Immune checkpoints and cancer in the immunogenomics era

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Abstract

Immune checkpoints have been the subject of a wave of new studies. Among these checkpoints are cytotoxic T-lymphocyte-associated antigen 4, checkpoints programmed death-1 and programmed death-ligand 1; their blockades have been approved by the Food and Drug Administration for therapy of melanoma and other types of cancers. Immunogenomics, which combines the latest nucleic acid sequencing strategy with immunotherapy, provides precise information about genomic alterations (e.g. mutations) and enables a paradigm shift of immune checkpoint therapy from tumor types to molecular signatures. Studying these critical checkpoints in relation to genomic mutations and neoantigens has produced groundbreaking results. This article examines these studies and delves into the relationships between immune checkpoint blockade and tumors harboring certain genomic mutations. Moreover, this article reviews recent studies on resistance to immune checkpoint therapy.

Key words: CTLA-4; PD-1; PD-L1; immune checkpoint blockade; immunogenomics; MMR deficiency; POLE; somatic; mutation; resistance to checkpoint blockade; neoantigen

Introduction

Although immunotherapy for cancer was launched more than 100 years ago, there had not been much success until the 1st landmark clinical trials reported in 2012 that employed regimes (antibodies), against the immune checkpoints programmed death-1 (PD-1) and programmed death-ligand 1 (PD-L1) [1, 2]. These regimes along with the blockade of cytotoxic T lymphocyte antigen 4 (CTLA-4) have taken center stage in the immunogenomics era and radically changed the outlook of cancer immunotherapy. It is the evasion of host CD4⁺ and CD8⁺ T cells that allows tumor cells to become immune tolerant in the tumor microenvironment and escape immune surveillance. Certain types of human cancers harbor numerous genetic and

epigenetic alterations that generate neoantigens, which can be recognized by the host immune system [3], and induce upregulation of endogenous immune checkpoints that normally terminate immune responses after antigen activation [4].

Utilizing single-cell and whole-genome exome sequencing approaches along with a clonal approach; a link between genetic mutations in cancer, neoantigens and immunotherapy has been revealed. This immunogenomic strategy has demonstrated that certain patients with somatic mutations exhibit robust responses to immune checkpoint blockade and thus helps fuel breakthroughs in cancer treatment. This review will briefly summarize recent advances in immune checkpoint therapy powered by immunogenomics in cancer.

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Table 1. Checkpoint blockade, alkylating agent and PARP inhibitor

Drug	Function	Main interactors
Ipilimumab	Increases T-cell activation and propagation	CTLA-4 [4–6]
Nivolumab	Blocks immune checkpoint receptors	Programmed Cell PD-1 receptors [1, 2, 10]
Pembrolizumab	Blocks immune checkpoint receptors	Programmed Cell PD-1 receptors [10, 56, 57]
Temozolomide	Alkylating agent used to treat tumors in the CNS	Alkylate or methylate DNA and the 1st line drug for glioblastoma [58–60]
Veliparib	Target cancers with DNA- repair defects	PARP [33, 34, 61]
Olaparib	Target cancers with DNA repair defects	PARP [33, 34, 62]

Immune checkpoints

Immune checkpoints are receptor-based signal cascades that negatively regulate T cells and cause immune tolerance, which allows tumors to evade and escape immune surveillance. The most prominent of these checkpoints are CTLA-4, PD-1 and PD-L1. Blockade of these immune checkpoints with specific monoclonal antibodies has emerged as a revolutionary therapy for cancer (Table 1). Immune checkpoint therapy has been shown to be a promising approach of controlling advanced unresectable melanoma, but it has recently been expanded to treat other malignancies.

CTLA-4

CTLA-4 is a crucial immune checkpoint that has been extensively studied [4–6]. CTLA-4 is expressed on regulatory T (Treg) cells and on both activated CD4⁺ and CD8⁺ T cells. CTLA-4's ligands are B7-1 and B7-2 (CD80 and CD86), which are expressed on antigen presenting cells (APCs), especially dendritic cells (DCs). Interestingly, CD28 on T cells also share B7-1 and B7-2 as its ligands. As part of the immune response, immature DCs capture and process antigens before evolving into mature DCs that present antigens to CD4⁺ T cells. When B7-1 and B7-2 bind to CD28 on CD4⁺ T cells, CD4⁺ T cells become T helper 1 (Th1) cells and produce interferon gamma (IFN- γ), tumor necrotic factor alpha (TNF- α) and interleukin 2 (IL-2), which promote proliferation of CD8⁺ T cells [7]. However, when the B7 presented by an APC binds to CTLA-4, IFN- γ , TNF- α and IL-2 production are reduced and T-cell proliferation is suppressed. When CTLA-4 and CD28 compete to bind with their ligands, B7-1 and B7-2 are much more likely to bind with CTLA-4 because CTLA-4 has a greater affinity to B7-1 and B7-2 than CD28 does (Figure 1) [8].

In the tumor microenvironment, tumors produce factors that suppress CD4⁺ T cells mainly through the upregulation of CTLA-4. The blockade of CTLA-4 has become a great immunotherapy strategy for cancers. Ipilimumab, used as an anti-CTLA-4 drug, was the 1st checkpoint antibody to be approved by Food and Drug Administration (FDA) for treatment of melanoma [9]. Anti-CTLA-4 has been used in the treatment of many carcinomas beyond melanoma. These include cancers of the bladder, blood, colon and rectum, lung, ovary, urothelial tract and uterus.

PD-1/PD-L1

PD-1/PD-L1 is an important pair of immune checkpoints [1, 2, 10]. PD-1 belongs to the same family of receptors as CTLA-4 and CD28. It is inducibly expressed on B cells, DCs, monocytes, natural killer cells and CD4⁺ and CD8⁺ T cells. Recently, PD-1 has also been found on tumor-associated macrophages (TAMs)

Figure 1. Impaired immunorecognition of tumor cells by CTLA-4 and PD-1/PD-L1. (A) Both CD28 and CTLA-4 bind to their cognate ligands B7 on APCs, on which the major histocompatibility complex (MHC) II present tumor antigen peptides to T-cell receptor (TCR) complex inducing T cell cytotoxicity activity. The CD28 binding triggers but the CTLA-4 binding suppresses T-cell activation and subsequent anti-tumor immunity. However, CTLA-4 has much higher binding affinity to B7 than that of CD28 resulting in a defect in tumor antigen presentation to T cells. CTLA-4 blockade interrupts interaction of CTLA-4 with B7 restoring antigen presentation capacity of APCs and T-cell activation. (B) PD-L1 expressed on tumors interacts with PD-1 on T cells and blocks T cells' attack on them. PD-L1 or PD-1 blockade inhibits their interaction thus relieving suppression of PD-1 and PD-L1 and leading to killing tumor cells by armed cytotoxic T cells. Note: +, stimulatory response; $\ominus$ inhibitory response.

[11]. PD-1 binds to its ligands, PD-L1 and PD-L2, which are not only expressed on APCs but also on the exterior of tumor cells allowing these cells to evade immune surveillance (Figure 1). When PD-L1/2 bind to PD-1, T cells are prevented from effectively recognizing and killing tumor cells and TAMs are not able to exert their phagocytosis function thus enhancing tumor immunity [11]. Therefore, the PD-1 and PD-L1 checkpoints are great targets for cancer immunotherapy, and their blockades have shown dynamic and durable responses in a series of malignancies [12, 13]. Recent data indicate that patients harboring tumors that overexpress PD-L1 react better to anti-PD-1-directed therapy. For example, Topalian et al. reported that 36% of patients with different solid tumors that expressed PD-L1 showed an objective response to nivolumab, a monoclonal antibody that blocks PD-1 receptors. However, none of the patients with PD-L1-negative tumors demonstrated a response [14].

Somatic mutations, neoantigens and immune checkpoint therapy

Recent advances in next generation sequencing combined with single-cell and whole-genome exome sequencing have enabled the diagnosis and treatment of cancers at the cell and molecular levels. Cancer cells can acquire somatic mutations that encode nonself immune antigens, also called neoantigens, which can be recognized by the host immune system (Figure 2) [3]. Because

Figure 2. MMR deficiency causes high somatic mutation load and neoantigen expression. Intrinsic or extrinsic factors cause DNA damages, which are immediately repaired by DNA repair machineries. DNA MMR deficiency leads to the failure of DNA repair and subsequent accumulation of somatic mutations and tumorigenesis. Intriguingly, the accumulated somatic mutations can induce expression of neoantigens that can be recognized by the host immune system, and upregulation of CTLA-4, PD-1 and PD-L1, which are targets of immune checkpoint therapy. Thus, high levels of somatic mutation load, neoantigen expression and immune checkpoint upregulation can be used as great indicators for immune checkpoint therapy.

mutational burdens are the engines of neoantigens, if tumors accumulate a large amount of somatic mutations before or after chemotherapy, the surrounding immune cells may either become very reactive and finally get exhausted or become immune tolerant due to overexpression of immune checkpoints. In any case, tumor cells can now proliferate, grow, invade or metastasize to distal organs. Conversely, tumors with these somatic mutations can be susceptible to immune checkpoint blockades, thus becoming better targets for immunotherapy.

DNA mismatch repair, somatic mutation, neoantigens and checkpoint therapy

DNA mismatch repair (MMR) is a critical pathway that is able to correct DNA mismatches during DNA replication and prevents mutations from becoming permanent in dividing cells [15]. MutS homolog 2 (MSH2), MSH6, mutL homolog 1 (MLH1) and PMS1 homolog 2 (PMS2) are MMR proteins that are involved in DNA repair and the loss of these proteins can result in frameshift mutations and the creation of novel microsatellite fragments [16]. Microsatellite instability (MSI) is thus used as a phenotypic evidence that the MMR of a cell is deficient. MSI-high (MSI-H) leads to an accumulation of somatic mutations in tumor cells. MSI colorectal cancer (CRC) accounts for approximately 15% of sporadic CRC [17]. It has been postulated that MSI CRC displays an active Th1/CTL immune microenvironment because of the recognition of many tumor neoantigens [17]. In addition, MSI CRC tumors have a high level of expression of the checkpoint molecules PD-1, PD-L1 and CTLA-4 [18]. It has been suggested that somatic mutations associated with MMR deficiency are strongly correlated with a heightened response to treatment, such as pembrolizumab (an antibody that targets the PD-1 checkpoint), in CRCs [14]. On the other hand, patients with CRC in the study with proficient MMR had no response to pembrolizumab. Recently, pembrolizumab has been approved by FDA to treat MSI-H tumors [12].

Although the inactivation of MMR within human cells has primarily been linked with CRCs, MMR deficiency has been identified in cancers of the urothelial tract, uterus, biliary tract, stomach, pancreas, ovary, prostate and small intestine. MMR deficiency is also associated with high checkpoint expression and a better response to checkpoint blockades. For example, a patient with high-grade urothelial cancer of the renal pelvis was found to have a high load of mutations along with the MMR-deficient phenotype due to the absence of MSH2 and MSH6 [19]. An elevated expression level of PD-1 was detected, but the patient experienced complete remission and had no severe side effects from the PD-L1 inhibition treatment. Another study compared PD-L1 expression in MMR-deficient endometrial carcinomas (ECs), which included Lynch Syndrome-associated and MLH hypermethylation cases, with MMR-proficient cases [20]. Overall,

it was found that 53% of MMR-deficient ECs showed a high expression of PD-L1. Among MMR-deficient ECs, 70% of Lynch Syndrome-associated ECs and 30% of MLH hypermethylation-associated ECs exhibited a high expression of PD-L1. In contrast, only 10% of MMR-proficient ECs expressed very low amounts of PD-L1. These findings suggest that MMR-deficient ECs associated with Lynch Syndrome are great targets for the immune checkpoint therapy.

However, MMR deficiency in some cancers may not lead to upregulation of immune checkpoints. Instead, its resultant somatic mutations generate a large amount of neoantigens, which can incorporate into human leukocyte antigens (HLAs) and make tumors susceptible to checkpoint blockades. Czink et al. brought to a light an intriguing case that involved a woman who bore a tumor that was MMR deficient and exhibited MSI [21]. Although the woman was not treated until an advanced stage of disease, she showed a robust and lasting response to pembrolizumab. What makes this situation unique is that the tumor did not display common features that are predictive of responsiveness to immunotherapy such as high expression levels of immune checkpoints. Elevated levels of expression of the HLA classes I and II antigens were identified in the patient, which led the researchers to put forward the notion of using MSI status and HLA classes I and II antigen expression in tumors to determine eligibility for immune checkpoint blockade even when traditional predictive markers are not detectable.

DNA polymerase epsilon and PD-1 checkpoint therapy

In addition to MMR deficiency, alterations of the polymerase epsilon (POLE) gene, which encodes the catalytic subunit of DNA replicase, also cause a hypermutated phenotype in CRC and ECs [22–24]. POLE is responsible for chromosomal DNA replication and DNA repair. POLE gene mutations were identified in less than 2% of CRC patients [25], and they occurred in 6–12% of patients with ECs [26]. Interestingly, POLE mutations mainly occurred in male CRC patients [25]. Importantly, the presence of POLE mutations led to better recurrence-free and disease-free survival compared to MSI-proficient tumors. A significant reduction in recurrence was observed in patients with stage II disease, indicating that POLE mutations can be a biomarker for CRC survival and recurrence. Moreover, POLE-mutant CRC had increased CD8⁺ lymphocyte infiltration and expression of cytotoxic T-cell markers and effector cytokines, similar to that identified in immunogenic MMR-deficient cancers. Furthermore, the effector cytokines such as IFN γ , CXCL9 and CXCL10, as well as immune checkpoints PD-1 and PD-L1 were upregulated in POLE-mutant CRC. Although there are no exact case reports regarding immune checkpoint therapy in CRC patients with POLE mutations, there was a case report on ECs with POLE mutation [23]. A patient with

ECs harboring a *POLE* mutation had an exceptional response to anti-PD-1 therapy. A large amount of somatic mutations was identified by whole exome sequencing. Similar to CRC with *POLE* mutations, *POLE*-mutant ECs exhibited significantly high levels of CD8⁺ T cells, T follicular helper cells, M1 macrophages and natural killer cells. Together, these findings strongly suggest that CRC and EC patients with *POLE* mutations can be subjected to immune checkpoint therapy.

Recently, *POLE* mutations have been discovered in lung carcinoma [27]. These patients with lung cancer showed similar immune profiles to those who had CRC and ECs. In *POLE*-mutant lung tumors, significant increased infiltration of CD4⁺, CD8⁺ T cells, Th1 cells, Th17 cells, natural killer cells and DCs was observed. Neoantigens were upregulated. Interestingly, *POLE* mutations were associated with gene mutations in the type I interferon pathway. Nevertheless, all of these findings suggest that *POLE* gene mutations are associated with a favorable outcome of lung cancer and are excellent biomarkers for immune checkpoint therapy.

Besides CRC, ECs and lung cancer, *POLE* mutations are also identified in glioblastoma and glioma. About 78% high-graded pediatric gliomas harbored mutations in *MSH6*, *MSH2*, *MLH1*, *PMS2*, *POLE* and *POLD1* genes [28]. There was a case report on a patient with glioblastoma whose *POLE* gene was hypermutated [29]. Analysis of the subclones of the tumor led to the discovery of mutational signatures that are related to DNA-repair defects. A high neoantigen load was detected in all tumors, which is consistent with the high mutation burden. The patient was treated with temozolomide (a chemotherapy drug used to treat brain cancer) followed by pembrolizumab (Table 1). After treatment with pembrolizumab, there were infiltrated lymphocytes in the central nervous system (CNS) indicating active immunosurveillance.

BRCA1 and BRCA2 mutations and checkpoint therapy

Breast cancer 1 (*BRCA1*) and 2 (*BRCA2*) are both tumor suppressor genes, which produce proteins that aid in repairing DNA double-strand breaks via the homologous-recombination repair pathway [30]. *BRCA1/2*-driven tumors are most likely to form when normal cells only have one copy of the gene (heterozygous) but become cancerous by the somatic loss of the *BRCA* allele that remains [31]. *BRCA1/2* mutations increase the risk of breast and ovarian cancers and constitute 20–25% of hereditary breast cancers [32]. Moreover, breast cancers, especially triple-negative breast cancers (TNBCs), arising from *BRCA1/2* mutations typically show a poor prognosis. Although (ADP-ribose) polymerase (PARP) inhibitors (e.g. veliparib and olaparib) [33, 34] (Table 1), which cause severe DNA damage and subsequent apoptosis, showed efficacy in a small portion of patients; high rates of relapse and drug resistance have been observed. Thus, there is an urgent need to pave new therapeutic avenues for *BRCA1/2*-mutated cancers. Recent studies suggested that *BRCA1*- or *BRCA2*-mutant TNBCs are associated with a high burden of mutations and noticeable immune cell infiltrations including T lymphocytes [35, 36]. Abundant PD-L1 expression was not only detected in the infiltrated immune cells, but it was also expressed in tumor cells. The feature, of which high mutation load leads to high expression levels of immune checkpoints, provides a rationale for the usage of PD-L1 blockade. Indeed, in the MMTV^{cre}/*BRCA1*^{fox/fox}-p53^{+/-} mouse model that develops human *BRCA1*-mutated TNBCs-like triple-negative mammary tumors, PD-L1 was abundantly expressed, and

combined PD-1 and CTLA-4 blockade efficiently induced a strong immune response and attenuated tumor growth [35].

While PARP blockade has limited efficacy in TNBCs with *BRCA1/2* mutations, it may exhibit a synergistic effect when combined with immune checkpoint blockades. In a *BRCA1*-deficient ovarian cancer mouse model, it was found that CTLA-4 blockade markedly increased CD8⁺ T cell activity and secretion of IFN γ [37]. PARP inhibition clearly augmented Th1 response leading to increased production of IFN γ and TNF α in the tumor microenvironment leading to lasting effects on T cells and enhanced anti-tumor immunity. As a consequence, the lifespan of the mice was extended.

Epidermal growth factor mutations, non-small cell lung cancers and immune checkpoint therapy

Epidermal growth factor (EGFR) is one of the receptor tyrosine kinases and plays a critical role in pathophysiology [38]. EGFR mutations have been identified in many cancers, especially, non-small cell lung cancers (NSCLCs). These mutations cause the hyper-activation of EGFR and subsequent transformation, malignancy and metastasis. Also, overactive EGFR can cause activation of Akt and mitogen-activated protein kinases [24, 38, 39], which are critical for tumor growth and metastasis, and immune suppression by enhancing Treg activation [40, 41]. Since conventional chemotherapies have limited clinical efficacy, target therapy using EGFR inhibitors and immunotherapy especially immune checkpoint therapy, have become choices for new therapeutic avenues. However, NSCLCs can acquire resistance to EGFR inhibitors such as tyrosine kinase inhibitors [42]. Thus, checkpoint therapy has recently drawn more attention.

Based on two early clinical trials which showed that PD-1 and PD-L1 blockades induced favorable tumor regression and prolonged disease stabilization in 20–25% of cancers including NSCLCs [1, 2], a study investigated the relationship between the effects of PD-1 on the tumor microenvironments in EGFR-driven lung cancers [43]. It was shown that EGFR activation induced PD-L1 expression in human bronchial epithelial cells and reduced the CD8⁺ T cell/Treg cell ratio leading to an immune suppressive tumor microenvironment. Blockade of PD-1 slowed down the tumor growth and significantly enhanced survival. Cellular analysis revealed that PD-1 blockade activated T cells and stimulated their effector activity. A later study examined the association of PD-L1 expression with the EGFR mutation [44]. It was found that high PD-L1 expression was associated with EGFR mutations in resected human NSCLC tumor tissues. Interestingly, this study also suggested that higher levels of PD-L1 were found in women than in men, indicating a sexual preference [44]. In addition, this study suggested that high PD-L1 expression was significantly associated with poor survival of NSCLC patients. Supporting this notion was a related study, which examined the association of PD-L1 and EGFR status in patients with advanced NSCLC [45]. It was found that high PD-L1 expression was associated with EGFR mutations and that high PD-L1 expression was significantly associated with poor survival of patients with wild-type EGFR. Conversely, two studies suggested that high PD-L1 expression was significantly correlated with a favorable survival rate in stage I patients with lung squamous cell cancer [46] or in the early stages of NSCLCs [47]. The discrepancy here might be caused by EGFR statuses, lung cancer stages or the sizes of cohorts. Nevertheless, the induction of PD-L1 expression by EGFR activation was also examined in an *in vitro* study using human NSCLC cell lines. EGFR

Figure 3. Potential mechanisms of checkpoint resistance. (A) Mutation, deletion and heterozygosity (LOH) in beta-2-microglobulin gene of cancer patients result in the failure of assembling MHC or HLA class complexes and reduction of their neoantigen presentation capacity to TCR complex as well as a great decrease in clinical efficacy of immune checkpoint blockade. (B) Mutations in Janus kinase 1 (JAK1) gene or JAK2 gene lead to the failure of transducing IFN γ signaling to their downstream events including signal transducer and activator of transcription 1 and anti-tumor immunity of IFN γ .

mutation not only stimulated EGFR activation but also induced PD-L1 expression through extracellular signal-regulated protein kinases 1 and 2 (ERK1/2) activation [48].

Resistance to immune checkpoint blockade

Despite success of immune checkpoint therapy in a specific subset of advanced cancers, the common feature is that many patients with cancer do not respond to checkpoint blockade. It is understood that this unresponsiveness is due to tumors with low or undetectable expression of checkpoints or due to the lack of tumor-infiltrating lymphocytes in the tumor microenvironment. However, the fact is that certain tumors with high levels of PD-1 do not respond to checkpoint blockade [49]. One of the resistance mechanisms is that in certain tumors beta-2-microglobulin (B2M), which is a key factor for assembling all MHC class I or HLA class I complex and is essential for antigen presentation [50], is mutated or deleted [49] (Figure 3). Moreover, analysis of the biopsies of the same melanoma patient cohorts revealed that loss of heterozygosity (LOH) in B2M was 3-fold enriched in 30% nonresponders compared to 10% responder. These abnormalities of B2M led to defects in antigen presentation and IFN γ pathway activation resulting in non-responsiveness to PD-1 or CTLA-4 blockade. Another mechanism is that tumors harbor point mutations in the genes of Janus kinase 1 (JAK1) and 2 (JAK2), of which their encoding proteins are essential for IFN γ signaling and CD8 $^{+}$ T cell activation [51] (Figure 3). Moreover, a recent study conducted in an inducible melanoma animal model suggested that the resistance to immune checkpoint therapy largely depends on the tumor microenvironment. In this microenvironment, tumor-infiltrated lymphocytes underwent apoptosis triggered by the Fas-ligand, which was released by myeloid-derived suppressor cells [49].

In clinic practice, it is often found that patients who originally responded well to checkpoints blockade acquired resistance to the therapy. One of the potential mechanisms is that after responding to checkpoint blockade, somatic mutations in tumors were markedly reduced leading to a striking decrease in neoantigen production [52].

Closing remarks and future directions

The combination of immune checkpoint therapy with immunogenomics has revolutionarily shaped immunotherapy framework for many advanced cancers and allowed for a paradigm shift of immune checkpoint therapy from tumor types to molecular signatures. However, the major limitation of mono-therapy is the presence of primary resistance or acquired resistance in a certain group of patients who previously responded to the therapy. In this review, we deliberated on CTLA-4, PD-1 and PD-L1 and their blockade but had limited space to touch on other immune checkpoints such as T-cell immunoglobulin mucin 3 [53] and lymphocyte activation 3 [18]. We also discussed several potential mechanisms involving this resistance. Future studies, in addition to what has been discussed in this article, need to elucidate on whether immune metabolomics and microbial status in the gut as well as signaling pathway networks associated with tumor-intrinsic and extrinsic factors play a profound role in the success of immune checkpoint therapy. Moreover, to achieve greater success with checkpoint therapy, combination therapy or synergic therapy will need to be implemented in order to reduce non-responsiveness and improve clinical efficacy. Furthermore, there is an urgent need for optimal management of treatment-related side effects such as autoimmunity and tumor hyperprogression in some patients who have been treated with immune checkpoint blockade [54, 55].

Key Points

- CTLA-4, PD-1 and PD-L1 are prominent immune checkpoints that lead to immune tolerance in cancer patients.
- MMR deficiency leads to defective DNA repair, generation of microsatellite fragments and accumulation of somatic mutations.
- High somatic mutation burden is the engine of neoantigens, which can be recognized by the host immune system and can also induce expression of immune checkpoints.
- MMR deficiency, MSI, POLE mutations, high somatic mutation load and high neoantigen burden are excellent indicators for immune checkpoint therapy
- Mutations, deletions and LOH in the B2M gene, mutations in the genes encoding JAKs or apoptosis of CD4 $^{+}$ and CD8 $^{+}$ T cells lead to resistance to immune checkpoint therapy.

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