cell lysis. Anti-tumor activity of mIL-12 mRNA was dependent on CD8 + T cells, not CD4 + T cells, NK or NKT cells. Th1 immune response genes or genes responsible for DC abundance and antigen presenting were both upregulated by mIL-12 mRNA, leading to IFN-γ and cytotoxic T cell-medicated immune response. A novel treatment for patients with

solid tumors, MEDI1191 (human IL-12 mRNA) is under evaluation in a phase I trial (NCT03946800) [83].

IL-15 Yu et al. evaluated the efficacy of IL-15 combined with αPD-L1 in CT26 tumor bearing mice. Mice after combinational treatments displayed less tumor nodules and longer survival time relative to mice in mIL-15 or αPD-L1 monotherapy. mIL-15 alone increased PD-1 expression on CD8 + T cells and CD8 + $CD44^{high}$ memory T cells. However, mIL-15 promoted IL-10 secretion. Mice in combining mIL-15 with αPD-L1 group displayed higher percentages of CD8 + IFN-γ + T cells in spleen and less IL-10 secretions by CD8 + T cells compared with mice in monotherapy or control group, moreover, CT26 specific cell lytic activity of CD8 + T cells was most significant in mIL-15 plus αPD-L1 therapy [84]. Knudson et al. found that N-803 (ALT-803), an IL-15 superagonist, enhanced anti-tumor effectiveness of αPD-L1 in mice bearing MC38-CEA tumors. Suppressed tumor growth and extended survival was induced in combinational treatments, and the anti-tumor effect was reliant on CD8 + T cells and NK cells. They found that in comparison with αPD-L1 alone, N-803 + αPD-L1 treatment induced a smaller number of G-MDSCs and Tregs, and promoted more NK cells infiltration and significant activation characterized by increase of NKG2D, Ki67 and GrB expressions on NK cells in spleen and tumor. Cytotoxic function of NK cells in N-803 + αPD-L1 therapy was more potent than that in αPD-L1 alone. Besides, N-803 + αPD-L1 therapy induced more significant CD8 + T cells activation ($CD44^{hi}CD62L^{hi}$ TCM) and proliferation. N-803 + αPD-L1 therapy induced more T cells expressing GrB and effect cytokines than αPD-L1 therapy. Consistently, levels of serum immunostimulatory cytokines, such as IFN-γ and TNF-α in combinational group was much higher than the αPD-L1 group [85]. Shi et al. generated a novel immunocytokine (LH01) containing αPD-L1 fused to IL-15 complex. LH01 treatments present more significant efficacy than αPD-L1 monotherapy in MC38 or CT26 tumor model. LH01 induced the increase of CD8 + T cells and NK cells in tumor, while reduced Tregs [86].

IL-21 Liu et al. engineered IL-21-αHSA, a human serum albumin (HSA) fused to the C-terminus of rhIL-21 with longer half-life. The effect of αPD-1 in MC38 tumor bearing mouse model was enhanced by IL-21-αHSA. The ratio of CD3+/CD45 + cells, CD8+/CD3 + cells, NK cells/CD45 + cells and the percentage of Ki67 + CD8 + T cells in combinational treatments were significantly increased than αPD-1 monotherapy [87].

Boosting of the effector responses of innate immunity

Innate immune cells modified the cytotoxic function of effect cells via expressing activating receptors or by blocking inhibitory pathways, such as TIM-3, TIGIT, LAG-3, VISTA and CD47. ICIs have been designed to target not only T cells but also NK cells or myeloid cells, and more rational combinational therapy for CRC treatments focuses on harnessing the potential of innate immunity and T cell functions (Fig. 6).

Blockade of immune checkpoint

T-cell immunoglobulin and mucin-domain containing-3 (TIM-3) By isolating tumor infiltrating lymphocytes (TILs) from CRC patients, Liu et al. found that TIM-3 + PD-1 + CD8 + TILs were the predominant population which displayed exhausted phenotype with little production of IFN-γ, TNF-α and IL-2, thus combination of TIM-3 and PD-L1 blockade was applied in DC and T coculture in vitro. In contrast to PD-L1 blockade alone, combinational treatments in conjunction with DC-targeted cancer cell vaccines reduced frequency of Tregs, but increased TNF-α + IFN-γ + CD8 + T cells population, and in the combinational group more CD8 + T cells undergo proliferation and exhibit potent cytolytic activity than those in the PD-L1 blockade group [88].

T-cell immunoglobulin and ITIM domain (TIGIT) TIGIT could induce CD3 + T cell dysfunction in CRC, and the combination of αTIGIT and αPD-1 had a synergistic efficacy in tumor inhibition in MC38 tumor bearing mouse model compared with TIGIT or PD-1 blockade monotherapy [89]. Johnston et al. explored the role of TIGIT in anti-tumor immune responses with mice bearing CT26 tumor. Combination of TIGIT and PD-L1 blockade induced tumor reduction and showed long lasting immunity even tumor rechallenge. TIGIT cooperates with PD-1/PD-L1 to selectively inhibit functions of tumor-infiltrating CD8 + T cell in tumor, and the anti-tumor responses displayed in the CD8 + T cell-dependent manner [134]. Zhang et al. found that highly expressed TIGIT is associated with exhaustion of tumor-infiltrating NK cells in mice or patients with CRC. Blocking TIGIT with antibodies prevent NK cell exhaustion, assisted anti-tumor immunity and sustained immunological memory. Therapeutic efficacy of co-blockade of TIGIT and PD-L1 in mice bearing CT26 tumor was dependent on NK cells. Blocking TIGIT in T cell deficient SCID mice, CD107a, TNF-α, IFN-γ or CD226 expressions were increased on tumor-infiltrating NK cells, indicating αTIGIT serves to avert NK cell exhaustion. NK deletion leads to decreased infiltration tumor-infiltrating CD8 + T cells expressing

IFN-γ or TNF-α, increased CD8 + PD-1 + T cells and reduced CD107 + CD8 + T cells within tumor, suggesting αTIGIT enhances adaptive immunity in a NK-cell dependent manner [90]. Han et al. explored the molecular mechanism behind the synergetic therapeutic efficacy of αTIGIT and αPD-1 combination in CT26 or MC38 tumor bearing mice, and found that combing TIGIT blocking antibodies with mIgG2a and PD-1 blocker elited more effective anti-tumor efficacy, exhibiting reduced tumor growth and increased complete responses rate. Blocking FcγRIV in combinational therapy comprised anti-tumor effectiveness of anti-TIGIT: mIgG2a antibody. However, depletion of intratumoral Tregs or TIGIT + cells did not reduce the efficacy of TIGIT blocker. The TIGIT blocker induced myeloid cell activation with production of CXCL10, CXCL11, IL-23 and TNF-α and upregulation of MHC class II, CD86, or CD40 expression on APC. In CT26 tumor bearing mice, combined TIGIT blocking antibodies with mIgG2a and PD-1 blocker induced increased gene expression (*CD45*, *CD3*, *CD11b*, *CD8b*, *Foxp3*, *IFN-γ*, *Perforin*, and *Granzyme B*) in tumor, and distinctly induced persistent granzyme B and perforin production, unlike predominant IFN-γ-secretion by anti-PD-1 blockade [91]. Grapin et al. investigated optimized fractionated radiotherapy (3 × 8 Gy RT) with αPD-L1 and αTIGIT in CT26 or MC38 tumor bearing mice, and showed combinational therapy (RT + αPD-L1 + αTIGIT) exhibited more effective than RT + αPD-L1. RT promoted immune cells infiltration, such as the increase of total T cells, CD4 + T cells, CD8 + T cells, CD8+/Treg ratio, CD8+/granzyme cells, TAM1/TAM2 ratio, but the decrease of myeloid cells, TAM2 and MDSCs. Further investigation revealed that differentially expressed genes were mainly involved in GAS-STING pathway, CD8 + T cell activation and differentiation, IFN-γ production and response pathways [92]. Clinically, Thibaudin et al. found that atezolizumab (αPD-L1) and tiragolumab (αTIGIT) restores TILs function in some patients with MSS CRC [135].

Lymphocyte activation gene-3 (LAG-3) Beyrend et al. reported that αPD-L1 inhibited MC38 tumor growth with significantly increased infiltrating CD8 + T cells in tumor, although they have found that CD4 + or CD8 + TAI expressed both activating (ICOS) and inhibitory (LAG-3, PD-1). Furthermore, TAI cells were recognized in tumors from five colorectal cancer patients, and αLAG-3 in combination with αPD-L1 enhanced survival and tumor growth delay [77]. Notably, Ballesteros-Briones et al. generated Semliki Forest virus (SFV) vectors expressing αPD-L1 (SFV-aPD-L1), and intratumoral injection of SFV-aPD-L1 led to complete regressions and even induces abscopal effects. However, CD137 and LAG-3 on CD8 + TILs were increased after SFV-aPD-L1 treatment. Combination of SFV-aPDL1 and αCD137 mAb showed a potent antitumor effect than SFV-aPD-L1 monotherapy, but αLAG-3 plus SFV-aPD-L1 did not display more potent effect than SFV-aPD-L1 alone [72].

V-domain Ig-containing suppressor of T cell activation (VISTA) Sasikumar et al. developed an oral immune checkpoint inhibitor CA-170 to selectively inhibits PD-1 and VISTA pathway. CA-170 treatment not only induced more proliferating CD4 + or CD8 + T cells within tumor than αPD-1 monotherapy, but also result in higher expression of co-stimulatory molecule OX-40 on CD8 + T cells in tumor and elevated intracellular levels of granzymeB in CD8 + T cells in blood [93]. Schaafsma et al. found that mice with large CT26 tumors (> 600mm^3) showed complete resistance to αPD-1/αCTLA-4 treatments, but supplement with αVISTA in combinational therapy (αPD-1/αCTLA-4/αVISTA) led to tumor rejection in more than 50% of the mice. Underlying mechanisms was further explored using single-cell RNA sequencing, multiplex immunohistochemistry, and flow cytometry. αVISTA treatment reduced myeloid-mediated suppression in tumor and did not induce CD45 + immune cells infiltration. scRNA-seq on tumor-specific CD8 + T cells displayed highly distinct pathways between αPD-1/αCTLA-4 and αVISTA therapy, the former increased the expansion of progenitor exhausted CD8 + T cell subsets, and the latter upregulate costimulatory genes and reduced the expression of regulators that maintain T cell quiescence. For the first time they reported one of checkpoint inhibitor could affect CD8 + T cell quiescence, suggesting T cells quiescence may represent a novel target for research or clinical treatment [94].

CD47 Ni et al. developed radiosensitizers (called IMD@Hf-DBP/αCD47), which contains TLR7 agonist, imiquimod (IMD) and hydrophilic αCD47, to investigate its anti-tumor efficacy in CT26 tumor bearing mice. Tumor growth after αPD-L1 alone treatment did not display significantly growth inhibition; however, IMD@Hf-DBP/αCD47 plus αPD-L1 completely eradicate primary and distant tumors after radiation, companied with the increase of IFN-γ + cytotoxic T cells in splenocytes. Besides, M2 macrophages (F4/80 + CD86-CD206+) were repolarized to M1 macrophages (F4/80 + CD86 + CD206-) by IMD, and αCD47 promoted macrophage phagocytosis. CD4 + and CD8 + T cells, NK cells and B cells were significantly increased in both primary and distant tumors in IMD@Hf-DBP/αCD47 therapy compared with the αPD-L1 group [95]. Hsieh et al. found that CD47 and PD-L1 expressions were

up-regulated in CRC by radiotherapy dependent on the ataxia telangiectasia and Rad3-related (ATR) activity, and triple therapy (RT/αSIRPa/αPD-1 or RT/αCD47/αPD-1) induced higher complete response rates than αPD-1 alone in both local and abscopal tumors and longer survival time in MC38 tumor bearing mice. Of note, RT/αSIRPa/αPD-1 therapy displayed significantly better efficacy than RT/αCD47/αPD-1 therapy. RT/αSIRPa/αPD-1 therapy reduced M2 macrophage and did not increase MDSCs, reversing adaptive immune resistance. TAA cross-presentation was significantly enhanced by triple therapy, characterized with profoundly upregulated CD86 expression on DCs and Mo-Macrophages. Further, TAA-specific CD8 + T cell was primed and activated and T cell clonality and clonal diversity was also increased by triple therapy. Dendritic cell was responsible for CD8 + T cell priming and STING activation was critical for the profound anti-tumor effect of triple therapy [96].

Mitigating immunosuppression in TME

Multiple resistance mechanisms to immunotherapy have been identified. The immunosuppressive TME plays a pivotal role in αPD-1/αPD-L1 therapy resistance in certain CRC patients. Tumors-derived immunosuppressive factors not only directly impair the functions of effector T cells but also inhibit innate immune cells, thereby hindering their ability to maintain robust anti-tumor immunity. Targeting these factors is one of strategies to overcome immunotherapy resistance (Fig. 7).

Blockade of immunosuppressive factors

Adenosinergic pathway Leone et al. found that CPI-444, an A2aR antagonist, modestly inhibited tumor growth and promoted survival in MC38 or CT26 tumor bearing mice. αPD-1 and CPI-444 combination induced more remarkable tumor regression and better survival rate. Blocking A2aR with CPI-444 in CT26 tumor bearing mice reduced PD-1 and LAG-3 expression on activated CD8 + CD44 + effector T cells and CD4 + FoxP3 + Tregs in tdLNs. CPI-444 significantly promoted effector function of tumor-infiltrating T cells, characterized with increased population of CD8 + T cells expressing TNF-α and IFN-γ. Besides, activation marker 41-BB within the TME and transcription factors T-bet expression on infiltrating CD8 + T cells were both increased by CPI-444. CPI-444 has the capacity to enhance both tumor-specific immune response and adaptive immune memory [97]. Willingham et al. reported that combining CPI-444 with αPD-L1 or αCTLA-4 abolished tumors in MC38 and CT26 tumor bearing mice, and anti-tumor effect was CD8 + T cells dependent. The antitumor effectiveness of CPI-444 was ascribable to the elevation of active GITR + IL7Rα + T cells and promotion of a Th1 gene expression signature in responsive tumor, including *CD8a*, *CXCL9*, *CXCL10*, *EOMES*, *IFN-γ*, *GZMA*, *GZMB*, and *TBX21*. Recovery of T cell function is a crucial mechanism whereby CPI-444 induces antitumor response. T cell inactivation induced by adenosine analogs NECA was restored by CPI-444. Upon activation of A2AR, the increased intracellular cAMP induced the phosphorylation of CREB (cAMP response element-binding protein), but CPI-444 suppressed the phosphorylation of CREB in PBMCs. Furthermore, pretreatment PBMCs with NECA suppressed T cell receptor (TCR) activation, resulting in downregulated pERK expression. Conversely, blockade of A2AR using CPI-444 completely restored the pERK induction [98]. Kim et al. showed that AB680 (a selective CD73 inhibitor) combined with αPD-1 could exhibited more significant tumor inhibition compared to each individual therapy. They further revealed distinct cellular alternation by single-cell RNA sequencing. Interestingly, five major cellular lineages (myeloid cells, granulocytes, T cells, NK cells and B cells) in sorted CD45 + TILs from CT26 tumor baring mice were clarified and the abundance of myeloid cells exceeded other populations. AB680 and αPD-1 differentially reshaped intratumoral immune microenvironments. αPD-1, not AB680 treatment significantly reduced myeloid cells and $Ikzf2^{high}$ CD4 + Tex cells, whereas αPD-1 and AB680 both increased $CD69^{high}$ CD8 + T cells and reduced $Malat1^{high}$ Tregs. TCR diversity of Entpd1(CD39 gene)-negative T cells and Pdcd1(PD-1 gene)-positive T cells were also increased after αPD-1 treatment. AB680, but not αPD-1, profoundly induced $Ccr2^{high}Tlr2^{high}$ M1 macrophage, and $Cx3cr1^{high}/Csf1^{high}/Nt5e$ + M2 macrophage was reduced by both AB680 and αPD-1. Moreover, αPD-1 induced Tregs depletion in AOM/DSS induced CRC model, and AB680 therapy increased CD8 + T cells activation in vitro [99].

IDO Phan et al. designed attenuated *Salmonella typhimurium* to deliver an shRNA plasmid targeting IDO (shIDO-ST) and assessed anti-tumor efficacy in MC38 or CT26 tumor bearing mice. Significant inhibition on tumor growth have been observed in shIDO-ST group in comparison with shSrc-ST control. However, they found that αPD-1 plus shIDO-ST did not show greater effectiveness than shIDO-ST alone, and the mechanism need to be clarified [136]. Shi et al. found that STING agonist (diABZI) and IDO inhibitor (1-MT) enhanced anti-tumor efficacy of PD-1 blockade, displayed more profound inhibition of tumor progression. Furthermore, CD8 + T cells (CD8 + IFN-γ + TILs) and dendritic cells (CD11b + CD86 + cells) were increased and the

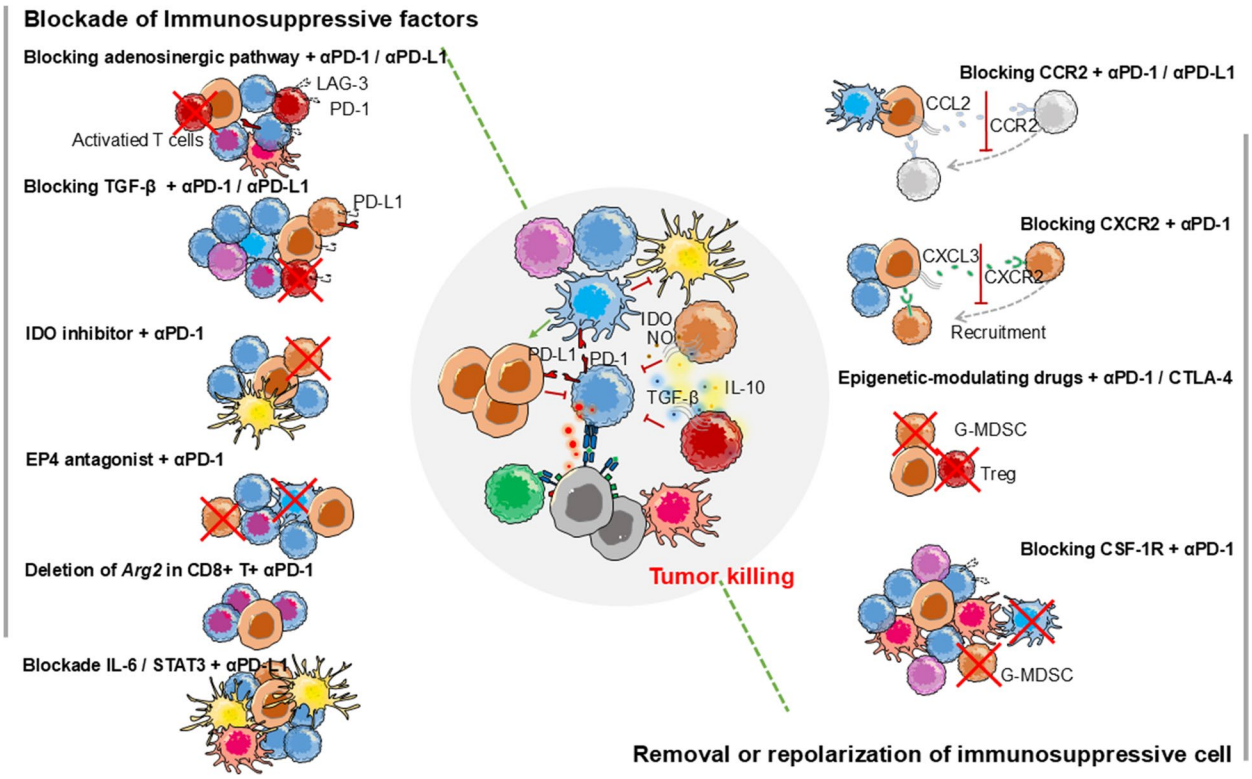

Fig. 7 Mitigating immunosuppression in TME. Immunosuppressive factors in tumors can directly alter T cell effector functions, preventing them from sustaining efficient anti-tumor immune responses. Blockade of immunosuppressive factors, such as TGF-β, adenosinergic pathway and prostaglandin E4 receptor and IL-6 or deletion of *Arg2* in CD8+T cells has been demonstrated to increase the efficacy of PD-1/PD-L1 blockade in CRC, besides, removal of MDSCs and Tregs or repolarization of TAMs by blocking CXCR2, CSF-1R or with epigenetic-modulating drugs was achieved in several combinational therapy against CRC. Mitigating immunosuppression includes blockade of immunosuppressive factors and removal or repolarization of immunosuppressive cell. The cellular alternation in combinational therapy were listed as follow: Blocking adenosinergic pathway + PD-1/PD-L1 blockade: reduced PD-1 and LAG-3 expression on activated CD8+ CD44+ effector T cells and CD4+FoxP3+ Tregs, increased CD8+ T cells expressing TNF-α and IFN-γ and M1 macrophage, and reduced Tregs. Blocking TGF-β + PD-1/PD-L1 blockade: enhanced the CD3+, CD4+ and CD8+ T cells infiltration and activation, and decreased PD-L1 expressions on $CD45^{neg}$ cells and immune cells. EP4 antagonist + PD-1/PD-L1 blockade: increased cytotoxic CD8+ T-cell and reduced MDSCs and M2 macrophages. Deletion of *Arg2* in CD8+T + PD-1/PD-L1 blockade: enhanced CD8+ T cell activation and inflammatory cytokine induction. Blockade IL-6/STAT3 + PD-1/PD-L1 blockade: more cytotoxic CD8+ T cells and $CD11c+I\text{-}Ad^{high}$ mature dendritic cells accumulation, suppressive macrophage to pro-inflammatory subtype, and enhanced functionality and proliferation of cytotoxic CD8+ T cells. Blocking CCR2 + PD-1/PD-L1 blockade: blocking recruiting monocyte and TAMs by tumor-derived CCL2. Blocking CXCR2 + PD-1/PD-L1 blockade: blocking recruiting MDSCs into tumor through binding CXCR2. Epigenetic-modulating drugs+ PD-1/PD-L1 blockade/CTLA-4: decreased Foxp3+ Tregs and G-MDSCs. Blocking CSF-1R + PD-1/PD-L1 blockade: enhanced T cell infiltration, induced CD8+ T cell dependent immune response, inhibited the recruitment of TAMs and reprogrammed TAMs to M1 phenotype and decreased MDSCs but increased CD45+ cells and T cells in tumor

infiltration of MDSCs were decreased by combinational STING agonist and IDO inhibitor [100].

TGF-β signal Local radiotherapy (RT) in combination with αCD137 plus αPD-1 induced abscopal effects in mice bearing MC38 tumor, and additive TGF-β blockade induced more potent abscopal effects than RT + αPD-1 + αCD137 combination, with increased survival rate 87% from 37% and higher serum IFN-γ level. CD8+, but not CD4 + T cell population in tumor was increased in MC38 tumor bearing mice, especially frequency of granzyme-B + CD8 + TILs were increased [101]. Nakanishi et al. found that atypical PKCs expression was decreased in human serrated tumors and that PKCs deletion induced spontaneous serrated tumorigenesis. Mouse lines with deletion in PKCa in IECs was generated with crossing $Prkci^{fl/fl}$ and $Prkcz^{fl/fl}$ mice. Deletion of aPKCs in model mice promoted serrated tumor development in colon, with stromal activation and stroma-derived growth factors upregulation (Egfr, Areg, and Ereg), indicating TGF-β signaling play a vital role in stromal activation in CRC. Besides, immune suppression in intestinal was confirmed by

the increase of PD-L1 + CD45 + cells. TGF-β signaling inhibitor galunisertib with PD-L1 blockade was used in $Prkci^{fl/fl}$ and $Prkcz^{fl/fl}$ mice and synergistic efficacy was observed in combinational therapy, characterized with reduced tumor number and size, load, aggressiveness, and more intramural CD8 + T cell infiltration. However, CD11b + myeloid cells were slightly reduced and the proportion of Tregs was not significantly changed [102].

Tauriello et al. developed metastatic intestinal tumors by crossing mice carrying conditional alleles of four main colorectal cancer mutations ($Apc^{fl/fl}$, $Kras^{LSL-G12D}$, $Tgfbr2^{fl/fl}$ and $Trp53^{fl/fl}$) in intestinal stem cells. Metastatic intestinal tumors presented a low mutation rate, significant exclusion of T cells, and an activated stromal environment driven by TGF-β. PD-L1 blockade induced limited anti-tumor immune response, whereas blockade of TGF-β signaling with Galunisertib enhanced the efficacy of anti-PD-L1 therapy in mice. Combinational therapy boosts the infiltration of CD3+, CD4 + and CD8 + T cells into tumor, elevated T-bet and IFN-γ expression in CD4 + Th cells and enhanced GZMB production in CTLs. Galunisertib treatment alone induced CD44 + CD62L-and CD69 + CD62L- populations in CD4 or CD8 + population, suggesting Th cells and CTLs has been activated by TGF-β blockade [103].

Ozawa et al. designed Bintrafusp alfa, a frist-in-class bifunctional fusion protein targeting TGF-β and PD-L1, and delivering Bintrafusp alfa in MC38 tumor bearing mice led to suppressed tumor growth in systemic or subcutaneously delivering. Bintrafusp alfa bind to the surface of nonimmune cells, CD8 + and CD4 + TILs, Tregs, M-MDSCs and G-MDSCs in the TME. PD-L1 expressions on $CD45^{neg}$ cells and immune cells (TILs, Tregs, M-MDSCs and G-MDSCs) were all decreased. Furthermore, the infiltration of cytotoxic CD8 + T cells into tumor was significantly augmented with the administration of Bintrafusp alfa [104].

Endoglin serves as a coreceptor for TGF-β ligands, and Schoonderwoerd et al. found that combinational targeting endoglin (TRC105) and αPD-1 is more effective in reducing tumor burden than αPD-1 alone in the AOM/DSS induced CRC mouse model, moreover, it was observed that the anti-tumor efficacy of combinational therapy was better than αPD-1 alone in MC38 or CT26 tumor bearing mouse model. Therapeutic effects of TRC105 plus αPD-1 were dependent on FcγR-mediated ADCC and CD8 + T cells. Further, they found that intratumoral CD8 + T cells, the ratio of CD8+/Foxp3 + and CD8 + granzyme B + T cells was increased by combinational therapy, and the number of intratumoral CD25+/Foxp3 + cells was reduced by TRC105 [105].

Prostaglandin E4 receptor (EP4) Lu et al. developed a selective EP4 antagonist TP-16 and showed that combination therapy (TP-16 and αPD-1) led to more significant tumor inhibition and prolonged survival than αPD-1 in CT26 or MC38 tumor bearing mice, with increased cytotoxic CD8 + T cells, reduced CD11b + Gr1 + MDSCs and CD11b + CD206 + M2 macrophages. TP-16 plus αPD-1 effectively decreased PD-L1, p-STAT3 and p-AKT expressions; besides, pro-tumor cytokines and chemokines IL-6 and CXCL1 was inhibited by combinational therapy. Gene expression pattern in combinational therapy was changed to inflammation- and immunity-related pathways, especially, T cell cytolytic and activation associated genes (*Gzmb*, *Tnfa*, *Ifng*, *Prf1*, *CD25*, *CD69*, *CD107a* and *CD178*) were upregulated. They also found TP-16 promoted anti-tumor efficacy of αPD-1 in AOM/DSS-induced colorectal cancer model. HE staging showed that combinational therapy increased cytotoxic T cell infiltration and reduced p-STAT3, p-AKT and Arg-1 expressions [106].

Arginase 2 (Arg-2) The targeted deletion of Arg2 in CD8 + T cells strongly promoted the efficacy of PD-1 blockade in inhibiting growth and extending survival in MC38-OVA tumor bearing mice. Further, deletion of Arg2 in CD8 + T cells displayed enhanced cell activation and cytokine induction. The frequencies of $CD69 + CD62L^{low}$ cells in $Arg^{-/-}$ CD8 + T cells and productions of IL-2 and IFN-γ were higher than that in WT CD8 + T cells, indicating that more CD8 + T cells were activated after Arg2 deletion. Transcriptome analysis revealed overexpression of critical genes associated with CD8 + T cell function, involved in cytotoxicity, IFN-γ signaling, cytokines and inflammatory response, and IL-2 signaling pathway. During adoptive transfer experiments, both OT-I or $Arg2^{-/-}$ OT-I T cells were administered into WT hosts bearing MC38-OVA tumor, and it was noted that $Arg2^{-/-}$OT-I cells displayed the enhanced antitumor potential [107].

IL-6/STAT3 Ohno et al. found that the anti-tumor effect of PD-L1 blockade was more potent in IL-6-deficient CT26 tumor bearing mice than WT CT26 tumor bearng mice. Administration of anti-IL-6R mAb significantly inhibited CT26 tumor growth in combination with αPD-L1, suggesting IL-6 was involved in anti-tumor efficacy of PD-L1 blockade. Moreover, lack of IL-6 promoted cytotoxic CD8 + T cells and CD11c + I-Ad^{high} mature dendritic cells accumulation in tumor than PD-L1 blockade alone [108]. Proia et al. also found Danvatirsen, a

therapeutic STAT3 antisense oligonucleotide (STAT3 ASO), plus PD-L1 blockade significantly suppressed tumor growth than either monotherapy alone in MC38 or CT26 tumor bearing mice. Combinational therapy significantly reversed the suppressive macrophage to pro-inflammatory subtype, and enhanced functionality and proliferation of cytotoxic $CD8^+$ T cells [109].

Removal and polarization of immunosuppressive cells

C-C motif chemokine receptor 2 (CCR2) Residual tumor after incomplete RFA (iRFA) led to metastases and poor survival in CRC patients with liver metastases; In line with these findings, iRFA promoted tumor progression and compromised the efficacy of αPD-1 in MC38 and CT26 tumor bearing mice. Immune analysis revealed that the number and proportion of infiltrating myeloid suppressor cells were significantly increased in residual tumor. From a perspective of cellular mechanisms, myeloid suppressor cells, including monocyte and TAMs, were recruited by tumor-derived CCL2, and TAMs stimulate CCL2 production by tumor cells through TNF-α/TNF-α receptor dependent manner. Blockade of CCR2 on tumor cells or using $CCL2^{-/-}$ tumor cells salvaged the antitumor efficacy of PD-1 blockade [110].

C-X-C chemokine receptor type 2 (CXCR2) Upregulated interferon regulatory factor 2 (IRF2) expression or targeting CXCR2 increased the efficacy of MC38 CRC cells expressing oncogenic *KRAS* to PD-1 blockade. Oncogenic *KRAS* on CRC cells inhibited IRF2 expression, thus promoted CXCL3 secretion. Increased CXCL3 recruited CD45 + CD11b + Gr-1 + myeloid-derived suppressor cells (MDSCs) into tumor through binding CXCR2 on MDSCs, especially CD11b + Gr-1 + MDSCs presented inhibitory function in T cell proliferation and activation; conversely, the number of T cells was increased after depletion of MDSCs with αGr-1, suggesting that migrated MDSCs in oncogenic *KRAS* mutation tumor induced immune-suppressive microenvironment [111]. Moreover, the combination of the CXCR2 inhibitor SB265610 and αPD-L1 exhibited more effective than αPD-L1 alone in inhibition of the metastasis of *KRAS* mutant CRC. In CT26-HOXA7 tumor bearing mouse model, combinational therapy profoundly decreased MDSCs infiltration but increased CD8 + T cells infiltration [112]. Bergeron et al. have found mice treated combined non-homogenous intratumor ionizing radiation (PI16/2) with αPD1 and with SB225002 (CXCR2 blockade) exhibited better tumor control (CR = 13/23) and survival than those from PI16/2 plus SB225002 group (CR = 13/23). However, the infiltration of CD8 + T cells and NK cells were not affected by CXCR2 blockade, indicating that the CXCR2-recruited immunosuppressive cells hampered the efficacy of αPD-1 [113].

Epigenetic-modulating drugs Blocking PD-1 and CTLA-4 in mice bearing CT26 tumor could eradiate tumor, while combination of PD-1/CTLA-4 blockade and a DNA methyltransferase inhibitor (5-azacytidine, AZA) plus a HDAC inhibitor (entinostat, ENT) induced stronger anti-tumor efficacy than PD-1/CTLA-4 blockade treatment. Cellular levels alteration included the decreased Foxp3 + Tregs and G-MDSCs in the combinational group (AZA + ENT + PD-1/CTLA-4 blockade), and AZA and ENT did not increase frequency of CD8 + T cells in tumor, suggesting that the function of effector T cells was inhibited by suppresser cells. After depletion of Tregs with αCD25 or G-MDSCs in combination with αLy6G in 4T1 tumor bearing mice, blocking αLy6G in combination with PD-1/CTLA-4 blockade showed similar anti-tumor efficacy as AZA + ENT + PD-1/CTLA-4 blockade [114].

Macrophage colony stimulating factor-1 receptor (CSF-1R) Triple combination treatment (oncolytic viruses, CSF-1R inhibitor-PLX3397 and PD-1 blockade) in mice bearing CT26 or MC38 tumor cells exhibited more significant tumor regression and longer survival time than αPD-1. Specific immune memory response to CT26 tumor was induced. Combinational therapy not only augmented T cell infiltration and but also triggered CD8 + T cell dependent immune response. Besides, the combinational therapy suppressed the migration of TAMs to the tumor site and reoriented TAMs to M1 phenotype. Combinational therapy downregulated immunosuppressive genes (*Cd68*, *Cd206*, *Msr1* and *Arg1*) and increased the expression of pro-inflammatory gene *iNOS*. Further mechanism exploration showed that triple combinational treatments induced IFN-γ + CD8 + T cells expansion, restrained multiple co-inhibitory molecular PD-1, LAG-3, TIGIT and TIM-3 expressions but increased expression of co-stimulatory ICOS. *CCL5*, *CXCL10*, *Gram B*, *perforin* and *IFN-γ* genes that regulating T cell recruitment and activation or response were enhanced [115]. Holmgaard et al. treated MDSCs with CSF-1R blockade, and tumor growth inhibition with prolonged survival time in CT26 tumor bearing mice was observed in the combination of dual CSF-1R inhibition (PLX647) and PD-1/CTLA-4 checkpoint blockade, whereas the individual components had no remarkable effect on tumor growth. A prominent anti-tumor efficacy with CSF-1R inhibition and blockade of PD-1/CTLA-4 in B16-IDO murine cancer model was observed, and the anti-tumor efficacy was dependent on CD4 + and

CD8 + T cells. Besides, CD11b + $Gr1^{int}$ MDSCs was significantly decreased, whilst CD45 + cells and T cells in tumor was significantly increased following the combined therapeutic treatments, especially TILs was mainly IFNγ + CD8 + and CD4 + effector T cells but not Treg cells [116]. Lv et al. also found that a novel CSF-1R inhibitor C19 promoted the anti-tumor efficiency of αPD-1 in MC38 tumor bearing mice or murine orthotopic model and the anti-tumor effect was dependent on CD8 + T cells. C19 induced TAM-derived CXCL9 generation to recruit CD8 + T cells [117].

Clinical trials

Several clinical trials have focused on the combination of innate immune activators with PD-1/PD-L1 inhibitors (Table 2), paving the way for CRC treatment in future. According to the current published data from clinical studies, the objective response rate (ORR) range of the different combination treatment regimens was 2 −50% [137–146], with well tolerated, without significant toxicity. Microsatellite instability-high (MSI-H)/mismatch repair-deficient (MMRd) metastatic colorectal cancer will get better clinical benefit. However, the ORR of microsatellite-stable (MSS) or mismatch repair proficient (MMRp) CRC was less than 10% in phase I/II trials. In the EPOC1503/SCOOP trial, the irORR with Napabucasin and Pembrolizumab reached 50.0% in cohort A (MSI-H) and 10.0% in cohort B (MSS). Among the common grade 3 or higher treatment-related adverse events, few patients were reported fever in 10.0%, decreased appetite in 7.5% and diarrhea in 5.0%.

Given that combining the two drugs does not bring the desired clinical efficacy, a multidrug combination chemotherapy regimen has been explored. A study evaluated Epacadostat (an oral, selective inhibitor of IDO1) in conjunction with Pembrolizumab and chemotherapy in patients suffering from advanced or metastatic solid tumors (ECHO-207/KEYNOTE-723, NCT03085914). The ORR of Group A (Epacadostat + Pembrolizumab + mFOLFOX6) was 55.6%, and Group G (Epa + Pembrolizumab + 5-FU and Platinum Agent) was 45.5%. Across all treatment groups was 31.4%. 78.6% of patients experienced Grades 3 and 4 treatment-emergent adverse events (TEAEs). The improvement of clinical efficacy also increased the number of toxic and side effects in this study.

Several other clinical trials that have been completed but have limited clinical benefits, such as Pembrolizumab + Poly-ICLC in MRP colon cancer (NCT02834052), GVAX (With Cyclophosphamide) and Pembrolizumab in MMR-p advanced colorectal cancer (NCT0298152), Pexa-Vec Oncolytic Virus in conjunction ICIs inhibition in refractory colorectal cancer (NCT032060734), ONCOS-102 (of an Adenovirus Vector Expressing GM-CSF) in combination with Durvalumab in participants with advanced peritoneal malignancies, and INCAGN01949 (an anti-OX40 agonist antibody) in combination with immune therapies (Nivolumab/Ipilimumab) in advanced or metastatic malignancies (NCT03241173). The results of targeting of TLR9 (NCT03507699), Microbiome (NCT05350501), CD40 (NCT03329950), iPSC-derived NK cell product (NCT03841110), poliovirus receptor-related immunoglobulin domain containing (PVRIG) (NCT03667716), A2aR/A2bR (NCT03629756), CD73 (NCT03454451), EP-4 (NCT03658772) and CSF-1R (NCT02777710) have not yet been disclosed.

In summary, the clinical activity of the combinations of innate immune activators with PD-1/PD-L1 inhibitors was limited with prolonged disease stabilizations. The mechanistic exploration on the identification of dominant tumor types, molecular markers indicative of sensitive populations, and the interplay between the cancer-immunity cycle and TME will facilitate the development of optimized clinical treatment strategies and inform additional studies in the additional cohort.

Future perspectives and conclusion

Clinical therapy for CRC has already entered a new era of personalized cancer medicine, and therapeutic response assessment attracted considerable attention. Identification of immune profiles for CRC patients will help choosing reasonable regime in the clinic. Nevertheless, the crucial function of a specific immune cell subset throughout the initiation and progression of CRC has yet to be elucidated. The assessment to tumor environments will be facilitated by using single-cell sequencing on cells components in TME, molecular subtype classification, or mass spectrometry-based flow cytometric analysis.

As we have summarized, PD-1/PD-L1 blockade therapy combined with innate immune activation significantly enhances the efficacy of PD-1/PD-L1 blockade treatment for CRC. The combination with other therapies, such as chemotherapy, radiotherapy, microwave ablation, and cryotherapy, not only markedly decreases the tumor load but also releases a substantial amount of tumor antigens. Dendritic cells and other antigen-presenting phagocytes engulf, process, and present these antigens, thereby inducing an antitumor immune response.

Manipulation of the gut microbiota is a promising therapy to enhance the efficacy of PD-1/PD-L1 blockade treatment in CRC patients. In contrast to conventional chemotherapy or targeted treatments that may inflict greater harm on the body, the gut microbiota strategy is marked by its gentle impact, causing minimal

Table 2 Clinical trials targeting innate immunity and blockade of PD-1/PD-L1 in colorectal cancer

Combinational regime	Title	Phase	Clinical Treatments	Identifier	Status
TLR3/PD-1	Rintatolimod and Pembrolizumab for the Treatment of Refractory Metastatic or Unresectable Colorectal Cancer	Phase 2	Rintatolimod Pembrolizumab	NCT04119830	Withdrawn
	Pembrolizumab + Poly-ICLC in MRP Colon Cancer	Phase 1 Phase 2	Pembrolizumab Poly-ICLC	NCT02834052	Completed
TLR4/PD-1	Study of Intratumoral Ipilimumab and TLR4 Agonist GLA-SE in Combination With Systemic Nivolumab and Chemotherapy (ISILI)	Phase 1	FOLFOX regimen Nivolumab Ipilimumab GLA-SE	NCT03982121	Withdrawn
TLR7/8/PD-1	A First-in-human Study Using BDC-1001 as a Single Agent and in Combination With Nivolumab in Advanced HER2-Expressing Solid Tumors	Phase 1 Phase 2	BDC-1001 Nivolumab	NCT04278144	Active, not recruiting
	REVEAL Study of NKTR-262 in Combination With NKTR-214 and Nivolumab in Patients With Locally Advanced / Metastatic Solid Tumor Malignancies (REVEAL)	Phase 1 Phase 2	NKTR-262 Bempegaldesleukin Nivolumab	NCT03435640	Terminated
TLR8/PD-1	A Study of SBT6050 Alone and in Combination With PD-1 Inhibitors in Subjects With Advanced HER2 Expressing Solid Tumors	Phase 1	SBT6050 Pembrolizumab Cemiplimab	NCT04460456	Unknown status
TLR9/PD-1	Combined Immunotherapy and Radiosurgery for Metastatic Colorectal Cancer	Phase 1	Liver radiation therapy Nivolumab Ipilimumab CMP-001	NCT03507699	Completed
Microbiome/PD-1	EO2040 in Combination With Nivolumab, for Treatment of Patients With Minimal Residual Disease of Colorectal Cancer (CLAUDE)	Phase 2	EO2040	NCT05350501	Completed
	FMT Combined With Immune Checkpoint Inhibitor and TKI in the Treatment of CRC Patients With Advanced Stage	Phase 2	Fecal microbiota transplantation Sintilimab Fruquintinib	NCT05279677	Unknown status
GM-CSF/PD-1	Study of GVAX (With CY) and Pembrolizumab in MMR-p Advanced Colorectal Cancer	Phase 2	CY GVAX Pembrolizumab	NCT02981524	Completed
	Exploratory Study of TG02-treatment as Monotherapy or in Combination With Pembrolizumab to Assess Safety and Immune Activation in Patients With Locally Advanced Primary and Recurrent Oncogenic RAS Exon 2 Mutant Colorectal Cancer	Phase 1	TG02-treatment Pembrolizumab	NCT02933944	Terminated
	Galinpepimut-S in Combination With Pembrolizumab in Patients With Selected Advanced Cancers	Phase 1 Phase 2	Galinpepimut-S Pembrolizumab	NCT03761914	Active, not recruiting
	Radiotherapy in Combination With Sintilimab, GM-CSF and Fruquintinib in Patients With MSS mCRC	Phase 2	Hypofractionation Radiotherapy Sintilimab GM-CSF Fruquintinib	NCT05292417	Unknown status

Table 2 (continued)

Combinational regime	Title	Phase	Clinical Treatments	Identifier	Status
GM-CSF/PD-L1	A Phase I/II Study of Pexa-Vec Oncolytic Virus in Combination With Immune Checkpoint Inhibition in Refractory Colorectal Cancer	Phase 1 Phase 2	Durvalumab Tremelimumab Pexa-Vec	NCT03206073	Completed
	A Study to Investigate ONCOS-102 in Combination With Durvalumab in Subjects With Advanced Peritoneal Malignancies	Phase 1 Phase 2	ONCOS-102 Durvalumab	NCT02963831	Completed
	Surufatinib Combine With Immunotherapy and Chemotherapy for Second-line Treatment in Advanced Colorectal Cancer	Phase 1 Phase 2	Surufatinib Camrelizumab Irinotecan GM-CSF	NCT04929652	Unknown status
CD40/PD-1	A Study of CDX-1140 (CD40) as Monotherapy or in Combination in Patients With Advanced Malignancies	Phase 1	CDX-1140 CDX-301 Pembrolizumab Chemotherapy	NCT03329950	Completed
OX40/PD-1	A Study Exploring the Safety and Efficacy of INCAGN01949 in Combination With Immune Therapies in Advanced or Metastatic Malignancies	Phase 1 Phase 2	INCAGN01949 / Nivolumab Ipilimumab	NCT03241173	Completed
IL-2/PD-1	A Study of Gene Edited Autologous Neoantigen Targeted TCR T Cells With or Without Anti-PD-1 in Patients With Solid Tumors	Phase 1	NeoTCR-P1 adoptive cell therapy Nivolumab IL-2	NCT03970382	Suspended
	CD8 + T Cell Therapy and Pembrolizumab in Treating Patients With Metastatic Gastrointestinal Tumors	Phase 1	Adoptive Immunotherapy Aldesleukin Cyclophosphamide / Pembrolizumab	NCT02757391	Terminated
	A Study of SAR444245 Combined With Other Anticancer Therapies for the Treatment of Participants With Gastrointestinal Cancer (Master Protocol) (Pegathor Gastrointestinal 203)	Phase 2	THOR-707 Pembrolizumab Cetuximab	NCT05104567	Terminated
IL-2/PD-1/PD-L1	FT500 as Monotherapy and in Combination With Immune Checkpoint Inhibitors in Subjects With Advanced Solid Tumors	Phase 1	FT500 Nivolumab / Pembrolizumab Atezolizumab / Cyclophosphamide Fludarabine IL-2	NCT03841110	Completed
	FT536 Monotherapy and in Combination With Monoclonal Antibodies in Advanced Solid Tumors	Phase 1	FT536 Cyclophosphamide Fludarabine IL-2 Avelumab Pembrolizumab Nivolumab Atezolizumab	NCT05395052	Terminated

Table 2 (continued)

Combinational regime	Title	Phase	Clinical Treatments	Identifier	Status
IL-15/PD-L1	QUILT-3.050: NANT Colorectal Cancer (CRC) Vaccine: Combination Immunotherapy in Subjects With Recurrent or Metastatic CRC	Phase1 Phase 2	Avelumab, bevacizumab, capecitabine, cetuximab, cyclophosphamide, 5-fluorouracil, fulvestrant, leucovorin, nab paclitaxel, nivolumab, lovaza, oxaliplatin, stereotactic body radiation therapy, ALT-803, ETBX-011, ETBX-021, ETBX-051, ETBX-061, GI-4000, GI-6207, GI-6301, and haNK.	NCT03169777	Withdrawn
IL-15/PD-1/PD-L1	QUILT-3.055: A Study of Combination Immunotherapies in Patients Who Have Previously Received Treatment With Immune Checkpoint Inhibitors	Phase 2	N-803 + Pembrolizumab N-803 + Nivolumab N-803 + Atezolizumab N-803 + Avelumab N-803 + Durvalumab N-803 + Pembrolizumab + PD-L1 t-haNK N-803 + Nivolumab + PD-L1 t-haNK N-803 + Atezolizumab + PD-L1 t-haNK N-803 + Avelumab + PD-L1 t-haNK N-803 + Durvalumab + PD-L1 t-haNK	NCT03228667	Active, not recruiting
TIM-3/PD-1	A Study of TSR-022 in Participants With Advanced Solid Tumors (AMBER)	Phase 1	TSR-022 Nivolumab TSR-042 TSR-033 Docetaxel Pemetrexed / Cisplatin Carboplatin	NCT02817633	Recruiting
PVRIG/PD-1	COM701 (an Inhibitor of PVRIG) in Subjects With Advanced Solid Tumors.	Phase 1	COM701 Nivolumab	NCT03667716	Completed
LAG-3/PD-1	Study of Nivolumab and Relatlimab in Patients With Microsatellite Stable (MSS) Advanced Colorectal Cancer	Phase 2	Nivolumab Relatlimab	NCT03642067	Active, not recruiting
	A Study of XmAb®22841 Monotherapy & in Combination w/ Pembrolizumab in Subjects w/ Selected Advanced Solid Tumors (DUET-4)	Phase 1	XmAb®22841 Pembrolizumab	NCT03849469	Completed
	A Study of Nivolumab Alone or Nivolumab Combination Therapy in Colon Cancer That Has Come Back or Has Spread (CheckMate142)	Phase 2	Ipilimumab Nivolumab Cobimetinib Daratumumab BMS-986016	NCT02060188	Active, not recruiting
CD47/PD-1	A Study of ALX148 With Cetuximab and Pembrolizumab for Refractory Microsatellite Stable Metastatic Colorectal Cancer	Phase 2	ALX148 Cetuximab Pembrolizumab	NCT05167409	Active, not recruiting

Table 2 (continued)

Combinational regime	Title	Phase	Clinical Treatments	Identifier	Status
A2aR/A2bR/PD-1	An Open Label Study Evaluating the Efficacy and Safety of Etrumadenant (AB928) Based Treatment Combinations in Participants With Metastatic Colorectal Cancer. (ARC-9)	Phase1 Phase 2	Etrumadenant Zimberelimab mFOLFOX-6 regimen Bevacizumab Regorafenib AB680	NCT04660812	Active, not recruiting
	A Study to Evaluate the Safety and Tolerability of Immunotherapy Combinations in Participants With Advanced Malignancies	Phase 1	Etrumadenant Zimberelimab	NCT03629756	Completed
CD73/PD-1	CPI-006 Alone and in Combination With Ciforadenant and With Pembrolizumab for Patients With Advanced Cancers	Phase 1	CPI-006 / Pembrolizumab	NCT03454451	Completed
IDO/PD-1	Azacitidine Combined With Pembrolizumab and Epacadostat in Subjects With Advanced Solid Tumors (ECHO-206)	Phase1 Phase 2	Azacitidine Pembrolizumab / Epacadostat / INCB057643 INCB059872	NCT02959437	Terminated
	A Study of Epacadostat in Combination With Pembrolizumab and Chemotherapy in Participants With Advanced or Metastatic Solid Tumors (ECHO-207/KEYNOTE-723)	Phase1 Phase 2	Epacadostat Pembrolizumab Oxaliplatin Leucovorin 5-Fluorouracil Gemcitabine nab-Paclitaxel Carboplatin Paclitaxel Pemetrexed Cyclophosphamide Cisplatin	NCT03085914	Completed
TGF-β/PD-1	Study of Safety and Tolerability of BCA101 Monotherapy and in Combination Therapy in Patients With EGFR-driven Advanced Solid Tumors	Phase 1	BCA101 Pembrolizumab	NCT04429542	Recruiting
	Study of NIS793 and Other Novel Investigational Combinations With SOC Anti-cancer Therapy for the 2 L Treatment of mCRC (daNIS-3)	Phase 2	NIS793 Bevacizumab Modified FOLFOX6 FOLFIRI Tislelizumab	NCT04952753	Active, not recruiting
TGF-β/PD-L1	M7824 in Patients With Metastatic Colorectal Cancer or With Advanced Solid Tumors With Microsatellite Instability	Phase 1 Phase 2	Anti-PD-L1/TGFbetaRII Fusion Protein M7824	NCT03436563	Active, not recruiting

Table 2 (continued)

Combinational regime	Title	Phase	Clinical Treatments	Identifier	Status
EP-4/PD-1	Grapiprant and Pembrolizumab in Patients With Advanced or Progressive MSS Colorectal Cancer	Phase 1	Grapiprant / Pembrolizumab	NCT03658772	Completed
	Phase 1a/1b Study of TPST-1495 as a Single Agent and in Combination With Pembrolizumab in Subjects With Solid Tumors	Phase 1	TPST-1495 Pembrolizumab	NCT04344795	Active, not recruiting
Arg-2/PD-1	Arginase Inhibitor INCB001158 as a Single Agent and in Combination With Immune Checkpoint Therapy in Patients With Advanced/Metastatic Solid Tumors	Phase1	INCB001158 Pembrolizumab	NCT02903914	Completed
	A Study to Evaluate the Safety, Tolerability, and Anti-tumor Activity of INCB001158 Plus Epacadostat, With or Without Pembrolizumab, in Advanced Solid Tumors	Phase1 Phase 2	INCB001158 / Epacadostat / Pembrolizumab	NCT03361228	Terminated
Epigenetic modulators/ PD-1/PD-L1	A Study of Enhancing Response to MK-3475 in Advanced Colorectal Cancer	Phase 1	Oral CC-486 Romidepsin MK-3475	NCT02512172	Completed
	Combining Epigenetic And Immune Therapy to Beat Cancer. (CAIRE)	Phase 2	Durvalumab / Tazemetostat	NCT04705818	Recruiting
CSF-1R/PD-L1	Evaluation of Safety and Activity of an Anti-PDL1 Antibody (DURVALUMAB) Combined With CSF-1R TKI (PEXIDARTINIB) in Patients With Metastatic/Advanced Pancreatic or Colorectal Cancers (MEDIPLEX)	Phase 1	Pexidartinib Durvalumab	NCT02777710	Completed

damage. This mode of intervention is also distinguished by its pinpoint precision, targeting the intestinal tumor microenvironment directly. Additionally, through judicious screening of the gut microbiota in CRC patients who show responsiveness to PD-1/PD-L1 immunotherapy, it is feasible to deliver personalized supplements of metabolic substances or microbial assemblages to non-responders, thereby calibrating the therapeutic benefits of PD-1/PD-L1 blockade treatment.

Based on numerous preclinical data, the combination of other treatments with PD-1/PD-L1 blockade therapy indeed improves the efficacy of αPD-1/αPD-L1 in the treatment of CRC. The function of drugs used in combinational therapy vary in their target cells. Taking ROS as an example, studies have shown that ROS can induce apoptosis and immunogenic death of colon cancer cells [129], and then the released tumor antigens are phagocytosed and presented by antigen-presenting cells, activating anti-tumor immunity and inducing immune memory. However, literature also indicates that MDSCs suppress T cell function and promote tumor progression by producing ROS and NO [147]. Nevertheless, studies have found that ROS can enhance the therapeutic efficacy of αPD-L1 in CRC treatment [148]. Therefore, ROS acts like a double-edged sword, with their specific effects mediated by the type of cell involved. Thus, it is necessary to observe the role of supplemented drug/therapy in combinational therapy from a more macroscopic perspective.

In preclinical research and clinical trials, the combination of innate immune activation has demonstrated enhanced efficacy when paired with PD-1/PD-L1 blockade for the treatment of CRC. However, significant limitations and challenges are inherent in this combinational approach. Firstly, not all strategies that couple activated innate immune stimulation with αPD-1/αPD-L1 therapy achieve success, due to the complex cellular and molecular mechanisms that have yet to be fully understood. Secondly, αPD-1/αPD-L1 therapy is known to elicit a range of immune-related adverse effects, and when it is combined with innate immune activation, there is a heightened risk of exacerbating these side effects. In dealing with these complications, clinicians often turn to steroids treatments or cytokine-antibody blockade strategies, which, unfortunately, may undermine the efficacy of αPD-1/αPD-L1 therapy. In contrast, microbiota-based immunotherapies are emerging as a potentially more tolerable and effective alternative. Lastly, although the synergistic effects of innate immune activation with PD-1/PD-L1 blockade have been remarkable in preclinical studies, the transition from bench to bedside requires considerable additional time and effort. A primary challenge lies in the lack of target specificity in combined drug therapies, which can lead to systemic toxicity. To circumvent this issue, the advancement of nanodelivery systems that can be precisely activated by photothermal therapy at the tumor site to release therapeutic agents is heralding an exciting new frontier in the field of cancer treatment.

In addition to innate immunity, adaptive immune responses, such as tumor vaccine, CAR-T therapy have shown preventive and curative effect, and clinical treatments for CRC in future cannot ignore the role of adaptive immunity in addition to innate immunity.

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Authors' contributions
Q.X.: Writing - Original Draft, Investigation, Visualization; X.L.L.: Writing - Review & Editing; R.Y.L.: Writing - Review & Editing; J.X.P.: Supervision; J.L.: Supervision. All authors reviewed the manuscript.

Data availability
No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate
Not applicable.

Competing interests
The authors declare no competing interests.

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