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Immunotherapy of Metastatic Colorectal Cancer: Prevailing Challenges and New Perspectives

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Abstract

Patients with recurring or metastatic colorectal cancer (mCRC) have strikingly low long-term survival, while conventional treatments such as chemotherapeutic intervention and radiation therapy marginally improve longevity. Although, many factors involving immunosurveillance and immunosuppression were recently validated as important for patient prognosis and care, a multitude of experimental immunotherapies designed to combat unresectable mCRC have, in few cases, successfully mobilized antitumor immune cells against malignancies, nor conclusively or consistently granted protection, complete remission, and/or stable disease from immunotherapy – of which benefit less than 10% of those receiving therapy. After decades of progress, however, new insights into the mechanisms of immunosuppression, tolerance, and mutation profiling established novel therapies that circumvent these immunological barriers. This review underlines the most exciting methods to date that manipulate immune cells to curb mCRC, including adoptive cell therapy, dendritic cell vaccines, and checkpoint inhibitor antibodies – of which hint at effective and enduring protection against disease progression and undetected micrometastases.

Keywords

Metastatic colorectal cancer; T-cell; Cytotoxic T-Lymphocyte; Helper T-Cell; Regulatory T-cell; Immunotherapy; Dendritic cell; Macrophage; Myeloid-derived suppressor cell; Vaccine; Antibody therapy; Adoptive T-cell therapy; Allogeneic stem-cell transplant; Ipilimumab; CTLA-4; PD-1/L1; Immunosuppression; Immunosurveillance; Biomarker; Antitumor immunity; Adoptive Immunotherapy

INTRODUCTION

Colorectal cancer (CRC) represents 10% of malignancies worldwide, and remains the fourth leading cause of cancer-related mortalities [1]. Although surgical resection of localized tumors greatly improves patient survival past 5 and 10 years [2], liver metastases will develop in over half of all CRC patients further lowering survival [3–5]. A minority of Stage

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III CRC patients benefit from adjuvant chemotherapy (i.e., 5FU/oxaliplatin regimens), yet all endure toxicity [6]. Where chemotherapy and radiation fail, priming the immune system to inhibit metastatic spread may improve patient survival without severe toxicity.

Harnessing the immune system has successfully eradicated malignant cells in animal models, and even though human tumors are more complex, some Phase III trials have been successful [7]. Immunotherapy introduces modulatory agents, such as adjuvants, antibodies, and/or immune cells to initiate or amplify an existing antitumor response. Ipilimumab is an exciting example where immune cell activating antibodies prolong the lives of melanoma patients [8].

The topic of CRC immunotherapy was explored elegantly in previous reviews; this review, however, mainly discusses the inherent immunosuppressive barriers in the local tumor environment that obstruct current immunotherapeutic intervention. In particular, this review will 1) briefly overview the prognostic impact of common tumor-infiltrating immune cells in CRCs, 2) critique the most current and promising therapies that orient the immune system against CRCs, and 3) suggest which immunosuppressive barriers can be targeted to improve immunotherapy efficacy.

ANTITUMOR IMMUNITY

Before the culmination of seminal studies performed by Freidman, Galon, and Pagès, who demonstrated that effector T-cells infiltrate CRCs and benefit patient survival, Jass and colleagues provided direction for these investigators by connecting the mechanism of protective immunity to infiltrating lymphocytes in advanced rectal cancer [9]. Many studies concur with the supposition that immunosurveillance [10, 11] inhibits CRC tumorigenesis and progression [9, 12–15]. Antitumor activity is highly coordinated, requires specifically primed lymphocytes to eradicate aberrant cells, and is evidenced by high densities of tumor-infiltrating lymphocytes (TILs) in early stage CRCs [16, 17].

Cytotoxic Immune Response

Rudimentary anti-tumor T-cell responses are driven by anti-proliferative interferon-gamma (IFN- γ) [18]. This cytokine is secreted upon proteasomal degradation and subsequent presentation of antigens to T-cell receptors (TCRs) by major histocompatibility complex (MHC) class I [19]. Cytotoxic CD8⁺ T-lymphocytes (CTLs) are a major source of IFN- γ [20, 21] as well as apoptosis inducing granzyme B [22] and require cooperative interactions from Type-1 polarized CD4⁺ T (T_H1)-cells [23]. High densities of CD8⁺ effector memory T-cells in CRCs associate with prolonged survival and lack of early signs of metastasis [13], suggesting T_H1 polarization and associated CTLs quell metastasis after curative surgery [15].

CXCR3 Ligands—A complex network of chemokines and cognate receptors dictate the type and density of infiltrating T-cells into tumors [24, 25]. The majority of CTLs and active T_H1 cells express CXCR3, the receptor for a trio of IFN- γ -induced chemokines, CXCL9, CXCL10, and CXCL11 [26–29]. These chemokines home CXCR3-expressing cells towards inflammation.

CCR5 Ligand—CCR5 is co-expressed with CXCR3 on T_H1-cells and CTLs in CRC invasive margins [30], however, is absent from CRCs with lymphatic and liver metastasis and low densities of CD8⁺ T-cells [31, 32]. Its ligand, CCL5 [33], is released from stored secretory compartments upon CD8⁺ T-cell stimulation [34], functions to attract T-cells towards inflammation [35], and is strongly secreted from specific CRC subsets [36].

Natural Killer Cells in CRC

Among the arsenal of cytotoxic lymphocytes, natural killer (NK)-cells and NK T-cells recognize and eliminate MHC negative tumor cells [37, 38]. However, their association with cancer progression is inconsistent across multiple cancer types. Recently, Sconocchia and colleagues demonstrate that certain cancer types harbor low densities of infiltrating NK-cells; breast cancers, melanomas, hepatocellular carcinomas, renal cell carcinomas, and CRCs all stain poorly for NK-cell markers [39, 40]. These cells are not found in CRC-associated liver metastases [41] and have no prognostic relevance for CRC patients [42–44]. These studies suggest that evasion from NK-cell detection and elimination is an obligate prerequisite for tumorigenesis [43]. The prognostic impact of TILs on CRC patient survival is summarized in Table 1.

MULTIDIMENSIONAL IMMUNOSUPPRESSION

Tumor eradication and host tolerance are dictated by the immune system. The type, polarity, and density of infiltrating immune cells can either reject or accept aberrant cell growth. Each CRC contains a unique composition of immune cells, including those that quench the tumor fighting capacity of effector T-cells described above. Other infiltrating immune cells produce proliferative signals and accelerate metastatic disease.

Immune-Regulating T-cells

In healthy tissues, regulatory FoxP3⁺CD4⁺CD25⁺ T-cells [45, 46] (T_{regs}) suppress effector T-cells and protect against autoimmune disease through an array of immune quenching factors that are not limited to, TGF-β, IL-10, and cytotoxic T-lymphocyte antigen 4 (CTLA-4) [47–49]. These factors silence memory CD8⁺ T-cell activity and IFN-γ production [50]. For example, T_{regs} powerfully suppress T-cells isolated from CRC patients [51] and depletion of T_{regs} via anti-CD25 antibody augments CRC rejection in mice [52].

Contradicting the studies mentioned above, T_{regs} may benefit CRC patients. FoxP3⁺ cell densities in CRCs positively correlate with patient survival [53–55] and absence of metastases [56]. These data question whether colon and rectum residing FoxP3⁺ cells truly represent T_{regs}. FoxP3 is transiently expressed by intratumoral effector T-cells upon TCR stimulation [57], suggesting that FoxP3 is a poor biomarker for immunosuppressive T-cells. Alternatively, T_{regs} may protect against tumor progression in barrier organs by suppressing tumor promoting inflammation initiated by foreign antigens [58]. Perhaps the true prognostic relevance of T_{regs} lies with their abundance to cytotoxic CD8⁺ T-cells; tumor progression may be slowed or halted when CD8⁺ T-cells far outnumber T_{regs}.

Considering all the studies mentioned above, it is unlikely that only one factor supplied by T_{regs} solely inhibits of immunosurveillance, nor is it likely that only one cell type promotes

immunosuppressive environments. Therefore, effective immunotherapy requires blockage of other cell types and their soluble factors. Without clear understanding, we risk over-valuing unreliable immunosuppressive markers and fail personalizing therapy. A better approach is to consider the holistic immune contexture of the tumor environment.

The major hole in immunotherapy research is the lack of mouse models with multidimensional immunosuppression. Mice engineered with a specific immune modulating mechanism do not remotely represent the complexity of the human tumor environment.

Tumor-promoting Myelogenous Cells

Macrophages residing in human tumors affect disease progression. These myelogenous cells polarize to either promote acute inflammation and adaptive T-cell immunity or immunosuppression and tumor escape [59]. Therefore, the prognostic relevance of tumor-associated macrophages (TAMs) in CRCs is debatable.

Tumor-Associated Macrophages—TAMs represent major components of the colorectal intratumoral immune milieu and are classified into heterogeneous subgroups by an array of secreted cytokines [60, 61]. Angiogenesis and metastasis promoting cytokines link these cells to CRC progression and poor prognosis [62]. Immature TAMs, termed myeloid-derived suppressor cells (MDSCs) facilitate immunosuppression, inflammation, and angiogenesis [63–66] by secreting VEGF, and T- and NK-cell inhibiting (and T_{reg} expanding) nitric oxide, reactive oxygen species, arginase, IL-10, and IL-6 [67, 68]. In contrary, the presence of TAMs at CRC invasive margins associate with improved survival [69]. Therefore, fully ascertaining the link between TAMs/MDSCs and patient survival will identify their mitigating effects on therapy.

Dendritic Cells—In the periphery, protective immunity requires antigen presentation by dendritic cells (DCs) [70]. These cells are equipped with molecular receptors that take up microbial components and program lymphocytes with the resulting information. Programming requires concurrent ligation of CD28 or OX40 on T-cells to CD80/86 [71]. Like TAMs, DCs form heterogeneous populations that dictate immune responses either away or against tumor antigens [72]. DCs activated without maturation signals will promote tolerance and immunosuppression through the induction of T_{regs} [73].

DC analysis in CRCs is limited to low density immunohistochemistry and tissue microarrays. These methods analyze immune cells within small tissue fragments [74]. Immunohistochemistry is very useful for studying spatial arrangements of immune cells in solid tumors, however only identifies cells by a limited number of markers. Tissue microarrays utilize more markers, but require fragmenting tissues, thereby destroying spatial arrangements of immune cells. Markers such as CD83 [75], S-100 [76], CD208 [61, 77] represent DCs in the stroma and invasive margins. However, no CRC study conclusively characterized the maturation status of DCs via cell surface markers or cytokine secretion using multidimensional approaches. As potential targets for immunotherapeutic intervention, much remains to be learned about the role of DCs in CRC progression. A summary of common immunosuppressive mechanisms is listed in Table 2.

Novel methods that improve immune infiltrate profiling and prognostic accuracy are encouraging, however counting T-cells, macrophages, and DCs alone does not optimally predict which patients will respond to therapy because the phenotype and activity of infiltrates are equally critical. We are very far from identifying all the immunosuppressive mechanisms, yet our currently knowledge will soon extent and improve the quality of patient lives.

The diversity of CRC environments requires research to develop multiple methods that directly manipulate immune infiltrate phenotype and activity. However, these methods must overcome three major obstacles to eradicate tumors. 1) Interruption of the immunosuppressive environment will strengthen the impact of existing anti-tumor responses. This can be done using simple molecules that target specific immune modulating pathways. 2) Encouraging CTLs to migrate to the invasive margin will augment antitumor immunity. Tumor rejection may simply require overpower the immunosuppressive environment by introducing an overwhelming number of T-cells, cytokines, and cytotoxic mediators. And, 3) Repolarizing myelogenous cells to secrete antitumor cytokines will suppress T_{regs} and encourage tumor antigen specific immunity. Tumor-fighting DCs may vaccinate patients to produce tumor fighting memory T-cells. Immunosuppressive barriers that may inhibit immunotherapy are depicted in Figure 1.

DIRECTING THE IMMUNE SYSTEM AGAINST METASTATIC CRC

The reminder of this review discusses methods that treat advanced CRC patients through immune manipulation, and includes perspectives of how immunosuppression stymies these methods. In the context of cancer, immunotherapy broadly encompasses any immune manipulation that eliminates tumor cells. Immunotherapy promises to eradicate microscopic metastases more precisely than a surgeon's scalpel and provide lasting protective immunological memory. The current arsenal used to prime the immune system against occult metastases is limited; however major breakthroughs are propelling research to investigate novel avenues.

Antibody therapy

The monoclonal antibody therapy anti-CTLA-4, or ipilimumab, is prominently used to improve metastatic melanoma patient survival [78]. As a checkpoint blocker, anti-CTLA-4 prevents CTLA-4 from outcompeting the T-cell co-stimulation receptor, CD28. Its effectiveness requires existing immunosuppressed intra-tumoral T-cells, making it the obvious choice for treating highly infiltrated CRCs. However, a pilot study reported no objective responses in 3 colon cancer patients [79], and a larger study using tremelimumab – another anti-CTLA-4 antibody – could not report any conclusive findings other than increased incidence of immune enterocolitis in 45 treatment-refractory CRC patients [80]. Other checkpoint proteins may be more appropriate for CRC therapy.

Programmed death receptor-1/ligand-1 blockade—Programmed death receptor-1/ligand-1 (PD-1/PD-1L) are members of the CD28 and B7 families and induce tolerance through regulation of T-cell. Activated CD8⁺ T-cells express PD-1; however PD-L1/L2 expressing tumor cells, circulating MDSCs [64], and DCs can impart unresponsiveness and

exhaustion of immune-directed T-cell activities [81]. An initial study reported only marginal responses to anti-PD-1 antibody where one CRC patient demonstrated complete response [82]. This study speculated that anti-PD-1 encourages lymphocyte migration into tumors and tissues [82]. Two larger studies included 19 patients with advanced CRC treated with anti-PD-1 [83] and 18 treated with anti-PD-L1 [84], but did not observe equitable clinical responses when compared to other cancers. Considering other tumor types, the first study witnessed objective responses in nine of the 25 patients with PD-L1-positive tumors and none with PD-L1 negative tumors.

A recent study demonstrated that defective mismatch repair CRCs strongly upregulate PD-1 and CTLA-4, as well as other immune checkpoints: PD-L1, lymphocyte activation gene-3 (LAG-3) and indoleamine 2,3-dioxygenase (IDO) [85••]. While these tumors rarely metastasize, these data provide evidence that multidimensional immunosuppression is important in more aggressive tumors. Two interesting notes: approximately 30% of intratumoral CD4⁺ FoxP3⁺ T-cells expressed LAG-3 [86–88], and IDO expression in CRCs is associated with liver metastasis, tumor progression, and decreased overall survival [89, 90]. These data suggest that strong anti-tumor T-cell responses are concomitantly surmounted by multiple mechanisms of immunosuppression.

Questions still remain as to why patients with metastatic melanoma and other cancers benefit from checkpoint blockade while CRC patients do not. The simple answer is previous CRC studies did not reach statistical significance; fortunately larger clinical trials are underway. The complex answer is checkpoint blockers may not provide durable or prolonged protection. For example, ipilimumab allows T_{reg} numbers to rebound after treatment and fails to increase CRC-specific T-cell abundance in peripheral blood [79], tremelimumab treatment fostered misdirected/nonspecific responses with mostly ineffective or undesirable consequences [80], anti-PD-1/PD-L1 antibodies may only be effective against PD-1/PD-L1 positive tumors [83], and finally, blocking only one axis of immunosuppression may doom almost every CRC clinical trial [83, 84].

Adoptive T-cell Therapy

Adoptive cell transfer (ACT) introduces overwhelming numbers of tumor-targeting effector T-cells to circumvent the need to reverse tolerance to tumor antigens. Therapy requires the transfer of autologous T-cells – specific to tumor antigens or transfected to encode tumor directing T-cell receptors (TCRs) – directly to the patient. However, recruiting T-cells to tumor beds is challenging because T-cell infiltration is mitigated by immunosuppressive pressures.

Early attempts to elicit protective immunity against CRCs through adoptive transfer have been effective. Six out of 19 patients exhibited complete or partial response after their lymphocytes were isolated, propagated, sensitized to autologous tumor antigen, and then stimulated with a streptococcal immunopotentiator before transferred back [91]. Fourteen CRC patients received reinfusion of IL-2 stimulated TILs, however did not demonstrate meaningful clinical results other than preserved TCR ζ - and ϵ -chain expression [92]. Three out of 14 gastric and colon cancer patients achieved clinical response with IL-2 stimulated

T-cells. These patients stably expressed TCR ζ -chain on peripheral blood T-cells, which is normally down-regulated with disease progression and immunosuppression [93].

These data suggest that effective adoptive transfer of IL-2 stimulated T-cells must avoid immunosuppressive preprogramming. To achieve this, sentinel lymph node-derived CD4⁺ T_H1-cells were expanded instead of TILs. Intriguingly, 4 out of 9 stage IV CRC patients achieved complete remission [94]. The study was successful because lymphocytes were acquired from lymph nodes that first received drainage from tumors and therefore were segregated from the immunosuppressive environment [94]. T-cells residing in sentinel lymph nodes have greater proliferative potential upon stimulation [95] and should be the standard mode of therapy. To criticize, however, harvesting sentinel lymph nodes complicates surgery and may not yield enough T-cells.

Chimeric antigen receptors—The presumption that TILs are defective through immunosuppressive preprogramming calls for novel approaches that refocus adoptive T-cell therapy. Engineering T-cells to express chimeric antigen receptors (CARs) specific for carcinoembryonic antigen (CEA) – commonly expressed by colorectal tumors – benefits some advanced staged CRC patients. CARs are antigen-specific heavy and light chain antibodies genetically attached to cytoplasmic signaling molecules, such as 4-1BB, OX40, Luk, and TCR ζ -chain [96] and designed to relay activation signals to T-cells without peptide presentation. CAR therapy is advantageous when MHC class I is downregulated [97].

However, an unfortunate case study describes treatment failure for a patient with ERBB2 overexpressing colon cancer. T-cells recognized ERBB2 on normal lung cells and triggered high levels of tumor necrosis factor-alpha (TNF- α) and IFN- γ , pulmonary toxicity, and death [98]. Another study transferred CEA-specific T-cells retrovirally transduced to express murine TCR; although one patient demonstrated regression of lung and liver metastases, all patients demonstrated severe transient inflammatory colitis [99].

These studies highlight how ubiquitously expressed self-antigens in the lungs and throughout the gastrointestinal tract are risky targets for immunotherapy. Future efforts need to consider the perils of targeting self-antigens, or at least weigh the consequences of treatment against clinical benefit.

Lymphodepletion—Suppressor cells, as well as endogenous lymphocytes, effectively add another layer of immunosuppression by serving as sinks for T-cell promoting cytokines IL-7 and IL-15 [100]. Therefore, increased access to homeostatic cytokines may be critical for successful ACT. Lymphodepleting chemotherapy prior to immunotherapy reduces the number of circulating T_{regs}, promotes successful engraftment of adoptively transferred T-cells, and improves the overall survival of melanoma patients [101]. Cyclophosphamide decreased peripheral FoxP3⁺ T_{regs} in all six metastatic CRC patients and increased IFN- γ producing T-cells after 22 days [102]. Local ablation of immune cells using fludarabine, cyclophosphamide, and/or temozomide may dramatically increase the durability of ACT by reducing immunosuppressive pressures and widening a niche for tumor-specific T-cells.

This treatment may best suit tumors devoid of beneficial CD8⁺ T-cells, and not mismatch repair defective CRCs, which are highly infiltrated by CD8⁺ T-cells.

Allogeneic CD34⁺ stem-cell transplantation

Allogeneic CD34⁺ stem-cell transplantation (allo-SCT) is common for treating blood-related diseases; however early attempts with CRC have achieved mild success [103, 104]. A preparative regimen of lymphodepleting drugs is administered before matched donor hematopoietic stem-cells (HSCs) are transferred to cancer patients. The ensuing curative graft-versus-tumor (GvT) effect is driven by donor cytotoxic T-cells that proliferate *in vivo* and target malignancies [105]. Injecting 111-Indium-labeled lymphocytes via the hepatic portal artery confirmed that donor CD3⁺, CD19⁺, and CD56⁺ lymphocytes home to liver metastases [106].

Successful allo-SCT requires local cytokine production. Neutralization of TNF- α and IL-1 β in target epithelium inhibits acute GvT effect in mice [107], suggesting cytokine-mediated cytotoxicity obviates multiple layers of immunosuppression. One allo-SCT patient with CRC demonstrated increased tumoral expression of HLA-class I-associated β 2-microglobulin molecule – an indicator of CD8⁺ T-cell activity – but, not severe enough for any meaningful clinical benefit. Donor cytotoxic T-cells, however, inadvertently targeted the recipient's lymphoid system [108]. Three out of 15 metastatic colon cancer patients receiving allo-SCT experienced disease stabilization or partial remission. Responders harbored intra-tumoral CEA-specific CD8⁺ T-cells [109]. Interestingly, patients receiving HSCs from unrelated donors engrafted CD3⁺ cells faster than those receiving HLA-identical HSCs [110], suggesting non-perfect matching may induce more aggressive GvT effects.

This approach has many obstacles. First, complete T-cell engraftment lags behind myeloid and B-cells and can take over 60 days. Fortunately, patients who fail engraftment can receive infusion of CD3⁺ T-cells [111]. Second, an overwhelming majority of patients who benefit from engraftment eventually succumb to disease, suggesting allo-SCT fades or becomes immunosuppressed by the tumor environment. Third, a study unrelated to gastrointestinal cancer observed striking up-regulation of IDO activity in colon tissues of patients receiving allo-SCT. This is expected because subsequent tryptophan depletion is a hallmark of intense gut inflammation [112]. Therefore, therapeutic inhibition of IDO activity during allo-SCT must be explored. And forth, the number of T-cells generated after transplant is limited; large and advanced tumors may require an absurd number of generated T-cells.

Anti-tumor Vaccines

The ideal vaccine is easy to administer, offers prolonged protection, and induces relatively low toxicity; however no vaccine has induced reproducible clinically relevant regression of mCRC. Induction of memory T-cells activates the anti-tumor cascade and provides prolonged protection against existing micrometastases [113]. Cancer vaccines must effectively break immunological tolerance and induce or amplify antigen-directed T-cell assaults.

Initial efforts to break tolerance to CRC antigens were directed against CEA. When delivered by DNA vaccine, or “naked” plasmid DNA, CEA is presented by MHC class I/CTL pathways [114]. A clinical trial did not detect relevant CEA-specific antibody responses in all 17 metastatic CRC patients, yet 4 patients demonstrated proliferation of peripheral lymphocytes [114]. This proliferation, however, was most likely triggered by CEA expressed by normal tissues. Overcoming tolerance to self-antigens is challenging, especially in profusely immunosuppressive environments, and requires adjuvants to intensify vaccination. Directing an immunological attack against self-antigens while ignoring healthy tissues is fraught with many unidentified obstacles.

Pathogen Derived Adjuvants—Inherent tolerance against self-antigens can be broken using bacterial or viral immunopotentiators. Diphtheria toxin (DT) conjugated to beta-human chorionic gonadotropin (β -hCG) peptide – often expressed by CRCs – induced humoral immune responses in 73% of IV CRC patients. Higher antibody responses to β -hCG associated with increased survival; however patients who mounted stronger antibody responses to DT antigen did not benefit from treatment [115]. Another study treated 161 patients with DT conjugated to gastrin-17 (G-17), a growth factor that contributes to gastrointestinal tumor growth. Three percent of patients achieved partial responses while 32% achieved stable disease, and those who generated antibodies to G-17 survive longer [116]. A promising study utilized adenovirus serotype-5 (Ad5) – known to trigger robust T-cell responses – to deliver CEA. This vaccine induced cell-mediated T-cell responses in 61% of advanced CRC patients; however the small study size could not conclude any survival advantage [117].

Alternatively, immune responses can be directed against tumor antigens by combining irradiated autologous tumor cells with pathogen derived adjuvants. Colon cancer patients survived longer when given autologous tumor cell-bacillus Calmette-Guérin (BCG) vaccines. [118]. A trial consisting of 254 colon cancer patients reported that these benefits are limited to stage II disease [119]. Two other studies used Newcastle disease virus (NDV) as an adjuvant because viral challenge induces production of interferons and RANTES (CCL5) and encourages chemotaxis of cytotoxic lymphocytes. Both studies boasted delayed disease reoccurrence and improved survival for colon cancer patients [120, 121]. No explanation was given to why rectal cancer patients did not benefit from either BCG or NDV. These studies strongly suggest that both the route of antigen presentation and the condition of immunostimulation dictate the efficacy of anti-cancer vaccines. These principles were applied to the next generation of vaccines.

Cytokine Adjuvants—Specific cytokines activate the adaptive immune response and establish T-cell memory. Among multiple signaling layers of antigen presentation and coreceptor ligation, cytokines mobilize immune cells. Granulocyte macrophage colony-stimulating factor (GM-CSF) – an immune stimulatory cytokine – enhances proliferation of T-cells and IFN- γ and IL-2 secretion in multiple myeloma patients immunized with autologous myeloma immunogen [122]. GM-CSF enhanced antibody response to CEA antigen and protected against metastatic CRC. All nine patients subcutaneously immunized and treated with GM-CSF demonstrated stronger dose-dependent IgG antibody responses,

T-cell proliferation, and IFN- γ secretion when compared to those not given GM-CSF [123]. Another study inserted epithelial cellular adhesion molecule (Ep-CAM) – commonly expressed on CRC cells – into a replicative-deficient recombinant avipoxvirus to ensure the antigen is presented through MHC class I and CTL pathway. All 6 patients received GM-CSF, and five generated Ep-CAM-specific IFN- γ ⁺ T-cells and no IL-4 secreting T-cells [124].

Dendritic Cell Vaccines—The previous studies suggest that successful vaccination against tumor antigens may be dependent on GM-CSF-mobilized immune cells. Introduced earlier in this review, DCs reside in dermal tissues and lymph nodes, present antigens, induce protective T-cell responses, and reprogram immunosuppression in the tumor environment and elsewhere in the body. Early attempts to direct a memory response against antigen-presenting tumor cells using DC-based vaccines have been promising [113]. DCs require harvesting, propagation, and specific *ex vivo* manipulation to be coaxed into effective primers of Type-1 T-cells [125].

A series of clinical trials successfully activated CTL responses by vaccinating CRC patients with antigen loaded DCs. Patients with metastatic tumors receiving CEA-pulsed GM-CSF/IL-4-derived DCs (GM/IL4-DCs) demonstrated no signs of autoimmune disease or acute toxicities [126]. Other studies used alternative means to generate DCs with similar results: Flt3-ligand [127] and IL-13 [128]. Either way, the majority of recent DC vaccines are generated using GM-CSF, IL-4, and CEA peptide [129–132].

Theoretically, antigen loading via mRNA introduces a broader range of antigenic epitopes than peptide loading, thereby generating a more potent T-cell repertoire. In contrast however, mRNA transfected GM/IL4-DCs did not produce CEA-reactive T-cells in CRC patients [133]. Regardless of the loading method, GM/IL4-DCs with CEA class I HLA-A2-restricted peptide, CAP-1, or with mRNA encoding CEA, triggered similar antigen-specific T-cell activities *in vitro* [134].

The method of antigen loading may not be most important for effective DC vaccination. Inactivated DCs remain ineffective at promoting protective T-cells and can induce tolerance to target antigens [135]. Immunopotentiators optimize DC-based vaccines and enhance production of the type-1 T-cell mediator IL-12. These include toll-like receptor agonists poly I:C, LPS, Pam3Cys and R848 [136–138]. The final study to be discussed in this review designed a vaccine with hyperstimulatory potential by loading DCs with a poxvector containing genes for CEA and other costimulatory molecules, including CD80. Amazingly, this vaccine induced CEA-specific immune T-cells in 10 out of 12 patients [139]. A summary of all the clinical studies mentioned in this review is listed in Table 3.

Combined, these studies fortify the assumptions that 1) naturally processed peptides via class I MHC to CTLs are sufficient for T-cell priming, 2) DCs can be generated with effective stimulating capacity through multiple means, and 3) properly activated DCs induce protective immunity and avoid inducing tolerance to tumor antigens. DCs vaccines are efficient at generating tumor-specific CD8⁺ T-cells *in vivo*; however the question still

remains to whether these are effective at countering the multiple layers of immunosuppression inherent in the tumor environment.

CONCLUSION

The population of colorectal tumors is heterogeneous in regards to immune cell infiltrates. This explains why directly manipulated only one immune mechanism has had limited success. Most studies described in this review administered experimental treatments to groups of mCRCs only to witness diseases relapse. Confounding treatment, particular mutations within cancer genes distinctly construe different tumor subtypes with unique histology and gene expression. Antibody therapy can only unlock one layer of suppression at a time, CAR T-cell therapy targets only one antigen, and DC-based vaccines activate CTLs that eventually become muted once migrated to the tumor. This Sisyphean cycle of trial and failure mainly proves that tumor environments eventually return to an immunosuppressive state. Improved perception of the multiple immunosuppressive forces interwoven throughout each tumor environment, as seen with mismatch repair defective tumors [85••], will facilitate experimental immunotherapies that progress past the trial stage. Effective immunotherapy needs to counter all layers of immunosuppression, therefore patients may benefit from combinational therapy, barring severe toxicities (Figure 2).

Currently, chemotherapy may provide a better option for altering the tumor environment. Major findings are limited to mouse studies, however MDSCs are inhibited by 5-fluorouracil, which relinquishes its suppressive effects on T-cells [140]. In contrast, irinotecan – a topoisomerase inhibitor [141] used in a cocktail with 5-fluorouracil and leucovorin called FOLFIRI – reverses the inhibitory effects of 5-fluorouracil on MDSCs [142•]. Therefore, the specific effects of combinational chemotherapy on the tumor environment should be fully understood to provide immunotherapies the potency to abrogate all suppressive barriers. Effective immunotherapy must reprogram the tumor environment by either disrupting immune cells that induce tolerance or stromal and tumor cells that anchor immunosuppression.

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Figure 1. Common immunosuppressive barriers in CRC that are potential targets for immunotherapy

Myeloid-derived suppressor cells (MDSCs, brown) release T_{reg} (orange) promoting factors, IL-10, TGF- β , and IDO (grey, purple, and blue), and T-cell suppressing arginase and nitric oxide (olive and brown), A. T_{regs} compete with other T-cells for coreceptor ligation by expressing CTLA-4 and LAG-3. CTLA-4 binds to CD80/CD86 and LAG-3 binds to MHC class II, thereby blocking the transmission of stimulatory signals from DCs (purple) and other antigen-presenting cells (APCs) to effector T-cells, B. T_{regs} and endogenous lymphocytes serve as sinks and limit the amount of homeostatic cytokines, IL-7 and IL-15

(light blue and red), that are available for effector T-cells, C. Chronic T-cell stimulation through viral challenge and cancer antigens perpetuates PD-1 expression leading to effector T-cell exhaustion, D.

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Figure 2. Immunotherapies used to treat metastatic CRC

Immunotherapies designed to direct cytotoxic T-cells to eradicate colorectal tumor cells. Antibody therapy against CRC has two popular targets. Anti-CTLA-4 (purple) liberates CD28 to ligate to CD80/CD86 for CTL (red) activation. Anti-PD-1 (purple) prevents tumor cells (gray) and DCs (violet) from inducing tolerance, unresponsiveness, and exhaustion of T-cell activities. Adoptive cell transfer requires autologous peripheral or sentinel lymph node T-cells (T_H1 -cells, blue) to be isolated and propagated *in vitro* via IL-2 and antigen (red curved bar) stimulation, then delivered back into the patient. Allo-SCT requires donor hematopoietic stem-cells (HSCs, mustard) to be introduced into cancer patients. HSCs mature into lymphoid progenitors (PL, green) and then into functional T-cells (and other lymphocytes) to induce a GvT effect. This effect requires donor CTLs to proliferate *in vivo* and target malignancies. DC vaccines program the immune system to mount a durable memory response against tumor antigens. DCs are derived *ex vivo* from peripheral immune cells precursor, then propagated and challenged with tumor antigens. Mature and activated DCs are delivered back into the patient and migrate to the lymph nodes to prime and educate naive T-cells against tumor antigens. In the lymph node, primed T_H1 cells release IFN- γ (dark green) to activate CD8⁺ T-cells. Once mature, CTLs migrate to tumor cells and release cytotoxic mediators (dark red) upon recognition of antigen-MHC complex (light green) on the tumor cell surface.

Table 1

Ability of common lymphocyte markers to predict CRC patient survival

Marker	Reference	Survival	Patient Benefit
CD3/Lymphocytes	[143, 144]	CS	Inconclusive
	[15, 145–149]	DFS	Positive
	[15, 143, 145, 147, 150, 151]	OS	Positive
CD8	[12, 14, 17, 143, 144, 152]	CS	Positive
	[14, 17, 145, 153–156]	DFS	Positive
	[14, 16, 143, 145, 153, 155–160]	OS	Positive
FoxP3	[54, 55, 143]	CS	Inconclusive
	[155]	DFS	Inconclusive
	[143, 155, 160–162]	OS	Inconclusive
CD56/CD57	[44]	OS	Inconclusive

CS, Cancer-specific survival; DFS, Disease-free survival; OS, Overall survival

Table 2

Effect of common immune regulating mechanisms on T-cells

Marker	Effect on effector T-cells
T _{regs}	Down-regulate immune responses by secreting modulating cytokines and expressing modulating receptors [46].
MDSCs	Promote angiogenesis and expansion of T _{regs} and suppress effector T-cells [67, 68].
Immature DCs	Promote immunosuppressive secretion from T-cells [73].
PD-1	Chronic viral challenge and cancer perpetuates PD-1 expression and exhausts T-cell [81].
CTLA-4	Prevents CD28 binding to co-receptors CD80 and CD86 during T-cell activation [49].
LAG-3	Expressed by T _{regs} and completes with CD4 binding to MHC class II, thereby inhibiting T _H 1-cells activation [86•].
IDO	Inhibits T-cell proliferation [89].

CTLA-4, cytotoxic T-lymphocyte antigen 4; DC, dendritic cells; IDO, indoleamine 2,3-dioxygenase; LAG-3, lymphocyte activation gene-3; MDSCs, myeloid-derived suppressor cells; PD-1, programmed death receptor-1; T_{regs}, regulatory T-cells

Table 3

Summary of important studies that targeted immune cells for treating metastatic CRC

Immunotherapy	Targeted Antigen	Adjuvants/immune modulator	Study population and size	Remarks	Reference
Ipilimumab	CTLA-4	None	3 colon cancer	T _{regs} were depressed early in treatment, but no increase in CD8 ⁺ T-cell responses.	[79]
Tremelimumab	CTLA-4	None	45 treatment-refractory CRC	One patient demonstrate partial response, while others experienced enterocolitis.	[80]
MDX-1106	PD-1	None	14 advanced CRC and 25 other cancers	One patient demonstrated durable complete response. Inflammatory colitis or adverse events occurred in 3 patients.	[82]
BMS-936558	PD-1	None	19 advanced CRC	No objective responses in CRC patients. One CRC patient died from treatment.	[83]
BMS-936559	PD-L1	None	18 advanced CRC	No objective responses in CRC patients.	[84]
AIT (Lymph node and peripheral lymphocytes)	Sonicated tumor extract	T-cell growth factors and OK-432 (Streptococcal preparation)	19 gastric or colorectal cancers with metastasis to the liver	2 patients demonstrated complete response, 4 with partial.	[91]
AIT (Tumor-associated lymphocytes)	Autologous tumor	IL-2	14 stage IV gastric and colorectal cancer	3 patients were protected from TCR- ζ down-regulation and experienced clinical response.	[93]
AIT (TILs)	Untargeted	IL-2	14 metastatic CRC	No difference was observed from control group.	[92]
AIT (CD4 ⁺ T-cells)	Autologous tumor	IL-2	16 CRC	4 of 9 stage IV patients experienced complete remission.	[94]
CAR (PBLs)	ERBB2	Anti-CD3 and IL-2	1 colon cancer	Patient died from respiratory distress.	[98]
CAR (PBLs)	CEA	Anti-CD3 and IL-2	3 metastatic CRC	1 patient demonstrated regression of lung and liver metastasis. All patients experienced severe inflammatory colitis.	[99]
Lymphodepletion	Untargeted	Cyclophosphamide	27 CRC	Increased IFN- γ -producing T-cells.	[102]
Allo-SCT	Untargeted	Busulfan and fludarabine	4 metastatic CRC	No significant toxicities were detected.	[103]
Allo-SCT	Untargeted	Busulfan and fludarabine	1 chemoradiotherapy resistant CRC	Severe inflammatory cell infiltration into mucosa. Patient did not achieve remission.	[104]
Allo-SCT	Untargeted	Fludarabine and total body irradiation	3 CRC and 4 other cancers	3 patients demonstrated stable disease for at least 18 months.	[106]
Allo-SCT	Untargeted	Fludarabine and total body irradiation	1 CRC	Patient died of pneumonia 4 months after transplant. Most metastases were necrotic with few remaining tumor cells.	[108]
Allo-SCT	Untargeted	Fludarabine and total body	15 metastatic CRC	3 patients demonstrated stable disease and 1	[109]

Immunotherapy	Targeted Antigen	Adjuvants/immune modulator	Study population and size	Remarks	Reference
		irradiation		with partial response.	
Allo-SCT	Untargeted	Fludarabine and total body irradiation	6 colon cancer and 12 other cancers	2 colon cancer patients demonstrated regression. 4 patients died from transplant.	[110]
Allo-SCT	Untargeted	Combinations of cyclophosphamide, busulfan, fludarabine, and total body irradiation	39 refractory metastatic CRC	6 stable disease and 2 partial response.	[111]
DNA vaccine	CEA	None	17 metastatic CRC	No clinical response. 4 patients demonstrated increased numbers of circulating CEA-specific lymphocytes.	[114]
Peptide vaccine	β -hCG	Diphtheria toxoid	77 metastatic CRC	The 56 patients who generated antibodies generally lived longer.	[115]
Peptide vaccine	G-17	Diphtheria toxoid	161 CRC	3 partial responses and 32 stable disease. Patients who generated antibodies generally lived longer.	[116]
Peptide vaccine	CEA	Ad5	25 advanced CRC	12 patients lived past 12 months. Did not report impact on survival.	[117]
Autologous tumor cell vaccine	Autologous tumor cells	BCG	80 CRC	Colon cancer patients demonstrated improved survival. Rectal cancer patients did not improve.	[118]
Autologous tumor cell vaccine	Autologous tumor cells	BCG	128 colon cancer	Vaccinated patients demonstrated fewer recurrences.	[119]
Autologous tumor cell vaccine	Autologous tumor cells	NDV	23 metastatic CRC	Vaccinated patients demonstrated fewer recurrences.	[120]
Autologous tumor cell vaccine	Autologous tumor cells	NDV	25 metastatic CRC	Vaccinated patients demonstrated improved metastasis-free and overall survival.	[121]
Peptide vaccine	CEA	GM-CSF	9 CRC	All patients receiving GM-CSF generated IFN- γ secreting T-cells. No autoimmunity was reported.	[123]
Peptide vaccine	Ep-CAM	GM-CSF and ALVAC	12 CRC	The GM-CSF patients strongly generated anti-Ep-CAM-specific Type-1 responses.	[124]
DC vaccine	CAP-1	GM-CSF and IL-4	21 CRC	1 patient demonstrated minor response and 1 demonstrated stable disease. No observable autoimmunity.	[126]
DC vaccine	CAP-1	GM-CSF and IL-4	17 metastatic CEA-expressing cancer	No difference between peptide and mRNA loading.	[134]
DC vaccine	CAP-1	GM-CSF, IL-4, PGE ₂ , TNF- α , IL-1 β , and IL-6	16 metastatic CEA-expressing cancer	8 of 11 receiving peptide demonstrated T-cell responses. None of the mRNA group responded.	[133]
DC vaccine	CEA	GM-CSF, IL-4, PGE ₂ , TNF-	7 CRC and 2 lung	CEA-reactive CD8 ⁺ T-cells were expanded. No	[129]

Immunotherapy	Targeted Antigen	Adjuvants/immune modulator	Study population and size	Remarks	Reference
		α , IL-1 β , and IL-6	cancer	tumor regression was observed.	
DC vaccine	CEA	GM-CSF, IL-4, PGE ₂ , and TNF- α	10 metastatic CRC	CEA-reactive CD8 ⁺ T-cells were expanded in 7 patients. 2 patients demonstrated stable disease for at least 12 months.	[130]
DC vaccine	CEA	GM-CSF, IL-4, TNF- α , and IFN- α	10 metastatic CEA-expressing cancer	2 patients responded with positive delayed-type hypersensitivity test.	[131]
DC vaccine	CAP-1	GM-CSF, IL-4, TNF- α , and IFN- α	7 stage III CRC	4 of 7 patients generated CEA-specific T-cell responses.	[132]
DC vaccine	CEA	Flt3L	9 colon, 1 rectum, and 2 lung	2 patients demonstrated complete response, and 2 with stable disease.	[127]
DC vaccine	CEA and other peptides	GM-CSF, IL-13, Klebsiella-derived cell wall fraction, and IFN- γ	11 advanced CRC	3 patients generated CEA-specific T-cells.	[128]
DC vaccine	CAP-1	GM-CSF, IL-4, and poxvector containing B7.1 (CD80), ICAM-1 (CD54), and LFA-3 (CD58)	11 CRC and 3 non-small lung cancer	10 patients generated CEA-specific T-cells. No toxicities were reported.	[139]

AIT, Adoptive Immunotherapy; Allo-SCT, allogeneic stem-cell transplantation; ALVAC, replicative-deficient recombinant avipoxvirus; BCG, bacillus Calmette-Guérin; β -hCG, beta-human chorionic gonadotropin CAP-1, carcinoembryonic antigen peptide-1; CAR, chimeric antigen receptor; CEA, carcinoembryonic antigen; CRC, colorectal cancer; CTLA-4, cytotoxic T-lymphocyte antigen 4; DC, dendritic cells; ERBB2, Ep-CAM, epithelial cellular adhesion molecule; Flt3L, Flt3-ligand G-17, gastrin-17; GM-CSF, granulocyte macrophage colony-stimulating factor; ICAM-1, intercellular adhesion molecule-1; IFN, interferon; LFA-3, leukocyte function-associated antigen-3; NDV, Newcastle disease virus; PBLs, peripheral blood lymphocytes; PD-1/PD-1L, programmed death receptor-1/ligand-1; PGE₂, prostaglandin E₂; TCR, T-cell receptor; TILs, Tumor-infiltrating lymphocytes; TNF, tumor necrosis factor; Tregs, regulatory T-cells