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Review

Evolving Roles for Targeting CTLA-4 in Cancer Immunotherapy

Yinghao Zhao[a] Wei Yang[a] Yuanyuan Huang[b] Ranji Cui[a] Xiangyan Li[c] Bingjin Li[a]

[a]Jilin Provincial Key Laboratory on Molecular and Chemical Genetic, the Second Hospital of Jilin University, Changchun, [b]Department of pediatric, the First Hospital of Jilin University, Changchun, [c]Jilin Provincial Key Laboratory of BioMacromolecules of Chinese Medicine, Jilin Ginseng Academy, Changchun University of Chinese Medicine, The Affiliated Hospital to Changchun University of Chinese Medicine, Changchun, Jilin, China

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Abstract
Cytotoxic T lymphocyte-associated antigen-4 (CTLA-4) is a membrane glycoprotein expressed by activated effector T cells (Teffs) and participates in the repression of T cell proliferation, cell cycle progression and cytokine production. Currently, antibodies targeting CTLA-4, ipilimumab and tremelimumab are widely used as a therapeutic approach in a variety of human malignancies. However, their detailed mechanism remains unclear. Therefore, in this review, we focused specifically on recent findings concerning the role of CTLA-4 in immune response and also discussed clinical studies of targeting CTLA-4, alone or in combination with other therapies for the treatment of cancers. CTLA-4 blockade is used as a therapeutic approach for the treatment of cancer through competing with CD28-positive costimulation for binding to their shared B7 ligands or exhibiting direct inhibitory effect on signaling molecules in the cytoplasmic tail. At present, antibodies for targeting CTLA-4 or in combination with other therapies significantly reinforced the anti-tumor effect and improved the prognosis of malignant disease. In addition, severe adverse events of targeting CTLA-4 therapy could be a challenge for the development of this therapeutic strategy. This review may provide some new insights for clinical studies of targeting CTLA-4.

Introduction

Immunotherapy is proved to be a promising therapeutic strategy against human malignancies. Cytotoxic T lymphocyte-associated antigen-4 (CTLA-4, CD152) is a membrane glycoprotein expressed by activated effector T cells (Teffs) and participates in the repression of T cell proliferation, cell cycle progression and cytokine (IL-2, IFN-γ) production [1, 2].

Y. Zhao and W. Yang contributed equally to this work.

Xiangyan Li
and Bingjin Li

Jilin Provincial Key Laboratory on Molecular and Chemical Genetic
The Second Hospital of Jilin University, Changchun (China)
E-Mail xiangyan_li1981@163.com, libingjin@jlu.edu.cn

CTLA-4 exerts its inhibitory function mainly through multiple mechanisms including competition with CD28-positive costimulation for binding to their shared B7 ligands (CD80/CD86) on the antigen-presenting cells (APC), as well as direct inhibitory effects through the cytoplasmic tail which associates with signaling molecules [3]. Currently, targeting CTLA-4 by antibodies is used as a therapeutic approach in a variety of human malignancies, with the aim of blocking the inhibitory effects of CTLA-4 in T cells. Anti-CTLA-4 immnomodulating monoclonal antibodies (mAbs), ipilimumab and tremelimumab are used, either alone or in combination with chemotherapy, vaccine or other antibodies (anti-PD-1 [4, 5] or anti-OX40 [6, 7]), in the preclinical studies and clinical trials of malignancies, including melanoma [8-12], non-small cell lung cancer (NSCLC), breast cancer, prostate cancer [13], pancreatic cancer [14], hepatocellular carcinoma [15], mesothelioma [16].

In this review, we focused specifically on important findings concerning the role of CTLA-4 in immune response and also discussed clinical studies of targeting CTLA-4, alone or in combination with other therapies for the treatment of cancers.

Role of CTLA-4 in immune response

Structure and expression

CTLA-4 was first identified by differential screening of a murine cytolytic T cell cDNA library, which has similar structures as T cell surface molecule CD28 with similar functional properties [17-19]. Like CD28, CTLA-4 localizes on band q33-q34 of human chromosome 2, and on band C of mouse chromosome 1, which encodes a 223-amino-acid protein containing one variable-like domain flanked by two hydrophobic regions. The sequence homology between CD28 and CTLA-4 is about 20%, but they share a 27% (murine) to 30% (human) identity at the amino acid level [20, 21]. Human CTLA-4 contains a leader peptide and three domains, an extracellular V domain of 116 amino acids, a transmembrane region of 37 amino acids and a 34 amino acid cytoplasmic tail [22], which contains two tyrosine-based motifs at position Y_{201} VKM and Y_{218} FIP. Several intracellular proteins bind to the Y_{201} VKM sequence including lipid kinase phosphatidylinositol 3-kinase (PI3K) [23], the phosphatases SH2 domain-containing protein tyrosine phosphatases (SHP-2), the serine threonine phosphatase PP2A[24, 25] and clathrin adaptor proteins activator protein 1 (AP-1) and AP-2[26] (Fig. 1A).

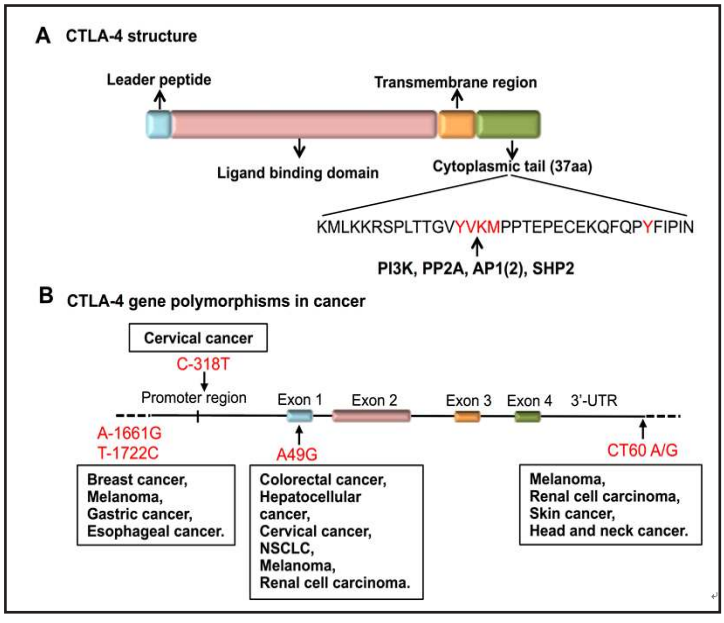

Fig. 1. CTLA-4 structure and its gene polymorphisms in cancer. (A) The CTLA-4 protein consists of three domains (ligand binding region, transmembrane region and cytoplasmic tail) and a leader peptide, which corresponds to exon 2, exon 3, exon 4 and exon 1 in the CTLA-4 gene, respectively. The cytoplasmic tail of CTLA-4 contains two tyrosine-based motifs. The YVKM motif constitutes a binding site for the lipid PI3K, the phosphatases PP2A and SHP2 and the clathrin adaptor protein AP-1 and AP-2. (B) The gene polymorphisms with the CTLA-4 gene have been associated with susceptibility to different types of malignances.

CTLA-4 protein was found to be primarily localized in intracellular vesicles and may cycle between intracellular stores and the cell surface.CTLA-4, a transmembrane glycoprotein,is induced largely on activated T cells, including memory and regulatory T cells [27] but expressed marginally on resting T cells [17, 28]. Specially, CTLA-4 is also expressed on different types of non-T cells, either normal [29, 30] or neoplastic cells [31]. In addition, Foxp3 amplified and stabilized CTLA-4 expression, which normally elaborated by conventional T cells upon TCR stimulation and capable of negative feedback regulation of T-cell activation [32]. Regulatory CD4+ T cells (Tr cells), the development of which is critically dependent on X-linked transcription factor Foxp3 (forkhead box P3), prevent self-destructive immune responses. Despite its important role, molecular and functional features conferred by Foxp3 to Tr precursor cells remain unknown. It has been suggested that Foxp3 expression is required for both survival of Tr precursors as well as their inability to produce interleukin (IL)-2 and independently proliferate after T-cell-receptor engagement, raising the possibility that such 'anergy' and Tr suppressive capacity are intimately linked. Here we show, by dissociating Foxp3-dependent features from those induced by the signals preceding and promoting its expression in mice, that the latter signals include several functional and transcriptional hallmarks of Tr cells. Although its function is required for Tr cell suppressor activity, Foxp3 to a large extent amplifies and fixes pre-established molecular features of Tr cells, including anergy and dependence on paracrine IL-2. Furthermore, Foxp3 solidifies Tr cell lineage stability through modification of cell surface and signaling molecules, resulting in adaptation to the signals required to induce and maintain Tr cells. This adaptation includes Foxp3-dependent repression of cyclic nucleotide phosphodiesterase 3B, affecting genes responsible for Tr cell homeostasis.

Several studies showed that persistent expression of CTLA-4 in the neoplastic cells from cancer patients contributes to the progression of both hematological [33-35] and solid tumors. Different CTLA-4 expression levels in different type of tumors might influence clinical outcome. For example, CTLA4 overexpression was detected in non-squamous type NSCLCand was related to patient age and histological differentiation, but not to the prognosis of clinical outcome [36].High CTLA-4 mRNA levels were associated with good clinical outcome and longer period of time to treatment onset in B-cell chronic lymphocytic leukemia (CLL)[37]. However, the report showed that breast cancer patients with higher CTLA-4 mRNA levels had obvious axillary lymph node metastases and a higher clinical stage [38]. Patients with high tumor CTLA-4 expression in mesothelioma [39], nasopharyngeal carcinoma [40], melanoma [41] and NSCLC [36]had a poorer prognosis than those with low expression, which suggested CTLA-4 as a potential target for tumor immunotherapy.

CTLA-4 polymorphisms in cancer

More than 100 single nucleotide polymorphisms (SNPs) of CTLA-4 have been identified, for example, rs231775 G>A, rs3087243G>A, rs4553808A>G, rs5742909 C>T, rs733618 A>G, rs16840252 C>T polymorphisms, which play an important role in some autoimmune diseases and cancers [42-44]. Recent studies have suggested thatSNPs [+49 (rs231775), CT60 (rs3087243), −1661 (rs4553808), and −318 (rs5742909)] in the promoter region of *CTLA-4* gene may modulate the gene expression and are associated with susceptibility to different types of malignances, such as colorectal cancer [45, 46], breast cancer [43], hepatocellular carcinoma [47, 48], cervical cancer [48-50], melanoma [51], head and neck cancer [52], NSCLC[53, 54], renal cell carcinoma [55]and others [56] (Fig. 1B). However, some studies showed that lack of association between CTLA-4 polymorphisms and some types of cancer, including esophageal cancer in a Chinese population [57, 58] and breast cancer in a North Indian population [59]. It is required to confirm the findings in large-scale and multi-populations studies. Knowledge of the CTLA-4 polymorphism may provide useful information for donor selection and indications for individual application of immunotherapy in cancer.

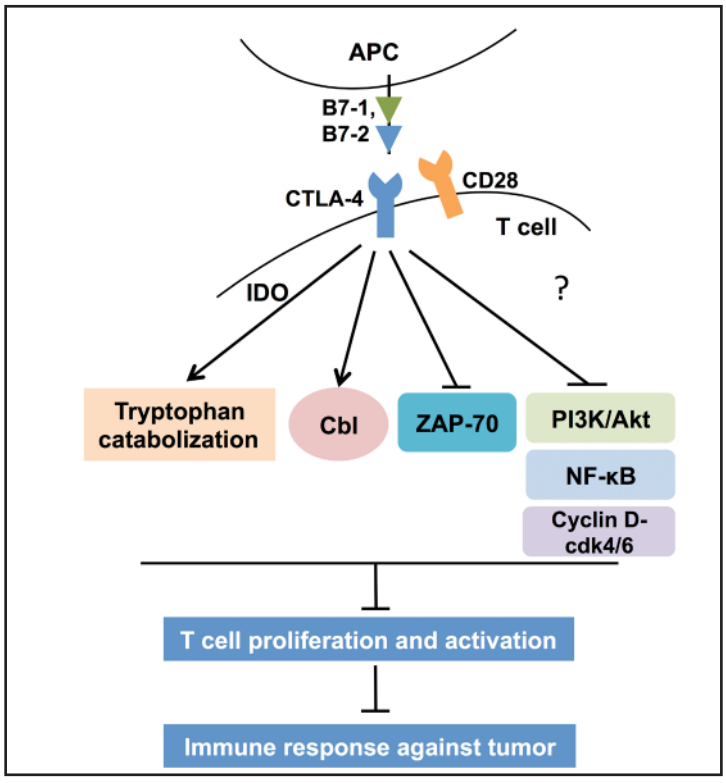

Fig. 2. Model describing the mechanisms of CTLA-4 function in cancer. CTLA-4 shares the same B7 ligands as CD28, including B7-1 (CD80) and B7-2 (CD86) with negative effects on T cell activation. After T cell receptor (TCR) activation, CTLA-4 induces IDO (indoleamine-2, 3-dioxygenase), promotes Casitas-B-lineage lymphoma (Cbl)-b protein expression, suppresses the formation of zeta-associated protein of 70kDa (ZAP-70) and also induces inhibition of PI3K/Akt, cyclin D3-cdk4/6 and NF-κB to negatively regulate T cell proliferation and activation by producing inhibitory signals to weaken the immune response against tumor.

Mechanisms of function

CTLA-4, a membrane receptor for cytotoxic T cells, shares the same B7 ligands as CD28, including B7-1 (CD80) and B7-2 (CD86) [60, 61], but it has negative effects on T cell activation [62, 63].After T cell receptor (TCR) activation, CTLA-4 is up-regulated and binds B7 with a higher avidity than T lymphocyte receptor CD28, resulting in reduced T cell proliferation and lessened cytokine secretion [64-68]. In the early phase of tumorigenesis, CTLA-4 could decrease the T cell activation by producing inhibitory signals to weaken the immune response against tumor [69].Additionally, CTLA-4 triggers reverse signaling through B7 to induce ndoleamine-2, 3-dioxygenase (IDO), which leads to catabolization of the amino acid tryptophan and subsequently results in inhibition of T cell proliferation [70, 71]. Moreover, CTLA-4was reported to promote the expression of Casitas-B-lineage lymphoma (Cbl)-b protein [72] or suppress the formation of zeta-associated protein of 70 kDa (ZAP 70)[73, 74] to negatively regulate T cell activation. Additionally, recent studies revealed that CTLA-4 induced inhibition of PI3K/Akt pathways, cyclin D3, cyclin-dependent kinases (cdk4/cdk6) and nuclear transcription factor (NF-κB)[75-77] (Fig.2).In spite of extensive researches on CTLA-4, mechanism of CTLA-4 interacting with its ligands or its downstream targets still needs to be further investigated.

Targeting CTLA-4 in cancer immunotherapy

CTLA-4 has a critical role in regulating the immune responses for tumors and considered as a potential target for tumor immunotherapy. A number of strategies for anti-CTLA-4 therapy relied on enhancing costimulation, including the use of irradiated tumor cells expressing GM-CSF to enhance crosspriming of T cells by APC, dendritic cells pulsed with peptides or RNA to afford immunization in conjunction with an APC [78]. Preclinical studies have confirmed that blockade of CTLA-4 potentiated therapeutic immunity against cancer [79-81]. At present, antibodies for targeting CTLA-4 (ipilimumab and tremelimumab)

Table 1. Clinical trials of CTLA-4 blocking antibodies or in combination with other therapies in human malignancies

Treatments	Phase	Tumor type	No. of patients	Dose	NCT number	Refs
Alone						
Ipilimumab	II	Advance melanoma	217	0.3, 3, 10mg/kg	NCT00289640	[83]
Ipilimumab	II	Advanced melanoma	155	10mg/kg	NCT00289627	[84]
Ipilimumab	III	Advanced melanoma	951	10mg/kg	NCT00636168	[85]
Ipilimumab	II	Metastatic renal cell carcinoma	61	3mg/kg followed by 1mg/kg or 3mg/kg	-	[86]
Ipilimumab	III	Castration resistant prostate cancer	799	10mg/kg	NCT00861614	[88]
Ipilimumab	I	Non-Hodgkin lymphoma	18	1,3mg/kg	NCT00089706	[87]
Tremelimumab	II	Malignant mesothelioma	29	15mg/kg	NCT01649024	[93]
Tremelimumab	II	Melanoma	251	15mg/kg	-	[90]
Tremelimumab	II	Refractory metastatic colorectal cancer	47	15mg/kg	-	[92]
Combination with other therapies						
Ipilimumab+ Carboplatin/Etoposide	II	Extensive-stage small cell lung cancer	42	10mg/kg	NCT01450761	[96]
Ipilimumab+ Carboplatin/Paclitaxel	I	Non-small cell lung cancer	15	3,10mg/kg	NCT01165216	[95]
Ipilimumab+ radiation therapy	I/II	Castration resistant prostate cancer	83	3,5,10mg/kg	NCT00323882	[101]
Ipilimumab+ radiation therapy	I	Solid tumors	35	3mg/kg	NCT02239900	[99]
Ipilimumab alone /+nivolumab (PD-1 antibody)	II	Advanced melanoma	142	3mg/kg	NCT01927419	[104]

or in combination with other therapies significantly reinforced the anti-tumor effect and improved the prognosis of malignant disease (Table 1).

Alone

Ipilimumab, the first anti-CTLA-4 human monoclonal antibody (IgG1) in humans, has been approved by FDA for the therapy of advanced melanoma patients in early 2011 [8, 82]. The randomized, double-blind and multicenter phase II trials revealed that ipilimumab elicited a dose-dependent effect on efficacy and safety measures in pretreated patients with advanced melanoma, lending support with encouraging long-term survival at a dose of 10 mg/kg. Moreover, a pharmacokinetic analysis performed demonstrated that ipilimumab had linear pharmacokinetics over the dose range 3 mg/kg to 10 mg/kg [83, 84]. The efficacy of ipilimumab was further validated by a randomized, double-blind, phase III trial after complete resection of high-risk stage III melanoma [85]. A phase II study of renal cell carcinoma demonstrated that ipilimumab induced cancer regression in some patients, even if they are not responded to other immunotherapies [86]. Moreover, blockade of CTLA-4 signaling using ipilimumab was well tolerated at the doses of 1 and 3 mg/kg, and had anti-tumor activity in patients with B-cell lymphoma [87].However, a phase III trial showed no significant difference between ipilimumab group and the placebo group in terms of overall survival in patients with metastatic castration-resistant prostate cancer [88]. Although it did not show similar benefit as ipilimumab, clinical trials for tremelimumab demonstrated acceptable tolerability and clinically meaningful activity in patients with melanoma [89-91], refractory metastatic colorectal cancer [92], hepatocellular carcinoma [15] and malignant mesothelioma [93].

Combination with other therapies

Combination of CTLA-4 blockade with other therapies, such as chemotherapy, radiation therapy and other immunotherapy has been widely used for the treatment of a variety of cancers. Clinical studies revealed that the recommended dose of ipilimumab in phased combination with chemotherapies was identified as 10 mg/kg and had a better treatment benefit in combination with chemotherapeutic agents, such as carboplatin, etoposide and paclitaxel in patients with lung cancer [94-96] and advance melanoma [97, 98]. Moreover,combining radiotherapy and ipilimumab was feasible and well tolerated with limited toxicity for solid tumors [99]. The report showed that radiation with doses ranged from 45-61.2 Gy in patients with stage IIIA NSCLC after 3 cycles of neo-adjuvant chemotherapy including ipilimumab with the last two cycles was well tolerated with no observed grade≥3 toxicity including NSCLC [100].In patients with metastatic castration-resistant prostate cancer, the combination of ipilimumab with radiotherapy (8Gy/lesion, up to 3 lesions) showed synergistic antitumor activity with disease control and manageable adverse effects [101].In addition, the efficacy of ipilimumab with other immunotherapies was evaluated in a number of clinical trials. Combination therapy with CTLA-4 and PD-1/PD-1L

potently could reverse immunosuppression and eradicate tumors via an intricate interplay between IFN-ɣ/IFN-ɣR and IL-7/IL-7R pathways [75]. Nivolumab and pembrolizumab, specifically targeting PD-1, are widely studied antibodies for the treatment of cancers [102, 103]. For example, combination of nivolumab plus ipilimumab led to improved outcomes compared with ipilimumab alone in patients with advanced melanoma [104-107], metastatic osteosarcoma [108], colorectal cancer [109], recurrent glioblastoma [110] and renal cell carcinoma [111] with significantly longer overall survival and manageable safety. Recently, oncolytic viruses that selectively infect tumor tissues are a novel and promising technique for stimulating antitumor immunity. One study showed that measles virus(MV) vectors encoding antibodies against the T-cell inhibitory factors CTLA-4 and PD-1 improved therapeutic outcome in melanoma xenografts, suggesting rapid translation of combing immune checkpoint modulation with oncolytic viruses into clinical application [112]. The combination of ipilimumab and anti-receptor activator of NF-κB (RANK) ligand (RANKL) denosumab in metastatic melanoma was recently reported [113]. A phase I clinical trial of ipilimumab in combination with bevacizumab (an antibody that inhibits angiogenesis) in patients with metastatic melanoma showed augmenting immune recognition in the tumor microenvironment through enhancing lymphocyte infiltration [114]. In addition, tremelimumab in combination with other therapies was also investigated and showed better clinical outcomes [93, 115].

Adverse events

Although treatments of targeting CTLA-4 have produced promising activities against malignant diseases, the drug-induced adverse events, termed "immune-related adverse events, (irAEs)" have been observed in some patients due to non-tumor-specific T cells activated by CTLA-4 blockade. IrAEs can have an impact on multiple organs and systems including skin, gastrointestinal tract, liver, endocrine system, eye, kidney, nervous system, pancreas and others, which have been reported in up to 72% of patients receiving ipilimumab [116-118]. Anti-CTLA-4-associated IrAEs are common and typically low grade and manageable, but can also be serious and life threatening. The skin and gastrointestinal tract are most frequently affected, while hepatic, endocrine, and neurologic events are less common [119]. Rash is generally the first irAEs to manifest and appears after the first or second dose of ipilimumab, which is different from those seen with other anticancer agents [120, 121]. Colitis and diarrhea are the most common gastrointestinal irAEs after the first few months of ipilimumab treatment, which can be very serious irAE, particularly in rare cases with the perforation of gastrointestinal tract. A phase III trial in patients with advanced melanoma demonstrated that the adverse events of ipilimumab were mild, with sporadic life threatening cases [122]. Approximately one-third of patients treated with ipilimumab had diarrhea, while colitis was observed in 7-22% of patients [123]. Recently, a study reported that anti-CTLA-4 antibodies might induce a severe and extensive form of inflammatory bowel disease, which suggests to be avoiding nonsteroidal anti-inflammatory drugs (NSAIDs) in patients treated with anti-CTLA-4 [124]. Hepatic irAEs are rare and may manifest as acute hepatitis pattern or as a biliary pattern, which can be reversed by corticosteroids [125]. In addition, autoimmune hypophysitis has been reported in up to 17% of patients with melanoma and renal cell carcinoma treated with anti-CTLA-4 therapy, which may related with enlarged pituitary gland and hormonal deficiencies [126].The risk of irAEs was dependent on the dosage, with incidence of all-grade irAEs being evaluated to 61% for ipilimumab 3 mg/kg and 79% for ipilimumab 10mg/kg. The median time of onset of irAEs was about 10 weeks after the onset of treatment [118]. Severe adverse events of CTLA-4 blockade could have a limitation of the development of this therapeutic strategy, which could be a challenge for future researches that patients are able to benefit from ipilomumab therapy with adequate control of toxicities.

Conclusions and Perspectives

Currently, immunotherapy has been widely used in many forms of tumors, providing a new therapeutic strategy for cancer patients. Multiple studies demonstrated that persistent expression of CTLA-4 on tumors contributed to the progression of both hematological and solid tumors, which produced inhibitory signals to weaken the immune response. Therefore, blockade of CTLA-4 is becoming an attractive approach in enhancing immune efficacy against malignancies and improving the prognosis of tumor patients. In present, the antibodies targeting CTLA-4, ipilimumab and tremelimumab have been tested in many preclinical and clinical trials on a variety of tumors, which could provide new insights for understanding therapeutic effect of CTLA-4 blockade. However, the side effects of CTLA-4 antibodies were observed to be more common and more severe compared with PD-1 antibodies in one clinical trial. Therefore, multiple agents blocking CTLA-4 or in combination with other therapies are under development to reduce severe adverse events, which could be novel therapeutic strategy for cancer patients.

Disclosure Statement

The authors declare to have no conflict of interests.

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