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Recent Advances in the Clinical Development of Immune Checkpoint Blockade Therapy for Mismatch Repair Proficient (pMMR)/non-MSI-H Metastatic Colorectal Cancer

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Abstract

Metastatic colorectal cancer (mCRC) continues to be associated with a poor prognosis, and there remains a significant unmet need for novel agents and treatment regimens. Major breakthroughs have been made with immune checkpoint blockade therapy in several disease types, including DNA mismatch repair deficient (dMMR)/microsatellite instability-high (MSI-H) tumors. To date, however, immune checkpoint monotherapy has not shown significant clinical activity in the treatment of patients with mismatch repair proficient (pMMR)/non-MSI-H mCRC. The immune resistance mechanisms in pMMR/non-MSI-H mCRC have not yet been clearly elucidated. Significant efforts are currently focused on identifying effective combination immunotherapy regimens for the treatment of patients with pMMR/non-MSI-H mCRC. The combination of atezolizumab with cobimetinib had shown promising clinical activity in an early-phase clinical trial. Unfortunately, the IMblaze 370 (COTEZO) phase III trial of atezolizumab/cobimetinib combination in patients with mCRC failed to show significant improvement in overall survival in patients treated with the atezolizumab/combimetinib combination in comparison to regorafenib alone. This review summarizes the recent major advances in the clinical development of immunotherapy regimens for patients with pMMR/non-MSI-H mCRC.

Keywords

Colorectal cancer; immune checkpoint; mismatch repair; microsatellite instability; PD-1; pMMR; MSI-H; non-MSI-H; immunotherapy

Introduction

Colorectal cancer (CRC) is a major public health problem in the U.S. and globally. In the U.S., 140,000 new cases of CRC will be diagnosed in 2018, and nearly 50,000 deaths will be attributed to this disease.[1] Metastatic CRC (mCRC) is usually associated with poor prognosis, with 5-year survival rates in the 5%-8% range. However, over the past 10 years, marked improvements have been made as the median overall survival (OS) is now in the

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range of more than 30-32 months. Historically, chemotherapy has been the mainstay approach for patients with mCRC. Significant advances have been made in the number of chemotherapy regimens that are now offered to patients with mCRC, but the results continue to fall far short of durable curative treatment of patients with mCRC. There is clearly an unmet need for new agents and/or treatment regimens. During the last decade, tremendous breakthroughs have been made in the clinical development of immune checkpoint blockade therapy with the recent approval of six immune checkpoint inhibitors by the U.S. Food and Drug Administration (FDA) for multiple tumor types (Table 1), including microsatellite instability-high (MSI-H) mCRC. With respect to mCRC, much focus has been recently placed on developing effective combination immunotherapy regimens for the treatment of patients with both MSI-H and microsatellite-stable (MSS) mCRC.

Effector immune function is under tight regulation by immune-inhibitor pathways, termed immune checkpoints, to maintain self-tolerance and minimize collateral tissue damage upon immune reaction in the peripheral tissues.[2-4] Multiple immune checkpoints have been identified, and intense scientific research has focused on targeting these various checkpoint pathways for clinical application. The two immune checkpoint pathways that have received the most attention include programmed cell death 1 (PD-1) and cytotoxic T-lymphocyte associated antigen 4 (CTLA-4). Several immune checkpoint receptors and/or ligands are upregulated in tumor tissues, and they play a major role in immune evasion of tumor cells by suppressing tumor antigen-specific $CD8^+$ effector T cell function. Blockade of immune checkpoint signaling is an effective therapeutic strategy associated with highly durable tumor response and minimal toxicity for multiple tumor types, including DNA mismatch repair deficient (dMMR) and/or MSI-H mCRC.[5-8]

In this review, we summarize the recent advances in the clinical development of immune checkpoint blockade therapy in patients with DNA mismatch repair proficient (pMMR) and/or non-MSI-H mCRC.

Immune Checkpoint Blockade Therapy for dMMR/MSI-H mCRC

The DNA mismatch repair (MMR) system is one of the key DNA repair mechanisms whose primary function is to preserve the fidelity of DNA replication as it recognizes and repairs erroneous bases or insertion-deletion loops of newly replicated DNA strands.[9] Approximately 15% of all CRCs are dMMR/MSI-H and 75%-80% of these patients have acquired methylation of MLH1 promoter region that leads to silencing of MLH1 protein expression.[10] Only 2%-3% of all CRCs have germline mutations in one of the MMR genes, and defects in mismatch repair genes are associated with the familial CRC syndrome known as hereditary non-polyposis colorectal cancer (HNPCC) or Lynch syndrome.[11] There are several diagnostic methods to determine the status of mismatch repair defect in CRC tumor tissues. Immunohistochemistry (IHC) analysis with antibodies targeting MLH1, MSH2, PMS2, and MSH6 proteins can diagnose dMMR status in tumor tissues. MSI-H status can also be detected by polymerase chain reaction (PCR) with either the well-established NCI-panel, known as Bethesda (or NCI-panel) markers, or a pentaplex panel of mononucleotide repeat markers.[12-17] IHC with a panel of four IHC markers including MLH1, MSH2, PMS2, and MSH6 has a predictive value for the diagnosis of dMMR/MSI-H status that is virtually

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equivalent to that of MSI testing.[18, 19] Next generation sequencing (NGS) is another molecular approach that can be used to diagnose dMMR/MSI-H status.[20-23] Concordance between IHC and PCR methods has been confirmed across several platforms with CLIA-approved commercial assays entering the clinical environment.[20, 21, 24]

Length variations in microsatellites of coding sequence lead to frameshift mutations, which then result in the production of completely different C-terminal peptide sequences in dMMR cancer cells. These dMMR CRC cells carry a high level of somatic mutations and are, therefore, considered to be highly immunogenic.[25] dMMR CRC tissues are characterized by heavy infiltration of $CD8^+$ T cells and high expression of immune checkpoint signaling pathways, including PD-L1.[26] The use of anti-PD-1/PD-L1 antibodies to block PD-1 signaling has been associated with significant antitumor activity in the setting of MSI-H or dMMR mCRC.[5-8] Pembrolizumab and nivolumab are the two anti-PD-1 antibodies currently approved by the U.S. FDA for the treatment of patients with MSI-H or dMMR mCRC who have progressed on treatment with a fluoropyrimidine, oxaliplatin, and irinotecan (Table 1). However, as noted above, the subset of patients with dMMR/MSI-H mCRC accounts for only a small fraction of mCRC (< 5%).[12] The large majority of mCRC patients are pMMR/non-MSI-H, and in all of the major studies conducted to date, these patients have been non-responsive to immune checkpoint blockade monotherapy (Table 2). As a result, it is critical to determine the status of MSI-H or dMMR in tumor tissues so as to provide guidance for an appropriate treatment decision for patients with mCRC.

Mechanisms of Primary Resistance to PD-1 Inhibitor Monotherapy in pMMR/non-MSI-H CRC

Immune checkpoint blockade enhances the antitumor activity of tumor antigen-specific $CD8^+$ T cells and is associated with highly durable tumor response and manageable safety profile in the treatment of patients with dMMR/MSI-H mCRC.[5-8] However, immune checkpoint inhibitor monotherapy, including PD-1 blockade or anti-CTLA-4 therapy, is associated with virtually no activity in patients with pMMR/non-MSI-H mCRC (Table 2).[5, 27-29]

There are several potential underlying mechanism(s) that mediate primary immune resistance of pMMR/non-MSI-H mCRC to immune checkpoint inhibitor therapy (Table 3).[30-32] In general, these pMMR/non-MSI-H mCRC tumor cells have relatively low immunogenicity for $CD8^+$ T cell recognition due to low expression of tumor-specific antigen secondary to low mutation burden; have defects in antigen presentation machinery in CRC cells; and/or display overexpression of intrinsic immunosuppressive oncogenic pathways.[33, 34] $CD8^+$ effector T cells exist in a relatively anergic state as a result of activation of immune checkpoint pathway, defects in co-stimulatory pathway, or dysfunction of intracellular metabolism. The immunosuppressive status of the tumor microenvironment (TME) also leads to $CD8^+$ T cell anergy.

For these reasons, it may be necessary to combine PD-1 blockade with other therapeutic approaches aimed at increasing the immunogenicity of CRC tumors and/or modifying the immunosuppressive TME (Figure 1).[32] Currently, intense focus has been placed on

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identifying novel combination immunotherapy regimens for this specific patient population.[32, 35] Of note, there are several promising phase 2/3 clinical trials of PD-1 blockade in combination with other immune modulating agents in patients with pMMR/non-MSI-H mCRC (Table 4).

Reduced Expression of Tumor-specific Antigens for CD8+ T cell Immune Recognition

pMMR/non-MSI-H CRC tumor cells have a relatively low mutation load in comparison with immune-sensitive tumor types including melanoma, non-small cell lung cancer, and MSI-H mCRC.[10, 25] Given this lower mutational burden, these tumors have a much lower frequency of tumor-specific neo-antigens for $CD8^+$ T cell recognition. There are several potential strategies to enhance the presentation of tumor-specific antigens for $CD8^+$ T cell immune recognition in pMMR/non-MSI-H mCRC as highlighted in Figure 1.

Epigenetic modulation by DNA methylation and histone modifications determine the patterns of cellular gene expression, and taken together, these processes lead to gene silencing.[36-38] Direct inhibition of epigenetic modulation through the use of hypomethyating agents or HDAC inhibitors can lead to increased expression of certain tumor-specific antigens including cancer-testis (C-T) antigens (e.g., NY-ESO-1)[39] and non-synonymous somatic mutations, which can then be presented in an MHC-restricted pattern for $CD8^+$ T cell recognition.

Immunogenic cell death (ICD) is typically accompanied by the release of immunostimulatory damage-associated molecular patterns (DAMPs), which enhance immune recognition of tumor antigens.[40] Another strategy to enhance immune recognition of tumor antigens is via induction of ICD of cancer cells by chemotherapy (e.g., oxaliplatin-containing chemotherapy)[41], radiation[42, 43], or oncolytic virus therapy[44, 45].

Defects in Antigen Presentation Machinery

$CD8^+$ T cells recognize tumor-specific epitopes presented in complex with MHC class I molecules on the surface of tumor cells. Human leukocyte antigens (HLA) molecule and β2-microglobulin form a MHC class I complex. Tumor-specific antigens are processed by the antigen presentation machinery, including the peptide transporters associated with antigen processing (TAP), and form complexes with MHC class I molecules of HLA/β2-microglobulin. The loss of expression or mutation in any of these antigen presentation machinery leads to the loss of immune presentation of tumor-specific antigen, evading $CD8^+$ T cell surveillance of tumor cells.[46] A small subset of pMMR/non-MSI-H CRC cells have low expression or loss of HLA class molecules or β2-miscroglobulin.[33, 47] HLA loss of heterozygosity (LOH) is a key immune escape mechanism in non-small cell lung cancer (NSCL) on PD-1 blockade therapy.[48]

Intrinsic Immnosuppressive Pathways

Activation of the MAPK signaling pathway inhibits HLA expression in tumor tissues, and is correlated with decreased intratumoral T cell infiltration.[49, 50] Inhibition of MEK (Mitogen/Extracellular signal regulated Kinase), which is a key intermediate in the mitogen activated protein kinase (MAPK) pathway, results in upregulation of MHC expression and an

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enhanced antitumor immune response by PD-1 blockade in *in vivo* murine breast cancer models.[50-52] MAPK activation in the setting of *RAS* or *BRAF* mutations increases PD-L1 mRNA stability and upregulates PD-L1 expression in CRC tumors.[53]

Loss of PTEN expression is associated with decreased intratumoral T cell infiltration and increased expression of various immunosuppressive cytokines, including CCL2 and VEGF in melanoma.[54] The loss of adenomatosis polyposis coli (*APC*) or of PTEN is associated with upregulated expression of Dickkopf-related protein 2 (*DKK2*) in tumor cells. Increased DKK2 expression leads to inhibition of STAT5 signaling, which then results in suppression of $CD8^+$ T cell function, as has been documented in the murine MC38 CRC model.[55]

Activation of WNT/β-catenin signaling is correlated with a reduction in intratumoral T cell infiltration in metastatic melanoma.[56] This signaling pathway is also dysregulated in CRC, and may play a role as an immune escape mechanism in CRC.[33, 57] On this point, Grasso et al showed that activation of WNT/β-catenin pathway closely correlated with decreased intratumoral $CD8^+$ T cell infiltration in CRC tumors.[33]

Immune Checkpoint Signaling

In addition to PD-1 and CTLA-4 pathways, there are several other immune checkpoints that, when activated, lead to induction of T cell anergy, including T cell immunoglobulin and ITIM domain (TIGIT), T-cell immunoglobulin mucin domain 3 (TIM-3), and lymphocyte-activation gene 3 (LAG-3) (Figure 1).[58, 59] The targeting of immune checkpoints has become an active area of drug development, and several molecules that target these various checkpoint pathways are in early clinical development either as monotherapy or in combination with PD-1 blockade. One potential strategy for enhancing the antitumor immune activity of tumor antigen-specific $CD8^+$ T cells is combining PD-1 blockade with another novel immune checkpoint inhibitor. However, a critical issue to consider with this approach is the possibility of synergistic immune-related toxicities as a result of excessive off-target activation of effector $CD8^+$ T cell function. In fact, such an increased toxicity has been observed in combination trials of anti-PD-1 and anti-CTLA-4 inhibitors in patients with metastatic melanoma.[58-60] Co-stimulatory agonists are immune stimulating signaling pathways that potentiate $CD8^+$ T cell response, which include CD28, CD27, CD137, GITR, OX40, and ICOS. Agonist antibodies targeting these pathways are able to induce activating signals in $CD8^+$ T cells and enhance anti-tumor effect in murine models. Several of these agents are in early clinical development either as monotherapy or in combination with PD-1 blockade.[61]

Intracellular Metabolism of $CD8^+$ T Cells

Various subsets of T cells have distinct metabolic profiles for their function and differentiation. In particular, $CD8^+$ effector T cells require robust aerobic glycolysis for the production of interferon-gamma (IFN-γ) to induce cytolysis of tumor cells.[62] Modification of intracellular metabolic pathways in $CD8^+$ T cells is another potential strategy for enhancing the antitumor immune response by $CD8^+$ T cells.[63-65] Metformin is an oral agent that is widely used to treat type 2 diabetes. It has a wide range of biologic activities, one of which is to modify mitochondrial fatty acid oxidation in $CD8^+$ T cells and increase the

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generation of memory $CD8^+$ T cells, which are critical for sustained anti-tumor immunity.[66] Scharping et al reported that metformin inhibits oxygen consumption in tumor cells *in vivo*, resulting in reduced intratumoral hypoxia, which then leads to enhanced antitumor activity of intratumoral T cells induced by PD-1 blockade in murine MC38 CRC models.[67]

Tumor-infiltrating T cells display a persistent loss of mitochondrial function and mass secondary to progressive loss of PPAR-γ coactivator 1 α (PGC1α) due to chronic AKT signaling. Scharping et al showed that increased expression of PGC1α induces reprogramming of tumor-specific T cells with enhanced effector function in murine MC38 CRC model.[68]

Immunosuppressive Cells

Treg cells play a critical role in maintaining peripheral tolerance by the production of inhibitory cytokines, induction of cytolysis, metabolic disruption, and modulation of dendritic-cell (DC) maturation or function.[69] Tregs also play an important role in mediating immune evasion of cancer cells.[70] Tregs were originally identified as $CD4^+CD25^+$ T cells, and CD25-depleting antibody (e.g., daclizumab) has been evaluated in clinical trials. However, CD25 is also expressed in multiple other immune cells, and anti-CD25 antibody therapy caused the depletion of not only $Treg_s$ but also activated effector T cells.[71] CCR4 is expressed in a subset of Tregs, and depletion of $CCR4^+$ Tregs enhances $CD8^+$ T cells function, and antibody targeting CCR4 (e.g., mogamulizumab) is a promising approach to modify the immunosuppressive function of Treg.[70]

Myeloid-derived suppressor cells (MDSCs) are a heterogeneous population of immature myeloid cells, and they play a crucial role in supporting the immunosuppressive TME. MDSCs consist of two major subpopulations of cells: monocytic MDSCs and polymorphonuclear MDSCs (PMN-MDSC or granulocytic MDSC).[72] MDSCs inhibit $CD8^+$ T cells and NK cells and are also able to recruit other immunosuppressive cells, including Tregs.[72] MDSCs express high levels of arginase I, which converts L-arginine, an essential amino acid, into L-ornithine and urea. As a result, L-arginine levels are depleted in the TME, resulting in cell cycle arrest and anergy of T cells. MDSCs express high levels of indole amine 2, 3-dioxygenase 1 (IDO1), which is critical for the catabolism of tryptophan in the TME. MDSCs produce high levels of reactive oxygen species (ROS), which induce T cell apoptosis and impair T cell receptor (TCR) signaling. MDSCs secrete immunosuppressive cytokines such as TGF-β and IL-10.

Tumor-associated macrophages (TAMs) are a key cellular component of the TME.[73] M-MDSC or blood monocytes can be recruited to the TEM and differentiated into TAMs. M1 TAMs are involved in promoting antitumor immunity while M2 TAMs promote immunosuppressive effect.[74] Colony stimulating factor 1 receptor (CSF1R) is a tyrosine kinase transmembrane receptor for the CSF1 cytokine, and this signaling pathway plays an important role in the differentiation and function of TAMs. Of note, inhibition of the CSF1R pathway modulates the immunosuppressive TME and in so doing, overcomes an important immune escape mechanism. A recent early-phase clinical trial of FPA-008, an anti-CSF1R antibody, in combination with nivolumab has shown promising clinical activity in heavily pretreated patients with metastatic pancreatic adenocarcinoma and other solid tumors.[75]

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Immune Regulatory Cytokines

The cytokine milieu in the TME is critical for the differentiation and function of $CD8^+$ T cells.[76, 77] Pro-inflammatory cytokines, including IL-1β, IL-2, IL-6, IL-12, IL-15, TNF-α, IFN-γ, type I interferons (IFN-I), upregulates inflammatory responses. There are several anti-inflammatory cytokines, including IL-10 and TGF-β, that have been shown to exert immunosuppressive effects.[77]

IL-12 is a pro-inflammatory cytokine produced by dendritic cells and macrophages in the TME. It is now well-established that IL-12 signaling is critical for the differentiation and function of $CD8^+$ T cells. Activation of this pathway induces the subsequent production of IFN-γ by $CD8^+$ cells and NK cells.[78] Ohs et al reported that IL-12 treatment in combination with PD-1 blockade has synergistic antitumor activity by restoring natural killer cell activity in murine 4T1 lung cancer model.

TGF-β is an immunosuppressive cytokine produced by both tumor cells and immune cells, and it inhibits $CD8^+$ T cell differentiation while promoting Treg generation.[79] TGF-β inhibits T-cell activation by interfering with TCR signaling and suppresses the differentiation of $CD8^+$ T cells by inhibiting the expression of lineage defining transcription factors including T-bet.[79] A subset of CRC with poor prognosis have high expression of genes related with TGF-β signaling pathway in tumor stromal cells, and the inhibition of TGF-β signaling halts tumor progression in patient-derived CRC tumor organoid and xenografts models.[80] Mariathasan et al reported that the inhibition of TGF-β signaling with anti-TGF-β antibody in combination with anti-PD-L1 antibodies induced a strong antitumor immune response in the murine MC38 CRC model.[81] Anti-TGF-β treatment in murine tumor model showed significant decrease of TGF-β signaling in stromal cells.[81] The combination of anti-TGF-β antibody and anti-PD-L1 antibody showed a significant increase in the number of tumor-infiltrating $CD8^+$ T cells in murine MC38 CRC model. RNA-seq analysis revealed a significant increase in $CD8^+$ T effector cell signature in tumor tissues with this novel combination therapy.

Immunosuppressive Metabolism

Tryptophan is an essential amino acid that is required for T cell activation and function. IDO1 catabolizes tryptophan to kynurenine in the TME. Severe depletion of tryptophan by IDO1 suppresses T cell proliferation. Tryptophan metabolites cause T cell anergy and induce apoptosis.[82] IDO1 is overexpressed in CRC tumors, and IDO1 overexpression induces immune tolerance by suppressing T cell responses.[83-85]

There is a relatively high concentration of adenosine in the TME due to tissue breakdown and the hypoxic environment. CD73 and CD39 are ectonucleotidases that catabolize extracellular ATP to adenosine in the TME.[86] Adenosine binds to its cognate A_2a receptor (A_2aR), which then initiates immunosuppressive signaling in the TME.[87] Blockade of A_2aR activation has significant anti-tumor immunity in *in vivo* mouse models, and several agents targeting A_2aR are in active clinical development, including MEDI9447, CPI-444, PBF-509 and AZD4635.[87, 88]

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Combination Immune Checkpoint Blockade Therapy for pMMR/non-MSI-H mCRC

PD-1 Blockade plus anti-CTLA-4 Therapy

PD-1 blockade in combination with anti-CTLA-4 has been under active investigation in several immune-sensitive tumor types, including metastatic melanoma.[58-60] The clinical efficacy of dual immune checkpoint inhibitor therapy is associated with increased activity when compared to anti-PD-1 or anti-CTLA-4 monotherapy. However, dual immune checkpoint blockade is associated with a significantly higher level of severe immune-related adverse events (AEs) when compared to monotherapy.

CheckMate-142 is a phase II trial of nivolumab with or without ipilimumab for MSI-H mCRC (NCT02060188)[7, 89] This study also included small cohorts of patients with pMMR/non-MSI-H mCRC.[7, 89] pMMR/non-MSI-H cohorts enrolled patients with mCRC intolerant/progression on ≥ 3 systemic chemotherapy for metastatic disease. The 10 patients enrolled in the N1I3 cohort of pMMR/non-MSI-H mCRC received nivolumab 1 mg/kg in combination with ipilimumab 3 mg/kg every 3 weeks for 4 doses, followed by nivolumab 3 mg/kg alone every 2 weeks. A total of 10 patients enrolled in the N3I1 cohort of pMMR/non-MSI-H mCRC received nivolumab 3 mg/kg in combination with ipilimumab 1 mg/kg every 3 weeks for 4 cycles, followed by nivolumab 3 mg/kg alone every 2 weeks. The primary endpoint of this study was overall response rate (ORR) by RECIST1.1. One patient in the N1I3 cohort of pMMR/non-MSI-H mCRC had a partial response (PR), while no clinical activity was observed in patients treated in the N3I1 cohort of pMMR/non-MSI-H mCRC.[7, 89] Severe AEs (SAEs) was experienced in 70% of the N1I3 cohort and in 30% of the N3I1 cohort. Taken together, the limited clinical activity along with the high rate of SAEs indicates that the nivolumab/ipilimumab combination does not merit further investigation in patients with pMMR/non-MSI-H mCRC.

CCTG CO.26 is a phase II randomized study of durvalumab in combination with tremelimumab versus best supportive care (BSC) alone in patients with mCRC who failed standard systemic chemotherapy regimens without any other therapeutic options. This study is being conducted at various study sites of the Canadian Cancer Trials Group (NCT02870920).[90] A total of 180 patients were randomized in a 2:1 ratio to either the combination immunotherapy with durvalumab 1500 mg intravenously every 28 days and tremelimumab 75 mg intravenously every 28 days for first 4 cycles or BSC alone. The primary endpoint of this study is overall survival (OS). Study enrollment has been completed, and the preliminary results are anticipated in early 2019.

PD-1 Blockade plus MEK Inhibition

MEK is an important component of the MAPK signaling pathway. Various pre-clinical *in vivo* model systems have shown that inhibition of MEK leads to induction of apoptosis, upregulates HLA expression, and downregulates certain key immunosuppressive factors, such as PD-L1 and VEGF-A.[91] *In vivo* animal studies have also shown that PD-1 blockade in combination with MEK inhibition leads to significant synergistic antitumor activity in the murine syngeneic CRC model.[51, 91] MEK inhibition increases the number of tumor-

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infiltrating $CD8^+$ T cells and upregulates the expression of MHC class I molecules in tumor tissues, which subsequently enhances the immune recognition of tumor cells by $CD8^+$ effector T cells. One critical feature of MEK inhibition in combination with immune checkpoint inhibitor therapy is that T cell responses, upon relief from immune checkpoint inhibition, are also dependent on MAPK pathway activation, including MEK activity. To highlight the potential complexities involved, MEK inhibition can also be associated with a profound block in naive $CD8^+$ T cell priming, which raises added caution with respect to the appropriate dose and schedule of MEK inhibition to minimize the potential negative impact on T cell priming.

Based on compelling preclinical data, the combination of atezolizumab, an anti-PD-L1 antibody, plus cobimetinib, a MEK inhibitor, was evaluated in a phase I study. Bendell et al reported on the initial results of this trial in patients with chemo-refractory metastatic CRC.[92] A total of 84 patients received atezolizumab 800 mg every 2 weeks intravenously (IV) and cobimetinib 60 mg orally once a day. Fifty-nine of the 84 patients received cobimetinib 60 mg daily on a schedule of 21 days on/7 day every 28 days, and the other 21 patients received cobimetinib 60 mg daily on a schedule of 14 days on/14 days off every 28 days. The primary endpoints of the study were safety and tolerability, and secondary endpoints included ORR and PFS by RECIST1.1, and OS. In general, the combination of atezolizumab and cobimetinib was safe and relatively well-tolerated. Treatment-related grade ≥ 3 AEs occurred in nearly 40% of the enrolled patients. The most common treatment-related grade ≥ 3 AEs were increased blood CPK levels (5%), skin rash (5%), diarrhea (5%), and fatigue (5%). Seven patients were found to have a PR and the ORR was 8% by RECIST1.1. Of the 7 patients with a PR, 4 had MSS and 1 had MSI-low mCRC, while the MSI status of the remaining 2 patients was unknown. The median PFS was 1.9 months (95% confidence interval [CI], 1.8-2.3), and median OS was 9.8 months (95% CI, 6.2-14.1). Of note, the subset of patients with confirmed MSS mCRC (N = 42) had a 6-month PFS of 27% (mPFS, 2.5 months) and 12-month OS of 51% (mOS, 13.0 months), which is higher than what has been reported for regorafenib or TAS-102, which are the two agents currently approved in the U.S. for the treatment of chemorefractory mCRC.[93, 94] Increased activity of MAPK pathway appears to be associated with prolonged PFS and OS in patients treated with the atezolizumab/cobimetinib combination as 22 patients with high MAPK gene expression (> 50%) had an improved mPFS (7.3 months vs. 1.8 months) and mOS (18 months vs. 6.5 months) when compared to the 20 patients with low MAPK gene expression (≤ 50%). For this study, the activity of MAPK pathway was determined using the average mRNA expression of *CCND1, DUSP4, DUSP6, ETV4, ETV5, NT5E, SPRY2* and *SPRY4* genes in tumor tissues using mRNA sequencing (mRNA-Seq).[92]

COTEZO IMblaze370 is a Phase III, multicenter, open-label, three-arm, randomized study of atezolizumab in combination with cobimetinib in patients with mCRC who have received ≥ 2 prior lines of chemotherapy for metastatic disease (NCT02788279). This is a confirmatory study based on the promising results of the phase I trial described above, and the primary endpoint of this study is OS. The control arm of this study is regorafenib, a standard of care therapy in the chemorefractory disease setting, and the experimental arms are the atezolizumab/cobimetinib combination and atezolizumab monotherapy. Target enrollment for this study is 360 patients, and enrolled patients were randomized equally to

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one of three study cohorts (1:1:1): atezolizumab monotherapy vs. atezolizumab plus cobimetinib vs. regorafenib. Patients in the atezolizumab monotherapy arm received atezolizumab 1200 mg IV on day 1 of a 21-day cycle. For the combination arm, patients were administered cobimetinib 60 mg orally on a schedule of 21 days on/7 days off in a 28-day cycle and atezolizumab 840 mg IV on days 1 and 15 of each cycle on a 28-day cycle. Patients randomized to the control arm received regorafenib 160 mg once a day orally on a schedule of 21 days on/7 days off in a 28-day cycle. Study enrollment was completed by the end of 2016. The preliminary results of this study have been recently released, and they showed that the atezolizumab/cobimetinib combination failed to improve OS in comparison to regorafenib monotherapy. However, the specific details are not yet available for review and will be presented at upcoming meetings.

SELECT-4 is a phase I dose escalation study of selumetinib (AZD6244, ARRY-142886), a MEK inhibitor, in combination with durvalumab (MEDI4736), an anti-PD-L1 antibody, in patients with advanced solid tumors refractory to standard therapy or without any further standard therapy (NCT02586987).[95] This study evaluates an intermittent dosing schedule of selumetinib to allow maximal relief of T-cell checkpoint blockade by durvalumab, and the primary objective of the study is to investigate the safety and tolerability of intermittent dosing of selumetinib in combination with durvalumab. Selumetinib is administered as a 7-day monotherapy run-in, starting at a dose of 50 mg twice a day orally, with increasing doses until the maximum tolerated dose is reached, and then on a schedule of 1-week on/1-week off, every 4 weeks. Durvalumab is administered at a flat dose of 1500 mg IV once every 4 weeks. With the establishment of the recommended phase 2 dose (RP2D) of selumetinib/durvalumab combination, an expansion cohort of 30 patients with chemo-refractory pMMR/non-MSI-H mCRC has been enrolled to further evaluate safety and tolerability, and to provide a preliminary evaluation of the mechanism of action and clinical activity of selumetinib/durvalumab combination. Patient enrollment for this cohort has been completed.

It should be noted that SELECT-4 and COTEZO IMblaze370 incorporated different schedules of MEK inhibition given the different pharmacokinetic properties and safety profiles of the respective MEK inhibitor used in each study. It remains to be seen whether the promising clinical activity observed with the atezolizumab/cobimetinib regimen will be reproducible with different MEK small molecule inhibitors when combined with other PD-1 immune checkpoint inhibitors for the treatment of pMMR/non-MSI-H mCRC. The increased MAPK gene expression (>50%) in responding patients appears to be a promising biomarker, and further research is warranted to confirm its use as a potential predictive biomarker of response to PD-1 blockade in combination with MEK inhibition. As presented in Table 3, several clinical trials of PD-1 blockade in combination with MEK inhibition are currently on-going, and some of these combination studies are also investigating the potential role of cytotoxic chemotherapy including oxaliplatin- or irinotecan-containing regimens.

Combination of PD-1 Blockade and Chemotherapy

It has now been well-established that cytotoxic chemotherapy is able to induce immunogenic cell death (ICD) of cancer cells, which can then lead to enhanced antitumor immunity.[96, 97]

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Oxaliplatin induces immunogenic cell death of tumor cells by stimulating pre-apoptotic calreticulin exposure, which results in post-apoptotic release of high-mobility group box 1 (HMGB1) protein.[98] *In vivo* animal studies using the murine syngeneic CT26 CRC model system have shown that oxaliplatin treatment leads to enhanced synergistic antitumor activity of PD-1 blockade with MEDI4736, an anti-PD-L1 antibody.[99] The fluoropyrimidine 5-fluorouracil (5-FU) selectively eliminates tumor-associated myeloid-derived suppressor cells (MDSCs), increases IFN-γ production by tumor-specific $CD8^+$ T cells infiltrating the tumor, and promotes T cell-dependent antitumor responses *in vivo*.[100] Vascular endothelial growth factor (VEGF)-A in the TME induces expression of PD-L1. Of note, the combination of PD-1 blockade with the inhibition of VEGF pathway induces a strong synergistic antitumor effect in the murine CT26 CRC model.[101]

GP28328 study is a phase IB trial of atezolizumab in combination with bevacizumab with or without chemotherapy in patients with advanced solid tumors (NCT01633970).[102, 103] Arm B of this study evaluated the safety and efficacy of atezolizumab in combination with modified FOLFOX6/bevacizumab as the first-line treatment of patients with newly diagnosed mCRC. Atezolizumab was administered at a dose of 800 mg IV every 2 weeks, and bevacizumab was given at 10 mg/kg IV every 2 weeks. The primary objectives of this study were safety, tolerability, DLT, and maximum tolerated dose (MTD) of the combination. A total of 30 patients with mCRC were enrolled and evaluable for safety. Grade ≥ 3 AEs were observed in 67% of evaluable patients, including neutropenia (40%), diarrhea (13%), increased ALT (10%) and increased AST (10%), but only 17% were attributed to atezolizumab. The ORR by RECIST1.1 was 52% (95% CI, 30.6-73.2) among the 23 evaluable patients, and the mPFS was 14.1 months (95% CI, 8.7-17.1), which is better than the mPFS of 9.4 months that is usually associated with the FOLFOX/bevacizumab regimen.[104] These preliminary findings are promising as they suggest that cytotoxic chemotherapy can be effectively and safely combined with PD-1 blockade therapy in patients with mCRC. The increased response rates and PFS observed with this triple combination of chemotherapy, bevacizumab, and atezolizumab also refutes a widely held concern that cytotoxic chemotherapy with steroid premedication may impair the antitumor immune reaction induced by PD-1 blockade. It should be emphasized, however, that this data is preliminary and based on only a relatively small number of patients. This promising clinical activity requires further validation of the true synergistic clinical activity of atezolizumab in combination with FOLFOX/bevacizumab chemotherapy in a randomized phase 3 trial.

An analysis of tumor biopsy samples from the GP28328 trial showed that intratumoral infiltration of $CD8^+$ T cells and PD-L1 expression in tumors were both enhanced following administration of FOLFOX alone or the combination of FOLFOX/bevacizumab and atezolizumab.[103] These findings are consistent with earlier preclinical observations showing that oxaliplatin-containing chemotherapy induces immunogenic cell death with enhancement of $CD8^+$ T cell infiltration and is able to transform an immunologically cold tumor to an inflamed hot tumor.[98, 99, 103] Furthermore, increased expression of cytotoxic T-cell signature genes (e.g. *CD8A, IFNG, GZMB, EOMES*), which reflect the activity of intratumoral cytotoxic $CD8^+$ T cells, was observed with FOLFOX/bevacizumab chemotherapy in several patient tumor specimens.[103] Of note, patients with increased

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intratumoral infiltration of $CD8^+$ T cells with the study treatment of FOLFOX/bevacizumab in combination with atezolizumab had sustained responses and/or prolonged disease control.

KEYNOTE-651 is a phase I study of pembrolizumab plus binimetinib or pembrolizumab plus chemotherapy with or without binimetinib in the first- and second-line treatment of patients with pMMR/non-MSI-H mCRC (NCT03374254). Binimetinib (MEK162, ARRY-162) is a potent oral MEK inhibitor undergoing active clinical development. The primary objectives of this study are to determine safety and tolerability and to establish the RP2D of the following combinations: pembrolizumab plus binimetinib (Cohort A); pembrolizumab plus mFOLFOX7 (oxaliplatin 85 mg/m^2; leucovorin 400 mg/m^2; 5-FU 2400 mg/m^2) (Cohort B); pembrolizumab plus mFOLFOX7 and binimetinib (Cohort C); pembrolizumab plus FOLFIRI (irinotecan 180 mg/m^2; leucovorin 400 mg/m^2; 5-FU 2400 mg/m^2) (Cohort D); and pembrolizumab plus FOLFIRI and binimetinib (Cohort E). Each cohort has two parts: Part 1 is a dose-finding phase using the modified toxicity probability interval (mTPI) design, and Part 2 is a dose confirmation phase to further examine safety and clinical efficacy. Each cohort can proceed to Part 2 independently after a preliminary RP2D for that cohort has been identified in Part 1. In Part 2, approximately 16 additional patients per cohort will be treated at the doses identified using the mTPI design in Part 1 to ensure that at least 30 patients are treated at RP2D. This study will be open to enrollment in early 2018.

The overarching goal of KEYNOTE-651 is to maximize the synergistic clinical activity of PD-1 blockade in combination with MEK inhibition by including systemic chemotherapy to induce immunogenic cell death. However, one concern for this triple combination strategy relates to safety profile as chemotherapy, MEK inhibition, and anti-PD-1 therapy have potentially overlapping side effects, especially as it relates to GI toxicities. As presented in Table 3, several other phase 2/3 clinical trials are currently investigating PD-1 blockade in combination with systemic chemotherapy, including FOLFOX with anti-VEGF or anti-EGFR, in patients with pMMR/non-MSI-H mCRC.

Combination of PD-1 Blockade and CEA CD3 TCB

Carcinoembryonic antigen (CEA) is a glycosylated cell surface protein of the immunoglobulin supergene family that is expressed at low levels in normal tissues and overexpressed in multiple human epithelial cancers including CRC. This protein is expressed at 10 to 60 times higher levels in CRC tumor tissues than normal colonic mucosa, and it is highly expressed in >90% of mCRC tumor tissues.[105,106] Given its widespread expression in mCRC, CEA has been evaluated as a potential target for cancer immunotherapy, which has included the development of a CEA-based vaccine.

CEA CD3 TCB (RG7802, RO6958688) is a novel T-cell bispecific antibody targeting CEA on tumor cells and CD3 on T cells, and this agent binds simultaneously to tumor cells and T cells.[107] CEA CD3 TCB has displayed potent antitumor activity in *in vivo* preclinical models with increased intra-tumoral T cell infiltration.[107] Tabernero et al reported on the preliminary findings of a phase I study of CEA CD3 TCB as a single agent and in combination with anti-PD-L1 antibody atezolizumab in patients with CEA-expressing solid tumors.[108] In the monotherapy cohort (N = 80; 70 patients with mCRC), this bispecific

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antibody was administered at dose levels of 0.05 mg to 600 mg as monotherapy once a week. In the combination cohort (N = 45; 35 patients with mCRC), CEA CD3 TCB was administered at dose levels of 5 mg to 160 mg weekly in combination with atezolizumab 1200 mg IV once every 3 weeks. The majority of AEs was observed in the first 2 dose levels of CEA CD3 TCB, which were mainly grade 1/2, with grade ≥ 3 observed in 7.9% of patients treated on the monotherapy cohort and in 8.1% of those treated on the combination cohort. The most common treatment-related AEs (TRAEs) among patients treated with CEA CD3 TCB at dose levels ≥ 40 mg were infusion-related reaction (64% in the monotherapy arm; 49% in the combination arm), diarrhea (46% in the monotherapy arm; 61% in the combination arm), and pyrexia (56% in the monotherapy; 70% in the combination arm). Infusion-related reactions (24% in the monotherapy arm; 12% in the combination arm) and diarrhea (57% in the monotherapy arm; 18% in the combination arm) were the most common grade ≥ 3 TRAEs among patients treated with CEA CD3 TCB at dose levels ≥ 40 mg. Diarrhea is one of the main dose-limiting toxicities of this therapy, and this is most likely due to the low-level expression of CEA in normal colonic mucosa.[105] The incidence of grade ≥ 3 diarrhea was less in the combination arm where the highest dose of CEA CD3 TCB was 160 mg in comparison with 600 mg in the monotherapy arm. Five patients in the monotherapy arm experienced dose-limiting toxicities (DLTs), which included grade 3 dyspnea, grade 3 diarrhea, grade 3 hypoxia, grade 4 colitis, and grade 5 respiratory failure. Two patients in the combination arm experienced DLTs, which were manifested as grade 3 transient increase of ALT and grade 3 skin rash. Overall, the safety profile of CEA CD3 TCB was manageable as a single agent and in combination with atezolizumab.

Two of 31 patients with MSS mCRC treated at the dose level of 60 to 600 mg of CEA CD3 TCB in the monotherapy cohort (ORR of 6%) and 2 of 23 patients with MSS mCRC treated at the dose level of 5 to 160 mg CEA CD3 TCB in the combination cohort (ORR of 9%) had a partial response by RECIST1.1. The two patients with documented PR in the combination cohort were treated at the dose level of 160 mg, which resulted in an ORR of 18% (2 of 11) at the dose level of 160 mg. CEA CD3 TCB in combination with atezolizumab showed promising preliminary clinical efficacy in MSS mCRC with a manageable toxicity profile. As such, further clinical development is warranted in patients with CEA-expressing pMMR/non-MSI-H mCRC and/or other CEA-expressing solid tumors.

Combination of PD-1 Blockade with Radiation

Radiation induces immunogenic cell death (ICD) of tumor cells, enhances antigen presentation, and alters the TME within the irradiated field.[109] The immunogenic cell death induced by radiotherapy involves the cell surface exposure of calreticulin and the release of high mobility group box 1 (HMGB1), triggering dendritic cell (DC) engulfment of dying cells, antigen presentation, and production of interleukin (IL)-1β.[42, 109-111] Low-dose radiation modifies the differentiation of $iNOS^+$ M1 macrophages in the TME, which play a critical role in the recruitment of $CD8^+$ T cells into tumor tissues.[112] The abscopal effect refers to the ability of localized radiation therapy to trigger systemic immune effects at distant non-irradiated metastatic lesions, resulting in systemic antitumor effects.[113] Several preclinical studies have demonstrated enhancement of the abscopal effect when radiation therapy is combined with PD-1 blockade in various preclinical models, which is most likely

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due to release of tumor-specific antigens and damage-associated molecular patterns (DAMP).[113]

Segal et al reported on the results of a phase II study of pembrolizumab in combination with radiotherapy in patients with pMMR mCRC patients (NCT02437071).[114] Patients with pMMR mCRC who had been treated with more than 2 standard systemic therapies for metastatic disease underwent palliative radiation, followed by pembrolizumab 200 mg IV every 3 weeks. Local radiation therapy at a total dose of 10-50 Gy was administered in 1-10 fractions to metastatic lesions in lymph nodes (N = 7), liver (N = 5), lung (N = 3), or other sites (N = 8). The first dose of pembrolizumab was administered within one week after completion of radiation. The primary endpoint of this study was ORR by RECIST1.1 in non-radiated target lesions to evaluate the abscopal effect of radiation in combination with pembrolizumab. One of 22 patients had PR to give an ORR of 4.5%. The combination was well-tolerated with all AEs being only grade 1/2: the most frequent AEs were fatigue (23%), skin rash (15%), and nausea (15%). No grade ≥ 3 pembrolizumab-related AE was observed. A preclinical study by Twyman-Saint Victor et al provides intriguing findings that show that dual checkpoint blockade with anti-PD-1/PD-L1 and anti-CTLA-4 is required to induce synergistic antitumor immunity in combination with radiation.[115] This preclinical work provides important insights on why pembrolizumab in combination radiation in pMMR/non-MSI-H mCRC did not exhibit robust antitumor activity as would have been predicted. Resistance to the combination of radiation and anti-CTLA4 treatment was due to upregulation of PD-L1 on tumor cells, resulting in T-cell exhaustion in the *in vivo* murine model.[115] Radiation in combination with dual checkpoint blockade with anti-CTLA4 and anti-PD-L1/PD-1 showed significant synergistic antitumor activity in *in vivo* murine pancreatic cancer models.[115] Anti-CTLA-4 predominantly inhibits Treg cells, thereby increasing the $CD8^+$ T cell to Treg ratio ($CD8^+$/Treg). This preclinical data suggests that dual checkpoint blockade of anti-PD-1/PD-L1 and anti-CTLA-4 is required for maximal antitumor activity when these immune checkpoint inhibitors are to be combined with radiation therapy. There are at least 3 ongoing trials testing this hypothesis in the clinical setting with the combination of PD-1 blockade and anti-CTLA-4 in combination with palliative radiation in patients with pMMR/non-MSI-H mCRC (Table 3).

NSABP FC-9 is a phase II study investigating dual immune checkpoint blockade with durvalumab plus tremelimumab following palliative hypofractionated radiation therapy in patients with pMMR/non-MSI-H mCRC following progression on cytotoxic chemotherapy (NCT03007407). The primary objective of this study is to determine the clinical efficacy of the dual immune checkpoint blockade with durvalumab plus tremelimumab after palliative radiation. Tumor response at the site of non-radiated target lesions is assessed by RECIST1.1. Following 3 doses of hypofractionated palliative radiation of 9 Gy daily on days −2, −1, and day 0 prior to cycle 1 day 1, patients then receive the combination of tremelimumab 75 mg IV and durvalumab 1500 mg IV on day 1 of each cycle in 28-day cycle for the first 4 cycles. Beginning with cycle 5 through cycle 12, patients receive durvalumab 1500 mg IV alone on day 1 of each 28-day cycle. A total of 21 evaluable patients will be enrolled using a Simon two-stage design, and this trial is actively enrolling patients at NSABP study sites in the U.S.

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NCI 10021 is a phase II trial of durvalumab and tremelimumab with or without high or low-dose radiation therapy in patients with mCRC or non-small cell lung cancer (NCT02888743). The CRC cohort of this trial enrolls patients with pMMR/non-MSI-H mCRC who have progressed on ≥ 1 systemic chemotherapy. Enrolled patients receive either low-dose radiation (0.5 Gy twice a day for 2 days) or 8 Gy daily for 3 days, followed by durvalumab 1500 mg IV in combination with tremelimumab 75 mg IV for cycles 1-4 and durvalumab 1500 mg IV alone from cycle 5. This trial is actively enrolling patients at various NCI Experimental Therapeutics Clinical Trials Network (ETCTN) sites in the U.S.

PD-1 Blockade plus Epigenetic Modulation

Epigenetic modulation by DNA methylation, histone modifications, and nucleosome remodeling determines the patterns of cellular gene expression, and abnormal epigenetic modulation plays a critical role in oncogenesis and tumor progression.[36-38] Azacitidine is an inhibitor of DNA methyltransferase (DNMTi), and treatment with this agent alters DNA methylation status and allows the re-expression of previously silenced genes by DNA hypermethylation, including tumor-associated antigens. Epigenetic modulation by DNMTi modifies the expression of genes related to both innate and adaptive immunity and genes related to immune evasion in tumor tissues.[116-118] Chou et al reported that decitabine, a DNMTi, induced expression of NY-ESO-1 and other cancer-testis (C-T) antigens in CRC cells both *in vitro* and *in vivo*.[119, 120] Ghoneim et al showed that de novo DNA methylation in T effector cells promotes T cell exhaustion and DNMTi treatment enhances the rejuvenation of anergic T cells by immune checkpoint blockade.[121]

A phase II study of pembrolizumab in combination with azacitidine was completed in patients with chemo-refractory mCRC (NCT02260440).[122] Enrolled patients received pembrolizumab 200 mg on day 1 of each cycle, every 21 days, and azacitidine 100 mg subcutaneous injection daily on days 1-5 of each cycle, every 21 days. The primary endpoint of this study was ORR by RECIST1.1. Thirty-one patients were enrolled from January 2015 to January 2016, and further enrollment was stopped due to early stopping rule for futility. Thirty patients received at least one dose of the study treatment (median, 3 cycles; range, 1-8). Ten patients (10/30) could not complete the first 3 cycles due to rapid symptomatic tumor progression. One patient with pMMR mCRC achieved a PR after 4 cycles, and 3 patients had stable disease (SD) as their best response.[122] The ORR was 3% (1/30; 95% CI, 0.1-17%) with mPFS of 2.1 months and mOS of 6.2 months.[122]

Currently, there are several ongoing combination trials of PD-1 blockade with epigenetic modulation for pMMR/non-MSI-H mCRC, some of which include a double epigenetic combination of DNMTi and HDAC inhibitor (Table 3). Kim and colleagues reported that epigenetic modulation with azacitidine and entinostat, a HDAC inhibitor, resulted in a marked improvement of the antitumor activity of checkpoint inhibitors in an *in vivo* murine CT26 CRC model. The antitumor activity was mainly due to the inhibition of myeloid-derived suppressor cells (MDSCs), which resulted from the combined epigenetic modulation.[123] This preclinical data suggests that a double epigenetic combination of DNMTi and HDAC inhibitor may yield a more potent synergistic combination with PD-1 blockade in pMMR/non-MSI-H mCRC.

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Biomarkers

With the clinical development of active combination immunotherapy regimens, the development of companion biomarkers to identify the subset of patients with pMMR/non-MSI-H mCRC who would be responsive to these novel therapies has taken on greater importance.

PD-L1 expression in tumor tissues by IHC is a well-validated predictive biomarker for the treatment of patients with immune-sensitive tumor types, including melanoma and non-small lung cancer. The threshold of positive PD-L1 expression in tumor tissue has been variable among many different trials in individual tumor types.[124] Moreover, there is discordance among commercially available antibodies that are being used for the PD-L1 assays. Of note, there is no significant correlation between tumor PD-L1 expression and clinical response to PD-1 blockade in patients with dMMR/MSI-H mCRC.[5, 7, 8] Positive PD-L1 expression does not appear to reliably predict for clinical activity for PD-1 blockade monotherapy in pMMR/non-MSI-H mCRC, mainly due to the lack of any clinical activity observed in this setting.[5]

Targeted NGS has now become a routine diagnostic modality for the molecular characterization of tumors. Tumor mutation burden (TMB) is defined based on the total number of all synonymous and non-synonymous mutations by targeted NGS and reported as mutations per megabase (mut/Mb) unit.[125] Estimates of TMB by targeted NGS is correlated well with whole exome sequencing (WES).[125] In a phase 3 trial of nivolumab plus ipilimumab versus chemotherapy in the first-line treatment of non-small-cell lung cancer (NSCLC), PFS among patients with a high TMB (> 10 mutations per megabase) was significantly longer with nivolumab plus ipilimumab than with chemotherapy.[126, 127] Furthermore, there was no significant overlap between TMB and PD-L1 expression as a predictive biomarker for immune checkpoint inhibitor therapy in NSCLC.[125, 126, 127]

The intratumoral infiltration of cytotoxic $CD8^+$ effector T cell is considered to be a prerequisite for any meaningful tumor response to PD-1 blockade.[128] Immunoscore is an immune biomarker based on the densities of $CD3^+$ and $CD8^+$ cell infiltration in the tumor center and tumor invasive margin and has been validated as a prognostic biomarker in early-stage surgically resected CRC.[130, 131] However, immunoscore currently has a limited role in the metastatic setting as typical tumor samples of core needle biopsy for the diagnosis of mCRC are not adequate for an immunoscore analysis.[132]

Cytotoxic $CD8^+$ effector T cell gene signatures in tumor tissue measures the level of functional $CD8^+$ effector T cell infiltration in tumor tissues and is a potential predictive marker for potential combination immunotherapy with PD-1 blockade. Cytolytic activity of $CD8^+$ effector T cells is mediated by granzymes, perforins and IFN-γ. Thus, the high expression of these effector molecules in tumor tissues is correlated with the overall activity of $CD8^+$ T cells. The level of cytotoxic $CD8^+$ effector T cell function can estimated by the gene expression levels of *CD8A* (CD8a), *CD8B* (CD8b), *EOMES* (eomesodermin), *GZMA* (granzme A), *GZMB* (granzyme B), *IFNG* (IFN-γ), and *PRF1* (perforin 1) using RNA-seq.[103] Wallin et al reported that the gene signatures of cytotoxic $CD8^+$ effector T cell function

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was increased with FOLFOX treatment (2 among 3 patients with pMMR/non-MSI-H mCRC) and with FOLFOX/bevacizumab in combination with atezolizumab (2 among 4 patients with pMMR/non-MSI-H mCRC).[103]

T cell–inflamed gene expression profiles (GEPs) in tumor tissues measures the expression of 18 genes associated with $CD8^+$ effector T cell function, which include IFN-γ–responsive genes related to antigen presentation, chemokine expression, cytotoxic activity, and adaptive immune resistance: *GZMB, GZMK, CXCR6, CCL5, CD3D, CD3E, CD2, IL2RG, NKG7, HLA-E, CIITA, HLA-DRA, LAG3, IDO1, CXCL13, TAGAP, CXCL10,* and *STAT1*.[129] The level of gene expression was measured by the NanoString nCounter gene expression platform using RNA isolated from baseline tumor samples of pembrolizumab-treated patients. The preliminary predictive value of this assay for clinical benefit with pembrolizumab therapy was evaluated in multiple tumor types.[129] This assay is currently being evaluated in pembrolizumab trials.[129]

Conclusion

PD-1 and CTLA-4 pathways are two well-established immune checkpoint pathways. Immunotherapy with immune checkpoint inhibitors is now an effective therapeutic strategy that is associated with highly durable tumor responses and a manageable safety profile and has been approved for several cancers. Major breakthroughs have been made with immune checkpoint blockade therapy in the treatment of patients with dMMR/ MSI-H mCRC. Pembrolizumab and nivolumab are currently approved by the U.S. FDA for the treatment of patients with metastatic dMMR and/or MSI-H mCRC. As monotherapy, these immune checkpoint inhibitors have yet to show clinical activity in the setting of pMMR/non-MSI-H mCRC. However, recent advances have been made in the clinical development of PD-1 blockade-based immunotherapy in the treatment of patients with pMMR/non-MSI-H mCRC when these agents are combined with other immunotherapy agents and/or targeted agents. There are several ongoing combination immunotherapy trials with PD-1 blockade backbone for patients with pMMR/non-MSI-H mCRC. PD-1 blockade in combination with CEA CD3 TCB is in early-phase development with promising preliminary clinical efficacy. Systemic chemotherapy, especially oxaliplatin-containing regimen, is known to induce immunogenic cell death (ICD) of tumor cells, and PD-1 blockade in combination with systemic chemotherapy is an attractive combination strategy for further clinical development. pMMR/non-MSI-H mCRC is highly immune resistant, and it is critical to elucidate the immune escape mechanism(s) of pMMR/non-MSI-H CRC tumors to immune checkpoint blockade for the development of effective combination immunotherapies for patients with pMMR/non-MSI-H mCRC. Furthermore, it is critical to develop biomarkers that can predict clinical response to combination immunotherapies in patients with pMMR/non-MSI-H mCRC. The intratumoral expression of cytotoxic T-cell signature genes appears to be a promising biomarker in early clinical development worthy of further validation.

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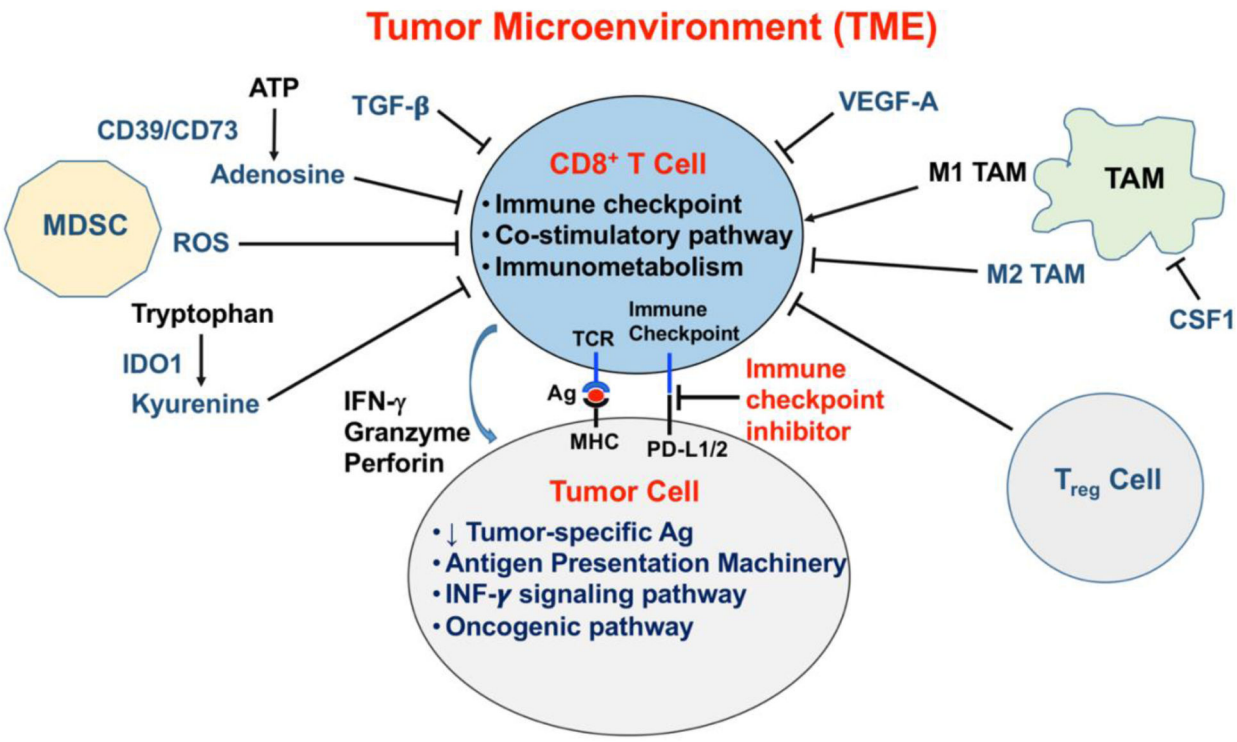

Figure 1. Potential Targets for Combination Immunotherapy with PD-1 Blockade in Patients with pMMR/non-MSI-H mCRC.

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Table 1.

Immune Checkpoint Inhibitors Approved by the U.S. FDA.

Agent	Target	Cancer Types
Pembrolizumab	PD-1	MSI-H or dMMR solid tumors
		Melanoma
		Bladder cancer
		Non-small cell lung cancer (NSCLC)
		Classical Hodgkins lymphoma
Nivolumab	PD-1	MSI-H or dMMR colorectal cancer
		Melanoma
		Non-small cell lung cancer (NSCLC)
		Bladder cancer
		Head and neck cancer (squamous cell cancer)
		Renal cell cancer
		Classical Hodgkins lymphoma
Atezolizumab	PD-L1	Bladder cancer
		Non-small cell lung cancer (NSCLC)
Avelumab	PD-L1	Bladder cancer
		Merkel cell carcinoma
Durvalumab	PD-L1	Bladder cancer
Ipilimumab	CTLA-4	Melanoma

MSI-H, microsatellite instability-high; dMMR, mismatch repair deficient

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Table 2.

PD-1 Inhibitor Monotherapy and Combination Therapy in Patients with pMMR/non-MSI-H mCRC.

Agent	Target Pathway	Number of Patients	Response Rate	References
Pembrolizumab	PD-1	28	0%	Le et al[5]
Nivolumab	PD-1	19	0%	Topalian et al[27]
BMS-936559	PD-L1	18	0%	Brahmer et al[28]
Nivolumab + Ipilimumab	PD-1/CTLA-4	20	5%	Overman et al[7, 89]
Atezolizumab + Cobimetinib	PD-L1/MEK	84	8%	Bendell et al[92]
Atezolizumab + FOLFOX6/Bevacizumab	PD-L1/Chemotherapy	23	52%	Bendell et al[102]
Atezolizumab + CEA CD3 TCB	PD-L1/CEA	23	9%*	Tabernero et al[108]
Pembrolizumab + Radiation	PD-1/Radiation	22	4.5%	Segal et al[114]
Pembrolizumab + Azacitidine	PD-1/Epigenetic	30	3%	Lee et al[122]

*18% (2 of 11) at the 160 mg dose level

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Table 3.

Mechanisms of Primary Resistance of pMMR/non-MSI-H CRC to PD-1 Inhibitor Monotherapy.

	Mechanism	Pathways	Strategies for Combination
Tumor Cell	Tumor-specific Antigen	Low frequency of non-synonymous mutation	Induction of ICD by chemotherapy, radiation, or oncolytic virus
		Low expression of cancer-testis antigen	Epigenetic modulation
	Antigen Presentation Machinery	Mutation or loss of HLA expression	MEK inhibition
		Mutation or loss of β2-microglobulin expression Defect in TAP	
	Mutations or loss of interferon-γ signaling pathway	Interferon-γ receptor	
		JAK1/2	
		Interferon regulatory factor 1	
	Immunosuppressive oncogenic pathway	MAPK pathway	MEK/ERK inhibition
		WNT/β-catenin pathway	Inhibition of WNT/β-catenin pathway[57]
		PI3K	PI3K inhibition
CD8+ T Cell	Immune checkpoint pathway	PD-1	Doublet of immune checkpoint inhibitors
		CTLA-4	
		TIM-3	
		TIGIT	
		LAG-3	
	Co-stimulatory pathway	4-1BB	PD-1 inhibition in combination with agonist of co-stimulatory pathway
		OX40	
		GITR	
		ICOS	
	Immunometabolism	Aerobic glycolysis	Metabolic reprograming.[63-65]
		Mitochondrial dysfunction	
TME	Immunosuppressive cells	MDSCs	Chemotherapy (5-FU); HDAC inhibitor
		TAMs	Anti-CSF1R
		Treg cells	Anti-CCR4; anti-CD25
	Immunosuppressive cytokines	TGF-β	Anti-TGF-β
		CSF-1	Anti-CSF1R
	Immunosuppressive metabolism	Depletion of tryptophan by IDO	IDO1 inhibitors
		Production of adenosine by CD39/CD73	A_2aR inhibitor; anti-CD39/CD73
	VEGF pathway	VEGF-A	Anti-VEGF
	Hypoxia	CCL28	Metformin[67]

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Table 4.

Ongoing Phase 2/3 Trials of PD-1 Blockade Combination in Patients with pMMR/non-MSI-H mCRC.

NCT Number	Study Title	Agents
PD-1 Blockade plus anti-CTLA-4		
NCT02870920	Durvalumab and tremelimumab and best supportive care vs best supportive care alone in patients with advanced colorectal adenocarcinoma refractory to standard therapies (CCTG CO.26)	Durvalumab + Tremelimumab
PD-1 Blockade plus Chemotherapy/Biologics		
NCT02375672	Study of pembrolizumab in combination with chemotherapy for patients with advanced colorectal cancer (HCRN GI14-186)	Pembrolizumab + FOLFOX
NCT02713373	Cetuximab and pembrolizumab in treating patients with colorectal cancer that is metastatic or cannot be removed by surgery (I 274515)	Pembrolizumab + Cetuximab
NCT03174405	Avelumab and cetuximab in combination with FOLFOX in patients with previously untreated metastatic colorectal cancer (Phase II AVETUX-CRC trial)	Avelumab + FOLFOX/Cetuximab
NCT03202758	Evaluation of the safety and the tolerability of durvalumab plus tremelimumab combined with FOLFOX in mCRC (MEDITREME)	Durvalumab + Tremelimumab + FOLFOX
NCT02291289	A study of biomarker-driven therapy in metastatic colorectal cancer (MODUL)	FOLFOX + Cetuximab; Atezolizumab; Vemurafenib; Trastuzumab; Pertuzumab; or Cobimetinib
PD-1 Blockade plus Epigenetic Modulation		
NCT02260440	A phase 2 study of pembrolizumab (MK-3475) in combination with azacitidine in subjects with chemo-refractory metastatic colorectal cancer (UPCI 14-118)	Pembrolizumab + Azacitidine
NCT03182894	Epacadostat in combination with pembrolizumab and azacitidine in subjects with metastatic colorectal cancer (UPCI 16-123)	Pembrolizumab + Azacitidine + Epacadostat
NCT02959437	Azacitidine combined with pembrolizumab and epacadostat in subjects with advanced solid tumors (ECHO-206)	Pembrolizumab + Azacitidine + Epacadostat
NCT02437136	Ph1b/2 dose-escalation study of entinostat with pembrolizumab in NSCLC with expansion cohorts in NSCLC, melanoma, and colorectal cancer (DNDX-275-0601)	Pembrolizumab + Entinostat
PD-1 Blockade plus Radiation		
NCT02437071	Assess the efficacy of pembrolizumab plus radiotherapy or ablation in metastatic colorectal cancer patients (MSKCC 15-069)	Pembrolizumab + Radiation
NCT03104439	Nivolumab and ipilimumab and radiation therapy in MSS and MSI-high colorectal and pancreatic cancer (MGH CC 17-021)	Nivolumab + Ipilimumab + Radiation
NCT03007407	Study of durvalumab and tremelimumab after radiation for microsatellite stable metastatic colorectal cancer progressing on chemotherapy (NSABP FC-9)	Durvalumab + Tremelimumab + Radiation
NCT03122509	A clinical trial of durvalumab and tremelimumab, administered with radiation therapy or ablation in patients with colorectal cancer (MSKCC 17-139)	Durvalumab + Tremelimumab + Radiation
NCT02888743	Durvalumab and tremelimumab with or without high or low-dose radiation therapy in treating patients with metastatic colorectal or non- small cell lung cancer (NCI/CTEP/ETCTN-10021)	Durvalumab + Tremelimumab + Radiation
NCT03102047	Study of durvalumab (MEDI4736) after chemo-radiation for microsatellite stable stage II-IV rectal cancer	Chemo-radiation + Durvalumab

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NCT Number	Study Title	Agents
PD-1 Blockade plus MEK Inhibition		
NCT02788279	A study to investigate efficacy and safety of cobimetinib plus atezolizumab and atezolizumab monotherapy versus regorafenib in participants with metastatic colorectal adenocarcinoma (COTEZO IMblaze370)	Atezolizumab + Cobimetinib vs. Atezolizumab vs. Regorafenib
NCT03377361	An investigational immuno-therapy study of nivolumab in combination with trametinib with or without ipilimumab in patients with previously treated cancer of the colon or rectum that has spread (CheckMate9N9)	Nivolumab + Trametinib +/− Ipilimumab
NCT03271047	Study of binimetinib + nivolumab plus or minus ipilimumab in patients with previously treated microsatellite- stable (MSS) metastatic colorectal cancer with RAS mutation (ARRAY-162-202)	Nivolumab + Binimetinib +/− Ipilimumab
NCT02060188	An investigational immuno-therapy study of nivolumab, and nivolumab in combination with other anti-cancer drugs, in colon cancer that has come back or has spread (CheckMate142)	Nivolumab + Ipilimumab; Cobimetinib; or Daratumumab
PD-1 Blockade plus Vaccine		
NCT03206073	A phase I/II study of Pexa-Vec oncolytic virus in combination with immune checkpoint inhibition in refractory colorectal cancer (NCI 17-C-0092)	Durvalumab + Tremelimumab + Pexa-Vec
NCT03050814	Standard of care alone or in combination with Ad-CEA vaccine and avelumab in people with previously untreated metastatic colorectal cancer (QUILT-2.004)	Avelumab + Ad-CEA + FOLFOX/Bevacizumab
NCT02981524	Phase 2 study of GVAX (with CY) and pembrolizumab in MMR-p advanced colorectal cancer (J16154)	Pembrolizumab + GVAX
PD-1 Blockade plus Targeted Agent		
NCT03258398	A study to evaluate eFT508 alone and in combination with avelumab in subjects with MSS colorectal cancer (eFT508-0006)	Avelumab + eFT508
NCT03332498	Pembrolizumab in combination with ibrutinib for advanced, refractory colorectal cancers (MCC-19091)	Pembrolizumab + Ibrutinib
NCT02851004	Special combination of BBI608 and pembrolizumab (EPOC1503)	Pembrolizumab + BBI608

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Table 5.

Potential Predictive Biomarkers for Immune Checkpoint Inhibitor Therapy in pMMR/non-MSI-H mCRC.

Biomarker	Specifics
PD-L1 expression in tumor tissues	Analysis of PD-L1 expression by immunohistochemistry (IHC); No significant role in the PD-1 inhibitor monotherapy for pMMR/non-MSI-H[5]
Tumor mutational burden (TMB)	The amount of non-synonymous somatic mutation in tumor tissue assessed by next-generation sequencing (NGS); No significant role in the PD-1 inhibitor monotherapy for pMMR/non-MSI-H[5]
$CD8^+$ T cell signature in tumor tissues[103]	Estimation of cytotoxic CD8+ effector T cell function by using gene signatures of *CD8A, CD8B, EOMES, GZMA, GZMB, IFNG,* and *PRF1* by RNA-seq
T cell-inflamed gene expression profiles (GEPs) in tumor tissues[129]	18 genes include IFN-γ-responsive genes related to antigen presentation, chemokine expression, cytotoxic activity, and adaptive immune resistance: *GZMB GZMK, CXCR6, CCL5, CD3D, CD3E, CD2, IL2RG, NKG7, HLA-E, CIITA, HLA- DRA, LAG3, IDO1, CXCL13,TAGAP, CXCL10,* and *STAT1.* The level of gene expression measured by the NanoString nCounter gene expression platform using RNA isolated from baseline tumor samples of pembrolizumab-treated patients