

## Review Article

# OX40 as a novel target for the reversal of immune escape in colorectal cancer

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**Abstract:** First-generation immunological checkpoint inhibitors, such as CTLA-4, PD-L1 and PD-1 exhibit significant advantages over conventional cytotoxic drugs, such as oxaliplatin and 5-FU, for the treatment of colorectal cancer. However, these inhibitors are not ideal due to their low objective response rate and the vulnerability of these treatment methods when faced with emerging drug resistant cancers. This study summarizes the immunological characteristics of colorectal cancer treatment, and analyzes the ways in which OX40 may improve the efficacy of these treatments. Activation of the OX40 signaling pathway can enhance the activity of CD4+/CD8+ T cells and inhibit the function of Treg. Simultaneously, OX40 can directly inhibit the expression of Foxp3, affect the inhibitory function of Treg, and inhibit the immunosuppressive factors in the tumor microenvironment so as to reverse immune escape and reverse drug resistance. Therefore, OX40 is an important target for treating colorectal cancer in “cold tumors” with less immunogenicity.

**Keywords:** OX40, immune escape, colorectal cancer, microenvironment

## Introduction

Colorectal cancer is tumor malignancy that occurs in the colon or rectum and is one of the top three causes of morbidity and top five causes of mortality worldwide [1]. Mortality in colorectal cancer is usually due to systemic metastasis of the cancer, as a result of treatment failing to address weak immunogenicity-related “cold tumor” immune escape and extremely efficient drug-resistant mutations of the cancer. The current approach for drug improvement is passive and inefficient. Newly developed drugs tend to disorder immune environments and contribute to the development of drug resistant tumors [2]. Optimal treatment strategies simultaneously address each of these factors by facilitating the efficient detection and killing of non-drug resistant tumor cells by immune cells. Research on tumor immunotherapy has revealed that cytotoxic T lymphocyte-associated antigen-4 (CTLA-4) antibodies and programmed death 1/programmed death ligand (PD-1/PD-L1) antibodies are effective tar-

gets for the treatment of colorectal cancer [3]. Additionally, solid tumors studies have revealed that combined application of checkpoint inhibitors, CTLA-4 and PD-1, yield significantly decreased chances of drug-resistant cancer development [4]. The emergence of drug resistant first-generation immunological checkpoint inhibitors urgently prompts the need for new immunomodulatory antibodies that break down tumor cell-mediated immune tolerance through multiple signaling pathways. Therefore, the immune costimulatory molecule OX40 (CD134) is a promising novel target for colorectal cancer immunotherapy.

First, this study reviews fundamental immunology concepts. The adaptive immune system is responsible for distinguishing tumor cells from normal cells. Tumor specific antigens present as peptides on MHC class I molecules for recognition by cytotoxic CD8+ T cells (or MHC class II molecules for recognition by CD4+ T cells). In order to trigger the CD8+ T cell response, tumor cell antigens are processed by dedicated anti-

gen presenting cells (APC), such as dendritic cells (DCs) and CD4+ helper T cell antigens, are presented to CD8+ T cells [5, 6]. The formation of tumor immunosuppressive microenvironments is attributed to two processes. Tregs, myeloid-derived suppressor cells (MDSC) and tumor-associated macrophages (TAM) are critical for the creation of tumor immunosuppressive microenvironments. TAMs play a critical role in this process by releasing immunosuppressive cytokines, such as IL-10, IL-4, TGF- $\beta$ , vascular endothelial growth factor (VEGF) and arginase. Another method of immune suppression by tumors is achieved through the upregulation of immunosuppressive receptors or ligands, such as CTLA-4, PD-1 and PD-L1. These factors interact to create immunosuppressive microenvironments. For example, vascular endothelial growth factor receptor (VEGFR) signaling can enhance the PD-L1/PD-L2 pathway [7, 8].

### Previously used cytotoxic drugs

**Oxaliplatin:** Oxaliplatin is a standard first-line treatment drug for colorectal cancer [9, 10]. Oxaliplatin treats colorectal cancer by activating the immune system APCs; this process is independent of T cell activation or MHC upregulation. Previous studies have found that co-culture of colorectal cancer cells in an oxaliplatin supernatant promotes the maturation of DCs and increases the proliferation of T cells [11]. However, oxaliplatin-associated death of colorectal cancer cells results in the production of HMGB1, which induces immunogenicity-related cell death in various colon cancer cell lines. Treatment with cisplatin has not been found to produce these outcomes as this process of immunogenicity-related cell death relies on TLR-4 [12, 13]. Other studies have validated the role of oxaliplatin as an immunogenic cell death-inducing factor. Oxaliplatin mediates the expression of calcium net protein and HMGB1, which neutralize antibodies to eliminate the expression of the HMGB1/calcium net protein/immunogenic cell death. This also confirms the importance of the HMGB1-TLR4 control shaft in oxaliplatin-mediated immune function [14]. Secondly, oxaliplatin reverses immunosuppression created by tumor growth. This could be mediated through PD-1/PD-L1, following DC exposure to oxaliplatin, resulting in enhanced stimulation of T cells. During this

process, there are no changes to the expression levels of MHC or costimulatory molecules, but the expression of PD-L2 and PD-L1 are decreased. This leads to elevated antigen-specific proliferation and enhances the recognition of tumor cells by T cells [15]. Joint treatment of colorectal cancer patients with IL-12 and oxaliplatin stimulate T lymphocyte and NK cell proliferation, which balances the ratio between effector cells and regulation/suppression cells. This increase in the CD8+/Tregs ratio and reduction in MDSC abundance enhances the immune response against colorectal cancer and eliminates liver metastases [16].

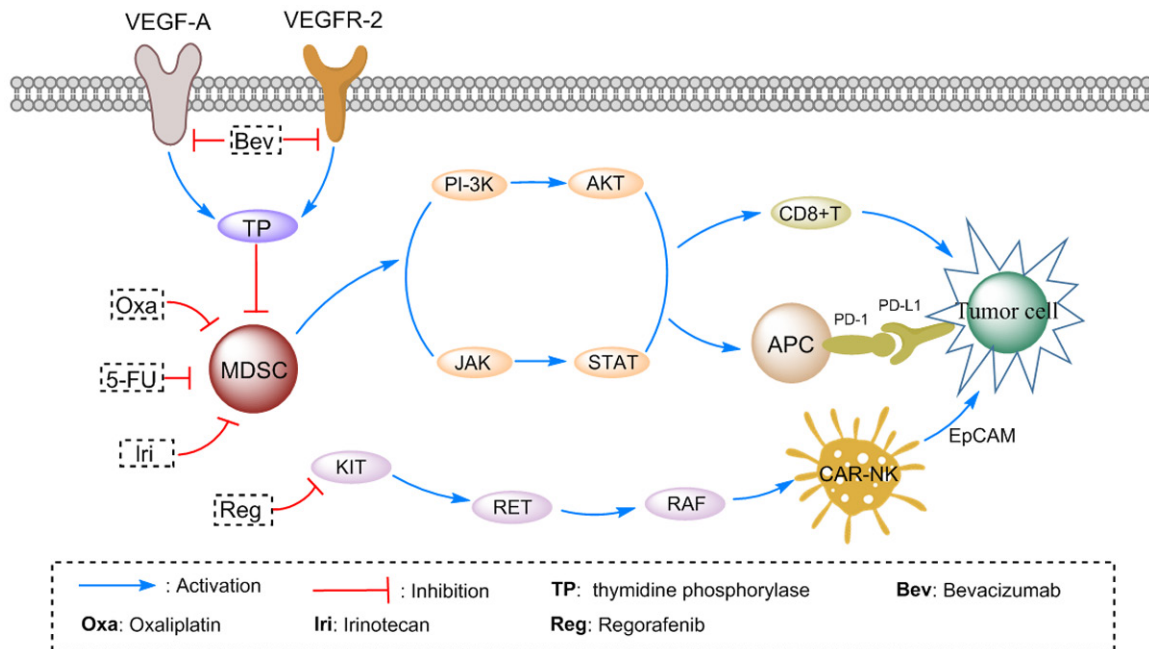
**5-fluorouracil (5-FU):** 5-FU is a basic drug used for the treatment of colorectal cancer [17]. 5-FU specifically has an influence on the immune system aside from its direct cytotoxic effects. 5-FU efficiently and selectively consumes the MDSC of mice in colorectal cancer and increases the expression of IFN- $\gamma$  produced by tumor-specific T cells [18]. In contrast with oxaliplatin, 5-FU does not induce immunogenic cell death (because it does not upregulate cadherin) and its anti-tumor activity is TLR4 independent. 5-FU effectively activates the immune system by enhancing the inhibition of anti-tumor immune functions [19].

**Irinotecan:** One of the most important drugs for the treatment of advanced colorectal cancer is irinotecan [20]. Melichar et al. found that irinotecan treatment increased the number of CD4+ and CD8+ cells in peripheral blood of 14 patients with metastatic colorectal cancer [21]. Additionally, Kim et al. demonstrated that irinotecan (as part of a FOLFIRI regimen) inhibited the immunosuppressive environment of tumors to permit the maturation of DCs, by using a DC vaccine transfected with a virus vector overexpressing CEA. Compared with the vaccine alone, the combination of DC vaccine and FOLFIRI enhances tumor-specific immune responses, as the number of CEA-specific IFN- $\gamma$  secreted lymphocytes increase. Although irinotecan does decrease MDSC and Tregs abundance, additional vaccine doses reverse this effect (**Figure 1**) [22].

### Current individualized anti-VEGF/EGFR drugs

**Anti-VEGF therapy:** Over the past 10 years, various methods of inhibiting vascular endothelial growth factor (VEGF) have been approved for

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**Figure 1.** Pathway analysis of Tumor cell and T cells: the pathways that involve MDSC cells, CD8+ T cells, Tumor cells, NK cells and chemotherapy drugs were constructed.

the treatment of colorectal cancer [23-29]. The main goal of anti-VEGF treatment is the restructuring of the tumor vascular system for enhanced drug delivery [30-32]. However, this therapeutic effect has not been efficiently achieved [33]. Nevertheless, pro-angiogenic factors are critical for the maintenance of the immunosuppressive tumor microenvironment. For example, pro-angiogenic factors stimulate Tregs in addition to producing immunosuppressive cytokines [34-36], thereby inhibiting the function of immune cells [37]. Counteracting these effects through treatment with VEGF facilitates normal immune system regulation and enhanced anti-tumor immune responses in the microenvironment [38, 39]. Treatment with bevacizumab enhances the anti-colorectal cancer effect of 5-fluorouracil on the VEGF-A/VEGFR-2 pathway by upregulating thymidine phosphorylase [40]. Additionally, treatment with venom peptide interrupts the MAPK signal pathway mediated by VEGFR-2 and COX-2, which in turn effectively inhibits VEGF-A related tumor growth [41]. VEGF-A, VEGF-C, VEGFR-2 and VEGFR-3 combined with the high expression CD4/CD8 in the associated matrix are good immune prognostic markers of colorectal cancer [42]. In another study, it was found that levels of peripheral blood (PB) DC1 and DC2 levels are nega-

tively correlated with VEGF serum levels in patients with colorectal cancer. It has been suggested that the interval number and impaired function of PBDC may be related with neoplasm staging and VEGF level [43, 44]. Additionally, the dosing of anti-VEGF therapy is critical, since studies have found that anti-VEGF therapy combined with tumor vaccination activates the immune system and inhibits tumor growth when anti-VEGF treatment is administered at 25% of the maximum dose. Compared with higher doses, lower doses lead to decreased MDSC infiltration. Lower doses of anti-VEGFR-2 also increase the distribution of the functional vascular system compared with higher doses. In conclusion, appropriate doses of anti-VEGF therapy normalize the vascular system of colorectal tumors and decrease the inhibition of the immune microenvironment [37, 39, 45, 46].

*Small molecule VEGF inhibitors:* Sorafenib and regorafenib are two types of multi-kinase inhibitors that can also be used to inhibit VEGF and have been used for the treatment of colorectal cancer. They have been shown to affect immunogenicity, but their net effect is unknown [47-51]. First, sorafenib inhibits the function of DC, decreases the induction of antigen-specific T cells [52, 53], and inhibits the function of NK

cells [54, 55]. Regorafenib is a third-line treatment for colorectal cancer but has a unique ability of reversing immune escape [56]. For example, regorafenib treatment enhances the responsiveness of adoptive chimeric antigen receptor modified T or NK cells (CAR-T or CAR-NK) in solid tumors. Regorafenib and CAR-NK-92 cells have been shown to exert synergistic effects on immune responses in human colorectal cancer xenografts in mouse models. Treatment with regorafenib enhances the ability of CAR-NK-92 cells to specifically recognize EpCAM-positive colorectal cancer cells and release cytokine killers, such as IFN- $\gamma$ , perforin and granular enzyme B [57].

*Anti-EGFR therapy:* Panitumumab and cetuximab have been proven to enhance immune activity in colorectal cancer patients [58]. A large number of preclinical studies indicate that cetuximab treatment can induce antibody-dependent cell-mediated cytotoxicity (ADCC) [59, 60]. Cetuximab is usually combined with chemotherapy but individualized gene expression is critical for its effect on immunity. Correale et al. found that EGFR expression is upregulated after chemotherapy, which subsequently leads to increased susceptibility of colorectal cancer cells to ADCC, independent of the k-ras pathway [61]. Cetuximab affects the proliferation and phagocytosis of colorectal cancer cells through DC. This suggests that CTL-dependent immunity is involved in the anti-tumor effects of cetuximab [62]. These studies also suggest that the order of administration is important for triggering an effective immune response. A critical finding presented in this study is that anti-EGFR therapy remains effective at suppressing immune escape even in cases of drastic mutations to the k-ras pathway. Compared with a standard FOLFOX regimen, the immune-activated chemotherapy regimen (with irinotecan and 5-FU administered on the first day and cetuximab administered on the third day) increases levels of CEA and thymidine synthase-specific CTL precursors in the blood of patients, as well as the number of killer T cells [63].

### **Potential immune costimulatory molecular drugs that reverse both immune escape and drug resistance**

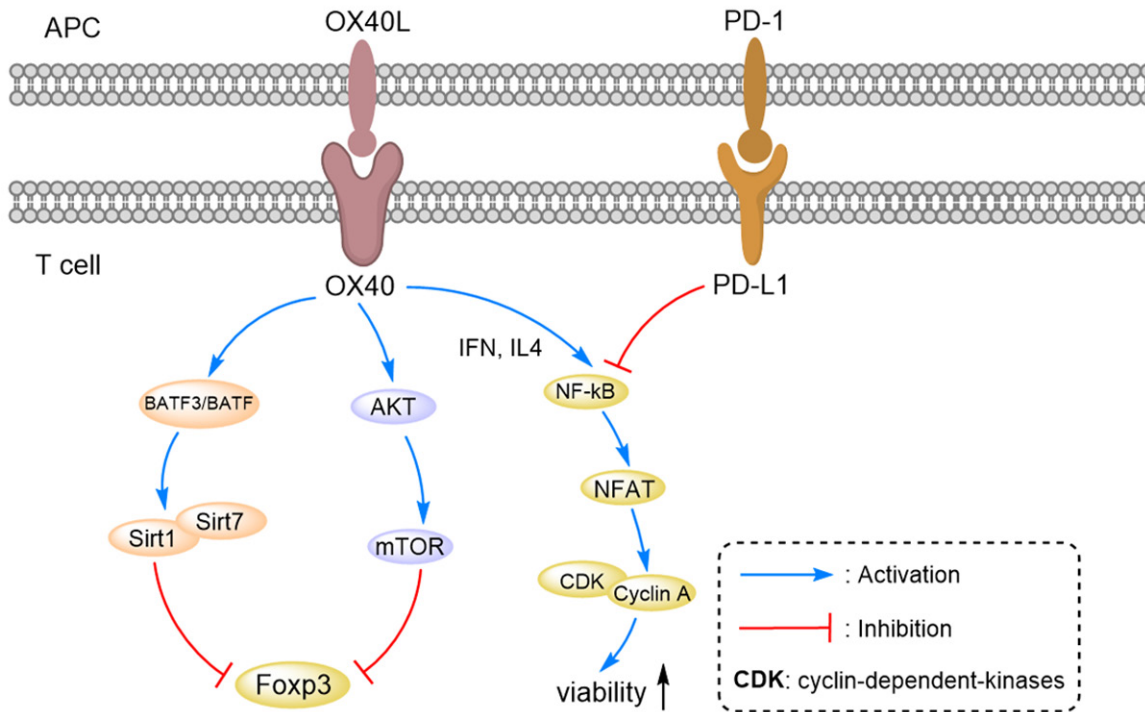
OX40 belongs to the tumor necrosis factor receptor superfamily and is expressed on the

surface of antigen-presenting cells, natural killer cells and mast cells [64]. The sole ligand of OX40, OX40L (CD252), activates T cells by stimulating OX40 and initiating activation signals, such as NF- $\kappa$ B and nuclear factor of activated T cells (NFAT) [65]. These signals increase the expression of cyclin A, cyclin-dependent kinase, cytokines and their receptors [66]. OX40 is expressed on the surface of tumor infiltrating lymphocytes (TILs) of various tumor tissues [67]. In colorectal cancer, the high expression levels of OX40 on TILs of mesenteric lymph nodes and other sites have been found to be positively correlated with overall survival, particularly when the expression of OX40 is elevated on the surface of CD4<sup>+</sup> T cells and on the surface of tumor-infiltrating Tregs [68, 69]. Activation of the OX40 signaling pathway enhances the activity of CD4<sup>+</sup>/CD8<sup>+</sup> T cells and inhibits the function of Tregs. However, T cell activation signals can be neutralized by inhibitory receptors, such as PD-1 or CTLA-4 [35, 70]. Previous animal studies have shown that OX40 is expressed on the surface of T cells between 24 and 96 hours after antigen recognition and that the presence of the OX40 agonist antibody increases the survival of different subtypes of effector T cells [71-73]. As illustrated in **Figure 2**, OX40 affects the inhibitory function of Tregs because OX40 inhibits the expression of Foxp3 directly, which inhibits the antagonistic effects of TGF- $\beta$  and is responsible for transforming T cells into Foxp3<sup>+</sup> Tregs [74-76]. Additionally, Tr1 cells (inhibitory CD4<sup>+</sup> T cells) induced *in vitro* can be blocked by the activation of OX40 [77]. However, the degree of OX40 activation depends on the immune microenvironment of T cells, since OX40 can only promote the proliferation of Treg in the absence of IFN- $\gamma$  and IL-4 [78, 79].

*Reversal of immune escape:* The OX40 signaling pathway can be activated by OX40L-Fc fusion proteins, specific OX40 antibody agonists, and transfected tumors and dendritic cells (DCs). OX40 antibody agonists directly improve the effector function of T cells and neutralize invasive Tregs [80, 81]. This is accomplished via antibody-dependent cell-mediated cytotoxicity (ADCC) or antibody-dependent cell-mediated phagocytosis (ADCP). Additionally, this process involves surface activated Fc  $\gamma$  receptors (human being: Fc $\gamma$ R I and Fc $\gamma$ R II a, mouse: Fc $\gamma$ R I, Fc $\gamma$ R III and Fc $\gamma$ R IV) of NK cells that recognize antibodies bound to antigens on



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**Figure 2.** Pathway analysis of APC cells and T cells: the pathways that involve OX40/OX40L, PD-1/PD-L1, mTOR/Akt, NK-κB/NFAT and Cyclin A/CDK were analyzed.

the surface of cell membranes and kill these specific cells. The antibody impacts the strength of the ADCC or ADCP response based on the following factors: (I) Antibody subtypes; for example, the ADCC effect of an IgG1 antibody is stronger than that of an IgG4 antibody [82-84]. (II) Glycosylation modification; for example, fucose can enhance the ADCC effect of natural killer cells. (III) Types of Fc receptors (and whether they are activated or inhibited) [85]. (IV) The density of macrophages in tumor lesions [86]. Additionally, the activation or depletion of OX40 antibodies depend on the expression of OX40 on the surface of different TIL subtypes. ADCC occurs only when NK cells are present and the activated Fc receptor is expressed [87]. Intravenous administration of OX40 antibody is more likely to activate peripheral lymphocytes, while intra-tumor administration can enhance the activation of tumor-specific immune responses and reduce systemic toxicity. ADCC can be further enhanced in combination with 4-1bb antibodies [88]. Decreasing the accumulation of bone marrow cells in tumors can weaken the tumor inhibition effect of the OX40 antibody [89]. However, OX40 antibody-mediated Tregs depletion does not account for

all anti-tumor effects, since it has been observed that OX40 antibodies directly activate CD8+ T cells and CD4+ effector T cells to elicit anti-tumor effects [90, 91]. In conclusion, Tregs depletion is a necessary but insufficient condition for OX40 antibody functioning, while the activation of effector T cells also play a critical role. This provides us with more useful details for future drug development efforts related to OX40 for the treatment of colorectal cancer.

*Reversing drug-resistance:* OX40 antibodies and OX40 Fc fusion shows strong tumor suppressive effects on low tumor load in mice. However, OX40 antibody treatment has a weak therapeutic effects on larger or metastatic tumors, which can be improved when OX40 is used in a combined drug regimen [92]. The current strategy is to enhance antigen release for improved immune suppression and providing assistance to adoptive T cells, including using cyclophosphamide to kill tumor cells, release antigens and mediate Tregs inhibition [93]. Since the OX40 signaling pathway significantly prolongs the survival of antigen-activated CD4+ and CD8+ T cells, the combination of OX40 agonists with certain combination therapies incre-

ases antigen load [94, 95]. For example, OX40L-Fc used in combination with chemotherapy and vaccines for the treatment of solid tumors [96]. Additionally, the survival of CD4+ and CD8+ effector T cells can be enhanced by inhibiting the function of Tregs (via depletion or attenuation of the inhibitory effect of Tregs). The most operable strategy is that OX40 agonists combined with immunosuppressants exert a synergistic effect for the treatment of metastatic colorectal cancer. PD-1 inhibitors combined with OX40 agonists significantly extend the survival time of 50% of experimental animals treated for colorectal cancer [97, 98]. Similarly, OX40 antibody combined with arginase inhibitors significantly enhance the function of CD4+ T cells and CD8+ T cells, thereby inducing tumor shrinkage [99].

### Summary and prospects

During recent years, many studies have explored potential treatment methods that modulate varying aspects of the immune response, in order to target cancers, such as colorectal cancer [100, 101]. The advantages of immunotherapy are clear: a lasting response, a lack of drug-resistance, generation of immune memory and a decrease in non-specific toxicity [102, 103]. However, immunotherapy is not suitable for every patient and may require a combination of multiple treatments to elicit an appropriate immune response and to address “immune-susceptible” tumors [104, 105]. For example, metronomic chemotherapy (high frequency, low dose chemotherapy) has gained increasing attention during recent years, since its administration has been shown to exert positive effects on the immune response [106-109]. Metronomic chemotherapy can improve CTL activity and reduce the quantity of immunosuppressive cells (Tregs and MDSCs) in the tumor microenvironment by adequately stimulating cytotoxic immune cells without exhaustion. Checkpoint suppression can further enhance the immune response against tumors by keeping T cells in an activated state. Combining checkpoint suppression with metronomic chemotherapy drug delivery can produce synergistic effects that enhance immune responses against tumors and eliminate metabolic competition. This allows for the elimination of treatment-resistant cancer cells, an effect that cannot be achieved with either treatment alone

[109, 110]. The OX40 antibody has broad prospects in combination with other therapies, such as surgery, radiotherapy, vaccines and immunomodulators. At present, more studies are needed to find the most effective combination schedule and optimal dose to balance the direct anti-cancer effect of conventional therapy in synergy with immunotherapy to achieve maximum effectiveness using OX40 for the treatment of colorectal cancer.

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### Disclosure of conflict of interest

None.

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