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Immune Checkpoints are Important Therapeutic Targets in Cancer Immunotherapy

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ABSTRACT

Immunotherapy has become one of the greatest advances in medical oncology over the last century; however, the optimal application for the treatment of different types of cancer remains an active area of investigation. Modern immunotherapy strategies augment the immune system and ideally, permit durable tumor-specific immune memory to target and kill cancer cells. This era began when first immune checkpoint inhibitor, ipilimumab, was approved. In fact, several monoclonal antibodies that mediate the immune checkpoint receptors have provided the most clinically meaningful improvement for cancer patients to date. Checkpoint blockade as monotherapy has demonstrated some encouraging results, although some combination strategies appear to augment those responses and may be particularly effective when administered earlier in the course of disease. Additionally, we have also discussed previous and ongoing clinical studies testing individual or combination immunotherapy in cancer patients. Overall, the goal of this review is to provide a summary and current status of immune checkpoints and their inhibitors as therapeutic approaches of cancer immunotherapy and highlight promising future directions.

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Keywords

CTLA, Immune checkpoint inhibitors, Immunotherapy, PD-1, Tumor, Tumor microenvironment.

Abbreviations

ADCC: Antibody-dependent cell-mediated cytotoxicity; ALL: Acute Lymphoblastic Leukemia; APCs: Antigen Presenting Cells; BCG: Bacillus-Calmette-Guerin; CAR: Chimeric Antigen Receptor; CCL8: C-C Motif Chemokine Ligand 8; CTLA4: Cytotoxic T Lymphocyte Antigen 4; DCs: Dendritic Cells; DC-LAMP: Dendritic Cell –Lysosomal Associated Membrane Protein; ECM: Extracellular Matrix; FDA: Food and Drug administrations; Hh: Hedgehog; HPV: Human Papilloma Virus; IC: Immune Check Point; ICI: Immune Check Point Inhibitors; ICOS+: Inducible Costimulatory Positive; IFNγ: Interferon gamma; IgSF: Immunoglobulin Superfamily; IL: Interleukins; IR: Inhibitory Receptor; ITIM: Immunoreceptor Tyrosine-Based Inhibitory Motif; ITSM: Immunoreceptor Tyrosine-Based Switching Motif; JAK/STATS: Janus Kinase/Signal Transducer and Activator of Transcription 5; LAG-3: Lymphocyte activation Gene 3 or CD223; LCMV: Lymphocytic Choriomenigitis Virus; mABS: Monoclonal Antibodies; MDSCs: Myeloid-Derived Suppressor Cells; MAPK: Mitogen-Activated Protein Kinase; NFκB: Nuclear Factor-κB; NK: Natural Killer; NO: Nitric Oxide; NSCLC: Non-

Small Cell Lung Cancer; OVs: Oncolytic Virus; PD1: Programmed Death 1; PD-L1: Programmed Death Ligand 1; PI3K/AKT: Phosphoinoside 3-Kinase (PI3K)/Protein Kinase B (AKT); PMN: Polymorphonuclear leukocyte; PSGL: P-Selectin Glycoprotein Ligand -1; SHP-1& 2: Src homology 2 domain containing protein tyrosine phosphatase 1 & 2; SLP: Synthetic Long Peptide; TAMs: Tumor-Associated Macrophages; TCR: T cell Receptor; TGF-β: Transforming Growth Factor Beta; Th1: T-Helper 1; TIGIT: T cell Immunoreceptor Tyrosine-Based Inhibitory Motif [ITIM] Domains; TIM-3: T cell Ig and Mucin Domain Containing -3; TME: Tumor Microenvironment; Tregs: T Regulatory Cells; VISTA: V-Domain Immunoglobulin (Ig) Containing Suppressor of T- Cell Activation; YSPSL: P Selectin Glycoprotein Ligand-Ig; ZAP70: Zeta Chain of T Cell Receptor Associated Protein Kinase 70.

Introduction

Immunotherapy is at the forefront of cancer treatment which has afforded cancer patients with the potential for long-term survival. Immunotherapy is used to strengthen the host immune system to identify and attack cancer cells in multiple targets. The goal of immune therapy is to overcome tumor and its microenvironment induced immunosuppression. This is associated with unique type of response, such as pseudoprogression, hyperprogression, long duration of response and also disease regression that continues after treatment stop. Unlike traditional cancer treatments such as chemotherapy and radiotherapy, immunotherapy stimulates immune cells and potentiates anti-tumor surveillance ability and eliminates most pre-malignant cells [1].

In past years, several inhibitory receptors (IR) or immune checkpoints (IC) have been discovered that negatively regulate the activation and function of T cells. Under normal conditions, both $CD4^+$ and $CD8^+$ T cells transiently express IR, which dampens the TCR (T cell receptor) signaling, restrains immune function and prevents autoimmunity [2]. Upon antigen clearance, IR expression is downregulated, and resting memory T cells maintain their low expression. During chronic antigen stimulation such as in cancer, there is sustained elevated expression of these receptors after initial activation rendering T cells into a dysfunctional or exhausted state. Different IR are coexpressed on exhausted T cells and their cognate ligands are upregulated on antigen presenting cells (APCs) and tumor cells [3]. Tumor cells abundantly express IC ligands, which works in conjunction with increased tumor-infiltrating Tregs (T regulatory cells) and myeloid-derived suppressor cells (MDSCs) to help tumor evade the active T cell responses. Tumor cells escape immunosurveillance by two major mechanisms: creating an immunosuppressive TME (tumor microenvironment) and immunoediting, which are also discussed in this review.

Immune checkpoints (ICs) inhibit T cell activation and function through diverse mechanisms but mainly via inhibiting TCR signaling. These ICs are of immense therapeutic importance, as blockade of these surface receptors can be used to reinvigorate T cells to promote tumor control. The discovery of several IC inhibitors (ICI) targeting cytotoxic T lymphocyte antigen 4 (CTLA-4) and programmed death-protein 1 (PD-1) represent a revolutionary milestone to cancer immunotherapy. Their discoverers, James Allison and Tasuku Honjo were awarded with 2018 Nobel Prize in Physiology or Medicine. Several other IC including LAG-3 (lymphocyte activation gene 3 or CD223), TIM-3 (T cell Ig and mucin domain containing-3), TIGIT (T cell immunoreceptor with immunoglobulin and immunoreceptor tyrosine-based inhibitory motif [ITIM] domains), PSGL-1 (P-selectin glycoprotein ligand-1) and VISTA/PD-1H (V-domain immunoglobulin (Ig)-containing suppressor of T-cell activation) have been discovered which work through diverse mechanisms. In this review, we have emphasized on the role of some of the major IC and clinical implications of their inhibitors in cancer immunotherapy.

Tumor Microenvironment: Acellular and Cellular Components

The TME is a cellular space harboring cancer stem cells, immune and non-immune cells, and acellular components that promotes tumor growth, metastasis, and survival. Cancer stem cells are capable of self-renewable and driving tumorigenesis [4]. The cellular component of TME encompasses MDSCs, tumor associated macrophages (TAMs), mast cells, granulocytes, dendritic cells (DCs), T cells, B cells, natural killer (NK) cells, cancer-associated fibroblasts, adipocytes and endothelial cells [5]. The acellular component of tumor microenvironment (TME) includes blood vessels, extracellular matrix (ECM), extracellular vesicles and cytokines.

The TAMs are reported to be pro-tumorigenic, and their infiltration is positively correlated to tumor malignancy [6,7]. TAMs are categorized into M1 and M2 phenotypes co-existing in TME. Heterogeneity of TAMs depend on the tumor type and can switch from one type to another depending on the TME. TAMs promote tumor invasion and metastasis by secreting ECM degrading enzymes, such as metalloproteinases (MMPs) and plasminogen activators, and are important factors in cancer invasion and metastasis, because invasion and metastasis of cancer cells require destruction of mesenchymal collagen or the endothelial basement membrane [8,9]. TAMs participate in neovascularization by secreting IL (interleukin)-8, CCL8, fibroblast growth factor (FGF) and vascular endothelial growth factor (VEGF). Clodronate-mediated macrophage depletion was reported to effectively prevent angiogenesis in mouse tumor models [10]. Another innate immune cell is myeloid-derived suppressor cells (MDSCs) whose number is significantly increased in cancer. MDSCs are heterogeneous population comprising myeloid progenitor namely granulocytic/polymorphonuclear MDSCs (PMN-MDSCs) and monocytic MDSCs (M-MDSCs) and immature cells and possess most potent immunosuppressive capacity [11]. MDSCs are capable of inhibiting T cell function *via* different suppressive mechanisms. MDSCs produce high level of nitric oxide (NO) that suppresses T cell proliferation either directly by inhibiting JAK/STAT5 pathway or by inhibiting antigen presentation by DCs [11]. It has been reported that MDSCs interferes with T cell activation [12], trafficking [13], non-responsiveness towards specific antigen

due to nitration of T cell receptor (TCR) complex and decreased expression of TCR-ζ-chain [14], and deprive T cells from essential amino acids [15].

Different T cell population exist within TME. Cytotoxic $CD8^+$ memory T cells kill tumor cells by recognizing specific tumor antigen and mounting an immune response [16]. $CD8^+$ T cells are supported by $CD4^+$ T helper 1 (Th1) cells that produce IL-2 and IFN-γ. Th2 cells support B cell response by producing IL-4, IL-5 and IL-33 [17]. On the other hand, Th17 cells producing IL-17A, IL-17F, IL-21 and IL-22 promote tumor growth. T regulatory cells (Tregs) are tumor promoters and exert an immunosuppressive function by producing IL-10, TGF-β and cytotoxic T lymphocyte antigen 4 (CTLA4). B cells are commonly found in draining lymph nodes and lymphoid structure adjacent to TME and have been reported to play a dual role [5]. In some cases, they regulate tumor cell survival and proliferation whereas in others, foster immune escape of tumor cells [5]. DCs are specialized antigen presenting cells (APCs) that initiate antigen-specific T cell response [18]. Presence of conventional DC1 (cDC1) in TME correlates with improved prognosis in different types of cancers [19,20]. Recent studies demonstrate that a high density of tumor-infiltrating DC-$LAMP^+$ DCs is associated with an immune contexture i.e., the spatial organization and density of immune infiltrate in TME, which is characterized by TH1 polarization and cytotoxicity activity. One of the studies suggested the presence of mature, DC-$LAMP^+$ DCs in TME to be a novel and powerful prognostic biomarker for high-grade serous ovarian carcinoma [20]. During tumor invasion, stromal cells act as a source of nutrients, oxygen, enzymes, and matrix-bound growth factors [21]. NK cells mount an anti-cancer immunity and produce immunomodulatory cytokines and chemokines that mold the immune cells and act as anti-cancer agent. The role of ECM in tumor development and spread has been well studied. ECM contains angiogenic factors and influence cancer cell migration by altering its elasticity, composition, and topography.

Cancer Immunoediting and Immunotherapy

The dual role of immune cells in malignancies have introduced the concept of immunoediting which comprise of three phases, elimination, equilibrium and escape [22]. During elimination phase, effector immune cells target and eradicate tumor cells. Cancer cell lysis occurs due to perforin secretion by cytolytic cells ($CD8^+$ T cells, NK cells, γδT cells, NKT cells) and antibody dependent cellular cytotoxicity (ADCC) [23]. Equilibrium phase is the longest phase where immune cells keep a check on tumor growth and metastasis. Tumor remains hidden in specialized niches in latent dormant state. Due to heterogeneity and genetic variability, tumor cells eventually become immune-evasive and escape the equilibrium. This initiates the onset of final phase of immunoediting during which tumor grows, metastasize and become detrimental [22].

Cancer immunotherapy is a novel promising modern-age treatment strategy, playing an increasingly vital role in cancer treatment [23]. Immunotherapy is a treatment, which harnesses host immune system to fight against cancer. It can be achieved either by stimulating the natural defense ability of the immune system so that it can recognize and attack cancer cells; or by synthesizing/ engineering immune system components to help restore immune system to find and attack cancer cells. There are several types of cancer immunotherapies, which are being extensively explored and used to treat cancer as depicted in Figure 1.

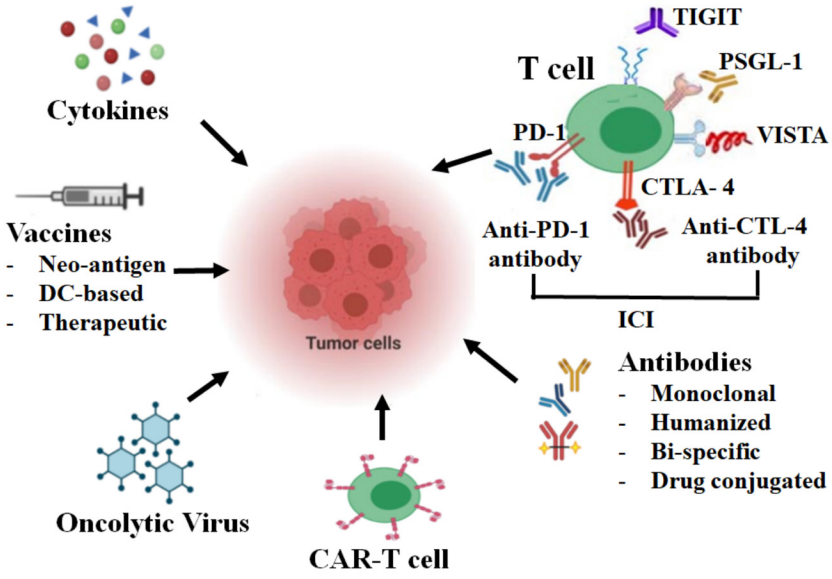

Figure 1: Different types of cancer immunotherapies. Examples of immunotherapy includes: immune checkpoint inhibitors (ICI) that prevent tumors from shutting off cancer-fighting cells by blocking checkpoints; antibodies designed to attack specific part of cancer cell and provide associated immunity; CAR (Chimeric antigen receptor)-T cell therapy which utilizes patient's own isolated T cells to eliminate tumor; oncolytic viruses that infect and destroy cancer cells thus making them visible to immune system; cancer vaccines that train T cells to respond to specific cancer antigens; cytokine treatment promote growth and activation of immune cells.

Several cytokines possess anti-proliferative or pro-apoptotic activity which limit tumor cell growth. IL-2 and IFN-γ have received The Food and Drug Administration (FDA) approval for the treatment of several malignancies such as renal cell carcinoma [24], follicular non-Hodgkin lymphoma [25], and metastatic melanoma [26]. Cancer vaccines help educate the immune system to recognize and eliminate cancer cells. Unlike prophylactic vaccines, which are generally administered to healthy individuals, therapeutic vaccines aim to treat late-stage disease by harnessing patient's immune system. FDA approved preventive vaccines include Cervarix against cervical cancers, Gardasil against HPV-related cancers, HEPLISAV-B against HBV-related liver cancer and therapeutic vaccines include Bacillus-Calmette-Guerin (BCG) against bladder cancer and Sipuleucel-T against prostate cancer [27]. Neoantigen-based vaccines prompt a strong immune response and reduce the possibility of resistance. Neoantigens are tumor-specific antigens that develop due to genetic instability of tumor cells leading to non-synonymous mutations [28]. Neoantigen vaccines mainly including nucleic acid, synthetic long peptides (SLP) and dendritic cell-based vaccines have been tested in clinical trial phase I and they presented promising results. Recent clinical studies on personalized therapeutic cancer vaccines predicted on neoantigens have been shown to be feasible, safe and immunogenic in melanoma and glioblastoma patients [29].

Chimeric antigen receptor (CAR) T-cell therapy is another promising way to fight cancer. The T-cells used in this therapy are engineered to express synthetic receptors that identify specific cancer cell antigens [30]. In 2017, FDA had approved two CART-cell therapies, one for the treatment of children with acute lymphoblastic leukemia (ALL) and the other for adults with advanced lymphomas [31]. Monoclonal antibodies (mAbs) are another popular immunotherapeutic, used against a variety of diseases due to their unique specificity, higher affinity and serum stability [32]. Anti-tumor mAbs function through various mechanisms such as directly targeting tumor cells, manipulating host response, delivering cytotoxic moieties and directing host cellular machinery against malignant cells [33]. Currently, there are various FDA approved mAbs for hematologic and solid tumors while many of them are undergoing clinical trials [34]. Oncolytic viruses (OVs) possess multi-modal mechanism of action, which target tumor cell, replicate in, and ultimately lyse tumor cells without affecting healthy cells [35]. Herpes virus based therapeutic, Imylgic, is an FDA approved therapy used in the treatment of advanced melanoma. According to BioCentury [36], currently there are two OVs in phase III trials, nine in phase II, at least eight in phase I development. Immune checkpoint inhibitors (ICI) are another advancement in the cancer therapeutics armamentarium. When immune checkpoint protein binds with partner proteins on other cells such as some tumor cells and then they send an "off" signal to the T cells, as a result it prevents the immune system from destroying the cancer. ICI block the negative regulators of T-cell functions (referred as immune checkpoints), thereby boosting T-cell activation [37]. ICI are drugs that act against several checkpoint proteins including CTLA-4 and PD-1 as depicted in Figure 2.

Immune Checkpoints (ICs)

The T lymphocytes are known for their antigen-directed cytotoxicity. After recognizing tumor-antigens, the active T cells proliferate, differentiate, and destroy tumor cells [38,39]. But recognition of antigen by T cell receptor is not enough to activate naïve T cells. The PD-1 receptor has two ligands: PD- L1 (B7-H1, CD274) and PD-L2 (B7-DC, CD-273) which are members of B7 and CD28 families. They play critical roles in T cell co-inhibition and exhaustion. B7 is a type of peripheral membrane protein found on APC. There are two B7 proteins: B7-1/CD80 and B7-/CD86. B7-1/CD80 is found on dendritic cells, macrophages and activated B cells, whereas B7-2/CD86) is found in B-cells. Both B7-1 and B7-2 interact with CD28 and CTLA-4. The

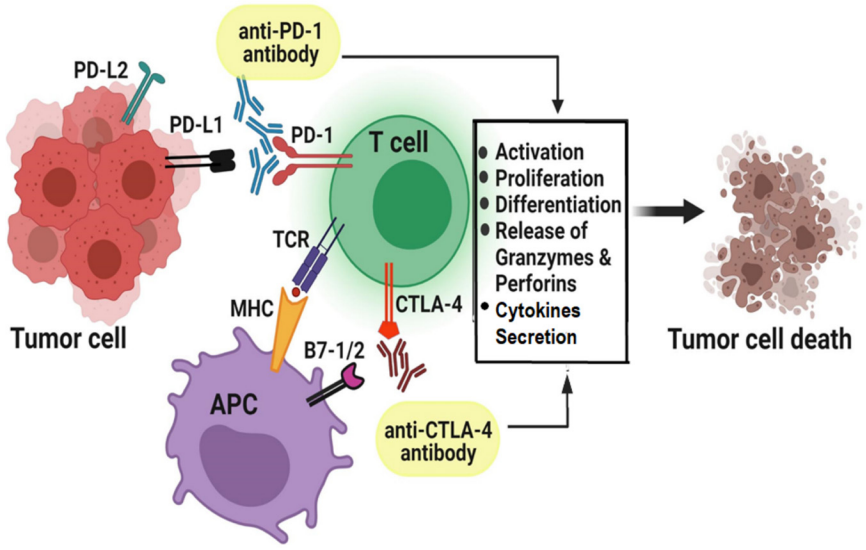

Figure 2: Blockade of CTLA-4 and PD-1 using antibodies. Blocking the interaction of cytotoxic T-lymphocyte antigen-4 (CTLA-4) with B7 proteins and programmed death-1 (PD-1) with PD-L1 using antibodies promote T cell activation and proliferation mounting an anti-tumor response thus leading to tumor cell death.

interaction between CD28 on T cell surface and B7 molecules (CD80 and CD86) on APCs provide the co-stimulatory signal required for T cell effector function. T cells infiltrate into TME, and encounter counter-defenses mounted by tumor cells and other cells present in TME [40]. T cell responses are regulated by both stimulatory and inhibitory signaling pathways. Under normal conditions, a perfect balance between T-cell activation and inhibition pathways is maintained to prevent autoimmunity, and identify and attack tumor cells [41]. The T cells in TME become dysfunctional due to immunosuppressive molecules and persistent antigen stimulation, leading to T cell exhaustion [42]. Extensive research on dysfunctional T cells suggested that exhausted T cells overexpress IR [40,43-45]. The term 'immune checkpoints' refer to these inhibitory proteins expressed by T cells that inhibit hyper-activation of T cells [1]. However, the same inhibitory proteins or receptors acts in favor of tumor cells by diminishing adequate immune response. Most of the ICs are initiated by ligand-receptor interaction. Hence, they can be easily blocked by antibodies or modulated by recombinant ligands or receptors. Seven of these drugs are approved by FDA to treat cancer. They block the proteins PD-1, PD-L1 and CTLA-4 (Table 1). In the following sections, we review our current knowledge of different IC and their inhibitors during cancer immunotherapy with a special focus on CTLA-4 and PD-1.

Table 1: Immune Checkpoint Inhibitors.

Site of Inhibition	Name of the inhibitors
PD-1	Pembrolizumab (Keytruda)
	Nivolumab (Opdivo)
	Cemiplimab (Libtayo)
PD-L1	Atezolizumab (Tecentriq)
	Avelumab (Bavencio)
	Durvalumab (Imfinzi)
CTLA-4	Ipilimumab (Yervoy)
	Tremelimumab (Ticilimumab)

Cytotoxic T Lymphocyte Antigen 4 (CTLA-4)

Mechanism of Action

CTLA-4 is a crucial T cell co-inhibitory receptor with structural and biochemical similarities to CD28 [46]. Unlike high basal levels of CD28 on conventional T cells, CTLA-4 is upregulated following antigenic activation. Interestingly, CTLA-4 is constitutively expressed in high levels by $CD4^+CD25^+$ regulatory T (Tregs) cells [47]. In naïve T cells, CTLA-4 is stored within cytosolic endosomes. CTLA-4 molecules are transported to the cell surface after TCR engagement and costimulatory signaling via CD28. Trafficking of CTLA-4 containing vesicles to plasma membrane depends on GTPase ADP ribosylation factor-1 and on phospholipase D activity [48]. Being a CD28 homologue, it competes with CD28 for its ligands (i.e., B7 molecules) thereby preventing CD28-mediated T cell activation. It has been reported that CTLA-4 binds to CD80 and CD86 molecules with 10 times higher affinity than CD28 [49]. Soluble CTLA-4 was reported to inhibit the proliferation of T cells co-cultured with APCs expressing B7 proteins [50]. Negative regulatory action of CTLA-4 is also evident in vivo. CTLA-4 knockout mice developed early autoimmune disease with massive lymphoproliferation and multiorgan destruction [51]. Despite several studies, it is still unclear which signaling pathways are initiated following interaction between CTLA-4 and its ligands. Both in vitro and in vivo studies showed relatively small changes in transcriptional profile after CTLA-4 engagement [52,53].

The main mechanism underlying tumor inhibition through CTLA-4 blockade is by competing with CD28 for B7 proteins. Since, tumor cells do not express B7 proteins (CD80 and CD86), presumably, anti-CTLA-4 inhibition takes place in tumor-draining lymph nodes where APCs present tumor antigen to T cells [54]. In addition, inhibition of CTLA-4 leads to expansion of tumor-neoantigen specific T cells. Blockade of CTLA-4 depletes intra-tumoral Tregs through ADCC and shifts immunosuppressive nature of TME in mouse models [55]. It has also been reported that CTLA-4 blockade broadens TCR repertoire with increased T cell diversity [56].

CTLA-4 Inhibitors in Cancer Immunotherapy

Recognition of CTLA-4 as a negative regulator of T cell response provided the rationale of blocking the interaction of CTLA-4 with B7 proteins and potentiate anti-tumor response. The approval of anti-CTLA-4 antibody (IgG1), ipilimumab in 2011 revolutionized cancer management and expanded the therapeutic arsenal. Professor Allison and his team developed an antibody against human CTLA-4, ipilimumab, for clinical testing [40]. Phase I and II showed immunologic and clinical effects of ipilimumab in patients with melanoma [57], ovarian cancer [58], renal cell cancer [59], and urothelial carcinoma [60]. Under phase III clinical trial, ipilimumab considerably improved the survival of patients with non-resectable stage III/IV melanoma [61] after which it received FDA approval in 2011. Data from multiple clinical trials demonstrated long-term survival in 22% of ipilimumab-treated patients with advanced melanoma [62]. An increase in T cell infiltrates in tumor tissue after anti-CTLA-4 blockade was found and gene array data revealed differences in T cell signaling [58]. The most notable difference was the increase in inducible costimulatory positive ($ICOS^+$) T cells. ICOS is a related member of CD28/CTLA-4 family. *In vivo* study in mice showed therapeutic effect of $ICOS^+$ CD4 T cells in CTLA-4 blockade where, CTLA-4 blockade efficacy was less than 50% in ICOS gene-targeted mice [63]. Further, it was also reported that combination therapy including ICOS agonist together with CTLA-4 blockade markedly enhanced the efficacy in mice [64]. Thus, ICOS is a stimulatory checkpoint, which plays a crucial role in anti-tumor effects of CTLA-4 blockade. Despite the successful outcome of ipilimumab mediated CTLA-4 blockade in melanoma treatment, it failed as a monotherapy for other cancers such as non-small cell lung cancer (NSCLC), small cell lung cancer and prostate cancer [47]. Animal models of less immunogenic cancers such as SM1 mammary carcinoma and B16 melanoma did not respond favorably to CTLA-4 targeted monotherapy [65]. Further studies assessed the potential of combining anti-CTLA-4 therapy with other approaches. Tremelimumab is another anti-CTLA-4

antibody (IgG2) received orphan drug designation by the FDA to treat mesothelioma. It showed tumor regression in patients with metastatic melanoma in Phase I and II trials however, it failed to improve overall survival rate in Phase III clinical trials [66].

Programmed Death-1 (PD-1)

Mechanism of Action

Programmed death-1 (PD-1) is a type I transmembrane glycoprotein belonging to the immunoglobulin superfamily (IgSF) CD28 and is encoded by *pdcd1* gene [67]. It was discovered in 1992 by Tasuku Honjo and his colleagues as a T cell membrane protein involved in cellular apoptotic pathway [68]. PD-1 is expressed on activated T cells, B cells, NK cells, DCs and monocytes. In addition to immune cells, PD-1 is widely expressed on non-immune cells (endothelial cells, mesenchymal stem cells, islet cells and reticular fibroblasts) and tumor cells. PD-1 interacts with its ligands, PD-L1 (B7-H1) and PD-L2 (B7-DC) present constitutively on APCs, leading to T cell inactivation, immune homeostasis and prevention of autoimmunity [69]. PD-1/PD-L1 pathway controls the induction and maintenance of immune tolerance within the tumor microenvironment. PD-1 regulates immune responses through intracellular inhibitory signaling in effector T cells and Tregs [3]. Upon ligand engagement, PD-1 is phosphorylated on two tyrosine residue and recruit two phosphatases, SHP-1 and SHP-2 that binds to immune receptor tyrosine-based inhibitory motif (ITIM) and immune receptor tyrosine-based switch motif (ITSM) motifs of PD-1 [70]. SHP-1 and SHP-2 are a cytosolic tyrosine phosphatase that regulates a board range of cellular functions and regulate multiple responses, including proliferation, differentiation, migration and invasion and controlled cellular functions in hematopoietic, non-hematopoietic tissue and solid tumor [71,72]. Phosphatases dephosphorylate downstream effector molecules (CD3 ζ-subunit and ZAP70), thereby inactivating the T cell activation. Redistribution of PD-1 from uniform cell surface expression to immunological synapse formed between APC and T cells occurs after antigen recognition [73].

The role of PD-1/PD-L1 axis in the negative regulation of T cell activation was unveiled when loss of *pdcd1* (mouse ortholog of PD-1) caused autoimmunity in mice. Multiple *in vivo* studies reported that mice lacking *pdcd1* gene and functional PD-1 protein developed lupus-like glomerulonephritis [74], cardiomyopathy [75] and accelerated type I diabetes mellitus [76]. PD-1 mediated signaling inhibits T cell glucose uptake, cytokine production and cell proliferation by inhibiting the expression of several transcription factors such as GATA-3, T-bet and Eomes [77,78]. It has been shown that PD-1 ligation controls T cell cycle by inhibiting the induction of cell survival factor Bcl-xL [79] and can induce a state of T cell dysfunction known as T cell exhaustion [80]. PD-1 on APCs can also regulate Treg differentiation and its immune suppression activity [81]. Unfortunately, tumor cells manipulate these mechanisms by upregulating PD1 ligands, evade immune responses and create TME that facilitates tumor growth and metastasis. Blockade of PD-1/PD-L1 axis may enhance anti-tumor immune response and act as an attractive therapeutic target in cancer.

Programmed Death-1 (PD-1) Inhibitors in Cancer Immunotherapy

Accumulating evidence indicates that the activity of PD-1 and its ligands PD-L1 or PD-L2 are responsible for T cell activation, proliferation, and cytotoxic secretion in cancer to degenerating anti-tumor immune responses. Furthermore, various signaling pathways can also regulate PD-1/PD-L1 axis in cancer cells and play an important role in tumorigenesis. These signaling pathways are (i) the mitogen-activated protein kinase (MAPK) pathway; (ii) Janus kinase (JAK)-signal transducer and activator of transcription (STAT) pathway; (iii) Phosphoinositide 3-kinase (PI3K)/protein kinase B (AKT) pathway; (iv) Wingless-related integration site (Wnt) pathway; (v) Hedgehog (Hh) pathway and, (vi) Nuclear factor (NF)- κB pathway [82]. PD-L1 overexpression has been suggested as a potential biomarker in patients with advanced melanoma, colon cancer, NSCLC, prostate cancer and renal carcinoma [69]. Although, most of the studies have been focused on PD-L1, PD-L2 is also overexpressed certain B cell lymphomas, such as mediastinal B cell lymphoma, follicular cell-B cell lymphoma and Hodgkin's disease [83]. The discovery of the role of PD-1 axis in negative regulation of T cells, paved the way to preclinical studies that examined various inhibitors to be used for cancer treatment. In recent years, the clinical trials involving anti-PD-1/PD-L1 has shown tumor regression in patients with melanoma, renal carcinoma, bladder cancer and non-small cell lung cancer [84,85].

The development of PD-1 axis directed mAbs were initiated in 2001 by Medarex (acquired by Bristol-Myers Squibb) [86]. The fully humanized anti-PD-1 mAbs pembrolizumab and nivolumab were the first FDA-approved targeted therapeutics against advanced or unresectable melanoma based on the findings from KEYNOTE and CheckMate clinical trials [87]. A randomized, controlled, phase III study on pembrolizumab showed significantly longer progression-free and overall survival in advanced melanoma patients [88]. Similar outcomes were reported in Phase II clinical trials of pembrolizumab in NSCLC patients with PD-1 expression on 50% of tumor cells [89] and in 2015, FDA approved it for the treatment of PD-L1 expressing NSCLC [47]. The phase III trial of nivolumab, demonstrated better overall survival rate at 1 year (72.9% vs 42.1%) in patients with metastatic melanoma when compared to the group treated with chemotherapeutic dacarbazine [90]. It should be noted that nivolumab is capable of blocking the interaction between PD-1 and both of its ligands, PD-L1 and PD-L2. Additional successful clinical trials showed broader clinical implication of these two anti-PD-1 mAbs. In 2016, the first humanized PD-L1 inhibitor, atezolizumab received FDA approval for urothelial cancer following the results of IMVigor trials [91]. The same year, it was approved for previously treated metastatic NSCLC based on the outcomes of POPLAR and OAK clinical trials [92,93]. Two other anti-PD-L1 humanized mAbs in the market are avelumab (IgG1λ mAb) and durvalumab (IgG1κ mAb). Avelumab became the first FDA approved treatment for Merkel cell carcinoma and received accelerated approval for treating metastatic urothelial carcinoma [94]. Likewise, durvalumab showed longer progression-free survival in NSCLC patients in

phase III PACIFIC trial and received full approval for treating phase III NSCLC [95]. PD-1/PD-L1 targeted therapeutics with FDA approval in multiple forms of cancers such as melanoma, NSCLC, urothelial carcinoma, renal cell carcinoma, head and neck squamous cell carcinoma, hepatocellular carcinoma, and Hodgkin's lymphoma have been summarized elsewhere [87]. Another approach to target PD-1 receptor is the fusion protein, AMP-224 composed of extracellular domain of PD-L2 fused to Fc region of human IgG1 [96]. In contrast to anti-PD-1 mAbs, AMP-224 does not act as a simple blocking agent because it binds to exhausted T cells but not to normally activated T cells. A pilot trial evaluated the feasibility, efficacy, and safety of AMP-224 in combination with cyclophosphamide [96]. An increased activity of AMP-224, functional T cell response and increased peripheral anti-tumor immunity was documented observed in patients with metastatic colorectal cancer refractory to standard chemotherapy.

A number of other IC have been identified that employ diverse mechanisms of inhibition including non-ITIM signaling domains, receptor competition and some unconventional signaling pathways [97].

Lymphocyte Activation Gene-3 (LAG-3)

Lymphocyte Activation Gene-3 **(**LAG-3) or CD223 is a type I membrane protein belonging to IgSF [46]. LAG-3 plays a key role in the activation of T cells and natural killer (NK) cells. LAG-3 shares homology with CD4 and is capable of binding MHC class II. It is a non-ITIM IR that may function through conserved KIEELE motif present in its intracellular tail to regulate negatively T cell homeostasis and cell proliferation [98]. LAG-3 has been reported to be highly expressed by exhausted $CD8^+$ T cells in chronic lymphocytic choriomenigitis virus (LCMV) infection, activated Tregs and tumor-infiltrating lymphocytes [99,100]. The first attempt of targeting LAG-3 was made by using soluble LAG-3-Ig fusion protein (Immutep, IMP321) in different mouse tumor models. High serum level of LAG-3 served as a good prognostic marker in human breast cancer expressing hormone receptors [101]. Phase I clinical trials with IMP321 increased the number of activated $CD8^+$ T cells in advanced renal cell carcinoma [102]. The combination therapy with paclitaxel in breast carcinoma increased the number of activated APCs and cytotoxic $CD8^+$ T cells and NK cells [103]. Anti-LAG-3 antibody blocks the interaction between T cell-expressed LAG-3 and major histocompatibility complex class II (MHC II) molecules on the surface of APCs and tumor cells. This prevents the negative regulation of T-cell activity that occurs via LAG-3-MHCII binding and enhances a cytotoxic T-lymphocyte (CTL)-mediated immune response against tumor cells. Antibodies tested in preclinical studies are TSR-033 and BMS-986016 both as a single agent as well as in combination with nivolumab [104].

T cell Ig and Mucin-Domain Containing-3 (TIM-3)

The T cell Ig and mucin-domain containing-3 (TIM-3) is a type I membrane protein containing N-terminal Ig variable domain, a highly glycosylated mucin domain and an intracellular tail with tyrosine-based signaling motif [105]. Ligation between TIM-3 and its ligand galectin-9 inhibits T cell responses and induces peripheral tolerance [106]. TIM family of genes includes eight members (TIM-1 to TIM-8) in mouse and three members (TIM-1, -3 and -4) in human [105]. TIM-1, -2 and -3 are expressed on T cells while TIM-4 is primarily expressed on APCs. TIM-3 is highly upregulated on exhausted Th1 cells and not expressed on Th2 cells [107]. It is also reported to be co-expressed with PD-1 on tumor specific $CD8^+$ T cells in mice. TIM-3 is expressed only on terminally differentiated Th1 cells whereas; PD-1 is expressed on all activated T cells. Anti-TIM-3 antibody showed modest therapeutic activity as a single agent but co-blockade with anti-PD-1 reversed T cell dysfunction, restored $CD8^+$ T cell cytokine generation and enhanced proliferation [108,109]. An ongoing multicenter, first-in-human phase I clinical trial is evaluating anti-TIM-3 antibody, TSR-022 in participants with advanced solid tumors [110]. The safety and tolerability of another anti-TIM-3 antibody, Sym023 has also been investigated in a phase I, open-label trial in patients with advanced solid tumor malignancies [111].

T Cell Immunoreceptor with Ig and ITIM Domains (TIGIT)

T cell immunoreceptor with Ig and ITIM domains (TIGIT) is a member of IgSF consisting of Ig variable domain, transmembrane domain and two ITIMs [112] and immune receptor present on some T cells and NK cells. TIGIT is upregulated by activated T cells, NK cells and regulatory T cells. Recent evidence indicated that TIGIT pathway regulates T-cell-mediated and NK cell-mediated tumor recognition in vivo and in vitro. In mouse tumor model, blocking both PD-1/TIGIT potentially increases tumor antigen specific $CD8^+$ T cell expansion and function in vitro and promotes tumor rejection [113]. It serves as a ligand for CD155 or CD112 and delivers a negative signal for T cell stimulation. Mechanistic studies had revealed that TIGIT may function both through ITIM motif and competition with CD155 for costimulatory ligand [97]. TIGIT is expressed on activated T cells, NK cells and Tregs. Mechanism by which TIGIT inhibits T cells in the TME are as follows: TIGIT binds to (i) CD1555 and stimulates inhibitory signals to T cells; (ii) CD155 on APC to simulate IL-10 production and decrease IL-12 production as a result it directly inhibits T cells; (iii) CD155 to disrupts CD226 homodimerization to impede CD226-mediated T cells activation; (iv) Tregs enhances it stability and their immunosuppressive functions and; (v) Fap2 protein to induced T/NK cell inhibition [111]. Several anti-TIGIT agents as a monotherapy or in combination with other agents are undergoing clinical trials. An ongoing clinical trial is evaluating anti-TIGIT mAb, BMS-986207 in combination with nivolumab and COM701 in advanced solid tumors [114]. A phase IA/IB dose-escalation study of the anti-TIGIT antibody tiragolumab used alone and/or in combination with atezolizumab showed preliminary safety and anti-tumor activity in NSCLC patients [115]. Preclinical data presented at the American Association for Cancer Research (AACR) 2020 Virtual Annual Meeting demonstrated that fully human anti-TIGIT antibody (EOS-448) blocked CD155-mediated T cell inhibition and induced cytotoxicity preferentially against Tregs in cancer patients [116].

P-selectin Glycoprotein Ligand-1 (PSGL-1)

P-selectin glycoprotein ligand-1 (PSGL-1) is well known as an adhesion molecule regulating immune cell trafficking. PSGL-1 is a 120 kDa mucin-like dimeric protein containing extracellular, transmembrane, and cytoplasmic domains [117]. It is expressed primarily on the surface of lymphoid and myeloid cells and is upregulated during inflammation to facilitate leukocyte rolling and tethering to the inflammatory sites. Although, PSGL-1 is highly expressed on resting T cells, selectin (P-, E- and L-selectin) binding ability is acquired only during activation and differentiation of effector T cells due to terminal glycosylation of PSGL-1 [118,119]. PSGL-1, primarily known for its role in cellular migration, has also been shown to function as a negative regulator of $CD4^+$ T cells in numerous diseases including cancer. PSGL-1 may promote $CD4^+$ T cell exhaustion pathways that favor tumor growth [116]. Recently PSGL-1 has emerged as a new player in the IC field. Tinoco *et al*. reported the negative regulatory function of PSGL-1. Ligation of PSGL-1 on exhausted $CD8^+$ T cells abrogated TCR signaling, inhibited T cell survival and upregulated PD-1 in *Selplg−/−* mice infected with LCMV (lymphocytic choriomeningitis virus) Cl13 [120]. There are reports indicating that PSGL-1 expression on Tregs inhibit effector T cell activity [121]. A few clinical studies have evaluated the use of a recombinant P-selectin glycoprotein ligand-Ig (YSPSL) in delayed graft function [122,123]. Safety and efficacy of SelK2, an anti-PSGL-1 mAb, was evaluated in a clinical trial focusing on blot clot prevention [124]. Currently, there are no clinical trial investigating PSGL-1 blockade in cancer, but a number of studies have targeted blockade of VISTA, a newly established ligand of PSGL-1.

V-domain Immunoglobulin Suppressor of T Cell Activation (VISTA)

V-domain immunoglobulin suppressor of T cell activation (VISTA) is a type I transmembrane protein belonging to IgSF which has a single extracellular Ig variable domain containing three cysteine residues [125]. It is constitutively and abundantly expressed on $CD11b^{high}$ myeloid cells, gastric cancer, prostate cancer, colorectal cancer, endometrial cancer, ovarian cancer, small cell lung cancer and expressed at lower levels on $CD8^+$, $CD4^+$ and Tregs cells [126]. Recent evidence also indicated that VISTA could regulate innate and adoptive antitumoral responses. Further, growing evidence indicates that VISTA blockade can increase the sensitivity of tumor cells to conventional IC based immunotherapy, such as CTLA-4 inhibitors [127]. It has been reported that blocking VISTA by antibodies has been associated with decrease VISTA interaction with V-stand immunoglobulin domain containing 3 (VSIG-3), leading to upregulation of IFN-γ IL-2, IL-17, CCL-5, CCL-3 and CXCL11 [128]. Enhanced anti-tumor immunity due to VISTA blockade in different tumor models was demonstrated by delayed or suppressed tumor growth. VISTA expression on $CD4^+$ T cells was reported to inhibit their activation and function [125]. In the cited study, VISTA mAb administration impaired tumor growth, increased the number of peripheral tumor-specific T cells, enhanced infiltration, and proliferation of tumor-reactive T cells in the TME. Also, VISTA blockade together with a peptide-based cancer vaccine synergistically acted to prevent tumor growth. In recent years, VISTA emerged as a negative checkpoint regulator with a new target for cancer immunotherapy. VISTA antagonists and mAbs are being clinically tested in cancer. CA-170, a small molecule inhibitor of VISTA and PD-L1 was investigated for the treatment of advanced solid tumors in patients in a Phase 1 trial [129]. The drug CI-8993 is a fully human IgG1κ mAb directed against VISTA ligand. It is currently under phase I clinical trial in patients with unresectable or refractory advanced solid tumor malignancy [130]. Thus, VISTA blockade either as a monotherapy or in combination with other ICI has indeed emerged as a promising therapeutic approach. Additional IC such as BTA (CD272), LAIR-1, Ceacam1, A2aR, OX-2 and their inhibitors have been reviewed elsewhere [46].

Combination Therapy

Recent evidence indicates that despite the remarkable efficacy of monoclonal antibodies to overcome immunosuppression induced by a tumor and its microenvironment in a number of malignancies, it has become that they are not sufficiently effective in many patients. Another strategy to enhance anti-tumor immune responses and improve clinical benefits is the combination therapy. ICI combined with another checkpoint inhibitor, chemotherapy, radiotherapy, epigenetic drugs, cancer vaccines and immunostimulatory agents have displayed immense success in several cancer types. Previous studies indicated that the radiotherapy elevates PD-L1 expression, indicating potentiality and rationality of combination therapy. Furthermore, despite the remarkable clinical efficacy of these agents in several malignancies, it has become clear that single immunotherapy is not sufficient for many patients. Initial evidence indicated that treatment with combined inhibition of PD-1 and CTLA-4 in melanoma and NSCLC has potential to further enhance the clinical benefits of monotherapies by combining agents with synergistic mechanisms of action. The Society for Immunotherapy of Cancer (SITC) Task Force was developed to identify and prioritize the most promising prospects for combinatorial approaches as well as address the challenges associated with developing these strategies including preclinical modeling, patient safety and toxicity and clinical implementation of these strategies [131]. Co-blockade of PD-1 and LAG-3 synergistically reversed T cell exhaustion in tumors [132]. The efficacy of PD-1 blockade was shown to be improved when tumor trafficking of MDSC was inhibited after anti-CXCR2 mAb therapy [133]. Depletion of granulocytic MDSC sensitized tumors to anti-CTLA therapy and induced $CD8^+$ T cell-mediated killing of tumor cells [134]. A number MDSC receptors such as PI3K and CSF1R, and suppressive factors, such as COX-2, ARG-1 and CXCR2 released in the TME are being targeted in combination with ICI. Various preclinical and clinical trials have shown synergistic effect between radiotherapy and IC blockade. Two ongoing phase III open label trials are evaluating the combined effect of nivolumab and radiotherapy in NSCLC [135] and glioblastoma [136]. An appropriate combination of chemo-drugs and ICI may enhance the overall anti-tumor effect, especially in less chemo-sensitive tumors. A recent phase II clinical study demonstrated the enhanced effect of chemo-drugs, pemetrexed and carboplatin when combined with pembrolizumab and it was later approved by FDA (KEYNOTE-021) [137].

The clinical benefits have been shown for combination of IC blockade with immunostimulatory cytokines or agonist against co-stimulatory molecules [138-140]. In recent years, combining ICI with CAR-T cell therapy is being extensively explored. A case study reported that combined therapy with CD19-specific CART (CART19) cells and PD-1 blockade increased anti-tumor response, expansion of CART19 cells and reduced co-expression of PD-1 and Eomes by CAR19 T cells [141]. An ongoing phase I trial of autologous CRISPR-edited CART cells with PD-1 and TCR genes knocked-out is being tested in patients with mesothelin-positive multiple solid tumors [142]. A number of preclinical and clinical studies have shown the efficacy of combination therapy; hence, combination therapy is considered to be an important future strategy for cancer treatment.

Personalized Combination Therapy

In recent few years, translational research has revolutionized the area of cancer patient treatment by developing new treatments and changes from an organ-centric concept towards deep molecular analysis, driving a personalized approach Molecular profiling of cancer patients, tumor DNA sequencing, computational modelling approach and *in silico* models of bioregulators of cancer have paved the way for personalized combination therapy [143]. The development of personalized vaccines to trigger *de novo* T cell responses against neoantigens have been shown to be feasible, safe and immunogenic in patients with melanoma, lung and glioblastoma [144]. Testing of personalized vaccines in combinations with PD-1 or PD-L1 inhibitions will be another approach in cancer therapy. In a Phase 1a study, a personalized RNA-lipoplex neoantigen vaccine encoding 20 neoantigen (RO7198457) along with atezolizumab (PD-L1 antibody) used on 132 patients with advanced stage solid tumors [145] increased antitumor activity with low graded systemic reactions of atezolizumab. Numerous studies utilizing different neoantigen vaccines and combination therapies are underway, with a goal of stimulating effective, tumor specific immunity in patients with cancer.

Challenges of ICI immunotherapy

Indeed, ICI therapy has shown enormous clinical benefits, but it is also associated with undesirable side effects. Activation of immune system by ICI is commonly followed by immune-related adverse events (irAE) affecting different organs and may be life threatening [146]. The onset of autoimmune complications and severity of irAE is variable depending on treatment modality and organs affected. The most frequent adverse effects to develop after ICI therapy was dermatological, gastrointestinal, hepatic, lung and renal complications [147]. Early detection of irAE and intervention strategy should be explored for management of ICI side effects. A major setback faced by ICI therapy is that only a fraction of patient's benefits from ICI while others experience disease relapse or fail to respond completely. Future insights into the expression levels of IR and their ligands, tumor mutational burden, neoantigen availability [148] and positive predictive biomarkers will help in identifying patient response to ICI therapy. Another challenge is understanding and overcoming tumor resistance to IC blockade. Several mechanisms including epigenetic modifiers of PD-1 pathway [149] and genetic defects in IFN-γ pathway-related genes [150] may be responsible for tumor escape from ICI therapy. Developing strategies to overcome tumor resistance or increase its sensitivity to ICI therapy remain an important area of investigation. Moving forward, many challenges need to be addressed to improve the efficacy and expand the reach of ICI therapy for cancer patients.

Conclusion

ICI as a cancer immunotherapy is a great breakthrough to the current existing cancer treatment modality. Several preclinical studies and clinical trials focused on IC blockade is a testimony to the significance of ICI in cancer treatment. In addition to CTLA-4, PD-1 and PD-L1, several other IC have been identified that could prove as successful targets of either monotherapy or combination IC blockade regimen. Next wave of clinical studies evaluating a combination of immunotherapy including IC blockade and their undesirable side effects are already underway. ICI immunotherapy holds both great opportunities as well as challenges. Indeed, ICI therapy has significantly improved the clinical outcomes in some, but not all the patients. The ongoing effort to address the aforementioned challenges that limit the ICI therapeutic efficacy will improve the anti-tumor response in patients and extend the reach of therapy to a large number of cancer patients.

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