



## 2016 Colorectal Cancer: Global view

# Dendritic cell-based cancer immunotherapy for colorectal cancer

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## Abstract

Colorectal cancer (CRC) is one of the most common cancers and a leading cause of cancer-related mortality worldwide. Although systemic therapy is the standard care for patients with recurrent or metastatic CRC, the prognosis is extremely poor. The optimal sequence of therapy remains unknown. Therefore, alternative strategies, such as immunotherapy, are needed for patients with advanced CRC. This review summarizes evidence from dendritic cell-based cancer immunotherapy strategies that are currently in clinical trials. In addition, we discuss the possibility of antitumor immune responses through immunoinhibitory PD-1/PD-L1 pathway blockade in CRC patients.

**Key words:** Colorectal cancer; Dendritic cell; Cancer immunotherapy; Cytotoxic T lymphocyte; Immune-checkpoint inhibitors

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**Core tip:** Dendritic cell (DC) is potent antigen-presenting cells that play a pivotal role in the induction of antitumor immune responses. Strategies for delivering antigens to DCs have been developed and used in clinical trials

in cancer patients, including colorectal cancer (CRC). Numerous reports indicate that the use of DC-based immunotherapy for CRC patients is promising to induce antigen-specific CTL responses. However, the immune suppression induced through CRC and the tumor microenvironment continues to be a major hurdle. Thus, the combination of DC-based immunotherapy with immune-modulating agents may be necessary to maximize antitumor immunity. These combinatorial therapies may have the potential for clinical benefit.

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## INTRODUCTION

Colorectal cancer (CRC) is a common cancer and remains one of the leading causes of cancer-related deaths worldwide. Although surgery is the only curative treatment available for localized disease, more than 20% of CRC patients are not eligible for surgery due to liver metastases at the time of diagnosis<sup>[1]</sup>. To date, surgery, neoadjuvant radiotherapy and adjuvant chemotherapy have improved the outcome of CRC patients; however, 50% of patients still die from recurrent or metastatic disease<sup>[1,2]</sup>. Indeed, the treatment of CRC patients with distant metastases or recurrence through surgery or chemotherapy currently remains limited. Therefore, alternative strategies, including immunotherapy, for treating advanced CRC have been considered<sup>[3]</sup>.

Recent studies have suggested that CRC is a good candidate for immunotherapy. As potential targets for cancer immunotherapy, human CRC cells express numerous numbers of tumor-associated antigens (TAAs), such as carcinoembryonic antigen (CEA)<sup>[4-6]</sup>, Wilms' tumor gene 1 (WT1)<sup>[7,8]</sup>, mucin 1 (MUC1)<sup>[4,9]</sup>, melanoma-associated antigen gene (MAGE)<sup>[10-12]</sup>, or p53<sup>[13]</sup>. Moreover, CRC is a heterogeneous disease with genetic and epigenetic characterizations, such as the mutation of oncogenes, microsatellite instability (MSI) phenotype, chromosomal instability (CIN) pathway, CpG island methylator phenotype (CIMP), and DNA hypomethylation<sup>[14]</sup>. For example, the MSI phenotype reflects various deficiencies in the DNA mismatch-repair system, leading to an increased mutation rate of oncogenes<sup>[15]</sup>. The CIN pathway in cancers reveals aneuploidy and chromosomal rearrangements<sup>[15]</sup>. Cancers with the CpG island methylator phenotype (CIMP) exhibit DNA methylation associated with the transcriptional inactivation of tumor-suppressor genes<sup>[15]</sup>. These genetic and epigenetic characterizations lead to multiple mutations of oncogenes, resulting

in immunogenic CRC. Therefore, some patients with CRC may be effective candidates for immunotherapy. Moreover, immunotherapy mediates a potent antitumor effect when combined with chemotherapy and/or radiotherapy<sup>[16-18]</sup>. Indeed, cancer immunotherapy targeting these TAAs can be combined with surgery, radiotherapy, and conventional chemotherapy for treating patients with CRC. Interestingly, given the success of immune-checkpoint inhibitors in several tumors, we believe that cancer immunotherapy may also be combined with immune checkpoint blockade agents to induce efficient antitumor immunity in CRC patients.

## ANTITUMOR IMMUNITY

T cells with the  $\alpha\beta$  T cell receptor (TCR) generally express CD4<sup>+</sup> or CD8<sup>+</sup> lineage markers and have primarily been classified as helper or cytotoxic subsets, respectively<sup>[19]</sup>. Major histocompatibility complex (MHC) class I molecules on cancer cells bound to antigenic peptide derived from tumor-associated antigens (TAAs) are recognized by the TCR of CD8<sup>+</sup> T cells. However, CD4<sup>+</sup> T cells recognize peptides in association with MHC class II molecules on antigen-presenting cells (APCs)<sup>[3,19]</sup>. The goal of cancer immunotherapy is to induce efficient antigen-specific cytotoxic CD8<sup>+</sup> T cells (CTLs). The induction of efficient CD8<sup>+</sup> CTLs requires helper functions mediated through CD4<sup>+</sup> T cells *via* the production of cytokines, such as interleukin (IL)-2 and interferon (IFN)- $\gamma$ , resulting in the maintenance of antigen-specific CD8<sup>+</sup> CTLs<sup>[20,21]</sup>. Therefore, the simultaneous interaction of the TCR of T cells with antigenic peptides/MHC class I and class II complexes on APCs is essential for the induction of CD4<sup>+</sup> and CD8<sup>+</sup> T cell-mediated antitumor immune responses. Moreover, antigen-specific CD8<sup>+</sup> CTLs respond to antigenic peptides presented by MHC class I molecules on cancer cells and identify and kill TAA-expressing cancer cells.

Dendritic cells (DCs) are potent APCs that play a pivotal role in the initiation, programming, and regulation of antitumor immune responses<sup>[20]</sup>. DCs capture antigens, resulting in a mature phenotype and the release of IL-12 from DCs. The exogenous antigens are processed by DCs, and antigenic peptides are presented on MHC class I molecules, a process known as antigen cross-presentation<sup>[20]</sup>. In addition, DCs also process endogenously synthesized antigens into antigenic peptides, presented to MHC class I molecules. However, exogenous antigens are also processed to antigenic peptides and complexed with MHC class II molecules<sup>[20,21]</sup>. Antigen presentation primarily occurs in the draining lymph node, where antigenic peptides are presented by DCs, resulting in the simultaneous activation of CD4<sup>+</sup> and CD8<sup>+</sup> T cells. Moreover, interactions between DCs and innate and innate-like immune cells, such as natural killer (NK), invariant natural killer T (iNKT), and  $\gamma\delta$  T cells,

can bypass the T helper arm in CTL induction<sup>[22,23]</sup>. NK, iNKT, and  $\gamma\delta$  T cells also have the ability to attack tumor cells directly<sup>[23]</sup>. Therefore, efficient induction of antitumor immunity *via* DC-based cancer vaccines may require interaction between DCs and innate and innate-like immune cells with central roles in DC-based cancer immunotherapy<sup>[23,24]</sup>.

Cancer immunotherapy, including peptide vaccines, whole tumor cell vaccines, viral vector vaccines, and adopted cell transfer therapy, have been developed to treat CRC patients<sup>[3]</sup>. In particular, peptide vaccines have been widely tested in clinical trials, reflecting the simple, safe, stable, and economical features of these vaccine types. However, there are several drawbacks to the peptide vaccines, including: (1) limitations due to the MHC type; (2) limited numbers of identified epitopes; and (3) impaired DC function in cancer patients<sup>[3,25]</sup>. Therefore, DCs have been loaded with multiple antigenic peptides<sup>[26-28]</sup>, whole tumor cell-mRNA<sup>[29]</sup>, whole tumor cell lysates<sup>[30]</sup>, and whole tumor-derived apoptotic bodies<sup>[31]</sup> or fused with whole tumor cells to form hybrid cells (DCs-tumor fusions)<sup>[32]</sup>. DC-tumor fusion cells process a broad array of TAAs, including both known and unidentified, and present these molecules by MHC class I and class II pathways in the context of co-stimulatory molecules<sup>[32,33]</sup>. In our laboratory, patient-derived DCs are generated through adherent mononuclear cells from a single leukapheresis collection after culture in the presence of granulocyte macrophage colony-stimulating factor (GM-CSF) and IL-4. Immature DCs are matured with penicillin-killed and lyophilized preparations of a low-virulence strain (Su) of *Streptococcus pyogenes* (OK-432) and with prostaglandin E2 (PGE2). Subsequently, a large number of DCs can be cryopreserved in ready-for-use aliquots for immunotherapy<sup>[27]</sup>.

## IMMUNOSUPPRESSION MECHANISMS

Although antigen-specific CTLs are induced in cancer patients, cancer cells often escape immune surveillance through several mechanisms, including (1) the down-regulation of certain antigens, TAP-1/2, MHC class I, or peptide-processing machinery in tumor cells<sup>[34,35]</sup>; (2) the induction of regulatory T cells (Tregs) producing proinflammatory and immunosuppressive cytokines, such as IL-10 and TGF- $\beta$ <sup>[36]</sup>; (3) the presence of immunosuppressive cells (*e.g.*, cancer-associated fibroblasts (CAFs), M2 macrophages, myeloid-derived suppressor cells (MDSCs), immunosuppressive tumor-associated macrophages (TAMs), tolerogenic DCs, and Tregs) in the tumor microenvironment<sup>[36]</sup>; (4) the production of multiple immune suppressive factors from tumor cells<sup>[37]</sup>; and (5) the expression of immune checkpoint blockade between tumor cells and activated T cells<sup>[38,39]</sup>. Although, activated CD8<sup>+</sup> T cells associated with clinical prognosis often infiltrate in CRC<sup>[40]</sup>, this benefit is controlled through immune suppressive cell

populations in the tumor microenvironment, promoting tumor escape from immune surveillance<sup>[14,41,42]</sup>. The direct production of immune suppressive factors, such as IL-6, IL-10, TGF- $\beta$ , vascular endothelial growth factor (VEGF), soluble Fas ligand (Fas-L), and indolamine-2,3-dioxygenase (IDO), by tumor cells also promotes the accumulation of heterogeneous populations of CAFs, M2 macrophages, TAMs, MDSCs, tolerogenic DCs, and Tregs<sup>[37]</sup>. These immunosuppressive cells in the tumor microenvironment inhibit antitumor immunity through various mechanisms, including the elaboration of arginase (Arg), nitrogen oxide (NO), and reactive oxygen species (ROS) from immunosuppressive cells<sup>[37]</sup>. Indeed, the tumor microenvironment is extremely complex and suppresses antitumor immunity, thus explaining why cancer immunotherapy is occasionally unsuccessful<sup>[41]</sup>. Therefore, the functional inhibition of Arg, NO, or ROS in immunosuppressive cells may augment antitumor immunity.

Programmed death 1 (PD-1) is expressed on the surface of activated T cells and inhibits T cell activation upon binding to the associated ligands PD-L1/PD-L2<sup>[3]</sup>. Moreover, cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) is another mechanism that inhibits T cell activation upon binding CD80/CD86 on DCs. CTLA-4 is expressed on naive or memory T cells. PD-1 is highly expressed in antigen-specific CTLs and activated DCs. PD-L1 is not constitutively expressed in some tumors but is induced in response to inflammatory signals, such as IFN- $\gamma$ , produced by antigen-specific CTLs. In contrast, the CTLA-4-mediated immune checkpoint is induced in T cells during the initial response to antigen. Therefore, antibodies can be used to block inhibitory ligand-receptor interactions by acting on tumor cells and DCs (*e.g.*, anti-PD-L1) or T cells (*e.g.*, anti-CTLA-4 or anti-PD1). Indeed, CRC cells express PD-L1 associated with CTL inactivation and Treg development in the tumor microenvironment, resulting in worse survival<sup>[43-45]</sup>. Combining the blockade of multiple immune inhibitory pathways may synergistically activate antitumor immunity.

## DC-BASED PASSIVE IMMUNOTHERAPY

DC-based cancer immunotherapy has been developed to induce TAA (*e.g.*, CEA, WT1, MAGE, or MUC1)-specific CTLs in patients with CRC. To date, various strategies for delivering TAAs to DCs have been developed and tested in clinical trials in cancer patients, including CRC (Table 1). In particular, as most CRC cells express CEA, CEA-targeted DC-based CRC immunotherapy has been reported.

### CEA

CEA is a so-called onco-fetal antigen abundantly present in a majority of CRC cases. Importantly, the elevated expression of CEA is associated with adenocarcinoma, particularly CRC. Therefore, CEA-

**Table 1 Clinical trials of dendritic cell-based cancer immunotherapy in patients with colorectal cancer**

Targets	Immunotherapy	Phase	Patients	Results	Ref.	Year
CEA	DCs loaded with CEA peptide (HLA-A2 restricted)	I	21 advanced CEA-expressing malignancies including 11 CRC	Skin punch biopsy at DC injection sites demonstrated pleomorphic, perivascular infiltration of cells consistent with a DTH response	Morse <i>et al</i> <sup>[46]</sup>	1999
	DCs loaded with CEA mRNA	I	13 patients with resected hepatic metastases of CRC	9 of the 13 CRC patients relapsed at a median of 122 d	Morse <i>et al</i> <sup>[47]</sup>	2003
	DCs modified with a recombinant fowlpox vector encoding CEA and a triad of costimulatory molecules [rF-CEA(6D)-TRICOM]	I	14 patients with HLA-A2 (11 CRC and 3 non-small cell lung cancer)	CEA-specific T cells responses were detected in 10 patients; 5 patients were stable through at least 1 cycle of immunization (3 mo)	Morse <i>et al</i> <sup>[48]</sup>	2005
	Fowlpox vector encoding CEA	I	14 patients (5 CRC, 3 lung cancer, and 1 urachal adenocarcinoma)	Of the 9 patients analyzed, all with stable disease ( <i>n</i> = 5) displayed increased NK activity	Osada <i>et al</i> <sup>[49]</sup>	2006
	Mature DCs induced by activation with a combination of OK-432, low-dose prostanoind, and IFN- $\alpha$ and loaded with CEA peptide		10 CRC patients	CRC patients with stable disease ( <i>n</i> = 8) exhibited increased levels of NK cell frequency and CEA-specific CTL activity with a central memory phenotype. Lack of CTL activity was found in 2 CRC patients with progressive disease, but NK cell proliferation was detected	Sakakibara <i>et al</i> <sup>[51]</sup>	2011
	DCs loaded with altered CEA peptide (HLA-A2 restricted) with Flt3 ligand	I	12 patients with HLA-A2 <sup>+</sup> malignancies (10 CRC and 2 non-small cell lung cancer)	CEA-specific CD8 <sup>+</sup> CTLs were detected in 7 patients; 1 patient with progressive metastatic CRC had a complete resolution of pulmonary metastasis and malignant pleural effusion at 4 mo after vaccination, and 1 patient with CRC developed a mixed response after vaccination, with regression of some but not all liver metastases	Fong <i>et al</i> <sup>[52]</sup>	2001
	DCs loaded with CEA peptide (HLA-A24 restricted)	I	10 advanced CEA-expressing malignancies including 7 CRC	2 patients (CRC and lung cancer) exhibited positive DTH reactions against CEA and remained stable for 6 and 9 mo, respectively	Itoh <i>et al</i> <sup>[53]</sup>	2002
	DCs loaded with CEA peptides (HLA-A2- or HLA-A24-restricted)	I	10 CRC patients (6 HLA-A24 and 4 HLA-A2) who had failed standard chemotherapy	CEA-specific CTLs were detected in 7 patients; 2 patients exhibited stable disease for at least 12 wk	Liu <i>et al</i> <sup>[54]</sup>	2004
	DCs loading with CEA peptide (HLA-A24 restricted)	I	8 patients with advanced CEA-expressing gastrointestinal malignancies (7 CRC and 1 gall bladder cancer)	4 patients developed CEA-specific CTL responses; a DTH reaction was observed in 1 patient, with skin biopsy at the injection site showing lymphocyte infiltration, and 3 patients, including 2 CRC, exhibited stable disease after vaccination	Matsuda <i>et al</i> <sup>[55]</sup>	2004
	DCs loaded with CEA peptide (HLA-24 restricted)	I	8 patients with CEA-expressing metastatic gastrointestinal or lung adenocarcinoma	Long-term stable disease or marked decreases in the serum CEA level was observed in some patients. CEA-specific immune responses were demonstrated in most of the patients in whom treatment was clinically effective	Ueda <i>et al</i> <sup>[56]</sup>	2004
WT1	DCs loaded with CEA peptide (HLA-2 restricted)	I	10 CRC patients with resection of liver metastases	CEA-specific CTLs were demonstrated in 7 patients; CEA-specific CTLs were detected in a resected lymph node in 1 patient	Lesterhuis <i>et al</i> <sup>[57]</sup>	2006
	DCs loaded with CEA altered peptide	I	9 patients with CEA-expressing malignancies (7 CRC and 2 lung cancer)	5 patients exhibited CEA altered peptide-specific CTL responses, and 3 patients exhibited CEA-specific CTL responses	Babatz <i>et al</i> <sup>[58]</sup>	2006
	DCs loaded with WT1 peptide (MHC class I and class II restricted)	I	3 advanced CRC	WT1-specific CTLs were detected and persisted for 2 yr with prolonged disease-free and overall survival	Shimodaira <i>et al</i> <sup>[8]</sup>	2015



MAGE	DCs loaded with MAGE-3 peptide (HLA-A2 or A24 restricted)	I	12 patients with advanced gastrointestinal carcinoma (6 stomach, 3 esophagus, and 3 CRC)	MAGE-3-specific CTL responses were observed in 4 patients. Tumor markers were decreased in 7 patients, and evidence of minor tumor regression was detected in 3 patients	Sadanaga <i>et al</i> <sup>[65]</sup>	2001
	DCs loaded with MAGE-3 or MAGE-1 peptides (HLA-A2 or A24 restricted)	I	28 patients with advanced gastrointestinal carcinoma, including 7 CRC	Peptide-specific CTL responses, tumor marker decreases, and minor tumor regressions were observed in some patients after vaccination	Tanaka <i>et al</i> <sup>[66]</sup>	2008
CEA and MUC1	DCs modified with CEA/MUC1 (PANVAC)	II	74 patients, disease free after CRC metastasectomy and perioperative chemotherapy	CEA-specific CTLs were detected	Morse <i>et al</i> <sup>[67]</sup>	2013
CEA, MAGE, and HER2	DCs loaded with CEA/MAGE/HER2/neu/pan-DR peptides (HLA-A2 restricted) and keyhole limpet hemocyanin (KLH) protein	I	13 advanced CRC	All patients exhibited progressive disease. CEA-specific CTLs were detected in 3 of 11 evaluated patients. Multiple TAAs-specific CTLs were induced	Kavanagh <i>et al</i> <sup>[70]</sup>	2007
Autologous whole tumor mRNA	DCs transfected with whole-tumor mRNA	I	15 advanced CRC received the immunotherapy and KLH intravenously	11 of the 13 CRC patients evaluated developed a positive KLH skin test, and 7 CRC patients exhibited CEA-specific responses	Rains <i>et al</i> <sup>[75]</sup>	2001
Autologous whole tumor cells	DCs-autologous whole-tumor fusion cells and IL-12	I	5 gastrointestinal tumors, including CRC	Among the 3 patients evaluated, 1 exhibited stable disease, and 2 exhibited progressive disease. No DTH-positive patients were detected in this trial. Good therapeutic responses in some patients with brain tumors were detected	Homma <i>et al</i> <sup>[78]</sup>	2005
Allogeneic whole tumor cell lysate	DCs loaded with allogeneic tumor cell lysate	I	6 advanced CRC (HLA-A2)	Antitumor immune responses in some patients and transient stabilization or even reduction of CEA levels were detected	Tamir <i>et al</i> <sup>[82]</sup>	2007
	DCs loaded with allogeneic melanoma cell lysate expressing at least one of six MAGE-A antigens	II	20 advanced CRC	1 patient experienced a partial response, 7 patients achieved stable disease, and 5 patients exhibited prolonged progression-free survival	Toh <i>et al</i> <sup>[83]</sup>	2009

DC: Dendritic cell; CRC: Colorectal cancer; DTH: Delayed-type hypersensitivity; CTL: Cytotoxic T lymphocyte.

targeted cancer immunotherapy has been developed. Morse *et al*<sup>[46]</sup> first conducted a phase I study using DCs loaded with an HLA-A2-restricted CEA peptide for the treatment of patients with 21 advanced CEA-expressing malignancies, including 11 CRC cases. One patient with ovarian cancer had a minor response, and one patient with breast cancer exhibited stable disease. Skin punch biopsy at DC injection sites demonstrated the pleomorphic, perivascular infiltration of cells consistent with a delayed-type hypersensitivity (DTH) response. This group also reported a phase II study of 13 patients with resected hepatic metastases of CRC, who received DCs loaded with CEA mRNA (DC/CEA mRNA). The administration of DC/CEA mRNA to CRC patients was feasible and safe. Nine of the 13 patients relapsed at a median of 122 days<sup>[47]</sup>. Furthermore, DCs modified with a recombinant fowlpox vector encoding CEA and a triad of costimulatory molecules [rF-CEA(6D)-TRICOM] was developed from the same group<sup>[48]</sup>. In this trial, 14 patients with HLA-A2 (11 with CRC and 3 with non-small cell lung cancer) were enrolled. CEA-specific T cells responses were detected in 10 patients. Five patients were stable through at least one cycle of immunization (3 mo)<sup>[48]</sup>. As recent reports indicate that DC-NK cell interaction plays a

critical role in the induction of antitumor immunity<sup>[23,24]</sup>, the same group conducted a phase I clinical trial of a vaccine consisting of autologous DCs loaded with a fowlpox vector encoding CEA<sup>[49]</sup>. Fourteen patients (5 CRC, 3 lung cancer, and 1 urachal adenocarcinoma) were enrolled in the trial; of the 9 patients analyzed, all with stable disease ( $n = 5$ ) exhibited increased NK activity. Therefore, NK responses following DC vaccination may correlate with clinical benefit, and evaluation of NK responses should accordingly be included as a biomarker for DC-based cancer vaccines in clinical trials<sup>[49,50]</sup>. Another recent clinical trial also supports the importance of NK activity in CEA peptide-loaded DC-based cancer vaccines. In this trial, mature DCs activated by a combination of OK-432, low-dose prostanoind, and IFN- $\alpha$  were used<sup>[51]</sup>, loaded with the CEA peptide and administrated to 10 CRC patients. Interestingly, the CRC patients with stable disease ( $n = 8$ ) exhibited increased levels of NK cell frequency and CEA-specific CTL activity with a central memory phenotype. Conversely, a lack of CTL activity was observed in those with progressive disease, even though NK cell proliferation was detected. To induce efficient CEA-specific CTL responses, another study developed altered CEA peptides restricted with HLA-

A2-loaded DCs, which were administered along with Flt3 ligand, a hematopoietic growth factor, to 12 patients with CRC ( $n = 10$ ) or non-small cell lung cancer ( $n = 2$ )<sup>[52]</sup>. After vaccination, the expansion of CEA-specific CD8<sup>+</sup> CTLs was detected in 7 out of 12 patients. Interestingly, 2 out of 12 CRC patients experienced dramatic tumor regression. One patient with progressive metastatic CRC had a complete resolution of pulmonary metastasis and malignant pleural effusion at 4 mo after vaccination, and one patient with CRC developed a mixed response after vaccination, with the regression of some but not all liver metastases. Clinical trials of DCs loaded with HLA-A24 restricted CEA peptides have also been reported. The vaccines were injected with adjuvant cytokines, such as natural human interferon alpha (IFN- $\alpha$ ) and natural human tumor necrosis factor alpha (TNF- $\alpha$ ), in patients with 10 advanced CEA-expressing metastatic malignancies, including 7 CRC cases<sup>[53]</sup>. Two patients (CRC and lung cancer) exhibited positive DTH reactions against CEA remained stable for 6 mo and 9 mo, respectively. Therefore, HLA-A24 and A2-restricted CEA peptide might be useful for inducing CEA-specific immune responses. Liu *et al.*<sup>[54]</sup> immunized 10 metastatic CRC patients (6 patients with HLA-A24 and 4 with HLA-A2) who failed standard chemotherapy with DCs loaded with HLA-A2- or HLA-A24-restricted CEA peptides. In this clinical trial, the DC vaccine was injected into one inguinal lymph node under sonographic guidance. After vaccination, CEA-specific T cells were detected in 7 out of 10 patients. Two patients exhibited stable disease for at least 12 wk. Matsuda *et al.*<sup>[55]</sup> also conducted a pilot study of DCs loaded with HLA-A24-restricted CEA peptide for 8 patients with advanced CEA-expressing gastrointestinal malignancies (7 CRCs and 1 gall bladder cancer). Four out of 7 patients developed CEA-specific CTL responses after vaccination. A DTH reaction was observed in 1 patient. Skin biopsy at the injected site showed the infiltration of lymphocytes. Three patients, including 2 CRCs, exhibited stable disease after vaccination. Reports from clinical trials using DCs loaded with HLA-restricted CEA peptide vaccines have also been reported in Japan, as 60% of the Japanese population and some Caucasians express HLA-A24. Ueda *et al.*<sup>[56]</sup> injected the vaccines into 8 patients with CEA-expressing metastatic gastrointestinal or lung adenocarcinomas positive for HLA-A24. In this trial, no definite tumor shrinkage was observed; however, long-term stable disease or marked decreases in the serum CEA level was observed in some patients after therapy. CEA-specific immune responses have also been demonstrated in most of the patients in whom treatment was clinically effective. Another study examining the vaccination of patients with resectable liver metastases from CRC using mature DCs loaded with HLA-A2-restricted CEA-peptide has been reported in the Netherlands<sup>[57]</sup>. A total of 10 CRC patients with

resection of liver metastases were treated, and the induction of CEA-specific T cells was demonstrated in 7 out of 10 patients. Interestingly, CEA-specific CTL responses were detected in a resected lymph node in one patient. CEA altered peptide (CEAalt) was also administered with DCs to induce antitumor immunity in patients with CEA-positive CRC ( $n = 7$ ) or lung cancer ( $n = 2$ )<sup>[58]</sup>. In this trial, 5 out of 9 patients exhibited CEAalt-specific CTL responses, and 3 of 9 patients exhibited CEA-specific CTL responses<sup>[58]</sup>. As CEA is typically produced in gastrointestinal tissue during fetal development, the immune system exhibits some degree of tolerance. Therefore, a break in tolerance is required to induce efficient CEA-specific immunity.

### WT1

The *WT1* gene possesses oncogenic functions and is highly expressed in various types of malignancies, including CRC<sup>[59]</sup>. Moreover, WT1 expression in CRC is significantly associated with tumor progression, lymph node metastasis, distant metastasis and clinical stage<sup>[60]</sup>. Therefore, the WT1 protein may be one of the most promising cancer antigens. Indeed, the National Cancer Institute (NCI) has ranked WT1 as the number 1 target for cancer immunotherapy based on several factors<sup>[61]</sup>. Moreover, *WT1* expression may be essential for maintaining the transformed characteristics of cancer cells. Tumor escape from immune surveillance, reflecting the downmodulation of WT1, is unlikely to occur<sup>[62,63]</sup>. Therefore, WT1-specific immune responses for the elimination of tumors may be induced in many types of cancers. Shimodaira *et al.*<sup>[8]</sup> conducted a phase I study to investigate the safety and immunogenicity of DCs loaded with WT1 peptides restricted by MHC class I and class II (DC/WT1-I/II) for advanced CRC patients. Standard treatment comprising surgical resection and chemotherapy was followed by 1 course of 7 biweekly administrations of DC/WT1-I/II with OK-432 in 3 CRC patients. Importantly, WT1-specific CTLs were detected after the first vaccination and persisted for two years with prolonged disease-free and overall survival (OS)<sup>[8]</sup>. The maintenance of long-term WT1-specific memory CD8<sup>+</sup> T cells through DC/WT1-I/II may be associated with clinical benefits in cancer patients<sup>[64]</sup>.

### MAGE

MAGE is a cancer-testis antigen aberrantly expressed in various types of human malignancies, including CRC. MAGE is not expressed in normal tissues except the testis. Thus, MAGE has been developed as a cancer immunotherapy target<sup>[10-12]</sup>. Sadanaga *et al.*<sup>[65]</sup> initially examined DCs loaded with MAGE-3 peptide in patients with gastrointestinal carcinomas, depending on the HLA haplotype (HLA-A2 or A24). Twelve patients with advanced gastrointestinal carcinoma (six stomach, three esophagus, and three colon) were enrolled. After vaccination, MAGE-3-specific CTL responses were

observed in 4 out of 8 patients. Tumor markers were decreased in 7 patients, and importantly, evidence of minor tumor regression was detected in 3 patients. This group also conducted clinical trials for CRC patients using MAGE-3 or MAGE-1 peptide<sup>[66]</sup>. Twenty-eight patients with advanced gastrointestinal carcinoma, including 7 CRCs, were administered mature DCs loaded with MAGE-3 or MAGE-1 peptide, depending on the HLA haplotype (HLA-A2 or A24). Peptide-specific CTL responses, tumor marker decreases and minor tumor regressions were observed in some patients after vaccination.

### **CEA and MUC1**

A recent report from a randomized phase II clinical trial also indicated the clinical benefits of TAA-targeted DC-based cancer immunotherapy for CRC patients<sup>[67]</sup>. The aim of this trial was to determine whether 1 of 2 vaccines based on DCs and poxvectors encoding CEA and MUC1 (PANVAC)<sup>[68]</sup> would lengthen the survival of patients with resected CRC metastases. A total 74 patients, disease-free after CRC metastasectomy and perioperative chemotherapy, were randomized to injections of DCs modified with MUC1 PANVAC (DC/PANVAC) or PANVAC with per injection GM-CSF. The results indicated no differences in the clinical outcomes [progression-free survival (PFS) or OS] between the 2 vaccine strategies. Although CEA-specific T cell responders after DC/PANVAC were more frequently detected compared with PANC, the clinical benefits were not significant<sup>[67]</sup>.

### **CEA, MAGE, and HER2**

HER2/neu is a proto-oncogene product overexpressed in CRC cells<sup>[69]</sup>. Therefore, Kavanagh *et al.*<sup>[70]</sup> conducted a phase I / II clinical trial administering a DC-based cancer immunotherapy targeting multiple TAAs, including CEA, MAGE, and HER2/neu, to patients with advanced CRC. The DCs were loaded with HLA-A2-restricted peptides derived from CEA, MAGE, and HER2/neu, pan-DR non-natural peptide optimized for both HLA-DR binding and TCR stimulation, and keyhole limpet hemocyanin (KLH) protein<sup>[71]</sup>. In this trial, 13 HLA-A2<sup>+</sup> advanced CRC patients received the immunotherapy. Although, all patients exhibited progressive disease, CEA-specific T cell responses were detected in 3 out of 11 evaluated patients. Moreover, this pilot study demonstrated the induction of immune responses to multiple TAAs in patients with advanced CRC.

### **DCs loaded with whole tumor cell-derived antigens**

DCs can present TAA-derived epitopes in various manners. Unlike antigenic peptide-loaded DCs, other strategies, such as DCs loaded with whole tumor cells (DC/whole tumor) through whole tumor lysates, apoptotic whole tumor cells, DNA, mRNA, or fusion with whole tumor cells, have been developed<sup>[72]</sup>. DC/whole tumor cells simultaneously induce numerous

TAA-specific CD4<sup>+</sup> and CD8<sup>+</sup> T cell responses that are at least theoretically more effective than antigenic peptide-loaded DCs<sup>[72]</sup>. Moreover, for DC/whole tumor-based immunotherapy, allogeneic tumor cell lines can also be used instead of autologous tumor cells to induce autologous tumor specific antitumor immunity. However, unlike defined antigenic peptides, whole tumor cell-based therapy is applicable to all patients, regardless of HLA type.

### **DCs transfected with mRNA**

We have previously reported that murine DCs transfected with MUC1 mRNA exhibited MUC1 expression on DCs in the context of co-stimulatory molecules, resulting in the induction of MUC1-specific CTL responses against CRC cells *in vivo* and *in vitro*<sup>[73]</sup>. Comparative studies have suggested that mRNA-transfected DCs are superior to other antigen-loaded DCs in inducing CTL responses<sup>[29,74]</sup>. In a clinical trial, DCs were transfected with whole tumor mRNA to induce antitumor immunity in CRC patients<sup>[74]</sup>. Fifteen patients with advanced CRC received the immunotherapy and KLH intravenously<sup>[75]</sup>. As a result, 11 out of the 13 CRC patients evaluated developed a positive KLH skin test, and 7 CRC patients exhibited CEA-specific responses.

### **Fusion of DCs with whole tumor cells**

The fusion of DCs with whole tumor cells generates a heterokaryon expressing DC-derived co-stimulatory molecules and a broad array of TAAs, including both known and unidentified molecules. Thus, this method offers several advantages for presenting antigenic peptides and subsequently inducing polyclonal antigen-specific CD4<sup>+</sup> cells and CD8<sup>+</sup> T cell-mediated antitumor immune responses, resulting in long-term antitumor immunity activation without inducing tolerance<sup>[76]</sup>. Moreover, this strategy circumvents the daunting task of identifying TAAs for individualized immunotherapy. Interestingly, DC-tumor fusion cells are potent immune stimulators compared with DCs loaded with either apoptotic tumor-cell fragments or tumor lysates in mice studies<sup>[77]</sup>. In DC-tumor fusion cells, TAAs access the endogenous antigen-processing pathway, whereas DCs loaded with apoptotic tumor-cell fragments or tumor lysates rely on the cross-presentation of the antigen, which is typically not efficient<sup>[33]</sup>. In a phase I study, DC-tumor fusion cell vaccines were also administered with IL-12 in 5 gastrointestinal tumors, including CRC<sup>[78]</sup>. Among the 3 patients evaluated, 1 patient exhibited stable disease, and 2 patients exhibited progressive disease. Moreover, no DTH-positive patients were detected in this trial. Immunotherapy through DC-tumor fusion cells with IL-12 induced no serious adverse events and provided good therapeutic responses in some patients with brain tumors. In addition, patients with elevated serum levels of anti-nuclear antibody (ANA) had significantly longer treatment periods than those without treatment in these trials<sup>[79]</sup>.

**Table 2** Clinical trials of immune checkpoint therapy in patients with colorectal cancer

Target	Immunotherapy	Phase	Patients	Results	Ref	Year
PD-1	Pembrolizumab, anti-PD-1 immune checkpoint inhibitor	II	11 MMR-deficient CRC, 21 MMR-proficient CRC, and 9 MMR-deficient non-CRC	The immune-related objective response rate and immune-related progression-free survival rate were 40% (4 of 10 patients) and 78% (7 of 9 patients), respectively, for MMR-deficient CRC and 0% (0 of 18 patients) and 11% (2 of 18 patients) for MMR-proficient CRC	Le <i>et al</i> <sup>[89]</sup>	2015

PD-1: Programmed death 1; CRC: Colorectal cancer; MMR: DNA mismatch repair.

### Allogeneic whole tumor cell lysate-loaded DCs

The use of autologous whole tumor cell lysates as a potential source of TAAs for DC loading has several potential advantages compared with defined antigenic peptides. DC-loaded whole tumor cell lysates and DC-tumor fusion cells express both known and unidentified TAAs, circumventing the daunting task of identifying TAAs. Moreover, DC-loaded whole tumor cell lysates also induce the simultaneous activation of polyclonal CD8<sup>+</sup> and CD4<sup>+</sup> T cells<sup>[80,81]</sup>. The activation of CD4<sup>+</sup> and CD8<sup>+</sup> T cells can provide robust assistance for the induction and maintenance of CD8<sup>+</sup> CTLs. However, autologous whole tumor cell-based immunotherapy is often limited by the availability of sufficient numbers of autologous tumor cells, which may not be obtained when surgery is not a component of the treatment. Therefore, an alternative approach involves a use of allogeneic tumor cell lines instead of autologous tumor cells. This approach is based on the fact that some TAAs, such as CEA, WT1 and MUC1, are shared among most tumors. We demonstrated that allogeneic CRC cell-loaded autologous DCs induce antigen-specific CTL responses in CRC patients *in vitro*<sup>[4]</sup>.

In clinical settings, autologous tumor lysate-loaded DC vaccines were used in advanced patients with CEA-positive CRC cells<sup>[82]</sup>. Six HLA-A2<sup>+</sup> CRC patients received the immunotherapy and tetanus toxoid antigen, hepatitis B, and influenza matrix peptides. The results revealed antitumor immune responses in some patients, and the transient stabilization or even reduction of CEA levels were also detected. Moreover, DCs loaded with allogeneic melanoma cell lysate expressing at least one of six MAGE-A antigens were examined in this phase II study<sup>[83]</sup>. Twenty patients with advanced CRC received a total of 161 vaccinations. One patient experienced a partial response. Seven patients achieved stable disease. Five patients exhibited prolonged PFS.

### IMMUNE CHECKPOINT THERAPY

DNA mismatch repair (MMR) is a group of genes encoding four proteins that play a key role in repairing mistakes and maintaining genomic stability<sup>[84]</sup>. Deficiencies in MM lead to MSI; thus, CRCs with MSI contain 10- to 100-fold more somatic mutations than metastatic CRC without MSI<sup>[85-87]</sup>. MSI reflects defective MMR in 15% to 20% of CRC patients<sup>[88]</sup>. Accumulating

evidence indicates that the neoantigens produced from mutated proteins in tumors with MSI are recognized by the immune system, inducing CTL infiltration in tumors<sup>[87]</sup>. In addition, CD8<sup>+</sup> CTL infiltration in CRC has a well-supported prognostic value<sup>[42]</sup>. However, the tumor microenvironment comprises not only CD8<sup>+</sup> CTLs but also immune regulatory cell populations. Recent evidence indicates that CD8<sup>+</sup> CTLs infiltration in tumors is associated with the therapeutic effects of immune checkpoint strategies<sup>[42]</sup>. Le *et al*<sup>[89]</sup> conducted a phase II trial to evaluate the clinical benefit of an anti-PD-1 immune checkpoint inhibitor, pembrolizumab, in 41 patients (11 MMR-deficient CRC, 21 MMR-proficient CRC, and 9 MMR-deficient non-CRC) (Table 2). Most patients (40 of 41) had previously received treatment of two or more lines of therapy; all patients then received pembrolizumab until either disease progression or unacceptable toxicity occurred. Pembrolizumab was well tolerated, and the immune-related objective response rates for MMR-deficient CRC and MMR-proficient CRC were 40% (4 of 10 patients) and 0% (0 of 18 patients), respectively. Moreover, the 20-wk immune-related progression-free survival rates were 78% (7 of 9 patients) and 11% (2 of 18 patients) for MMR-deficient CRC and MMR-proficient CRC, respectively. Additionally, patients with MMR-deficient non-CRC displayed responses similar to those of patients with MMR-deficient CRC<sup>[89]</sup>. Importantly, PD-1 blockade in patients with tumors with MSI has exhibited dramatic and durable responses, even in patients with colon cancer<sup>[89,90]</sup>.

### CONCLUSION

The goal of CRC immunotherapy is to induce efficient antigen-specific polyclonal CD4<sup>+</sup> and cytotoxic CD8<sup>+</sup> CTLs in patients. DCs are potent APCs that play a pivotal role in the induction of antitumor immune responses. Therefore, the use of DC-based immunotherapy for CRC patients is promising. However, the immune suppression synergistically generated from CRC and the tumor microenvironment continues to be a major hurdle. Here, we described the ability of DC-based therapeutic immunotherapies to activate antitumor immune responses in CRC patients. However, these strategies may require combination with immune-modulating agents to maximize antitumor immunity. The induction of antigen-specific polyclonal T cell activation may be



associated with the success of immune checkpoint therapeutic strategies. The combination of DC-based immunotherapy and simultaneous blockade of multiple immune checkpoints may have the potential for clinical benefit and should be evaluated<sup>[91]</sup>.

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