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10.2478/AMB-2024-0022

REVIEW

NEW APPROACH IN UNDERSTANDING COLORECTAL CANCER IMMUNOSUPPRESSION AND IMMUNOTHERAPY-BASED STRATEGIES IN THE TREATMENT OF MICROSATELLITE STABLE COLORECTAL CANCER

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Abstract. *Except the widely accepted use of immune checkpoint inhibitors in the treatment of microsatellite instability-high (MSI-H) mismatch repair-deficient (MMRd) CRCs representing about 5% of all metastatic (m)CRC patients, new strategies are applied to cure MMR-proficient (MMRp) mCRC patients. Tumor microenvironment (TME) is decisive for cancer development. The determination of some immunoeffective and immunosuppressive immune cells and some cytokines, chemokines and growth factors in the TME gives information about the use of immune checkpoint inhibitors in MMRp CRCs. The increased level of IL-6 in the serum and increased number of IL-6+ immune cells in TME, the increased number of IL-17+ Th17 cells, and of FoxP3+ cells are used to determine the use of anti-IL-6 antibody and of anti-FoxP3 antibody for treatment. The determination of high CD8+, high PD-1 expression and little or no Th17 cells appoint better response to anti-PD-1 therapy. The used combination therapies are: combination of immunotherapy with chemotherapy, with radiation therapy, with targeted therapy, with vaccines, oncolytic viruses and bispecific antibodies. Classical treatment of CRC patients has included chemotherapy, radiotherapy and surgery. Recently, immunotherapy has been added as a mainstay for therapy of CRC. The main checkpoint inhibitors used in CRC immunotherapy are pembrolizumab and nivolumab (anti-PD-1), durvalumab (anti-PD-L1), ipilimumab (anti-CTLA-4), favezelimab (anti-LAG3), etc. They are applied after fluorapyrimidine, oxaliplain, and irinotecan therapy. In conclusion, we may state that the future treatment of MSS CRC is in combination therapies, i.e. conventional and immunotherapies. We consider that immune infiltrate in TME must be assessed in order to determine combination therapies.*

Key words: *colorectal cancer, IL-6, Th17, FoxP3, checkpoint inhibitors, combination therapies*

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Received: *23 January 2023;* ***Accepted:*** *10 April 2023*

Colorectal cancer (CRC) accounts for 10-20% of malignant tumors worldwide [1, 2]. CRC is a leading cause of mortality and is the third most common cause for cancer-associated death [3]. About 25% of CRC patients have metastasis in regional lymph nodes at diagnosis. After chemotherapy, 50% of CRC patients develop peritoneal metastasis [3]. Recently, studies have highlighted the pivotal role of gut microbiota in anticancer therapy. Changes in the intestinal microbiota initiate CRC development through enhanced release of toxins produced by bacteria, disruption of the epithelial barrier and bacterial dysbiosis [4].

Chronic inflammation is a major risk factor for CRC [5]. Inflammatory bowel diseases (IBD) such as Crohn's disease and ulcerative colitis show an increased risk for development of colitis-associated cancer (CAC) [6]. Chronic inflammation plays essential role in all stages of carcinogenesis i.e. initiation, development and progression [7]. Tumor microenvironment (TME) is decisive for cancer development. TME includes complex communications between tumor cells and cancer-associated fibroblsts (CAFs), immune cells, endothelial cells, extracellular matrix (ECM) etc. These cellular interactions are modulated by many soluble factors like cytokines, chemokines, growth factors and ECM components. Balance between pro- and anti-inflammatory cytokines defines immune response in TME [8]. In TME the persistent exposure to pro-inflammatory cytokines supports tumor development through deregulated pathways, and resistance to therapy [3]. Myeloid-derived suppressor cells (MDSCs), immature and regulatory dendritic cells (DCs), immunosuppressive T helpers, tumor-associated macrophages (TAMs) M2 type etc. mediate pro-tumor immune responses and resistance to therapy [8, 9].

Elevated levels of cytokines such as TNF-α, IL-6 and IL-1β, chemokines like CXCL1 and CXCL2 and the lipid molecule prostaglandin E2 (PGE2) in the serum of CRC patients are associated with tumor development. These molecules are targets for anti-cancer therapy [2]. It has been shown that the serum levels of IL-6 [10], IL-6, TNF-α, and IL-17 [7] are elevated in CRC patients. The IL-6/JAK/STAT3 signaling pathway promotes CRC tumor development [11]. The NF-κB/IL-6/STAT3 activation initiates c-Myc and cyclin-D1 overexpression stimulates CRC progressions, tumor angiogenesis and resistance to chemotherapy [2, 12, 13].

The therapy based on IL-6/IL-6R/STAT3 signaling includes IL-6 ligand-blocking antibody that possesses anti-tumor and anti-inflammatory activities or **CNTO-328** [14]. The **siltuximab** (**CNTO-328**) blocks the IL-6/IL-6R/gp130 transduction pathway at the level of inhibiting the binding of IL-6 to the IL-6R [15]. That treatment has been applied in phase I and II trials and consequently it causes partial transient retardation of cancer cell proliferation and of the inflammatory response, but doesn't provide durable response [14]. Inhibition of IL-6 enhances the action of check point inhibitors in CRC treatment [16].

In addition, another antibody a humanized anti-IL-6R McAb of IgG1 class named anti-IL-6R Ab (**tocilizumab**) inhibits the mIL-6R and sIL-6R [17]. The inhibition of IL-6/sIL-6R signaling combined with other cancer immunotherapies appears to be more promising for treatment [18]. **The chemotherapy with carboplatin/doxorubicin combined with IL-6/STAT3 blockade** is helpful to overcome the tumor recurrence [19].

IL-6 can suppress the effective immune response and supports tumor immune surveillance. The JAK/STAT3 pathway is dysregulated in DCs and their antigen presentation is impaired [20]. IN addition IL-6 limits activation of CD8+ and CD4+ lymphocytes, represses the differentiation of T regulatory cells (Tregs) and promotes T helper 17 (Th17) cell appearance and changes Th1/Th2 balance towards Th2 [20, 21, 22].

Th17 CD4+ T helpers together with Tregs are the main cells in the gut microenvironment [23, 24]. IL-17A is a pro-tumorigenic cytokine, which is overexpressed in adenoma-carcinoma pathway and in dysplasia. Its overexpression in CRC is correlated with tumor size and shorter survival [25]. IL-17A stimulates tumor angiogenesis triggering vascular endothelial growth factor (VEGF) production [26]. In CRC TME IL-17A restricts immune surveillance, inhibits recruitment of CD8+ T cells and stimulates MDSC infiltration creating a pro-tumorigenic TME [27]. On the contrary, IL-17F possesses anti-tumor activities as the decrease of VEGF level [28]. The increase of pathogenic gut bacteria is associated with elevated IL-17A secretion that triggers gut inflammation with stimulation of the adaptive immune response (**Th1, Th17 and Tregs increase**) [23]. IL-17A functions as anti-bacterial host defense and initiates epithelial repair from one side and can somehow induces colon carcinogenesis [29]. Even IL-6 evokes Th 17 cell differentiation [30].

In CRC, increased number of **FoxP3+ T** cells correlated with both improved [31, 32] or with worsened prognosis [33]. In a large cohort of CRC patients it has been demonstrated that high FoxP3+ Tregs were associated with earlier pT stage, well or moderate differentiation of tumors, absence of venous or lymphatic invasion and with favorable prognosis [34]. On the

other hand, low numbers of FoxP3+ Tregs in stage II CRCs predicts high risk of progression [35].

CD4+CD25hi+FoxP3+ regulatory T cells (Tregs) produce mainly TGF-β, prevent autoimmunity and suppress the effective immune response [36]. Tregs are several subsets such as thymus Tregs (tTregs) that recognize self-antigens or peripheral Tregs (pTregs) in secondary lymphoid organs recognizing foreign antigens [37]. Furthermore, FoxP3 T cells expressing CD4 can be subdivided into central Tregs (cTregs) and effector Tregs (eTregs). In humans, eTregs secrete proinflammatory cytokines (IL-17, IL-2 and IFNγ) and are considered to be non-suppressive [37]. Better prognosis in CRC has been associated with increased infiltration of **non-suppressive FoxP3^{lo}CD45RA^{-} eTregs**. In contrast, increased numbers of suppressive **FoxP3^{hi} CD45RA^{-} Tregs** correlate with worse prognosis [33]. The limitation of these studies is that the percentage of non-suppressive Tregs remains unknown. TME in the colon contains abundant bacterial species such as Fusobacterium nucleatum associated with tumor invasion and Tregs infiltration [38]. Colonic microflora can divert the effective immune responses away from cancer cells [35]. Adoptive transfer of Tregs has been able to prevent bacteria-driven inflammation and carcinogenesis in CRC [34]. Tregs could suppress Th17 cell activities in TME that are pro-tumorigenic [36]. Therefore, **FoxP3+ Tregs can be in fact anti-tumorigenic in CRC. The depletion of Tregs can provoke autoimmune diseases and therefore is quite dangerous to be used for treatment.**

Except the widely accepted use of immune checkpoint inhibitors in the treatment of **microsatellite instability-high (MSI-H) mismatch repair-deficient (MMRd) CRCs** that represent about 5% of all metastatic (m)CRC patients, new strategies are applied to cure MMR-proficient (MMRp) mCRC patients [39]. Llosa and colleagues have investigated 44 immune-related genes in MMRd CRCs that have responded to immune-checkpoint therapy and compared them to the same genes in MMRp CRCs, which have not responded to such therapy. They have found **10 genes that predict immunosensitivity**. Something more, **MMRp CRCs exhibit high CD8+ T lymphocytes with up-regulated PD-1 and no Th17 cells** show better response to anti-PD-1 therapy [39,40]. The use of anti-IL17A antibodies such as **secukinumab** and **ixekizumab** in combination with anti-PD-1 therapy (**pembrolizumab**) leads to a significant benefit to MMRd CRC patients with high protein and mRNA expression of PD-L1 [41]. Therefore, the formula for treatment with checkpoint inhibitors in MMRp CRCs is high PD-L1, increased CD8+ T lymphocytes and no or low Th17 cells [41].

MSI-H phenotype is found in 15% to 18% of stage II CRC patients and is a favorable prognostic factor, while MSI-H/dMMR tumors in later stages (III + IV) are low in number (4% to 5%) [42a]. The MSI-H/dMMR phenotype is determined by length of polymorphisms of repeats of noncoding DNA sequences. This is a result of changes in DNA mismatch reparation (MMR) system and includes mutations in the MMR genes *MLH1*, *MSH2*, *MSH6* and *PMS2*. In sporadic CRCs MSI is a result of inactivation of MMR genes through somatic mutations or epigenetic silencing (*MLH1* promoter somatic hypermethylation) [42a]. In hereditary CRCs (Lynch syndrome) MSI is a result of germline mutations of MMR genes [43]. Therefore, for diagnosis of hereditary CRC except DNA dMMR testing it is necessary to test *MLH1* promoter hypermethylation and testing for *BRAF^{V600E}* mutation. In the case of *MLH1* promoter hypermethylation the tumors with or without BRAF mutation is referred as sporadic CRC. In addition, when we have loss of the *MLH1*/PMS2 couple or of MSH2/MSH6 couple without hypermethylation and *BRAF^{V600E}* mutation the patient should be considered for oncogenic counselling [44b].

Nowadays, two standard methods for MSI testing are validated: immunohistochemistry for detection of absence of MMR proteins using antibodies MMR genes MLH1, MSH2, MSH6 and PMS2; and PCR testing using two panels: the Bethesda panel (BAT25 and BAT26 – mononucleotide markers and D5S346, D2S123 and D17S250 – dinucleotide markers); and the Promega panel (BAT25, BAT26, NR21, NR24 and NR27 – mononucleotide markers. New generation sequencing (NGS) is in development nowadays. It determines MSI and common mutations i.e. *BRAF*, *RAS* and *HER2* amplification [44b, 45].

Classical treatment of CRC patients has included **chemotherapy, radiotherapy and surgery**. Recently, immunotherapy has been added as a mainstay for therapy of CRC. The Food and Drug Administration (FDA) has approved CRC immunotherapy for MSI-H tumors [46]. The current therapy of II and III stage CRC that are MSI-H/dMMR comprises of surgery and following adjuvant chemotherapy with fluoropyrimidine and oxaliplatin [44b] or radiation therapy.

In the near past other immunotherapies have been used. For example, **Bewacizumab** (Avastin®) targets angiogenic **VEGF/VEGFR** pathway, used as first line therapy alone or in combination with irinotecan, fluorouracil and leucovorin [47]. Another anti-VEGF/VEGFR2 antibody used for anti-angiogenic treatment is **Ramucirumab** (Cyramza®) [48]. Bewacizumab is used in the treatment of right-sided CRCs and **Cetuximab** – for left-sided ones [49]. Other group of monoclonal antibodies is that targeting **EGFR signaling**

pahway. Cetuximab (Erbitux®) and Panitumumab (Vectibix®) are used for the therapy of wild-type mutant KRAS and NRAS tumors [50]. Therefore, a growing body of evidence points to the association of TME and immunotherapy.

Therapy with immune check-point inhibitors is targeted against the immunosuppressive TME in CRC. The determination of companion biomarkers that identify patients with pMMR CRC that can be treated using immunotherapy, is with growing importance in novel combination therapies. The PD-L1 expression in tumor cells is not predictive for clinical activity and checkpoint blockade in pMMR CRC. The estimation of tumor mutational burden (TMB) by NGS has limited diagnostic value [51]. The immunoscore in TME based on the investigation of densities of CD3+ and CD8+ cell infiltration is a biological marker for effective use of checkpoint immune blockade [52, 53]. The assay of cytotoxic CD8+ effector T cell gene signature is currently under investigation in pembrolizumab trials [51]. Modern multiplex immunohistochemistry/ immunofluorescence (mIHC/IF) investigates cell markers in the TME. More than three markers are necessary to assess the immunoscore in CRC [53].

Multiple immune checkpoint molecules have been identified in CRC. Progammed death-1 (**PD-1**) receptor (CD279) is located on CD4+, CD8+ and B cells, on NK cells and macrophages [54]. While, **PD-L1** (B7-H1) and **PD-L2** (B7-DC) ligands are constitutively expressed on tumor cells, cancer-associated fibroblasts (CAFs), tumor-infiltrating macrophages and DCs [54]. The check-point inhibitors used in MSI-H/dMMR CRCs are **pembrolizumab** (Keytruda ®) and **nivolumab** (Opdivo®) – two **anti-PD-1** antibodies or **durvalumab** (**anti-PD-L1**) antibody [3]. They are applied after fluoropyrimidine, oxaliplatin and irinotecan [51]. Anti-PD-1 therapy enhances CD8+ T cell action. In MSS/MMRp CRC patients combined anti-PD-1 blockade is used with other therapies that aim to enhance the immunogenicity of tumors [53]. The cytotoxic T lymphocyte antigen (CTLA-4) expressed on T lymphocytes binds to B7-1 (CD80) and B7-2 (CD-86) the co-stimulatory molecules on DCs and competitively block CD28 [53]. **Ipilimumab** (Yervoy®) is an anti-CTLA-4 agent [55]. The combination therapy of **nivolumab** (anti-PD-1) and **ipilimumab** (anti-CTLA-4) is applied in MSS CRC patients with moderate improvement of overall survival (OS) [20]. Well known marker for immune checkpoint blockade is lymphocyte activation gene-3 (LAG-3) that interacts with MHC class II markers and realize negative regulation of T cells, B cells, NK cells and pDCs. **Favezelimab** (mk-4280) is an anti-LAG-3 antibody against LAG-3 receptor on T cells and **avelumab** (anti-PD-L1) are used in combination with anti-DCs vaccines [56].

One of the most successful combination therapies in pMMR/non-MSI-H mCRCs is atezolizumab (anti-PD-L1) with chemotherapy 5-fluorouracil +oxaliplatin+ foliac acid (FOLFOX) and anti-VEGF (bevacizumab) with response rate in 52% [57]. Other checkpoint inhibitor is T cell immunoglobulin and mucin-containing protein-3 (TIM-3) (**BMs-986258**). TIM-3 is expressed on T helper1 (Th1) and CD8+ cytotoxic T cells (CTL) [53]. The T cell immunoreceptor with immunoglobulin and ITIM domain (TIGIT) is expressed on activated T cells, Tregs and NK cells [58].

CRC is a “cold” tumor, having low number of neoantigens and decreased number of immune cells in TME [59]. In **KEYNOTE 177 trial** in patients with dMMR CRC the application of checkpoint inhibitors anti-PD-1 (pembrolizumab or nivolumab), anti-PD-L1 (atezolizumab), and anti-CTLA-4 (ipilimumab) has been successful as frontline therapy. About 50% of patients have 2-3 year overall survival. Part of these patients have had chemotherapy with fluoropyrimidine, oxaliplatin and irinotecan [55]. The **CheckMate-142 trial** a phase II study is using a first-line therapy with nivolumab (ant-PD-1) and ipilimumab (anti CTLA-4) in dMMR/CRC patients with response rate of 69% [60]. Response to pembrolizumab has been achieved in MSS mCRC patients showing DNA polymerase ε (POLE) mutation, responsible for a lead strand DNA replication [61]. These patients show increase in TME of cytotoxic T lymphocytes and effector cytokines [62].

New strategies are developed for treatment of pMMR MSS m CRC patients and include combination therapies. Novel therapies involve heightening of tumor mutation burden (TMB) and the number of neoantigens, use of interferon-γ (IFN-γ) therapies, inhibition of immune suppressive molecules, or transformation of TME in immunoeffective [63]. The used combination therapies are: combination of immunotherapy with chemotherapy, with radiation therapy, with targeted therapy, with vaccines, oncolytic viruses and bispecific antibodies [20,51].

Immunotherapy in combination with chemotherapy for treatment of pMMR/non MSI-H mCRC

Cytotoxic chemotherapy induces immunogenic cell death (ICD) of tumor cells that triggers the antitumor immunity. The main chemotherapy agent 5-fluorouracyl (5-FU) used for treatment of pMMR/MSS CRC induces apoptosis of MDSCs and stimulates recruitment of CTLs [64]. **Oxaliplatin** stimulates ICD and uncovers tumor-specific anigens making them recognizable from the immune system [65,66]. Oxaliplatin

enhances the antitumor activity of **PD-1 blockade** with anti-PD-L1 antibody (MED14736) [51, 67]. Another combination (AtezoTR i BE study) is frontline therapy **FOLFOXIRI** (irinotecan + oxaliplatin + leucovorin + 5-FU) applied with **bevacizumab** (anti-VEGF/VEGFR pathway) + atezolizumab (anti-PD-L1) is successful [68]. In the MODUL trial FOLFOX has been used in CRCs with BRAF mutations and followed by maintenance treatment with 5-FU + atezolizumab + bevacizumab with good progression free survival (PFS) and OS [69].

Immunotherapy in combination with anti-EGFR agents and chemotherapy and with anti-angiogenic agents

The contemporary therapy of metastatic RAS/BRAF wild-type CRC is chemotherapy combined with agents targeting the EGFR [63]. Cetuximab (anti-PD-1) is an IgG1 antibody that promotes expression of MHC class II molecules on DCs. The used combination is avelumab (anti-PD-L1) or cetuximab + FOLFOX (phase II AVETUX clinical trial) [70].

In a syngeneic murine colon cancer model a combination of pembrolizumab and lenvatinib leads to activation of CD8+ T lymphocytes via reduction of TAMs and activation of interferon pathway results in higher response rate [71]. The combination is used for treatment of MSS CRC [63].

Immunotherapy in combination with radiotherapy

The combination of radiotherapy with durvalumab and tremelimumab reduces the size of distant CRC tumors. Increased CD8+ T lymphocytes with their activation and proliferation has been observed [72]. Other clinical trials (NCT02992912, NCT02437071, NCT03122509) are investigating checkpoint inhibitors combined with different types of radiotherapy such as standard radiotherapy with stereotactic body radiation or radiofrequency ablation. This therapy has impact on immune response genes [73].

Immunotherapy combined with target therapies

Activation of signaling pathways RAS/BRAF/MEK/ERK pathways are commonly detected in CRC [63,74]. The used combinations are immunotherapy and RAS/BRAF/MEK/ERK inhibitors in pMMR/MSS mCRC patients. The frequently used agent is **sotorasib** (**KRAS G12C inhibitor**). This therapy has been applied in in CMS3 metabolic CRC subtype with *KRAS G12C* mutation and with low number of immune infiltrate [75]. It is combined with PD-1/PD-L1 inhibitors and sotorasib that induces a pro-inflammatory TME with increased CTL and NK cell activity [75]. *BRAF* mutations in CRC occur in about 15% of patients and the most frequent is $BRAF^{V600E}$ [63]. One successful therapy has been the combination of BRAF inhibitor **dabrafenib** and EGFR inhibitor **cetuximab** that resulted in longer overall survival [76].

MEK (MAPK (mitogen-activated protein kinase) inhibitors in mouse models cause upregulation of MHC class I molecules and increase of CD8+ T lymphocytes. MEK inhibitor (**trametinib**) in combination with anti-PD-1/PD-L1 and anti-CTLA-4 antibodies resulted in tumor regression [77].

Bispecific antibodies

The treatment with bispecific antibodies is directed against tumor antigens such as CEA, CEACAM, EpCAM, HER2 and CD276 antigen and against immune cells like T cells via the CD3 receptor [78]. Such agent is cibisatamab (RO6958688) antibody directed against CEA and CD3 used in MSS mCRC [79].

The treatment with **DC vaccines** (DCs isolated from the patient and enriched with tumor-associated antigens – TAAs from autologous tumor lysate. Disappointingly, the result of this treatment is a matter of debate [80].

The treatment with stimulators with ***IFN genes*** (STING) [81] and oncolytic viruses applied in the tumor itself are under investigation [82].

In **conclusion** we may state that the future treatment of MSS CRC is in combination therapies i.e. conventional and immunotherapies. We consider that immune infiltrate in TME must be assessed in order to determine combination therapies.

Disclosure Summary: *The authors have nothing to disclose.*

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