Advances of PD1 PD-L1 Signaling Pathway in Tumor Immunotherapy

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Abstract: Increasing clinical data has demonstrated the effectiveness of checkpoint blockade immunotherapy in cancer. Programmed death ligand-1 (PD-L1) and programmed cell death protien-1 (PD-1) as two promising targets of checkpoint blockade have received growing attention in treating various malignancies. This review introduces the basic biological mechanisms of PD-1 signaling regulation in T cells, the landscape of PD-L1 expression in tumor, and the advancement of PD-1/PD-L1 signaling pathway research in different cancer types. We will also discuss the limitations of antibody-based treatments and mechanisms related to PD-1/PD-L1 blockade resistance. In the end, we will also demonstrate potential solutions to overcome the challenges of current immunotherapy and the development of new therapeutic agents. Overall, immunotherapies targeting PD-1/PD-L1 pathway provide opportunities to boost anti-tumor immunity while there's still problems waiting to be solved to allow larger patient population to be benefited.

Keywords: PD1; PD-L1; signaling pathway; tumor immunotherapy
Tob Regul Sci.™ 2021;7(5):1087-1096
DOI: doi.org/10.18001/TRS.7.5.26

INTRODUCTION

The incidence and mortality of malignant tumors escalate over years, which have become one of the major threats to public health. Current methods of treatments, including surgical incision, radiotherapy, chemotherapy fail to bring substantial extension to patients' survival. In recent years, with the fusion and crosstalk development among oncology, immunology, molecular biology and other fields, cancer immunotherapy shed light on novel therapeutic targets and immunotherapy has been named 2013 "Breakthrough of the year" by Science. [1] Current checkpoint blockade therapies, like anti-PD-1/PD-L1 have brought long-term benefits for patients with advanced melanoma [2] and NSCLC [3]. Thus, tumor immunotherapy has attracted more and more attention from the public.

Evading immune destruction is a hallmark of cancer [4] and tumors cells can develop various mechanisms to escape immunosurveillance, such as downregulating immunomodulatory molecules, expressing immunoinhibitory ligands, secreting immunosuppressive cytokines and recruiting T regulatory cells. Programmed cell death ligand 1(PD-L1) expressed by many types of malignancies, plays a crucial role in cancer immunosuppression by interacting with programmed cell death receptor (PD-1) on activated T cells, B cells and NK cells, which serves as an immune checkpoint for maintaining homeostasis, and inducing T cell inactivation and subsequent apoptosis. PD-1/PD-L1 pathway provides a promising target for cancer immunotherapy and enhancing the efficacy of treatments. Antibody blockade of PD-1/PD-L1 checkpoint including nivolumab and pembrolizumab proved by FDA has studied in a broad spectrum of clinical trials and showed prominence antitumor effects in melanoma, non-small cell lung cancer (NSCLC), renal cell carcinoma (RCC), urothelial cancer, head and neck cancer, Hodgkin lymphoma, gastric cancer, colorectal cancer, and Merkel cell carcinoma. [5, 6, 7, 8] Despite the encouraging results, the response rate of PD-1/PD-L1 blockade treatment varies dramatically dependent on the certain malignancies and the stages of diseases and meanwhile the percentage of patients react to the treatment remains low. Thus, elucidating the mechanisms underneath is vital to explicate the factors contributing to PD-1/PD-1 blockade resistance and to develop new agents to improve the efficacy of treatments. Extensive studies have been conducted and revealed several mechanisms of the endogenous and exogenous induction PD-1 and PD-L1, the downstream signaling pathways and the interactions between PD-1 expression T cells with tumor and tumor stroma.The roles of PD-1/PD-L1 in cancer can be summarized as inhibiting conventional T cell activation, promoting T cell exhaustion, and contributing to adaptive resistance and thus reaching the goal of hijacking immune suppression. [17]

The preferentially expression of PD-1 by exhausted T cells was firstly described in lymphocytic choriomeningitis virus (LCMV) chronic infection mouse model and the its role in modulating immune response has been revealed since. [54] The upregulation of PD-1 in tumor infiltrating T cells and the overexpression of PD-L1 in tumor cells and tumor stromal have been demonstrated in various malignancies and the antitumoral effects of PD-1/PD-L1 has been reported in certain mouse models. Increasing data has shown that PD-1/PD-L1 inhibition may be not sufficient enough to restore the exhausted lymphocyte activity. Kamphorst et al. showed that CD28 co-stimulation served as an indispensable factor for PD-1+CD8+ T cell expansion and the effectiveness of PD-1 blockade therapy. [55] LAG3, another co-inhibitory receptor expressed by T lymphocytes, has been found synergistically regulating T cell function with PD-1 and the co-inhibition of LAG3 and PD-1 overcame the resistance of single antibody treatment. [56] The downstream target SHP2 of PD-1 has been shown strongly activated upon PD-L1 ligand engagement and the prevention of between SHP2-PD1 interaction may sensitize tumor to anti-PD-1 treatment. [57] Multiple inducers including cytokines and growth factors can result in the upregulation of PD-L1 in tumor cells and stromal. Some commonly oncogenic pathway activation is also able to upregulate PD-L1 expression in tumor cells, like PI3K and JAK/STAT pathways. [58,59] The mechanisms of cytokines, co-inhibitory and co-stimulatory molecules functioning on PD-1/PD-L1 pathway and the signal transduction downstream of PD-1 need to be further clarified for developing combinatory therapies.

THE REGULATIONS AND SIGNALING PATHWAYS OF PD-1 AND PD-L1

Programmed cell death-1 (PD-1) is a type I transmembrane glycoprotein belonging to CD28/CTLA-4/ICOS costimulatory receptor family, which maintains the peripheral tolerance and immune homeostasis. [9] PD-1 expression is induced upon primary stimulation in T cells, B cells, NK cells and other myeloid lineages. [10] PD-1 is encoded by Pdcd1 gene and its expression is strictly regulated under normal conditions. In T cells, upon TCR engagement initiate the downstream signaling cascade that activates the transcription factors involved in PD-1 gene expression. Family members of the nuclear factor of activated T cells (NFAT) has been shown its dephosphorylation and translocation into the nucleus are associated with PD-1 transcription regulation. (Figure1) T cells treated with NFAT inhibitor cyclosporine A (CsA) displayed a strong reduction of PD-1 expression. [11] IL-2, IL-7, IL-15, and IL-21 are also found directly induced PD-1 expression in purified T cells. It is shown that the induction of PD-1 by γc cytokines is limited to effector cells and memory T cells whose IFN-γ producing capability was drastically attenuated while stimulated with PD-L1 or PD-L2. [12] Epigenetic factors such as DNA demethylation and histone acetylation are also found positively regulating the PD-1 expression in viral infection mouse models. PD-1 induction is mostly studied in acute antigen stimulation scenario when the expression of PD-1 appears transient and down regulated over time. [13] Under chronic antigen stimulation, T cells persistently express PD-1, leading to T cell exhaustion.

Figure 1 PD-1 regulation in T cells

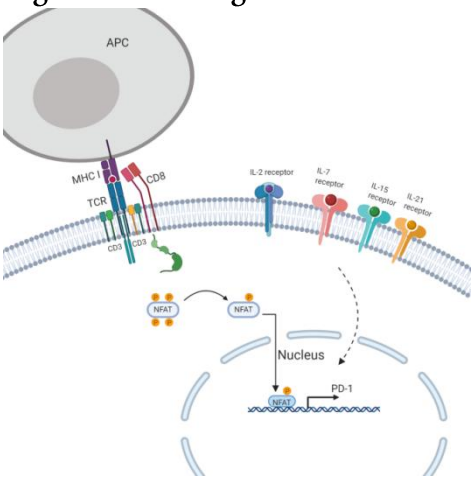
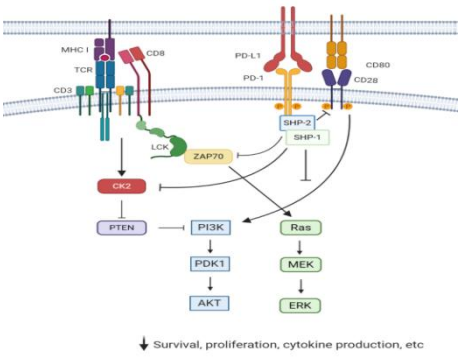


Figure 2 PD-1 PD-L1 signaling in CD8 T cells



Engagement of PD-1 to its ligand PD-L1 or PD-L2 renders reduction of cytokine production, decreased proliferation potential, downregulation of pro-survival protein and overall limited effector features. As a member of co-inhibitory family, PD-1 serves an antagonistic role of CD80-CD28 co-

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stimulation. [14] The mechanism of PD-1 inhibiting T cell signaling still remains opaque. There's study demonstrating the direct target of PD-1 is CD28 instead of TCR. [15] The C-terminal immunoreceptor tyrosine-based switch motif (ITSM) and immunoreceptor tyrosine-based inhibitory motif (ITIM) of PD-1 are phosphorylated upon the ligand engagement, which recruits scaffolding protein SHP1 and SHP2 phosphatases to close proximity to plasma membrane. [16-19] Shp2 phosphatase activity is trigger upon the interaction with PD-1, dephosphorylating the nearby targets including CD28, PI3K by stabilizing PTEN activity and MEK/ERK signaling. [14,20,21] (Figure2) Under intensive PD-L1 stimulation, PD-1 also presents direct dephosphorylation of TCR components like ZAP70 and SLP76. [15] The dephosphorylation of CD28 and PI3K also results in the diminished expression of pro-survival protein Bcl-xl, lowering the survival potential. [16] T cells encountered PD-L1/L2 alter their metabolic schematics. PD-1 signaling abrogates glycolysis and amino acid uptake while enhances lipid oxidation, preventing the development of effector T cells. [22] Overall, PD-1 signaling lead to restricted T cell effector features and favor T cell inertia and exhaustion.

PD-L1, also recognized as B7-H1, as one of the ligands of PD-1, is found commonly expressed by myeloid lineage immune cells, normal tissue and tumor cells and the overexpression of PD-L1 is closely associated with the poor prognosis of many types of malignancies such as squamous cell carcinoma of the head and neck, melanoma, and carcinomas of the brain, thyroid, thymus, esophagus, lung, breast, gastrointestinal tract, colorectum, liver, pancreas, kidney, adrenal cortex, bladder, urothelium, ovary, and skin (figure 3 and figure 4).[23,24] Inflammatory cytokine found in tumor microenvironment are strong inducers of PD-L1 expression. IFN-γsecreted by inflammatory immune cells serves as a positive regulator of PD-L1 expression by cancer cells. In a melanoma model, IFN-γ is found activated JAK1/JAK2-STAT1/STAT2/STAT3-IRF9 axis that binds to PD-L1 promoter region and controls its expression. [26] TNF-α AND IL-17 is another strong modulator of PD-L1 in tumor microenvironment. In human Prostate and Colon Cancer Cells, TNF-α and IL-17 are shown inducing PD-L1 expression independently through NFκB and AKT pathways. [27] Other cellular growth factors play roles in the regulation of PD-L1 in tumor cells and tumor stromal as well, such as EGF, TGF-βand GM-CSF. [23] In gastric cancer, GM-CSF level exhibits a positive relationship with PD-L1 expression in tumor infiltrating neutrophils which suppress T cell immunity. [28] PD-L1 expression in esophageal squamous cell carcinoma depends on epidermal growth factor receptor–mediated signaling. The mechanism of EGF regulation on PD-L1 expression is still not clear but EGFR–PI3K–AKT, EGFR–Ras–Raf–Erk, and EGFR–PLC-γ signaling pathways may be candidates of participating in PD-L1 regulation. [29] Cell intrinsic mechanism of regulating PD-L1 involves gene amplification, DNA demethylation and histone modification. CMTM6 is identified as another cell intrinsic regulator that is required for constitute induction and retention of PD-L1 on plasma membrane. With the absence of CMTM6, PD-L1 fail to be efficiently endocytic recycled and the surface expression can be lost. [30]

Figure 3 PD-L1 regulation in tumor cells

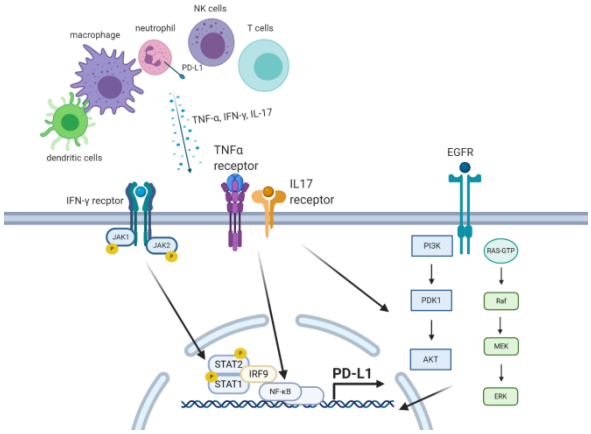
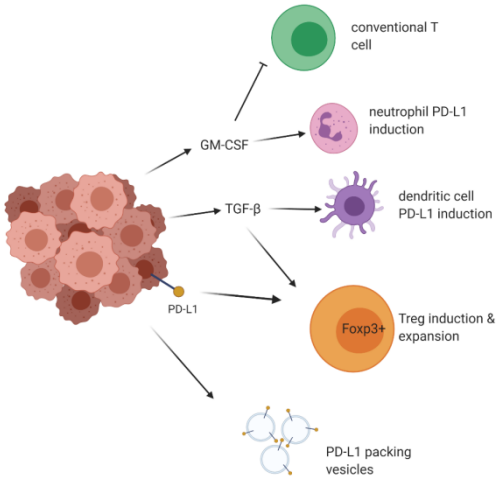


Figure 4 PD-L1



Besides the role of interacting with PD-1 on T cells and suppressing the activity, PD-L1 also servers in maintaining and expanding T regulatory population. In vitro, PD-L1 coated beads can drive the development of naïve CD4+CD62LhiFoxp3– T cells towards induced T reg (iTreg) and also maintain the expression of Foxp3 and iTreg suppressive ability.[31] A high correlation between tumor PD-L1 expression and Foxp3+ Treg infiltration is observed human breast cancer, glioblastoma and NSCLC.[32,33,34,35]

PD-1/L1 SIGNALING IN CANCER IMMUNE ESCAPE

Immune system plays a vital part in detecting and eliminating transformed cells to prevent abnormal neoplasm of tissues. In immune surveillance, by recognizing neoantigens or molecules due to cellular stress expressed by cancer precursors, both innate and adaptive immune cells are involved in cancerous cell destruction. Cancer cells in order to gain growth advantage, develop various mechanisms to constrain immune surveillance and favor tumorigenesis, which lead to a more comprehensive concept of immunoediting. It is considered that immunoediting includes three phases: elimination, equilibrium and escape. [36] During last phase, immune escape, tumor cells that have survived the early immune destruction exhibit lowered immunogenicity or/and elevated immunosuppression to attenuate the immune responses. The mechanisms in immune escape are also observed in checkpoint blockade resistant tumors. In low immunogenic tumors, lowered antigen presentation including diminished MHC I expression is one of the main mechanisms utilized by cancer cells to induce the loss of target of immune cells, resulting in overall immune silence. Nevertheless, in the tumors with high degree of T cell infiltration and inflammation, the dysfunction instead of absence of T cells is widely observed.

The examination of human melanoma lesions revealed a strong association between PD-L1 expression and tumor infiltration lymphocytes (TILs). [37] The persistent interaction between PD-L1 and PD-1 brings T cell in to exhaustion state and thus adaptive resistance of cancer cells. The components in tumor microenvironment (TME) implicating in PD-L1 dependent T cell inactivation include tumor cells, tumor stroma, inflammatory myeloid cells and T regulatory cells. Stromal expression of PD-L1 has been shown closely correlated with the outcome in colon cancer patients. [38] Tumor associated macrophages (TAMs) and dendritic cells (DCs) also participate in PD-L1/PD-1 axis immunosuppression, which also secret other pro-tumor cytokines. Besides membrane bound PD-L1, extracellular PD-L1 such as exosomal and soluble form PD-L1 serve as potent immunosuppressant. [39] Exosomes expressing PD-L1 can directly interact with T cells or fuse with the target cells to release the contents. In a NSCLC in vivo model, PD-L1 containing exosomes deficits the IL-2 and IFN-γproduction by T cells. [40]

While the PD-L1/PD-1 interaction has been widely studied, the intrinsic role of PD-L1 remains unclear. Azuma et al. demonstrated the “molecular shield” on PD-L1 signaling in cancer cells. [41] They found that cytotoxic cells preferentially lysed B7-H1 expressing cancer cells in B7-H1+ and B7-H1− mixed tumor model, and illustrated the indispensable role of the intracellular domain of B7-H1 in forming the molecular shield which operates the evasion of immune destruction. They also showed PD-1 could act as the ligand of B7-H1, which activated the anti-apoptotic signal in cancer cells. Other intrinsic roles of PD-L1 involves epithelial-to-mesenchymal transition (EMT), metastasis and resistance to therapy. [42] PD-L1 expression has been proved as a consequence of EMT in numerous cancer types. In NSCLC and gastric carcinoma, PD-L1 was upregulated under the presence of NF-κB signaling during EMT and co-expressed in VIMENTIN positive NSCLC tissues. [43,44] The vice versa relationship between PD-L1 and EMT was observed in renal cell carcinoma and esophageal cancer. In RCC, downregulation of PD-L1 was found accompanied by the decreased expression of pluripotency regulation marker genes and PD-L1 promoted the stemness of RCC cells. [45] Similarly, the overexpression of PD-L1 in esophageal cancer cell lines promoted the cell viability, migration and EMT phenotype. Oncogenic role of PD-L1 was also observed in glioblastoma multiforme where PD-L1 activated Ras/MEK/ERK signaling and thus promoted EMT. [46] In conclusion, the understanding of the intrinsic role of PD-L1 will help the development of combinatory therapy targeting both immune populations cancer cells to improve the efficacy of treatment.

CLINICAL RESEARCH OF PD-1/PD-L1 BLOCKADE

Cancer immunotherapy has become one of the most promising research directions and joined the standards of cancer care besides chemotherapy, radiotherapy and surgery. Aiming to develop patient specific treatment, to further dissect the mechanisms of immune escape and tumor microenvironment, and to combine immunotherapy with traditional cancer care to improve the efficacy of the treatment is the challenge waiting to be solved. Current FDA approved PD-1/PD-L1 inhibitors include Pembrolizumab, Nivolumab, Cemiplimab which target PD-1 on T cells and Atezolizumab, Avelumab, Durvalumab which target PD-L1. In the following section, we will review the clinical trials using either single inhibitor or the combination of multiple PD-1/PD-L1 inhibitors or combination of PD-1/PD-L1 inhibitors with other checkpoint inhibitors, shown as table 1.

The anti-tumor effects of anti-PD1 or anti-PD-L1 monotherapies has been demonstrated in advanced NSCLC and it’s also approved by FDA in the treatment of melanoma, urothelial cancer, head

and neck cancer, Hodgkin lymphoma, Microsatellite instability or mismatch repair deficient cancers and colorectal cancers while only a small population of patients have showed response to monotherapy, ranging from 10-30% and meanwhile a subset of patients displayed hyper-progression with accelerated disease progression. In a retrospective analysis of fifty patients treated with immunotherapy for different malignancies, 8% of patients exhibited 2-fold increase in tumor size and developed multiple bone and liver metastasis and very high tumor burden. [47] The hyperprogressive pattern has been investigated in advanced non-small cell lung cancer patients after being treated with PD-1/PD-L1 inhibitors. Among eligible patients treated with PD-1/PD-L1 inhibitors, fifty-six patients (13.8%) were classified as having hyperprogressive disease (HPD) with 50% increase in tumor growth rate after receiving the PD-1 monotherapy. To be noticed, hyperprogressive disease was significantly associated with higher metastatic burden before PD-1/PD-L1 inhibitors compared with non-HPD. [48] The acceleration of tumor growth kinetics (TGK) has also been observed in recurrent and/or metastatic head and neck squamous cell carcinoma (R/M HNSCC) patients after pembrolizumab and nivolumab treatment. It occurred that 29% of total 34 patients showed hyperprogression with one bearing distant metastases and HPD was closely associated with shorter progression-free survival (PFS) according to RECIST ($P = 0.003$) and irRECIST ($P = 0.02$) [49] This novel aggressive pattern of hyperprogression need to be further studied to reveal the mechanisms and specific patients subtypes prone to such pattern.

To overcome the limitation of monotherapy that targets only one specific pathway or cell group, combinatory therapies have been introduced to further improve the efficacy of PD-1/PD-L1 blockade treatment. Multiple studies have indicated the advantages of combinatory therapies over monotherapies, especially in the overall survival rate and disease progression free rate.CTLA-4 is another co-inhibitory pathway that can be targeted to boost the anti-tumor immunity and the monotherapy of CTLA-4 inhibitor has already demonstrated clinical benefits, while only on a limited group of patients. In phase 3 of Nivolumab or Nivolumab Plus Ipilimumab Versus Ipilimumab Alone in Previously Untreated Advanced Melanoma (CheckMate 067), greater percentage of patients received nivolumab plus ipilimumab or nivolumab alone showed sustained long-term overall survival at 5 years than those who received ipilimumab alone showed more durable long-term survival rate. [50] The toxicity of combined immunotherapy has also brought concern as the enhanced immune response to tumors can create a distinct treatment-related adverse events (TRAEs) compared to traditional therapies and thus lead to the termination of treatment. A meta-analysis covering 2626 patients showed that among 16 articles reported any grade TRAEs, the incidence was 95 and 76% in melanoma and NSCLC patients and among 17 articles reported grade 3 or higher TRAEs, the incidence was 55 and 33% in melanoma and NSCLC patients, respectively. Incidence of TRAEs resulted in severe adverse events and consequently the discontinuation of therapies. This study demonstrated that most patients received anti-PD1/PD-L1 plus anti-CTLA-4 combinatory therapy had at least one any grade TRAEs during treatment course, which was less commonly observed in patients received monotherapies. [51] Other ongoing combinatory therapy research involving PD-1/PD-L1 blocks consist of utilizing chemotherapy, radiotherapy and small molecule inhibitors while these can also associate with increased irAEs. For example, combining PD-L1 mAb (durvalumab) and EGFR inhibitor (osimertinib) induced a significantly higher risk of interstitial lung disease (2% [n = 23 of 1149], 2.8% [35 of 1207], and 38% [n = 13 of 34], respectively. [52,53] The complexity of tumor immune environment makes it challenging to probe biomarkers to define patient subsets who are more suitable for combinatory therapies. So, it is urgent to develop new methods to predict the outcome of combinatory treatments in patients more precisely.

Table 1 clinical trials

Study	Phase	Intervention/ Treatment/Arms	Disease/Conditions
NCT04203485	III	Experimental: Camrelizumab (anti-PD-1) Camrelizumab +	PD-L1 Positive NSCLC

		Apatinib Mesylate (EGFR inhibitor) Active Comparator: Pemetrexed/Paclitaxel injection+ Carboplatin	
NCT03546426	I	Pembrolizumab (anti-PD-1) + Autologous dendritic cells + Interleukin-2	PD-L1 Negative Mesothelioma, Malignant
NCT04470674	II	Experimental: Durvalumab (anti PD-L1) + carboplatin + Pemetrexed Active Comparator: Durvalumab	Kras Mutation Positive and PD-L1 High (≥ 50%) NSCLC
NCT03417882	II	Pembrolizumab (anti-PD-1) + GRN-1201 (cancer peptide vaccine)	Metastatic PD-L1+ NSCLC
NCT02791334	I	Experimental: LY3300054 (anti-PD-L1) LY3300054 + Ramucirumab (VEGFR2 inhibitor) LY3300054+ Abemaciclib (CDK4/6 inhibitor) LY3300054 + Merestinib (RTKs inhibitor) LY3300054 + LY3321367 (anti-TIM3)	Advanced Refractory Solid Tumors Microsatellite Instability-High (MSI-H) Solid Tumors Cutaneous Melanoma Pancreatic Cancer Breast Cancer (HR+HER2-)
NCT04139317	II	Experimental: Pembrolizumab (anti-PD-1) + Capmatinib (c-MET inhibitor) Active Comparator: Pembrolizumab	NSCLC With PD-L1≥ 50%
NCT02369874	III	Experimental: MEDI4736 (Durvalumab, anti-PD-L1) MEDI4736 + Tremelimumab (anti-CTLA4) Active Comparator: Standard of Care	Recurrent or Metastatic PD-L1-positive or -Negative Squamous Cell Carcinoma of the Head and Neck (SCCHN)
NCT02685059	II	Experimental: MEDI4736 as monotherapy followed by MEDI4736 in nab-paclitaxel (part 2) followed by MEDI4736 + epirubicin (part 3). Active Comparator: Nab-Paclitaxel Epirubicin Cyclophosphamide	Triple Negative Breast Cancer

NCT04165083 (MK-3475-01B/KEYNOTE-01B)	II	Experimental: Pembrolizumab + MK-4830 (anti-ILT4)	PD-L1 Positive Advanced Non-small Cell Lung Cancer (NSCLC)
NCT04199104	III	Experimental: Pembrolizumab + Lenvatinib (VEGFR1/2/3 inhibitor) Active Comparator: Pembrolizumab with Placebo	Head and Neck Squamous Cell Carcinoma
NCT02735239	I/II	Experimental: Durvalumab + standard chemotherapy (Oxaliplatin + Capecitabine) Durvalumab + Tremelimumab + standard chemotherapy Durvalumab + surgery + standard chemotherapy Durvalumab + surgery + standard chemo and radiotherapy Durvalumab, surgery, new standard of care chemotherapy FLOT (Oxaliplatin + 5-fluorouracil (5-FU) + Leucovorin + docetaxel)	Esophageal Cancer

DISCUSSION AND CONCLUSION

Current clinical and preclinical research on cancer immunotherapy falls greatly on re-activating T-lymphocytes and anti-tumor immune responses. To the date, immunotherapies aiming to boost T cell activity can be roughly classified into three categories: immune checkpoint therapy, adoptive T cell transfer therapy and cancer vaccines. [60] CTLA-4 and PD-1 are two most potent negative regulator expressed by activated T cells, which are originally considered as major players in preventing autoimmune diseases, and have been widely targeted in cancer immunotherapies. The importance of other negative regulators including B7-H3, TIM3 and V-domain immunoglobulin (Ig)-containing suppressor of T-cell activation (VISTA) [61] are gradually emerging and gaining more attention. Combinatory therapies that incorporate agents targeting multiple pathways have become the direction of future research. For example, patients accepted anti-CTLA4 and anti-PD1 therapies have shown survival benefits over the ones received monotherapy. [50] However, the success of combinatory therapies is accompanied by the risk of toxicity which may lead to the termination of treatment. Thus, better solutions need to be found to avoid such scenario.

Therapeutic cancer vaccines have shown limited effectiveness compared to checkpoint blockade therapies and have been facing more challenges as due to the cancer specific neoantigens, more personalized treatments are required. The checkpoint blockade shed light on providing new possibilities for cancer vaccines. [62] Particularly for patients who have had weak spontaneous immune responses to cancer vaccines, the activation of immune system by checkpoint can induce synergetic effects to maximize the anti-tumor immunity.

During the last decades, the breakthroughs of cancer immunotherapy has proven its efficacy, specificity and long-term benefits in patients. The burgeoning increase of innovative therapies aims to offer more effective and tolerable treatments to larger population of patients. The better understanding of the mechanisms of tumor immune escape and anti-tumor immunity serves a pivotal role in unleashing new possibilities of cancer immunotherapy.

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