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# Harnessing the immune system by targeting immune checkpoints: Providing new hope for Oncotherapy

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With the goal of harnessing the host's immune system to provide long-lasting remission and cures for various cancers, the advent of immunotherapy revolutionized the cancer therapy field. Among the current immunotherapeutic strategies, immune checkpoint blockades have greatly improved the overall survival rates in certain patient populations. Of note, CTLA4 and PD-1/PD-L1 are two major non-redundant immune checkpoints implicated in promoting cancer immune evasion, and ultimately lead to relapse. Antibodies or inhibitors targeting these two c-checkpoints have achieved some encouraging clinical outcomes. Further, beyond the canonical immune checkpoints, more inhibitory checkpoints have been identified. Herein, we will summarize recent progress in immune checkpoint blockade therapies, with a specific focus on key pre-clinical and clinical results of new immune checkpoint therapies for cancer. Given the crucial roles of immune checkpoint blockade in oncotherapy, drugs targeting checkpoint molecules expressed by both cancer and immune cells are in clinical trials, which will be comprehensively summarized in this review. Taken together, investigating combinatorial therapies targeting immune checkpoints expressed by cancer cells and immune cells will greatly improve immunotherapies that enhance host elimination of tumors.

## KEYWORDS

immune checkpoints, programmed cell death protein 1 (PD-1), programmed cell death 1 ligand 1 (PD-L1), cytotoxic T lymphocyte-associated antigen-4 (CTLA4), oncotherapy, T cell immunoglobulin and mucin-3 (Tim-3), lymphocyte activation gene 3 (Lag-3)

## Introduction

The concept of immunotherapy was first introduced by McFarland Burnett and Louis Thomas in 1957, who proposed that lymphocytes were the primary actors responsible for mediating immune surveillance and tumor clearance (1–3). In the ensuing decades, immunotherapy was advanced into several major areas of study, including tumor infiltrating lymphocytes (TILs), chimeric antigen receptor (CAR)-T cells, T cell receptor (TCR)-T cells, oncolytic viruses, cytokine therapy, therapeutic cancer vaccines, and immune checkpoint blocking antibodies. The goal of these treatments was to unleash the power of the host immune systems to fight back against the cancer.

With years of research and clinical trials, immune checkpoint blocking therapies have been approved by the FDA in the United States and elsewhere. Currently, administration of immunotherapies has outweighed traditional therapeutic regimes for a variety of reasons. Firstly, immunotherapy exclusively targets immune cells and tumor cells, thus leaving other cells unscathed. Secondly, the adverse effects of immunotherapy are comparatively less severe than traditional chemotherapy and radiotherapy-treatment regimens (4). However, tumor cells are highly adaptable and can become unresponsive to immunotherapies, and therefore they continuously evolve mechanisms that evade host immunity and promote tumor persistence. Furthermore, neo-antigens, a hostile tumor microenvironment, T cell exhaustion, and other factors contribute to immune checkpoint-blockade resistance (5). Nevertheless, combinatorial use of immune checkpoint blockade with a broad spectrum of chemicals and antibodies can overcome therapy resistance, and has since produced promising clinical results (6). In this review, we comprehensively summarize the recent immunotherapy literature, with a focus on immune checkpoint blockade.

## Immune checkpoints

As mentioned above immune checkpoints (ICPs) could overcome the limitations of conventional cancer therapy as chemotherapy and radiotherapy. Therefore, followed by the discovery of the first immune checkpoint named CTLA4, a number of ICPs, including both stimulatory and inhibitory molecules, have been identified. Systematic assessment on the correlation between the ICPs of different cancers and the treatment responses/outcomes revealed that the ICPs varied across different cancers due to the cancer heterogeneity. Meanwhile, ICPs could be potential prognostic factors and therapeutic targets in certain types of cancer (e.g. breast cancer, lung cancer and ovarian cancer) (7). Hence, we summarized the ICPs according to their pivotal role in oncotherapy. Here is the detailed information of the major ICPs (Figure 1).

## CTLA4

In response to foreign pathogens, T cell activation is mediated by the T cell receptor (TCR) along with the combination of co-stimulatory positive signals such as CD28 and inducible co-stimulator (ICOS), or inhibitory signals such as cytotoxic T lymphocyte-associated antigen-4 (CTLA4, also referred to as CD152) and programmed cell death protein 1 (PD-1). The final T-cell response is determined by the balance between the positive and negative signals (8). T cell activation requires 2 signals, including: 1) the engagement of the T cell receptor (TCR) with major histocompatibility molecules (MHC) on antigen presenting cells (APCs) and 2) co-stimulation *via* binding of B7(CD80/CD86) on APCs to CD28 on T cells (8). As the first immune checkpoint protein discovered, CTLA4 plays a pivotal role in immune regulation (8, 9). CD28 is predominately expressed in resting T cells, while CTLA4 is absent or scarcely expressed in resting T cells. However, following T-cell activation, CTLA4 is expressed (10). Further study revealed that the intracellular localization of CTLA4 in T cells plays an essential role in its function (11). In naive T cells, CTLA4 protein is retained in the Golgi apparatus of the cell, and therefore exerts no effect on T cell activation (12). Upon engagement of TCR with MHC, CTLA4 is translocated from the cytoplasm to the plasma membrane, where it competes with CD28 for binding with CD80/CD86 by trans-endocytosis (13). CTLA4 engagement inhibits T cell activation and proliferation to maintain immune homeostasis, and protect against aberrant immune responses to self-tissues (14). In addition, CTLA4 also induces T-cell motility and inhibits the binding of T cells with antigen presenting cells such as dendritic cells (DCs) (15). CTLA4 competes with CD28 for B7 ligands for the inhibition of effector T cells, and stimulates the transcription of Foxp3 for regulatory T cells (Tregs) (16). Furthermore, by binding to its ligands B7-1 (CD80) and B7-2 (CD86), CTLA4 also reduces T cell responses by an attenuation of the induction of memory T cells (17). CTLA deficient mice show multi-organ lymphocyte infiltration and tissue destruction, implying the critical role of CTLA4 in down-regulating T cell activation and preventing autoimmunity (18). A number of investigators have identified the cytoplasmic binding partners of CTLA4 (19). The endocytosis of CTLA4 depends largely on its adaptor clathrin, where it binds to clathrin-associated adaptor complex (adaptor protein-2, AP-2) to induce CTLA4 internalization (20). When CTLA4 is internalized, it cannot deliver a negative signal. The binding domain of CTLA4 contains a YVKM motif, and when it is phosphorylated at the tyrosine site of the YVKM motif upon T cell activation, AP-2 can disengage from the binding at the same tyrosine site of CTLA4 (20, 21). With the disengagement of AP-2, CTLA4 is activated and functions as a negative regulator of T cells.

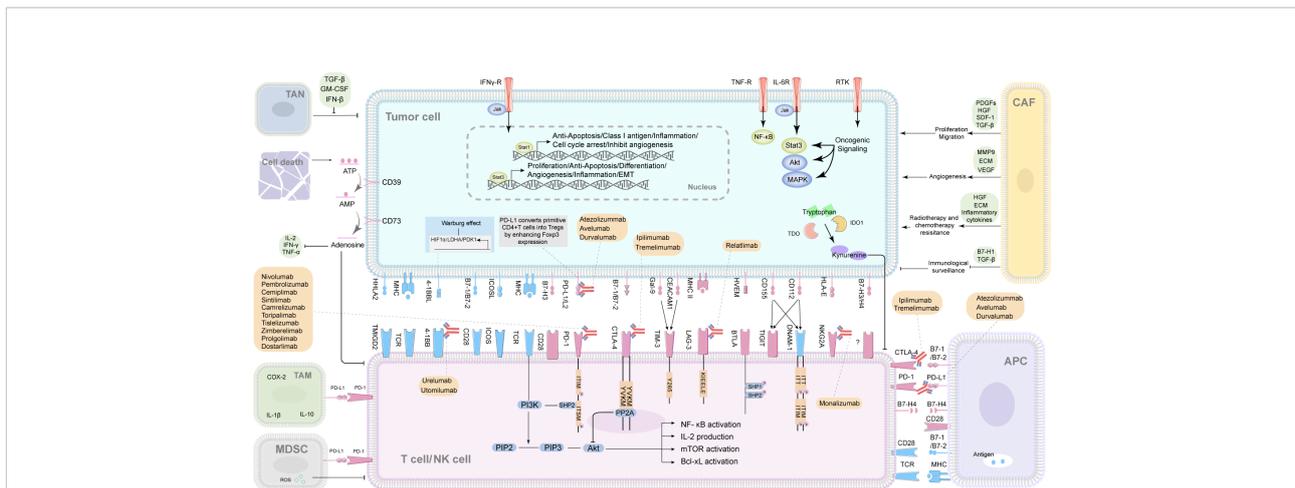


FIGURE 1

The interactions of immune checkpoints of cancers and immune cells, and the correlated immune checkpoint inhibitors (ICIs). TAN, tumor-associated neutrophil; TAM, tumor-associated macrophage; CAF, cancer associated fibroblast; MDSC, myeloid-derived suppressor cell; APC, antigen-presenting cell; NK cell, natural killer cell; ATP, adenosine triphosphate; AMP, adenosine monophosphate; CD, clusters of differentiation; IL, interleukin; IFN- $\gamma$ , interferon- $\gamma$ ; TNF- $\alpha$ , tumor necrosis factor; COX-2, cyclooxygenase-2; TGF- $\beta$ , transforming growth factor- $\beta$ ; GM-CSF, granulocyte-macrophage colony stimulating factor; IFN- $\beta$ , interferon- $\beta$ ; PDGFs, platelet-derived growth factors; HGF, hepatocyte growth factor; SDF-1, stromal cell-derived factor-1; MMP9, matrix metalloproteinase 9; ECM, extracellular matrix; VEGF, vascular endothelial growth factor; ROS, reactive oxygen species; TDO, tryptophan 2,3-dioxygenase; IDO1, indoleamine 2,3-dioxygenase 1; HHLA2, The B7 family ligand HERV-H LTR-associating protein 2; TMIGD2, transmembrane and immunoglobulin domain containing 2; B7-, B7 family. B7-1(CD80), B7-2 (CD86), B7-H1/PD-L1(CD274), B7-H2/ICOSL (CD275), B7-H3(CD276), B7-H4; 4-1BBL, 4-1BB ligand 4-1BB, CD137, a member of the TNF receptor superfamily, is an activation-induced T-cell costimulatory molecule; ICOSL, inducible costimulator ligand; ICOS, inducible costimulatory; MHC, major histocompatibility complex class I, E; NKG2A, also called KLRC1, killer cell lectin like receptor C1; ITT, ITT-like motif; ITIM, immunoreceptor tyrosine-based inhibitory motif; ITSM, immunoreceptor tyrosine-based switch motif; PIP2, phosphatidylinositol-3,4-bisphosphate; PIP3, phosphatidylinositol-3,4,5-trisphosphate; Y265, a highly conserved tyrosine in the intracellular tail of Tim-3; KIEELE, conserved regions of Lag3 cytoplasmic domain, KIEELE motif; NF- $\kappa$ B, nuclear factor kappa-B; Bcl-xL, B-Cell Lymphoma-extra-large; SHP1/2, src-homology domain 2(SH2)-containing protein tyrosine phosphatase-1/2.

CTLA4 also plays an important role in the regulation of regulatory T cells (Tregs) functions, including Treg suppressive function, TCR hyposignaling and the induction of anergy (22). Deficient CTLA4 expression by Tregs fails to expand effector CD4<sup>+</sup> T cells, even in the presence of interleukin-10 (IL-10) (23). Treg specific CTLA4 deficiency leads to reduced expression of CD80 and CD86 on dendritic cells (DCs), lymphocyte proliferation, T-cell mediated autoimmune diseases, and immunoglobulin E (IgE) production<sup>7</sup> (24). CTLA4 controls both the functional-development phase and the effector phase of Tregs (25). In addition, Tregs (which constitutively express CTLA4) regulate the infiltration of CD4<sup>+</sup> T cell into tumors *via* a CTLA4/CD80-dependent manner (26).

Previous studies revealed that CTLA4 is constitutively expressed on tumor cells, and, therefore, CTLA4 blockade might stimulate tumor cell apoptosis and lead to the regression of tumors (27). Because CTLA4-inhibited immune activation relies primarily on the stimulation and activation of Tregs, the therapeutic effect of CTLA4 blockade principally acts *via* the regulation of Tregs (28). Administration of neutralizing monoclonal antibodies against CTLA4 are able to selectively

deplete FOXP3<sup>+</sup> Treg cells, and thereby promote tumor immunity by activating effector CD8<sup>+</sup> T cells (29). Clinically, treatment with anti-CTLA4 monoclonal antibodies (mAbs) does not deplete Foxp3<sup>+</sup> Tregs, but does increase CD4<sup>+</sup> and CD8<sup>+</sup> T cell infiltration into tumors (30).

### PD-1 and PD-L1

Programmed cell death protein 1 (PD-1) was named based on its initial identification as a receptor inducing cell death in an activated T cell hybridoma (31), and is now recognized as a dominant negative regulator of T cells similarly to CTLA4 (32). Like CTLA4, PD-1 is absent from naïve and memory T cells, but is expressed upon TCR engagement (33). In contrast to CTLA4, PD-1 is expressed on the surface of activated T cells, and contains a conventional immunoreceptor tyrosine inhibitory motif (ITIM) and immunoreceptor tyrosine switch motif (ITSM). PD-1 engagement leads to the activation of the inhibitory phosphatase SHP-2, thus resulting in the inhibition of TCR mediated function, increased T cell migration, and

immune evasion within the tumor microenvironment (TME) (34). Unlike the ligand of CTLA4, one of PD-1's ligands known as programmed death-1 ligand 1 (PD-L1), is broadly overexpressed on tumor cells and infiltrating leukocytes (35); this allows for the induction of PD-1 mediated T cell exhaustion by tumor cells (36). However, this phenotype can be reversed *via* blockade of either PD-1 or PD-L1, resulting in elevated antitumor cytotoxic T cell responses and tumor regression (35). Because CTLA-4 inhibits T cells at the early stages in the lymph nodes, the CTLA4 deficient mice show severe lymphoproliferative disease, lethal lymphocytic infiltration in multiple organs and tissue destructions (37). However, because PD-1 regulates in the immune response in the peripheral tissues, the phenotypes of PD-1 and PD-L1 deficient mice are comparatively milder, as evidenced by delayed onset of inflammation and lack of severe organ destruction. These differences highlight the importance of the PD-1 pathway, specifically, in tumor immunotherapy (38, 39).

It is widely acknowledged that CTLA4 regulates T-cell proliferation in lymph nodes (40). Therefore, CTLA4 antibodies (ipilimumab and tremelimumab) can induce clinically unrestrained T-cell activation (41). And the major immune-related adverse events (irAEs) of CTLA4 blockade have been reported to be diarrhea, dermatitis, hepatitis, and endocrinopathies. However, PD-1 suppresses T cells primarily in peripheral tissues (40), and, hence, blockade of PD-1 or PD-L1 exerts less frequent and mild autoimmune adverse effects compared with CTLA4 mAb therapies (35). However, the therapeutic outcomes of PD-1 blockade on solid tumors have remained relatively poor, mainly due to the hostile TME, and the presence of immunosuppressive Tregs (42). PD-L1 has been implicated in the development and function of Tregs by enhancing the expression of Foxp3. It also converts naive CD4<sup>+</sup> T cells to Tregs, and stimulates the immunosuppressive function of Tregs (43). The expansion of Tregs could be regulated by PD1 and PD-L1 *via* Notch signaling pathway (44). Additionally, in myeloid tumor cells (K562) or T cells, the PD-L1 expression was able to convert Th1 cells into Tregs (45). Furthermore, PD-1 blockade stimulates the proliferation of PD-1<sup>+</sup> effector Treg cells, inhibiting the antitumor immunity (46, 47). Therefore, the efficacy of PD-1 blockade therapy may be predicted by the levels of PD-1 expression on effector T cells (Teff) and Tregs (48), and should be used as a biomarker to predict patient responsiveness.

## Tim-3

T cell immunoglobulin and mucin-3 (Tim-3; also known as HAVCR2) is broadly expressed on activated T cells, FoxP3<sup>+</sup> Treg cells, NK cells and monocytes (49). Tim-3, similarly to PD-1 and CTLA4, suppresses the immune response upon interaction with its ligand galectin-9 (49). The main functions of Tim-3 include

promoting CD8<sup>+</sup> T cell exhaustion, and inducing the expansion of myeloid-derived suppressor cells (MDSCs) and Tregs. Co-expressed with carcinoembryonic antigen cell adhesion molecule 1 (CEACAM1), the maturation and cell surface expression of Tim-3 is regulated by CEACAM1 by forming a heterodimer to mediate immune tolerance and T cell exhaustion (50). Blockade of Tim-3 accelerates the pathogenesis of autoimmune diabetes in nonobese diabetic (NOD) mice, and inhibits transplantation tolerance induced by co-stimulation blockade (51), highlighting Tim-3's immunoregulatory role. As a T helper type 1 (Th1)-specific cell surface marker, Tim-3 binds with its ligand galectin-9, causes the hyperproliferation of Th1 cells and the release of Th1 cytokine, and thereafter induces peripheral tolerance (52, 53). Activation of the Tim-3/galectin-9 pathway is also known to increase the frequency of CD11b<sup>+</sup>Ly-6G<sup>+</sup> myeloid cells, inhibits immune responses (54). Clinically, Tim-3 promotes T-cell exhaustion and is associated with the poor prognosis in hepatocellular carcinoma (HCC) patients (55). Tim-3 blockade also increases the pathological severity of the Th1-dependent autoimmune disease of experimental autoimmune encephalomyelitis (EAE) in mice (56).

Regarding Tregs, Tim-3<sup>+</sup> Treg is highly effective at inhibiting T-cell proliferation (57, 58), and the blockade of Tim-3 induces a reduction in the frequency of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Tregs (59). Experiments using murine tumor xenograft models suggest that Tim-3 is expressed on PD-1 expressing CD8<sup>+</sup> TILs. Functionally, Tim-3+PD-1+ TILs are severely exhausted, as evidenced by a failure to secrete interferon- $\gamma$  (IFN- $\gamma$ ), tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), and interleukin-2 (IL-2) (60). Therefore, compared with the inhibition of CTLA4 pathway or PD-1/PD-L1 pathway alone, combinatorial Tim-3 and PD-1 blockade has been demonstrated to be more effective (60). For example, combinatorial therapy reverses tumor-induced T-cell exhaustion and dysfunction in patients with colorectal cancer by increasing the proliferation of tumor antigen-specific CD8<sup>+</sup> T cells and decreasing immunosuppressive Treg populations (61). In patients with advanced melanoma, increased expression of Tim-3 and PD-1 correlates with tumor antigen-specific CD8<sup>+</sup> T cell dysfunction (62). A recent study using triple therapy (PD-1 blockade, Tim-3 blockade and anchored granulocyte-macrophage colony-stimulating factor (GM-CSF) vaccination) demonstrated tumor regression levels higher than 50% (63). This was associated with reduced apoptosis of CD8<sup>+</sup> TILs, decreased production of tumor-promoting cytokines, and improved cytotoxicity in bladder cancer (64) and prostate cancer (65). In another study, combinatorial Tim-3/Lag-3/PD-1 blockade significantly improved antitumor immunity in gastric cancer cell-T cell coculture models, suggesting the therapeutic potential of combinatorial therapy in gastric cancer patients (66). Tim-3 could also be used as a biomarker for cancer therapy responsiveness. In medullary thyroid cancer, a cohort study of 200 patients revealed that Tim-3, CTLA4 and PD-1/PD-L1 are promising biomarkers for tumor recurrence (67). In head and

neck cancer, increased PD-1<sup>+</sup> and Tim-3<sup>+</sup> CD8<sup>+</sup> TILs were inversely correlated with clinical outcome of cetuximab therapy (68). Ultimately, Tim-3 blockade has been proven in pre-clinical studies to be beneficial in improving current immune-checkpoint blockade therapies when applied in combination. These promising results provide precedence for assessing the use of Tim-3 blockade in clinical trials so as to improve clinical outcomes of cancer patients.

### Lymphocyte activation gene 3

Discovered in 1990, lymphocyte activation gene-3 (Lag-3; also referred to as CD223), is an immune checkpoint molecule expressed by activated T cells, Tregs, NK cells and B cells (69–72). Lag-3 is structurally homologous to the CD4 co-receptor and competes with CD4 for its binding with MHC class II (73). Inhibition of Lag-3 results in the expansion of T cells, B cells, macrophages, granulocytes, and DCs (74), and increases activation of CD8<sup>+</sup> T cells (75). With respect to Lag-3's regulatory functions, a study by Workman et al. revealed an inability of Vβ7/8<sup>+</sup> (T cells bearing Vβ7 and Vβ8 (Vβ7/8) T cell receptors) Lag-3-deficient T cells to expand, even when stimulated with staphylococcal enterotoxin B. Expansion of Lag-3 deficient T cells was subsequently restored by the constitutive expression of Lag-3 (76). Lag-3 is differentially expressed on induced Tregs and is required for Treg function. Upon activation, natural CD4<sup>+</sup>CD25<sup>+</sup> Tregs express Lag-3 to suppress effector cells, whereas CD4<sup>+</sup>CD25<sup>+</sup> Tregs from Lag-3 knockout mice exhibit reduced regulatory activity (77). Lag-3 is also a biomarker for active CD4<sup>+</sup>CD25<sup>high</sup>Foxp3<sup>+</sup> Tregs (78). CD49b and Lag-3 are stably and selectively co-expressed on CD4<sup>+</sup> type 1 Tregs (Tr1), which induce and maintain immune tolerance (79). Moreover, Lag-3 is expressed on CD11c<sup>low</sup>/B220<sup>+</sup>/PDCA-1<sup>+</sup> plasmacytoid DCs, and regulates their homeostasis (80). Lag-3 binds with MHC II expressed within plasma membrane lipid rafts of immature human DCs, and promotes their maturation and activation (81). Due to the pivotal role of Lag-3 in immune regulation, several diseases including autoimmune diseases, cancer, chronic viral infection, and parasitic infection are correlated with aberrant Lag-3 expression (82–84). Given the immunoregulatory role of Lag-3, the combinatory therapies targeting both Lag-3 and PD-1 have gained some encouraging clinical outcomes (85–87). Lag-3 and PD-1 contribute to the rapid trafficking of the immunological synapse upon T-cell activation, and lead to a synergistic inhibitory effect on T-cell signaling (88). Dual blockade of Lag-3 and PD-1 resulted in the clearance of multiple established tumors, which were resistant to PD-1 blockade alone (89). Another study in renal cell carcinoma revealed that the inhibition of PD-1 resulted in a significant upregulation of Lag-3, and the blockade of both immune checkpoint proteins leads to increased IFN-γ secretion,

emphasizing the significance and the potential of combination therapy (90). Therefore, to overcome the resistance of PD-1 blockade in resistant cancers, the synergistic blockade of Lag-3 and PD-1 may provide a promising therapy.

### TIGIT

Discovered by genome-wide association studies (GWAS), the co-stimulatory molecule CD226 together with T cell immunoreceptor with immunoglobulin and ITIM domain (TIGIT) forms a striking pathway similarly to the CD28-CTLA4 pathway, and is correlated with multiple autoimmune diseases (91, 92). TIGIT is expressed by the activated T cells, Tregs, and NK cells (93). The ligand for TIGIT is poliovirus receptor (PVR) CD155, which also binds to CD226. Hence, in the TIGIT-CD226 axis, TIGIT can bind and disrupt CD226 homodimerization, and therefore directly inhibits T-cell activation, proliferation, and effector function by competing with CD226 for binding to CD155 (94, 95). TIGIT has been shown to suppress antitumor immunity by increasing the frequency of Tregs that express the co-inhibitory receptor Tim-3 and that induce CD8<sup>+</sup> T-cell dysfunction (96). TIGIT<sup>+</sup> Tregs selectively suppress the responses of proinflammatory Th1 and Th17 cells *via* the upregulation of fibrinogen-like protein 2 and the secretion of IL-10 (97). TIGIT represses IFN-γ secretion, promotes the nuclear translocation of forkhead box O1 (FoxO1), inhibits protein kinase B (PKB, also called Akt) function, and restores suppressor function of Tregs (98). TIGIT also plays an important role in regulating NK cell responses. In mouse models, the blockade of TIGIT's interaction with its ligand PVR leads to increased NK-cell mediated cytotoxicity (99). In tumor models, TIGIT exerts its regulatory function and inhibits NK-mediated cytotoxicity on tumor cells to promote immune evasion (100). Antibody blockade of TIGIT could inhibit NK cell exhaustion and promote NK cell dependent tumor immunity (101). Clinically, dual blockade of TIGIT and PD-1 could increase the frequency of CD8<sup>+</sup> TILs and tumor antigen specific CD8<sup>+</sup> T cells in established melanoma patients (102). Dual PD-1 and TIGIT blockade treatment in glioblastoma multiforme (GBM) patients showed decreased effector T cell function, increased Tregs, and increased tumor infiltrating dendritic cells (TIDCs) (103). In B-cell non-Hodgkin's lymphoma patients, the response rates (RRs) of PD-1 blockade are comparatively low, and these patients display increased CD8<sup>+</sup> and CD4<sup>+</sup> T effector memory cells expressing TIGIT and PD-1 with limited IFN-γ, TNF-α, and IL-2 production (104). In gastric cancer patients, TIGIT and PD-1 were found to be upregulated on infiltrating CD8<sup>+</sup> T cells in tumor tissues, suggesting that TIGIT may serve as an emerging biomarker (105). In follicular lymphoma, increased numbers of TIGIT<sup>+</sup> T cells are associated with poor survival, therefore TIGIT may serve as a predictive marker for therapeutic clinical outcomes (106).

## BTLA

The B and T lymphocyte attenuator (BTLA) is an immunoglobulin superfamily member, which downregulates T-cell activation (69). BTLA contains a glycoprotein with two immunoreceptor tyrosine-based inhibitory motifs. The ligand for BTLA is B7x, a peripheral homolog of B7. BTLA is not expressed in naïve T cells, but it is expressed on Th1 cells upon activation (107). After crosslinking BTLA with antigen receptors, BTLA attenuates IL-2 secretion, and therefore it functions as an inhibitory receptor similar to CTLA4 and PD-1 (108). Blockade of BTLA in T-cells increased T-cell proliferation, and BTLA deficient mice display increased incidence and severity of autoimmune diseases, such as EAE. A recent report suggests that PD-1 and BTLA can suppress T cell signaling *via* SHP-1 and SHP-2 (109). BTLA also functions as a negative regulator of B cell receptor (BCR) signaling by binding with its ligand herpesvirus entry mediator (HVEM). Engagement of BTLA4 leads to recruitment of the tyrosine phosphatase Src homology 2 domain containing phosphatase 1, and reduces the activation of BCR-signaling pathways (108). Furthermore, BTLA4 engagement inhibits the activation of B cells by targeting Syk and B cell linker proteins (110).

BTLA is also expressed on DCs and macrophages (111, 112). BTLA plays a regulatory role in peripheral tolerance, evidenced by the ability of BTLA<sup>+</sup> DCs to induce CD8<sup>+</sup> T cell tolerance that could alleviate the severity of type 1 diabetes (113). Regarding Tregs, BTLA governs the cell differentiation and activation of Tregs (114). BTLA deficient mice have fewer Tregs and develop autoimmune diseases such as EAE. Interestingly, adoptive transfer of myelin oligodendrocyte glycoprotein fused reovirus protein  $\sigma$ 1-B220<sup>+</sup>CD5<sup>+</sup> Bregs, which express elevated BTLA levels, protects against EAE development, suggesting that BTLA plays a critical role in the activation of Tregs and Bregs (115). Therapeutically, several studies have explored the application of BTLA4 blockade in improving clinical outcomes in patients with colorectal cancer, epithelial ovarian cancer, lung cancer, gastric cancer, and other cancers (116–119). BTLA blockade inhibits IL-6 and IL-10 secretion, induces CD19<sup>hi</sup> B cells through AKT and STAT3 signaling pathways, and significantly improves the therapeutic outcomes in epithelial ovarian carcinoma patients (117). Another study revealed that up-regulation of BTLA occurs independently of functional exhaustion, which is induced by high antigen load. Combinatorial blockade of PD-1, Tim-3 and BTLA could enhance the expansion, proliferation, and cytokine production of CD8<sup>+</sup> T cells (120). Taken together, BTLA should be further explored to enhance the clinical responsiveness to immune checkpoint blockade therapies.

## Other immune checkpoints

In addition to the immune checkpoints mentioned above, other immune checkpoint molecules have been identified including B7 family molecules (B7-H3, B7-H4 and HHLA2), indoleamine 2,3-dioxygenase 1 (IDO-1), V-domain Ig suppressor of T cell activation (VISTA), and inducible T cell co-stimulator (ICOS). B7-H3, also known as CD276, is a newly discovered immune checkpoint protein, and plays a crucial role in the adaptive immune response in human cancers (121, 122). As a transmembrane glycoprotein, B7-H3 contains single variable and several constant immunoglobulin domains. It binds with its receptor CD28 which is universally expressed on T cells, B cells, monocytes, NK cells, and APCs including DCs and myeloid-derived suppressive macrophages (123, 124). Moreover, it is also constitutively expressed on the surface of tumor cells fibroblasts, tumor endothelial cells, osteoblasts and amniotic fluid stem cells (125). Upon interaction with CD28, B7-H3 ultimately leads to immune evasion by inhibiting the activation of T cells. B7-H3 deficient mice have increased frequencies of differentiated Th1 cells associated with severe inflammation in the respiratory system and the development of autoimmune encephalomyelitis earlier than wild type littermates (126). However, B7-H3 also possesses a co-stimulatory role by inducing IFN- $\gamma$  production, increasing T cell proliferation (121), and stimulating the differentiation of CD8<sup>+</sup> T cells that drive antitumor immunity (127). CD4<sup>+</sup>CD25<sup>+</sup>Tregs inhibit DC activation *via* up-regulation of inhibitory B7-H3 on DCs. B7-H3 engagement also results in decreased MHC-peptide complexes and impairs T cell activation (128). The expression of B7-H3 and B7-H4 has been reported to be correlated with metastasis by stimulating the expression of immunosuppressive IL-10 and TGF- $\beta$ 1 (129). Studies in tumor metabolism revealed that B7-H3 increases the expression levels of hypoxia-inducible factor 1  $\alpha$  (HIF1 $\alpha$ ), lactate dehydrogenase A (LDHA), and pyruvate dehydrogenase kinase 1 (PDK1), and therefore stimulates the Warburg effect by increasing glucose uptake and lactate production (130). These studies provide evidence that B7-H3 can regulate aspects of both cancer persistence and immune regulation, and should be considered as a potential target in the next stage of immune checkpoint blockade therapies for cancer.

B7-H4, also referred to as B7x and B7-S1, is a highly conserved immunoglobulin superfamily and belongs to the B7 family (131). It is ubiquitously expressed by tumor tissue and immune cells, including T cells, APCs, DCs, peritoneal macrophages and B cells (131, 132). B7-H4 negatively regulates T cell activation by decreasing T cell proliferation, inhibiting the production of IL-2 and inducing cell cycle arrest of T cells (133, 134). B7-H4 regulates pro-inflammatory T-cell responses by inhibiting CD4<sup>+</sup> T-cell proliferation and

differentiation to both Th1 and Th17 cells, inducing the production of IL-10, and increasing Treg activation (135). Deficiency of B7-H4 leads to elevated Th1 and Th17 responses (136). The expression of B7-H4 was shown to be up-regulated in various cancers, including breast, pancreatic, ovarian, lung, renal cell, and endometrial cancers (137–140). The expression levels of B7-H4 have also been recognized to constitute a prognostic indicator associated with poor overall survival in pancreatic cancer patients (137).

HHLA2 (HERV-H LTR-associating 2; also referred to as B7H5 and B7H7) belongs to the B7 family and is expressed by macrophages and DCs. As a novel co-stimulatory molecule, HHLA2 binds with CD28H (also known as Transmembrane and Immunoglobulin Domain Containing 2 (TMIGD2)), stimulates T cell proliferation and increases cytokine production *via* induction of the Akt signaling pathway (141–143). When combined with TCR and CD28 stimulation, it plays a co-inhibitory role comparable to PD-L1 (144). In T cells and NK cells, it also binds with KIR3DL3 (killer cell immunoglobulin-like receptor, three immunoglobulin domains and long cytoplasmic tail 3) to mediate tumor immune evasion independently from PD-L1 (145). HHLA2 inhibits T-cell proliferation and cytokine production including IFN- $\gamma$ , TNF- $\alpha$ , IL-5, IL-10, IL-13, IL-17A, and IL-22 (146). Therefore, it has been reported to be a novel prognostic predictor, similar to PD-1, in tumor immunotherapy (147, 148).

V-domain Ig-containing Suppressor of T cell Activation (VISTA; also referred to as PD-1H, DD1 $\alpha$ , c10orf54, Gi24, Dies1 and SISP1) is a transmembrane protein containing a single N-terminal immunoglobulin (Ig) V domain (149). Phylogenetically, VISTA is similar to PD-1, CD28, and CTLA4 (150). It is the most conserved molecule among the B7 family members, sharing similar domains with CD28 and CTLA4 within its cytoplasmic tail (149). Studies on VISTAs Src homology 2 (SH2) binding motif suggest that it functions as both a ligand and receptor in controlling immune responses (149, 151). VISTA is mainly expressed on cells of the hematopoietic lineage, including macrophages, DCs, monocytes, and circulating neutrophils (152). Albeit absent in B cells, it is unanimously expressed in naïve T-cells, CD4<sup>+</sup> memory T- cells and Tregs (150). Weak expression of VISTA is also observed in CD8<sup>+</sup> T cells and NK cells (149). VISTA deficiency results in enhanced activation of T-cells with increased production of IFN- $\gamma$ , TNF- $\alpha$  and IL-17, and contributes to the onset of overt autoimmunity (151, 153). Within the TME, VISTA plays a crucial role with increased expression observed on myeloid DCs, myeloid-derived suppressor cells (MDSCs) and Tregs (154). Blockade of VISTA decreases the number of MDSCs, increases activated DCs, and reduces the frequency of tumor specific Tregs (155).

The immune checkpoint molecules indoleamine 2,3-dioxygenase-1 (IDO-1) and tryptophan dioxygenase (TDO) belong to the tryptophan catabolic enzyme family. They

catalyze the degradation of tryptophan (Trp) into kynurenine (Kyn) (156, 157). IDO-1 plays an immunosuppressive role in various cancers (158) by suppressing CD8<sup>+</sup> T effector (Teff) cells and NK cells (159, 160). Further, it stimulates the proliferation of Tregs and MDSCs (161), decreases the ratio of Th17 cells versus Tregs, inhibits Th17 related cytokine production, and decreases the number of Th1 and Th22 cells (162, 163). Moreover, IDO-1 regulates the recruitment, polarization and phagocytosis capabilities of macrophages (164, 165). TDO has also been reported to be an immunosuppressive molecule capable of reducing CD8<sup>+</sup> T cell viability, stimulating the secretion of cytokines such as IFN- $\gamma$ , TNF- $\alpha$ , IL-10 and IL-17 by TDO reactive CD4<sup>+</sup> T-cells, and contributes to the tumor metastasis (166–168).

Inducible Co-Stimulator (ICOS), a homodimeric protein expressed on activated T cells, is an inducible T-cell co-stimulator (169). Specifically, it shares common signaling mechanisms with CD28, and increases T cell responses by stimulating T-cell proliferation and cytokine secretion (169). By binding with its ligand (B7-H2, B7RP-1), ICOS stimulates the response of T effector cells and T cell-dependent B cells (170). Further, this interaction promotes IL-10 secretion by effector T cells even in the presence of mature DCs (171). ICOS blockade attenuated T cell proliferation and inhibited Th effector responses by decreasing Th2 cytokine secretion (172). Inhibition of ICOS attenuates cytokine productions as IL-2, IL-4, IL-5, and IFN- $\gamma$ , and decreases the Th2 mediated mucosal inflammation (173). Deficiency in ICOS fails to form T follicular helper (Tfh) cells. Therefore, inhibition of ICOS decreases the immunosuppressive function of Tregs and decreases the expression of Tfh markers of the lymphoid tumor cells (174, 175). Beyond the canonical immune checkpoints of CTLA4 and PD-1/PD-L1, other immune checkpoints may, therefore, also be crucial in reducing immune responses in numerous cancers. Combination therapy with classical inhibition of CTLA4 and PD-1/PD-L1 may, thus, result in a durable clinical response in cancer therapy.

## Drugs targeting the immune checkpoints

Tremendous progress has been achieved in cancer immunotherapy over the past few decades. To date, the most widely applied immunotherapeutic drugs are antibodies that block immune inhibitory receptors such as CTLA4, PD-1, and PD-L1 (Supplementary Table 1). Although the FDA has approved these drugs to treat various cancer types, a large number of immune checkpoint inhibitors (ICIs) targeting other inhibitory receptors are in clinical development. In this section, we will summarize these drugs in cancer immunotherapy.

## CTLA4 inhibitors

Ipilimumab was the first FDA approved CTLA4 inhibitor to hit the market (176). In humans, a phase III clinical trial in 2010 (MDX010-20, NCT00094653) in patients with unresectable or metastatic melanoma evaluated the efficacy and safety of ipilimumab (3 mg/kg) and found it alone extended the median overall survival (OS) of patients by more than three months compared with the control group, showing that a single ICI can have potent antitumor effects (177). In 2011, ipilimumab was approved by the FDA for the treatment of late-stage (metastatic) melanoma patients (176), and 3 mg/kg administered once every 3 weeks (Q3W) for four cycles was adopted as the standard of care based on the results of several clinical trials (177–179). Besides melanoma, clinical trials using single-dose, 3 mg/kg ipilimumab have been conducted in patients with ovarian cancer (180, 181) and prostate cancer (182), but its effect was not as effective in these cancer types compared to the success rates for melanoma patients. Thus, use of ipilimumab in combination with other treatment modalities, such as additional immunotherapies, chemotherapies and radiotherapies, are being tested in the clinic to improve success rates in multiple cancers. For example, 10 mg/kg ipilimumab plus dacarbazine (a chemotherapeutic agent) prolonged the median OS from nine months to 11 months in patients with previously untreated metastatic melanoma compared with dacarbazine plus placebo (183).

Another CTLA4 inhibitor, tremelimumab, is a fully human mAb containing an IgG2 Fc domain in order to minimize Fc domain effector functions such as complement fixation and antibody-dependent cytotoxicity (184, 185). Tremelimumab alone has displayed potential antitumor effects in advanced melanoma patients in phase I, I/II and II trials (186–188). However, in a phase III trial in patients with unresectable stage IIIc–IV melanoma who had not received prior systemic treatment, tremelimumab was found to not be superior to standard of care chemotherapy regimens (median OS 11.8 months vs 10.7 months, hazard ratio (HR) 0.96) (189). These disappointing results led to early termination of the study and has limited available data of tremelimumab in melanoma patients. Besides melanoma, tremelimumab monotherapy has been studied in several other solid tumors, including metastatic esophageal and gastric adenocarcinoma, locally advanced or metastatic non-small cell lung cancer (NSCLC), treatment-refractory colorectal cancer (CRC), etc, but no satisfactory results have been obtained (190–193).

## PD-1 inhibitors

Currently there are three anti-PD-1 and three anti-PD-L1 antibodies approved by the FDA for treatment of more than 10 cancer indications. The first PD-1 inhibitor to enter the clinic

was nivolumab in 2006, and the first to receive FDA approval was pembrolizumab in 2014 for the treatment of advanced melanoma patients (194). Both of these PD-1 inhibitors contain human IgG4 Fc regions containing mutations in the stabilizing hinge region (195). Nivolumab was tested in 2010 in a phase I trial in 39 patients with advanced solid tumors that were treatment-refractory (196). These tumor types included metastatic melanoma, prostate cancer, NSCLC, renal cell carcinoma (RCC) and CRC. Different doses (0.3, 1, 3, 10 mg/kg) of nivolumab were used and antitumor activity was found in 1 CRC (complete response (CR), 3 mg/kg), 1 melanoma (partial response (PR), 10 mg/kg) and 1 RCC patient (PR, 10 mg/kg). The drug was well-tolerated and common AEs observed were reduced lymphocyte numbers, fatigue, and musculoskeletal symptoms (196). In a subsequent study, a multidose (ranging from 0.1 to 10 mg/kg) clinical trial with expanded patient numbers (N=296) was conducted in the same tumor types (197). CR or PR was observed in melanoma (28%), RCC (27%) and NSCLC (18%) patients, and phase III trials were then initiated for these indications (198–201). Considering the promising results from these trials, the FDA approved nivolumab for the treatment of refractory melanoma in 2014 and NSCLC in the following year (202). Since then, nivolumab has gained FDA approval for the treatment of multiple solid tumor types (Supplementary Table 1). Interestingly, in classical Hodgkin's lymphoma (cHL), the surface of tumor cells also overexpresses PD-L1/PD-L2 due to genetic alterations on chromosome 9p24.1 (203, 204). In a phase I study, nivolumab showed beneficial responses in 87% (20/23) of relapsed/refractory cHL patients (205). In a phase II study, the overall objective response rate (ORR) was 69% and median PFS was 14.7 months (206). Thus nivolumab was also approved for treating cHL patients in 2016 as the first PD-1 inhibitor (207).

Pembrolizumab was produced by grafting the variable region sequences of a mouse mAb onto a homologous human IgG4- $\kappa$  isotype framework containing a stabilizing S228P Fc mutation to reduce the immunogenicity of the mouse variable region (208). In a Phase I trial of patients with advanced ipilimumab-refractory melanoma, 26% of patients in the pembrolizumab group showed either CR or PR (209). Later in a Phase III trial in patients with advanced melanoma, the RR was significantly higher in pembrolizumab 10 mg/kg Q2W (33.7%) and Q3W (32.9%), compared with ipilimumab (11.9%) (210). In NSCLC patients (untreated, advanced), a Phase III trial showed the median PFS was 10.3 months in pembrolizumab, compared to 6.0 months in chemotherapy groups and the RR was 44.8% and 27.8%, respectively (211). Pembrolizumab was well tolerated, and the most common AEs reported were fatigue, pruritus, and rash. Similar to nivolumab, pembrolizumab is approved for treatment of patients with multiple cancer types (202).

Cemiplimab is another human IgG4 antibody that is approved for treating cutaneous squamous cell carcinoma

(cSCC), basal cell carcinoma and certain types of NSCLC (212, 213). In a meta-analysis comparing the efficacy of cemiplimab and pembrolizumab in advanced cSCC patients, cemiplimab treatment was associated with better OS (HRs ranging from 0.21–0.52) and PFS (HRs ranging from 0.49–0.55) (214). However, this conclusion should be interpreted with caution as its results were from indirect comparison without evidence from head-to-head clinical trials. The overall number of comparator studies included in this meta-analysis was low, and some studies enrolled small numbers of patients. While data from nivolumab or pembrolizumab trials treating locally advanced or metastatic vulvar SCC has been scarce, a phase III trial (NCT03257267) investigated cemiplimab in patients with recurrent/metastatic (RM) cervical cancer, who were resistant to first-line platinum-based therapy. Results from the overall population highlighted a 31% reduction in the risk of death as well as a 25% reduction in disease progression and an ORR of 16% ( $n = 50$ ) [6% ( $n = 19$ ) in the chemotherapy-treated cohort]. Further, the median OS in the cemiplimab group was 12 months compared to 8.8 months with chemotherapy, and the drug was well tolerated (215). According to these data, cemiplimab was granted priority review by the FDA for patients with RM cervical cancer who have experienced disease progression while on or after chemotherapy treatment regimes.

Besides the anti-PD-1 mAbs discussed here, a number of other anti-PD-1 mAbs have been approved by China or the European Union for treatment of different cancers, such as sintilimab, camrelizumab, toripalimab, tislelizumab, zimberelimab, progolimab, or dostarlimab; all of which have shown promising therapeutic effects for various cancer related indications (216).

In addition to PD-1 blocking agents, antibodies targeting PD-L1 also have potent antitumor qualities. Currently, three anti-PD-L1 antibodies, atezolizumab, avelumab, and durvalumab, are approved by the FDA to treat several cancers (217). Atezolizumab is a human IgG1 that has an N297A mutation to eliminate the unwanted fragment crystallizable (Fc)-mediated functions such as antibody-dependent cytotoxicity (ADCC) by glycosylation (218). Durvalumab also contains a modified Fc region to prevent depletion of PD-L1 expressing T cells (217). In contrast, avelumab owns a native Fc region which can bind to Fc- $\gamma$  receptors on NK cells to initiate ADCC (217). Their approved indications, time and dosage are summarised in [Supplementary Table 1](#).

## Non- CTLA4 and PD-1 ICIs

Despite the success of CTLA4 or PD-1/PD-L1 inhibition, a majority of patients develop drug resistance. Thus, the identification of novel and non-redundant pathways of immune inhibition is important for advancing therapies that promote anti-tumor immunity. In a phase III clinical trial of

anti-Lag-3 (relatlimab, 160 mg Q4W) combined with nivolumab (480 mg Q4W) for melanoma patients, patients receiving dual Lag-3/PD-1 blockade showed an increased PFS than those receiving nivolumab alone (median PFS 10.1 months vs 4.63 months, and PFS rates 47.7% vs 36.0% at 12 months). The combined regimen was well tolerated and total AEs were not significantly increased: 18.9% vs 9.7% grade 3/4 TRAEs and 14.6% vs 6.7% treatment discontinuations (219). Thus, this fixed-dose combination trial laid the foundation for dual inhibition apart from anti-CTLA4/anti-PD-1 pathways for clinical use and was recently FDA approved for patients with unresectable or metastatic melanoma (220).

Monalizumab is a humanized anti-NKG2A antibody that has the ability to enhance the anti-tumor activity of NK cells (221). In A20 tumor-bearing mice, combined treatment with anti-NKG2A and anti-PD-L1 mAbs rescued 75% of the mice from death compared to 40% in the anti-PD-L1 alone group (221). Although monalizumab alone was tested in patients with RM squamous cell carcinoma of the head and neck (SCCHN) in a phase II trial (222), the study did not meet its primary endpoint (no OR was observed) and it was terminated at the interim as ineffective (222). The safety profile of monalizumab was favorable, and it is now being tested in combination with durvalumab in a biomarker-based study in RM SCCHN patients. Cetuximab, an anti-EGFR mAb, promoted ADCC by binding to CD16/Fc $\gamma$ RIII (223), and the ADCC induced by cetuximab was enhanced by monalizumab through NK cell stimulation, providing greater anti-tumor activity than cetuximab alone (221). A phase II trial of monalizumab plus cetuximab in SCCHN patients showed a 31% ORR, with the most common AEs being fatigue (17%), pyrexia (13%), and headache (10%) (221). These results led to an ongoing phase III (INTERLINK-1) trial for RM HNSCC patients who underwent prior ICI- and platinum-based chemotherapy treatments. Although monalizumab has not yet gained FDA approval, it has shown benefit in enhancing the effects of other ICIs. However, future studies are needed to carefully define the suitable tumor types and to determine which type of combination therapy provides the optimal benefits.

In several preclinical cancer models, TIM-3 blockade displayed a slight advantage in terms of efficacy, especially when combined with PD-1 blockade (60, 224). Several-in-human Phase I/II trials have been initiated for numerous TIM-3 antibodies in patients with solid tumors or lymphoma (225). Most of them have been tested in conjunction with anti-PD-1/PD-L1 mAbs. TSR-022 (TIM-3 antibody, Tesaro) plus TSR-042 (anti-PD-1 antibody) demonstrated improved antitumor immunity in NSCLC patients who failed to respond to anti-PD-1 treatment alone previously (226). A SCLC patient who received LY3321367 (a novel TIM-3 mAb) 1200 mg Q2W as a single agent also achieved PR (227). All in all, these early data suggest that blocking TIM-3 alone or together with PD-1/PD-L1 is generally safe, well tolerated, and provides a slight

advantage in efficacy compared to anti-PD-1/PD-L1 therapies alone.

## Combinatorial use of immune checkpoint blockade and other drug therapies

Despite the PD-1/PD-L1 pathway being recognized as a key checkpoint in the immune response of various cancer types, most patients do not benefit from PD-1/PD-L1 pathway blocking therapies due to primary or acquired resistance (228). The immune system can paradoxically suppress or promote cancer development and growth. Therefore, removing or blocking the factors that promote immune tolerance and boost anti-tumor immunity may facilitate better responses to anti-PD-1/PD-L1 therapy. Based on this conception, combinations of anti-PD-1/PD-L1 mAbs with chemotherapies, targeted therapies, or CTLA4 blockade have been tested clinically and some regimes become the standard of care for several cancer types including metastatic melanoma, kidney, lung, head and neck, triple negative breast (TNB), and liver cancers. Herein, we will briefly discuss the progress that has made with dual combination of ICIs and alternative therapeutic regimes (Table 1).

### Combinatorial targeting of the CTLA4 and PD-1/PD-L1 pathways

Although CTLA4 and PD-1 are both immune checkpoints, they can suppress the activation of T-cells in non-redundant manners, and therefore dual blockade may work synergistically to enhance anti-tumor immune responses. Neoadjuvant ipilimumab plus nivolumab in patients with macroscopic stage III melanoma showed a 2-year estimated recurrence-free survival rate of 84% in all patients, a 97% estimated survival rate in patients who achieved a pathologic response, and a 36% estimated survival rate in non-responding patients (229). In addition to melanoma, many neoadjuvant ipilimumab plus nivolumab trials have been conducted in solid tumors, and have demonstrated enhanced responses in advanced RCC (230), microsatellite high/deficient mismatch repair colorectal cancer (mCRC MSI-H/dMMR) (231), HCC (232), NSCLC [at 12 months (233) and 24 months (234)], and malignant pleural mesothelioma (MPM) (235). Based on these clinical trials, the FDA has approved the combination of ipilimumab and nivolumab for the six cancer types mentioned above.

The combination of tremelimumab and durvalumab are being tested in several phase 3 trials (236–248). Although some results were unsatisfactory, this combination significantly improved OS in patients with advanced, unresectable HCC and NSCLC patients who had a high tumor mutation burden (TMB) (248, 249), showing the importance of selecting appropriate treatment regimens for specific patient subsets.

## Combinatorial use of anti-PD-1/PD-L1 and other ICIs

Early in 2022, the FDA approved a fixed-dose combination of nivolumab and relatlimab for the treatment of unresectable or metastatic melanoma based on a phase II/III study (NCT03470922) (220). Data from the clinical trial showed a significantly superior PFS (median 10.2 months) in the combination group over nivolumab alone (4.6 months,  $p=0.0055$ ). Combination therapy also reduced risk of death by 20% and improved OS rates versus nivolumab, although it did not reach statistical significance. The outcomes of patients with features often associated with poor prognosis such as high tumor burden, visceral metastases, increased serum LDH levels, or mucosal or acral melanoma, were improved in the dual-inhibition group compared with nivolumab alone (220). The safety profile of this combination was generally manageable and no novel or unexpected safety concerns were noted, although grade 3 or 4 TRAEs (such as fatigue, hepatitis, and adrenal insufficiency) were more frequently noted in patients receiving relatlimab-nivolumab than those receiving nivolumab (250). However, the efficacy of combined therapy in real-world patient needs to be further investigated since some populations of patients are often excluded from clinical trials, including patients with untreated or active brain metastases or with certain melanoma subtypes (e.g., uveal melanoma).

### Combinatorial use of anti-PD-1/PD-L1 blockade and chemotherapy

Chemotherapeutic drugs slow tumor growth primarily by inhibiting DNA replication, interfering with cellular metabolism, inducing cell cycle arrest, or inhibiting microtubule assembly (251). In addition, some cytotoxic drugs such as oxaliplatin and anthracyclines can induce cell death of immunogenic cells, thereby stimulating anti-tumor immune responses (252, 253). Based on these effects, chemotherapeutic drugs may be suitable partners for combinatorial administration with anti-PD-1/PD-L1 to achieve rapid and long-term control of cancers. A large number of clinical trials have tested the efficacy and safety of chemotherapy plus anti-PD-1/PD-L1, with FDA approved combinations summarized in Supplementary Table 1. In general, pembrolizumab combined with chemotherapy has had great clinical benefit for a wide range of cancer indications, followed by atezolizumab combined with chemotherapy.

### Combinatorial use of anti-PD-1/PD-L1 blockade and angiogenesis inhibitors

The hypoxic tumor microenvironment (TME) leads to the upregulation of vascular endothelial growth factor (VEGF) and angiopoietin 2 (ANGPT2), which are key mediators in angiogenesis (254). By inhibiting these pro-angiogenic pathways, angiogenesis inhibitors can promote vascular normalization, restore oxygenation within the typically

hypoxic TME, improve perfusion and oxygenation in tumors, and enhance the delivery of antitumor drugs (255, 256). Several tyrosine kinase inhibitors that target the VEGF-signalling axis have been approved in the United States and European Union, including sorafenib, sunitinib, pazopanib, axitinib, lenvatinib, and cabozantinib (257).

The FDA recently approved the use of nivolumab plus cabozantinib, pembrolizumab/avelumab plus axitinib, and pembrolizumab plus lenvatinib in advanced RCC; pembrolizumab plus lenvatinib in advanced endometrial carcinoma; atezolizumab plus bevacizumab (anti-VEGF mAb) in unresectable or metastatic HCC; and atezolizumab plus bevacizumab and chemotherapy in non-squamous and metastatic NSCLC based on the results of these clinical trials (258–264).

## Adverse effects of ICIs

With the use of ICIs for cancer treatment rising, several side effects associated with treatment have raised concerns. Compared to treatment with standard chemotherapy or other biologics, ICIs have a different toxicity profile and most of these AEs are caused by aberrant immune responses against normal self-tissues due to impaired self-tolerance from the loss of T-cell inhibition, a phenomenon known as immune-related adverse events (irAEs) (265). Three drugs (ipilimumab, nivolumab and pembrolizumab) were reported to account for nearly 60% of reported irAEs in patients receiving immune checkpoint blockade therapies (266).

irAEs can affect any organ system, most commonly the skin, colon and endocrine systems (267). The onset time usually ranges from a few days after treatment initiation to  $\geq 1$  year after completion of treatment, with a median time of 2–16 weeks (268–270). Most irAEs occurring soon after administration (3 weeks after initiation of therapy) of CTLA4 and PD-1 inhibitors typically involves the skin (271). Serious and often life-threatening AEs include pneumonitis and colitis (272). In general, there are fewer AEs associated with anti-PD-1/PD-L1 drugs than CTLA4 inhibitors (273), potentially due to their different mechanisms of action. CTLA4 is expressed on T cells (including Tregs), and its activation primarily suppresses the immune response in the early stages of T cell activation in lymphoid tissues. As CTLA4 blockade of Tregs would also result in the loss of immune suppression, CTLA4 inhibition would result in widespread, nonspecific activation of immune response that might explain the broad spectrum of AEs (274, 275). In contrast, PD-1 inhibitors target T cells more specifically in the tumor microenvironment and tissues, resulting in a more restricted spectrum of AEs (274, 276).

Colitis and hypophysitis often occur in patients receiving CTLA4 inhibitors, while less common AEs (pneumonitis and thyroiditis) may occur in patients using drugs targeting PD-1/PD-L1 pathway (271, 277, 278). A meta-analysis of the irAEs of

individual ICIs revealed that the most common side-effects associated with ipilimumab included dermatological, gastrointestinal, and renal toxicities; and side-effects associated with nivolumab often involved endocrine toxicities. Similarly, side-effects associated with pembrolizumab included arthralgia, pneumonitis, and hepatotoxicity; and irAEs associated with atezolizumab included hypothyroidism (279). Most AEs resolved without intervention or responded to appropriate treatment (280). Severe irAEs usually require the treatment of systemic glucocorticoids or other immunosuppressive medicines, such as anti-TNF blockers.

The combination therapy such as dual PD-1/PD-L1 and CTLA4 blockade can also lead to severe irAEs such as colitis, pneumonitis, hypophysitis, and thyroiditis (267). Therefore, in these combined regimens, the dose of ipilimumab is usually reduced, which may impair the efficacy of the combination therapy (281). In a preclinical study, prophylactic TNF blockade reduced the toxicity of dual PD-1/PD-L1 and CTLA4 blocking antibodies (282). When ICIs are combined with chemotherapies, the reported irAEs were consistent with those of each drug (283, 284). In order to minimize the potential toxicity of dual PD-1/PD-L1 and CTLA4 blockade, Dovedi et al. developed a bispecific anti-PD-1/CTLA4 antibody, MEDI5752 (285, 286). The antibody could inhibit the signal transduction through PD-1 axis and preferentially block CTLA4 on activated PD-1<sup>+</sup> T cells over PD-1<sup>-</sup> T cells, which means that CTLA4 is inhibited only in T cell populations that have already been exposed to the antigen. Therefore, the additive toxicity of this bispecific antibody is reduced compared with traditional dual blockers (285). In an ongoing in-human study in patients with advanced solid tumors, one patient with gastric cancer in whom five prior lines of chemotherapy had failed showed PR with 60% tumor reduction, and another patient with treatment-naïve renal clear cell carcinoma had PR with 68% tumor reduction. Both of the patients had manageable toxicity (285). However, more clinical studies are needed to determine the safety and efficacy of this bispecific agent or other combination therapies.

## Potential biomarkers of ICI response

We are now in an era of innovation in cancer immunotherapy that is transforming the field of clinical oncology. Sustained durable responses from ICIs provide new hope as a treatment option for patients previously diagnosed with terminal illnesses. However, only a small percentage of tumors are responsive to these therapies and the overall response rates are low. There might be several reasons for this (1). The corresponding TMB. High TMB accompanied by elevated neoantigen expression might induce cytotoxic responses against tumor cells (287, 288). Thus tumors with low TMB and poor immunogenicity such as prostate and pancreatic cancers would be more resistant to ICIs (287) (2). Intratumor

heterogeneity may lead to the selection of subclones that lack neoantigen expression and confer resistance to immune therapies (289, 290) (3). Genetic instability such as alterations in DNA-mismatch repair genes can increase immunogenicity and enhance response to ICIs (291, 292). For example, melanoma patients responded better to anti-PD-1 therapy when their tumor cells were rich in mutations in BRCA2, a gene important for homologous recombination in DNA repair (293). Mutation of B2M was also reported to be associated with initial resistance to anti PD-1 therapy in melanoma patients (294, 295) (4). Potential biomarkers may predict the response to ICIs. For example, the greater the number of tumor-infiltrating lymphocytes (TIL) in the tumor microenvironment, the better the anti-tumor effect (296); and tumors manifesting PD-L1 overexpression usually portend clinical outcomes superior to those of ICI compared with those with lower levels of ligand (297) (5). Complex interactions of immune cells and cytokines in the TME. For example, Tregs promote self-tolerance by inhibiting the function of T<sub>H</sub>1 through inhibitory cytokines and direct contacts (298), and in some cancers, the infiltration of Tregs suggest an immunosuppressive environment (299). Myeloid-derived suppressor cells (MDSCs) in the TME can promote tumor growth and immune evasion (300, 301) (6). Alterations to a patient's gut microbiome have also been associated with response or resistance to immune-checkpoint blockade, although the exact mechanism remains unknown (302) (7). The presence of tertiary lymphoid structures (TLS) might indicate improved survival in some tumor types (303, 304). Higher densities of TLS were associated with increased tumor-infiltrating CD8<sup>+</sup> T cells density and also with an activated and cytotoxic immune signature (304–306). The existence of mature TLS was associated with improved outcomes in cancer patients treated with ICIs, regardless of their PD-L1 status and tumor-infiltrating CD8<sup>+</sup> T-cell level after adjustment (307).

There are also other factors that could influence the anti-tumor effect of ICIs, including T-cell exhaustion (308), chromatin remodeling (309), and upregulation of alternative negative immune-checkpoint molecules (310). Resolving these issues is posited to elevate the efficacy of ICIs to a higher level. For example, personalized vaccines composed of an individual patient's tumor neoantigens might be taken up and presented by activated APCs, leading to the activation of specific T cells that target these neoantigens, and thereby enhancing the efficacy of PD-1/PD-L1 inhibitors (311). Targeting other immunomodulatory cells or cytokines such as MDSCs, anti-TGF- $\beta$ , and PI3K $\gamma$  are currently in clinical trials (311, 312). Since some results are not promising, we still need to advance our overall understanding of the molecular biology of tumors.

In addition, it is necessary to explore how to better predict patient responses to improve clinical outcomes. Although the expression of PD-L1 on tumor cells, TIL status, and the assessment of mutational burden are presently used markers

(290, 313–317), they often have poor specificity and sensitivity. Accurate prediction using biomarkers therefore remains a major clinical challenge and necessitates further investigation due to the complexity of antitumor immune responses and the heterogeneity among patients and tumors.

## Conclusions and perspective

The therapeutic landscape of oncology has been revolutionized with the advent of immunotherapies targeting immune checkpoints. A growing number of ICIs have been approved to treat different cancers. They can be used alone or in combination, or combined with other chemotherapies, vaccines and tumor immunotherapies, etc. Although these ICIs can bring survival benefits, their response rates and PFS are not high, and they also have a variety of adverse effects. A better understanding of the regulatory pathways of immune checkpoints will improve the success and efficacy of ICIs.

With in-depth investigations into immunotherapy, more novel immune checkpoints, regulators, and receptor-ligand pathways between tumor and host immune cells will continue to be discovered and further advance our understanding of the mechanisms leading to tumor immune evasion or resistance. Individualized therapeutic strategies based on a patient's genetic background may suppress overactive T-cell responses more robustly, and thereby assist in patient selection. Further research is urgently needed to elucidate the mechanisms underlying tumor biology and immunotherapy, how one ICI affects another, and the development of novel sequential treatment options, so as to facilitate the successful use of ICIs in treating cancer patients.

## Author contributions

YW and QZ (4<sup>th</sup> author) conceived and designed the research. LY and MS prepared the draft. QZ (3<sup>rd</sup> author) prepared the tables. QZ (3<sup>rd</sup> author) prepared the figure. YW and QZ (4<sup>th</sup> author) reviewed and edited the manuscript. All authors read and approved the final manuscript.

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## Conflict of interest

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## Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2022.982026/full#supplementary-material>

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