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ARTICLE

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ROLE OF IMMUNE CHECKPOINTS IN CANCER THERAPY

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The immune system developed certain mechanisms to control overreaction. The negative regulators of immune system are necessary for maintaining peripheral tolerance and inhibiting autoimmune diseases. Important immune checkpoint molecules of T cell are cytotoxic T lymphocyte antigen-4(CTLA-4), programmed cell death protein-1 (PD-1), lymphocyte activation gene-3 (LAG-3) etc. They exhibit their function by reducing tumor response and limiting T cell activity and proliferation. CTLA-4 and PD-1 have distinct mechanisms to inhibit immune and antitumor responses. They act in different stages of immune response. Cancer specific T cell shows co-expression of checkpoint receptors. Recent immunotherapy uses immunomodulatory monoclonal antibodies to block these checkpoint receptors for treatment of cancer. Ipilimumab, human monoclonal antibody blocking CTLA-4, was approved in 2011 for treatment of melanoma. Later other antibodies were developed to block PD-1 (nivolumab, pembrolizumab) and PDL-1, ligand of PD-1, (atezolizumab, durvalumab). After receiving several preclinical and clinical experimental successes, it was found that combination therapy of specific monoclonal antibodies with specific drugs or vaccines and combination of multiple immune checkpoint blockade are more effective than monotherapy for treatment of several cancers. Blockade of multiple checkpoint receptors leads to improved immune function against cancer.

Introduction

Several mechanisms regulate immune response to avoid unwanted inflammatory reactions and autoimmune diseases by maintaining equilibrium in the expression of co-stimulatory and inhibitory signals. These signals are known as 'checkpoint molecules'[1,2]. Tumor cells acquire several immune editing methods to evade host immune recognition and killing in tumor microenvironment (TME)[3,6]. T cell requires two signals for full activation into effectors state, one is T cell receptor (TCR) mediated peptide-MHC engagement and another is positive co-stimulatory signals which include interaction of CD28 on T cell with CD80 (B7.1) and/or CD86 (B7.2) on antigen presenting cell (APC)[5]. Several co-inhibitory checkpoint molecules are – cytotoxic T lymphocyte antigen-4 (CTLA-4), programmed cell death protein-1(PD-1), T cell immunoglobulin and mucin domain containing-3 (TIM-3), lymphocyte activation gene-3 (LAG-3) or T cell immunoreceptor with Ig and ITIM domains (TIGIT) etc.[2] In case of cancer and chronic viral infection when host fails to clear pathogens T cell become exhausted and continuous high antigenic loads result in up regulation of T cell inhibitory receptors[6-8]. These inhibitory signaling leads to reduced proliferative activity and effect or function and sometimes even deletion of the T cells. Activated T cells produce perforin, granzyme to kill tumor cells. Proinflammatory cytokines such as IL-2, IFN-γ are produced by several sub-type of T cell that helps in tumor control. Co-inhibitory signaling gradually restricts the production of effector molecules and makes T cell hyporesponsive. Checkpoint inhibitors can rescue the effector function and increases proliferation of T cell that will lead to better tumor response[6,9]. Expression of inhibitory receptors should not always an indication of immune exhaustion[10].

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Therapeutic approaches help to stimulate T cell response such as treatment with cytokines or vaccines[12]. These treatments were applied in nonrepresentative (patient groups with highest possible degree of homogeneity and having similar condition of a disease) and selected patient groups, where substantial toxicities are observed[11,14]. Recent years, immunotherapy with checkpoint inhibitors have shown great success in treating cancers[15]. Anti CTLA-4 and PD-1 monoclonal antibodies can induce immune responses against tumor and prolonged survival in patients but sometimes it is associated with immune related adverse events (IAEs)[13,16]. The occurrence of IAEs is lower in case of PD-1/PDL-1 blockade which is more effective in advanced melanoma[3,6,18]. CTLA-4 and PD-1 blockade acts cooperatively, inhibiting effector T cells and natural killer (NK) cells in peripheral tissues and also prevents T reg cells differentiation[19,20,21]. Combination of CTLA-4 and PD-1 blockade has shown an increase rate of anti-tumor responses and survival rate in melanoma patients as compared to blockade of one checkpoint alone[22].

Here we focus mainly on the immune checkpoint signaling pathways and the associated therapeutic strategies used in various cancer treatments to maintain immune responses through the suppression of these inhibitory targets using monoclonal antibodies singly or in combination therapy.

Immune Checkpoint Molecules:

There are several inhibitory checkpoint molecules involved in controlling harmful immune responses (Table1). Among them several checkpoint molecules belong to CD28 receptor and ligands family, which includes both co-stimulatory CD28, inducible T cell co-stimulator (ICOS) on activated T cells and co-inhibitory receptors CTLA-4, PD-1[23]. Several others co-inhibitory receptors are TIM-3, LAG-3 or TIGIT[24]. LAG-3 belongs to the immunoglobulin superfamily. TIM-3 is a member of TIM immune regulatory proteins family and TIGIT was identified again as a member of CD-28 family[24].

CTLA-4: CTLA-4 is a type 1 transmembrane protein of immunoglobulin superfamily[25]. As a leader checkpoint molecule CTLA-4 prevents activation of auto reactive T cell at initial stage mainly in lymph nodes[16].

Expression and Cellular Trafficking: Up regulation of CTLA-4 expression occurs by exocytosis after both TCR-MHC and CD-28-B7 binding, result in prevention of IL-2 production and reduction of T cell function[16]. CTLA-4 expression on T cell is regulated by graded feedback loop; but they are constantly endocytosed into clathrin coated vesicles by the help of AP2[27]. CTLA-4 is primarily found in trans Golgi network, endosomes, lysosomal vesicle and secretory granules[26] and transiently expressed on cell surface[27]. Two enzymes such as ADP-ribosylation factor 1 (ARF1) and phospholipase D play an important role in transporting CTLA-4 from Golgi to plasma membrane[26].

Ligand: CTLA-4 shares ligands with CD28, these are B7.1 (CD80) and B7.2 (CD86). B7.1 is expressed at very low level by non-activated DCs and upregulated upon activation of DCs[28]. CTLA-4 acts as homolog of CD28 with much greater binding affinity for both B7 ligands (500-2500 folds higher) than CD28 and helps to develop peripheral tolerance to self-proteins[29]. CTLA-4 may out compete CD28 by removing CD80/86 from cell surface by trans endocytosis[6].

Function: CTLA-4 regulates T cell proliferation, anergy, effector function and autoimmunity. It increases TCR mediated activation threshold of T cell[27]. Normally a threshold for activation of T cell is set higher than TCR stimulation from low affinity peptide-MHC (pMHC) interaction leads to positive selection in thymus. CTLA-4 signals elevate the threshold of stimulation of naive T cell[30]. CTLA-4 knockout mice develop CD4 mediated little polyclonal differentiation of lymphocyte and tissue infiltration in several organs within 3-4 weeks of birth[28,31].

Cell Extrinsic Function: CTLA-4 expressing Treg cells have suppressive function in several autoimmune diseases[32]. Studies suggested suppressive capacity of $CD4^+$ $CD25^+$ Treg cells and thymocytes became decreased in CTLA-4 deficient animals such as bone marrow chimeric mice[27]. CTLA-4 leads to the production of regulatory cytokines (such as TGF-β), inhibits T cell and APC function[27]. TGF-β also suppresses excessive lymphoproliferation of $CTLA^{-/-}$ cells and observed to inhibit inflammatory bowel disease (IBD)[32]. APC produces tryptophan degrading enzyme indoleamine 2,3-dioxygenase (IDO) which is a rate limiting enzyme of tryptophan metabolism. It can inhibit T cell by tryptophan depletion or by local aggregation of inhibitory metabolites[27,32] (Figure 1). CTLA-4 expressing Treg cells reduce ligands by the process of trans endocytosis, intracellular transport of CD80/86 from APC to CTLA-4 containing vesicle in T cell. Then these ligands undergo lysosomal degradation[27]. This process is blocked by anti CTLA-4 antibody or in deficiency of CTLA-4[33].

Cell Intrinsic Function: High avidity binding of CTLA-4 to CD80 and CD86 dampens T cell activation by out competing CD28 binding to same ligands. Overexpression in CTLA-4 deficient mice of truncated CTLA-4 transgene that lacks cytoplasmic domain (essential

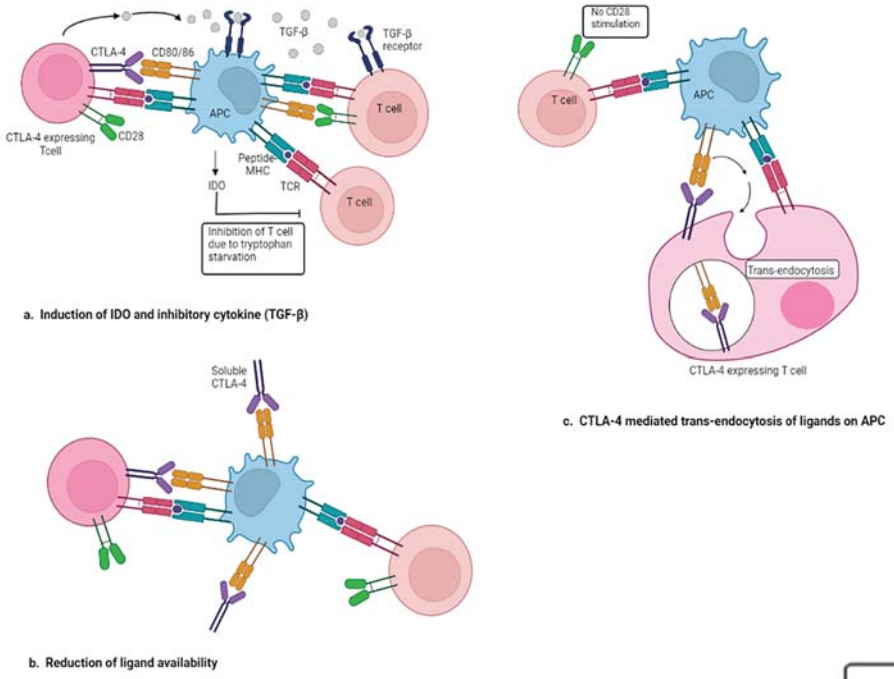


Figure1: Cell Extrinsic Function of CTLA-4.

a Interaction of CTLA-4 with its ligands initiates reverse signaling by activation of IDO, leads to tryptophan depletion and T cell inhibition. CTLA-4 signaling also promotes the production of inhibitory cytokines such as TGFβ.

b CTLA-4 restricts CD80/86 ligand availability, that reduces the chance of receiving CD28 co-stimulation from APC for T cell activation.

c Ligand sequestration is done by CTLA-4 through trans-endocytosis. Endocytosis decreases ligand availability for T cell activation.

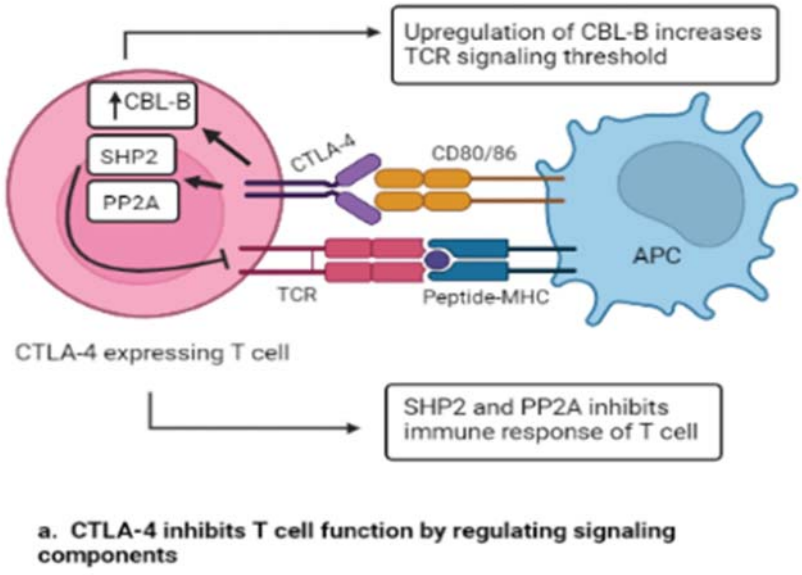
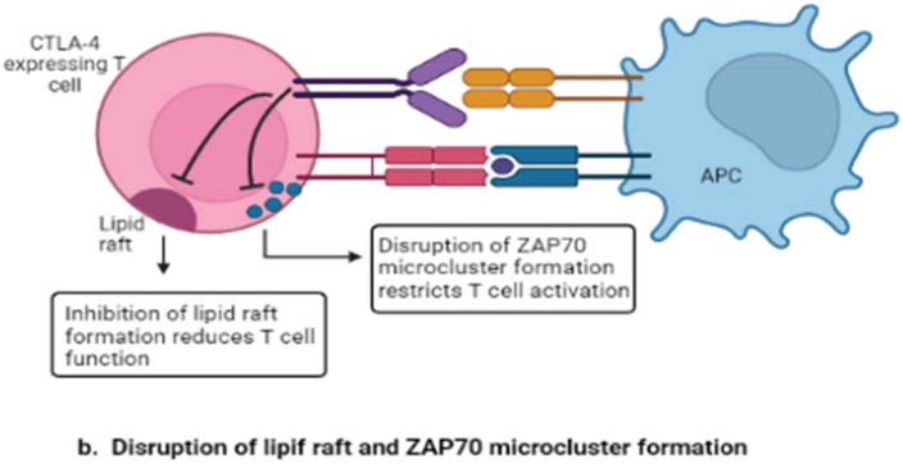


Figure 2: Cell intrinsic Function of CTLA-4

a. CTLA-4 regulates signaling components such as SRC homology-2 domain containing PTPase 2 (SHP2) and protein phosphatase2A (PP2A) to restrict T cell function by dephosphorylation. CTLA-4 can also promote the expression of CBL-B to increase threshold for TCR signaling.

b. Interaction of CTLA-4 with its ligand disrupts the formation of lipid raft and ζ chain of ZAP70 microcluster which are needed for TCR singnaling.



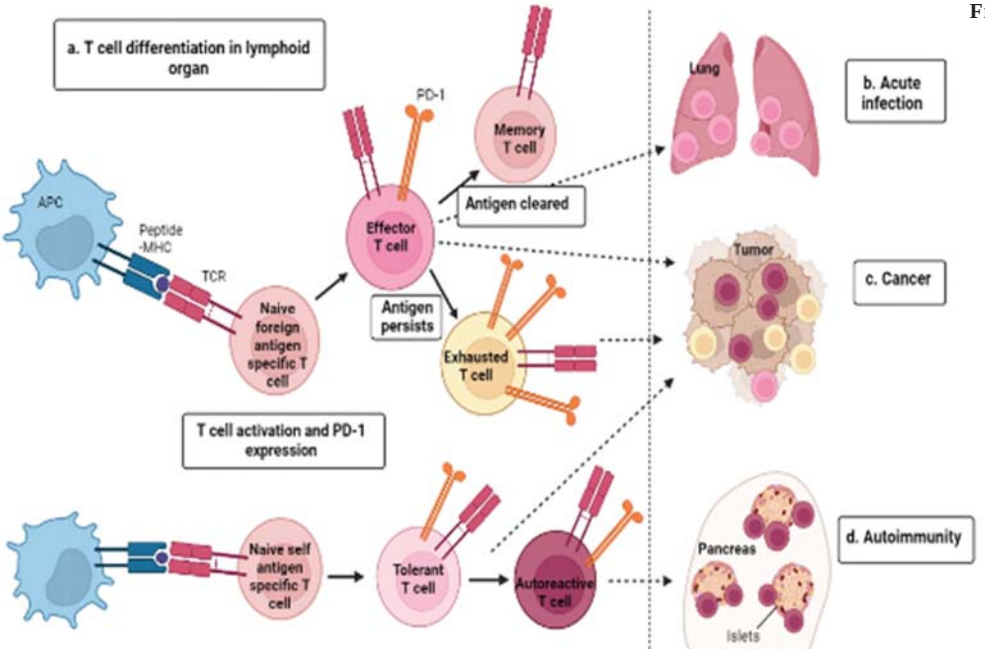


Figure 3: Function of PD-1

a. During infection (lung), PD-1 suppresses the hyperactivation of T cell response and prevents self-tissue damage by autoreactive immune response.

b. During cancer progression, PD-1 promotes dysfunctional T cell subset such as tolerant and exhausted. It helps cancer cell to escape immune mediated lysis.

c. Breakdown of peripheral tolerance leads to autoimmunity while autoreactive T cell infiltrates to target tissue (pancreas). Destruction of target tissue leads to inflammation and increased expression of PDL-1 to regulate PD-1 expressing autoreactive T cells. PD-1 plays important role to maintain T cell exhaustion.

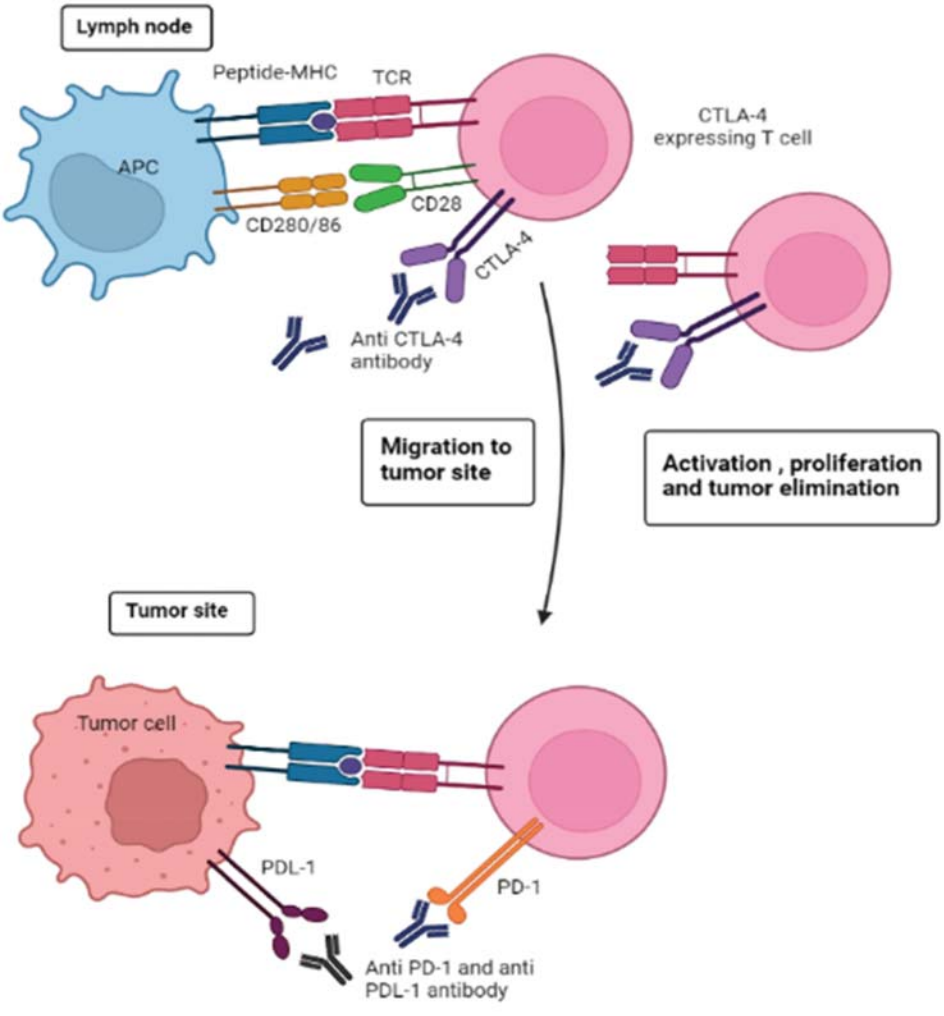


Figure 4: Mechanism of Immune Checkpoint Inhibitors

CTLA-4, PD-1 blockade enhance T cell activation in draining lymph node by removing their checkpoint molecules, increase effector T cell proliferation regardless of T cell receptor (TCR) specificity, diversify peripheral T cell pool and prevent T cell suppression via Treg cells by targeting numerous tumor antigen.

for signal transduction) leads to retardation of the disease[27,32]. Lipid rafts and micro clusters are formed immediately after TCR ligation and they are needed for TCR signaling. Studies showed lipid raft formation in naïve T cell is blocked by CTLA-4. Micro clusters of TCR complex are important site for tyrosine phosphorylation. They are formed by ZAP70 and adaptors such as LAT and SLP76. Interaction of CTLA-4 with its ligand in central supra-molecular activation cluster (cSMAC), shows blockade of CD28 binding in studies of lipid bilayer model. CTLA-4 can alter the location of lipid rafts[27,32] (figure 2). Phosphatases such as SHP-2 and protein phosphatase 2A (PP2A) are recruited by CTLA-4 to reduce the function of kinases such as FYN, LCK, ZAP-70 and dephosphorylates some major TCR signaling protein such as CD3 and linker for activation of T cells (LAT)[24,27]. CTLA-4 phosphorylation is done by SRC family kinases and resting lymphocyte kinase (RLK or TXK). This recruits phosphoinositide 3-kinase (PI3K) which allow T cell become anergic. CTLA-4 also exerts signal in ligand independent manner[27].

CTLA-4 may be expressed by tumor cells and associated with decreased survival in nasopharyngeal carcinoma. Apoptotic death of CTLA-4 positive melanoma cells was observed after treatment with CTLA-4 engaging reagent. Treatment of CTLA-4 expressing melanoma cells with recombinant forms of CD80 and CD86 ligands leads to direct killing of cancer cells by triggering cellular pro-apoptotic machinery[4,15].

PD-1: PD-1 is another important checkpoint molecule. It is thought that PD-1 pathway involves the regulation of activated T cells at later stage of immune response, mainly in peripheral tissue[16].

Expression and Ligands: Its expression is upregulated by cytokine, antigen stimulation. PD-1 has two ligands- PDL-1 and PDL-2. PDL-1 belongs to B7 ligand family and is expressed mainly by all B cell, T cell, some non-hematopoietic cells, cancer cell, APCs. On the other side it's ligand PDL-2 is mostly expressed by professional APCs and sometimes by cancer cells[3,24].

Function: PD-1 inhibitory pathway preserves tolerance, suppresses the hyperactivation of T cell response and prevents self-tissue damage by autoreactive immune response, thus maintaining immune homeostasis. PD-1 helps in shaping $CD4^+$ and $CD8^+$ cell responses, differentiation, fate and development of memory T cells. In absence of PD-1, $CD8^+$ T cell may produce pro inflammatory cytokines and cause tissue damage (Figure 3). In PD-1 deficient mice lethal immunopathology is induced by lymphocytic choriomeningitis virus (LCMV) infection[5]. PD-1 blockade leads to enhance $CD8^+$ T cell function during secondary response in lung infection. PD-1 signaling helps to regulate threshold of TCR signaling. In absence of PD-1 signaling, the populations of double positive T cell become increased. Destruction of target tissue leads to inflammation and increased expression of PDL-1 to regulate autoreactive T cells. The chance of development of more severe autoimmune encephalitis (EAE) symptoms are higher in experimental PD-1/PDL-1 knockout mice than PDL-$2^{-/-}$or control mice[5] PD-1 blockade helps in re-invigoration of exhausted T cell. The anergy and apoptosis of activated $CD8^+$ T cells are enhanced by binding of PD-L1 (on cancer cell) to PD-1, helps cancer cell to escape immune mediated lysis. Thus, expression of PD-L1 on cancer cell acts as an anti-apoptotic factor.

Cytoplasmic tail of PD-1 has two immunoreceptor tyrosine based inhibitory motif (ITIM), recruit protein tyrosine phosphatases such as SHP-1 and SHP-2, leads to CD3 dephosphorylation and inhibits Akt activation, limits T cell function[24]. Activated T cell secretes interferon gamma (IFN-γ) to upregulate this receptor and several other positive regulators of PD-1- FOXO-1, NFAT and B cell receptor (BCR) engagement[5]. Tumor cell can enhance expression of PDL-1 on their surface upon PD-1 recognition on T cell, results in T cell apoptosis. Interaction of PD-1 with PDL-1 inhibits autoimmune TILs, enhance self-tolerance, reduce TNF, interferon gamma (IFN-γ) and IL-2, which help in immune evasion of cancer cells[3]. Interaction of PD-1 and PDL-1 results in phosphorylation of tyrosine residues and recruits SHP-2[5].

PDL-1 on the surface of tmour cells can be upregulated by IFN-γ produced by activated T cells, helps tmour cell to escape immune response. PD-1/PDL-1 signal transduction pathway can diminish T cell mediated immune surveillance. In hepatocellular carcinoma, PD-1 positive DCs exhibit reduced ability to stimulate T cells[6]. Co-inhibitory checkpoint inhibits tumor-infiltrating CD4+ /CD8+ T cells and reduce production of cytokines (TNF, IFN-γ, IL-2), cancer cell escapes immunoreaction[6]. Checkpoint inhibitors unblock immune suppression of anti-tumor T cells, leads to T cell multiplication and permeation into TME and gives anti-tumor response.

Other Checkpoint Receptors:

TIM-3: TIM-3 is a member of TIM gene family. Ligands are C type lectin galectin (Gal-9), high mobility group protein B1 (HMGB1), carcinoembryonic antigen cell adhesion molecule1 (CEACAM-1) etc.[37] Gal-9 binds to TIM-3 and induce intracellular calcium influx of Th1 cells,

Table1: Immune Checkpoint Receptors and their Ligands

Receptor	Expressing Cells	Ligands	Ligand Expressing Cells	Reference
CTLA-4 (CD152)	CD4 (activated/exhausted,Tregs), CD8 (activated/ exhausted), some tumor cells	CD80 (B7.1), CD86 (B7.2)	DCs, macrophages, activated B, T cell	1
PD-1 (PDCD1)	CD4 (activated/exhausted, follicular), CD8 (activated/ exhausted), B cells, dendritic cells, NK cells, monocytes, maturing thymocytes, mast cells, Langerhans cells	PDL-1, PDL-2	APCs, $CD4^+$ T cells, B cells, non-lymphoid tissues, some tumor cells	2
LAG-3	CD4 (Treg and exhausted), CD8 (including exhausted), NK cells	MHC II molecules, LSECtin	APCs, liver, some tumor cells	34
TIM-3	CD4 (Th1, Th17, Treg), CD8 (exhausted and Tc1), DCs, NK cells, monocytes, macrophages	Gelectin-9, phosphatidyl serine, high mobility group protein B1, Ceacam-1	Endothelial cells, apoptotic cells, some tumor cells	36
TIGIT	CD4 (Treg, T helper cell), CD8, NK cells	CD155 (PVR), CD122 (PVRL2, nectin-2)	APCs, T cell, some tumor cells	1

triggers cell death[36,38]. Binding of adaptor protein Bat-3, promotes TCR signaling by recruiting Lck. Phosphotyrosine residues can recruit P85 adaptor protein, which activates PI3 kinases and induces NFAT and NF-κB activation in T cells[37,39,40]. TIM-3 signaling results in reduction of production of IFN-γ, induces autoimmunity[24]. Tumor induced T cell exhaustion can be reversed by applying TIM-3 monoclonal antibody in melanoma[36]. Administration of anti-TIM-3 antibody also leads to enhanced macrophage activation thus worsening autoimmune diseases (experimental autoimmune encephalomyelitis)[41].

LAG-3: LAG-3 is important checkpoint molecule, homolog of CD4, as it binds to MHC class II molecule with greater affinity. It may prevent CD4-MHC interaction, thus reduce $CD4^+$ T cell activity[37]. In TME other ligand such as LSECtin and Gelectin-3 bind to extracellular domain of LAG-3 to inhibit T cell and NK cell activity[34,37,43]. Another identified ligand for LAG-3 is fibrinogen-like protein 1 (FGL1), expressed by several cancer cells[37,44]. IL-2 and IL-12 promote expression of LAG-3 on activated T cells as 'exhaustion marker'[44]. Data suggested interaction of MHC-II and LAG-3 can suppress both Treg and APC function and proliferation[37,45]. In nonobese diabetic (NOD) mice model, knockout of LAG-3 results in increased number of infiltrating $CD4^+$ and $CD8^+$ T cells and accelerates the type1 diabetes[24]. So, blockade of LAG-3 leads to a great clinical benefit for immunotherapy.

Immune Checkpoint Inhibitors:

Immune checkpoint inhibitors (ICIs) are a variety of immunotherapy that hindersthe binding of immune checkpoint proteins with their corresponding cellular partnersthere by turning the immune response on. Ipilimumab (Yervoy) is first monoclonal antibody for treating human metastatic melanoma, got approval by Food and Drug Administration (FDA)[46]. Six monoclonal antibodies were approved for targeting PD-1 (nivolumab, pembrolizumab, cemiplimab) and PDL-1 (atezolizumab, durvalumab, avelumab) for treatment of several types of tumors (Table 2). These antibodies increase survival rate of patients, enhance tumor response and reduce tumor size[3]. Combination therapy of NAB-paclitaxel chemotherapy with atezolizumab drug gave promising result to increase overall survival in patient with TNBC. Combination of both anti CTLA-4 and PD-1 antibody exhibits effectivity of 60% in phase II trial of advanced melanoma than application of either antibody alone[3].

Mechanism of Function of Immune Check Point inhibitors:

There are several hypotheses regarding the mechanism (Figure 4). One is selectively blocking the active sites of CTLA-4, PD-1 so that they cannot exhibit their immunosuppressive function and thus enhance T cell activation in draining lymph node. This increases effector T cell proliferation regardless of T cell receptor (TCR) specificity, diversify peripheral T cell pool in melanoma and prevent T cell suppression via Treg cells by targeting numerous tumor antigen[16]. Approved anti CTLA-4, PD-1 antibodies exhibit partial or complete tumor responses in melanoma, lung cancer and some other through type 1 immune responses. They can directly bind to CTLA-4 expressing tumor cells lead to direct cytotoxicity. CTLA-4 blockade induces de novo antitumor responses, endogenous memory responses and enhance antibody

Table 2: Approved Immune Checkpoint Inhibitors

Drug	Brand Name	Target of Immune Checkpoint	Approved For	Reference
Ipilimumab	Yervoy	CTLA-4	Unresectable metastatic melanoma (as monotherapy), adjuvant therapy for melanoma stage III and as combination therapy with nivolumab for unresectable or metastatic melanoma	19, 24
Nivolumab	Opdivo	PD-1	Unresectable metastatic melanoma, metastatic non-small-cell lung cancer (NSCLC), renal cell carcinoma (RCC), urothelialcarcinoma, classical Hodgkin's lymphoma, head and neck squamous cell carcinoma (HNSCC)	47,46
Pembrolizumab	Keytruda	PD-1	Melanoma (metastatic and resectable), NSCLC, HNSCC, classical Hodgkin's lymphoma, primary mediastinal B-cell lymphoma (PMBCL), urothellial carcinoma, solid tumors with MSI-H and MMR aberrations	47, 50
Cemiplimab	Libtayo	PD-1	Metastatic cutaneous squamous cell carcinoma (CSCC)	47,50
Atezolizumab	Tecentriq	PDL-1	Urothelial carcinoma (metastatic), NSCLS (both monotherapy and combining with chemotherapy), as combination with chemotherapy in metastatic SCLC, combining with paclitaxel in treatment of triple negative breast cancer	19,47
Durvalumab	Imfinzi	PDL-1	Metastatic urothelial carcinoma, NSCLC (stage III unresectable)	47,48
Ipilimumab plus nivolumab	Yervoy plus Opdivo	Both CTLA-4 and PD-1	Metastatic melanoma, RCC, colorectal cancer with MSI-H and MRR aberrations	24, 46

response[46]. In mouse model anti PD-1 mediated tumor regression is associated with IFN-γ and granzyme B upregulation[6]. Effector state of immune response is targeted by anti PD-1 antibody because tumor infiltrating T cell with PD-1 receptor is suppressed by PDL-1 expressing tumor cell or tumor infiltrating T cell[16]. Inhibition of PD-1/PDL-1 pathway influences other types of cells such as dendritic cells (DCs), B cells[6]. PD-1 expressing tumor infiltrating regulatory B cells, that produce IL-10 are found in high proportion in hepatocellular carcinoma patients. PDL-1 expressions on both cancer cells and tumor infiltrating macrophages are indication of anti-tumor responses and contribute to local immunosuppression[6]. Abundant PDL-1 expression on tumor cell is found in several tumor types- melanoma, non-small cell lung cancer (NSCLC), ovarian cancer. Study showed PDL-1 expression on tumor cells is closely associated with anti PD-1 blockade response whereas no involvement of PDL-1 expressing TILs[16]. Resistance to checkpoint inhibitors involves several immune parameters such as expression of PDL-1, tumor microenvironment (TME), activation status of TILs, expression of tumor neoantigen, evaluation of tumor mutational burden (TMB)[3]. It is important to routinely apply standard radiologic evaluation criteria (response evaluation criteria in response evaluation criteria in solid tumors (RECIST) for monitoring responses to chemotherapy or targeted other therapies[17].

Preclinical Trial of Checkpoint Inhibitors in Immunotherapy

Monoclonal antibodies are produced by immune cells that bind to certain targets in the body. In cancer immunotherapy, immune checkpoint inhibitors are used to prevent the activity of cancer cells or to kill them[47]. PD-1 blockade in MCA sarcoma and MC38 adenocarcinoma exhibits better outcomes than in B16 melanoma and MB49 bladder cancer[49]. Monoclonal antibodies help to increase immune responses to immunogenic tumors, decrease toxicity, reduce tumor size, prevent growth of advanced tumors and metastases, and enhance patient's overall survival[3]. The possibility of treating mice with established B16-BL6 melanoma by combining anti CTLA-4 antibody with granulocyte-macrophage colony stimulating factor (GM-CSF) is reported[50]. Treatment of melanoma bearing murine model with combination of CTLA-4 and PD-1 blockade plus vaccination suggested the hypothesis that result of combinational blockade is more effective than single checkpoint blockade[24]. Enhancement of immune response is done by combining CTLA-4 blockade with several other therapies such as chemotherapy, radiotherapy, reduction of CD25+ Treg cells and CpG administration[28]. Combination blockade of CTLA-4 and PD-1 is enough to enhance survival and inhibit established tumor growth of both small and large size in case of MB49 bladder cancer

model[24]. Although these therapies are really important to combat against tumors however there is issue regarding the necessity of vaccination for highest clinical success[3]. PD-1/LAG-3 double knockout mice could mostly reject established tumors without any autoimmune disease side effects, but delayed tumor growth was observed in single knockout mice. The combined therapy was more effective by increasing secretion of effector cytokines include IFN-γ and TNF-α by tumor infiltrating cells[24].

Clinical Trials of Checkpoint Inhibitors in Immunotherapy

There are two CTLA-4 targeted human monoclonal antibodies (mAb). One is ipilimumabis IgG_1 antibody. Another is tremelimumab, IgG_2 antibody. They can bind and suppress CTLA-4 inhibitory signaling with affinities <1 nmol/L (Table 3). The total four doses of induction phase with 10 mg ipilimumab/kg every 3 weeks gave satisfactory result with best overall response rate (BORR) followed by a maintenance period of 12 weeks. The second phase III trial in patients with metastatic melanoma, OS was higher (11.2 months) with hepatotoxicity in patients treated with darcabazine + ipilimumab and lower OS (9.1 months) when treated with ipilimumab alone[51].

Phase II clinical trial has showed concurrent use of nivolumab with chemo and radiotherapy will be beneficial for treatment of stage III NSCLC[52]. Objective response rate (ORR) of 18.5% and 33% has been observed in treatment with pembrolizumab or atezolizumab respectively in phase I trial of patients with metastatic triple-negative

Table 3: clinical trial with immune checkpoint receptors

Target Disease	Trial ID and Phase	Agents With Dose	Follow Up	Results	Reference
Advanced or unresectable metastatic melanoma with at least one measurable, nonirradiated brain metastasis	NCT02320058 Phase II	Ipilimumab (BMS-734016): 3 mg/kg Nivolumab (BMS-936558): 1 mg/kg	≥6 months	57% and 56% of rate of intra and extracranial clinical benefit respectively, 12 months OS rate was 82%, Grade 3-4 AEs 55%	55
Previously untreated advanced melanoma	NCT01844505 Phase III	Ipilimumab (BMS-734016):3 mg/kg Nivolumab (BMS-936558):1 mg/kg	≥36 months	Not reached 3-year OS rate was 58%, Hazard ratio (HR) for disease progression or death 0.55, Grade 3-5 AEs 59%	56
Untreated advanced NSCLC	NCT02659059 Phase II	Ipilimumab (BMS-734016):1 mg/kg every 6 weeks Nivolumab (BMS-936558):3mg/kg every 2 weeks	≥6 months	44% of ORR in patients with TMBe≥10 mutations per megabase, 68% of 6 months PFS rate, Grade 3-4 AEs 29%	
Malignant pleural mesothelioma	NCT03048474 Phase II	Ipilimumab (BMS-734016): 1 mg/kg every 6 weeks, 4 times Nivolumab (BMS-936558): 240 mg every 2 weeks	>12 months	Not reached 12 months OS rate was 64%, ORR 38%, 6 months PFS rate was 50%, Grade 3-4 AEs 38%	58
Advanced or metastatic RCC	NCT01472081 Phase I	Nivolumab (BMS-936558): starting dose 2 mg/kg every 3 weeks with some plus either sunitinib: 50 mg/day (arm N+S) or pazopanib: 800 mg/day (arm N+P)	Arm N+S: 50 Months Arm N+P: 27.1 months	Arm N+S: 55% ORR, Median PFS 12.7 months, Grade 3-4 AEs 82% Arm N+P: 45% of ORR, Median PFS 7.2 months, Grade 3-4 AEs 70%	59
Previously treated MMR/MSI-H positive advanced colorectal cancer	NCT02060188 Phase II	Ipilimumab (BMS-734016):1 mg/kg Nivolumab (BMS-936558):3 mg/kg one time every 3 weeks	>9 months	Not reached 12 months OS rate was 85%, ORR was 55%, Not reached 12 months PFS rate 71%, Grade 3-4 AEs 32%	60

breast cancer (mTNBC)[53]. Combination of durvalumab and tremelimumab has reported ORR of 23% for advanced squamous and non-squamous NSCLC[19]. Phase III trial of ipilimumab plus nivolumab was investigated in patients having stage IV or recurrent NSCLC with high tumor mutation burden[54].

Immune Related Adverse Events:

Despite of promising efficiency, the majority of patients treated with immune checkpoint inhibitors is associated with immune related adverse events (IAEs) of different degrees[28]. These adverse events involve autoimmunity via hyper activated T cells. Commonly reported IAEs are dermatologic, such as vitiligo, rash, erythema[48]. Severe cardiovascular toxicity, gastrointestinal and endocrine disorders are also observed. Other IAEs include myocarditis, arrhythmias, myasthenia gravis, uveitis, thyroid dysfunction[3]. Patients treated with ipilimumab developed gastrointestinal IAEs (diarrhea, colitis) after 6-7 weeks. Life-threatening pneumonitis and classical Hodgkin lymphoma are reported in patients during treatment with either nivolumab or pembrolizumab in advanced melanoma and NSCLC[48]. Colocation of $CD8^+$ and $CD4^+$ T cells perivascular lymphoid aggregation with dying melanocytes suggests that IAEs involve loss of tolerance to melanocyte differentiation antigen[28].

Conclusion

CTLA-4 and PD-1 are important checkpoint molecules, have immune suppressive function. They play key role in maintaining peripheral tolerance, allow tumor to grow and escape immune response. Upon interaction with ligands CTLA-4 and PD-1 exhibit their function in different ways. Expression of ligands is found mainly in lymphoid tissues and in peripheral tissues for CTLA-4 and PD-1 respectively, suggested that CTLA-4 primarily acts early to induce tolerance whereas PD-1 acts in later stage to maintain long term tolerance. PD-1 helps in shaping $CD4^+$ and $CD8^+$ cell responses, differentiation. PD-1 Inhibitory pathway restricts immunopathological response by inducing inflammation and helps in Immune homeostasis. Inhibitors of CTLA-4, PD-1 and its ligand, PDL-1 help to restore immune responses against tumors and benefit patients in long term survival. Clinical evidence suggests combination therapy is more efficient over immune checkpoint monotherapy, however these are associated with IAE. Further clinical trials are necessary to determine which combination leads to better survival in patients with low grade of IAE. There are several ongoing clinical trials and have the potential to achieve best results for treatment of several cancers in future. ❐

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