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REVIEW**Combined anti-PD-1 and anti-CTLA-4 checkpoint blockade: Treatment of melanoma and immune mechanisms of action**

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Cytotoxic T-lymphocyte associated protein-4 (CTLA-4) and the Programmed Death Receptor 1 (PD-1) are immune checkpoint molecules that are well-established targets of antibody immunotherapies for the management of malignant melanoma. The monoclonal antibodies, Ipilimumab, Pembrolizumab, and Nivolumab, designed to interfere with T cell inhibitory signals to activate immune responses against tumors, were originally approved as monotherapy. Treatment with a combination of immune checkpoint inhibitors may improve outcomes compared to monotherapy in certain patient groups and these clinical benefits may be derived from unique immune mechanisms of action. However, treatment with checkpoint inhibitor combinations also present significant clinical challenges and increased rates of immune-related adverse events. In this review, we discuss the potential mechanisms attributed to single and combined checkpoint inhibitor immunotherapies and clinical experience with their use.

Keywords: PD-1 · CTLA-4 · Cancer · Immunotherapy · Melanoma

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Introduction

Malignant melanoma is the fifth commonest cancer in the UK, with an estimated 300 000 cases reported globally per annum [1,2]. Melanoma is described as the archetypal immunogenic cancer as supported by clinical observation of spontaneous tumor regressions and increased rates of melanoma in immunosuppressed individuals [3,4]. Melanoma carries a large mutational load, providing a range of tumor-specific antigens that can drive the host immune response. However, tumors, such as melanoma, can evade immunosurveillance via activation of different immune-inhibitory pathways including via immune checkpoint molecules and their downstream signals. Physiologically, checkpoint pathways play a role in immune homeostasis providing negative feedback stimuli to prevent autoimmune reactivity. The best characterized checkpoint pathways are those of negative regulatory molecules cytotoxic T-lymphocyte associated protein-4 (CTLA-4) and of the Programmed Death Receptor 1 (PD-1) and its ligands PD-L1 and PD-L2.

Checkpoint inhibitor (CPI) antibodies were designed to promote immune-mediated elimination of tumor cells. Antibody binding to either CTLA-4 (Ipilimumab) or PD-1 (Pembrolizumab and Nivolumab) results in abrogation of signaling in response to these inhibitory receptors' ligands in the tumor microenvironment (TME) or draining lymph nodes. Their approval for the management of melanoma has transformed prognosis in the last decade. This is compared with previously poor 5-year survival rates in advanced disease with limited palliative treatment options. CPIs were originally approved as monotherapies, however, more recent evidence revealed that dual immunotherapy can augment the efficacy of these treatments, perhaps due to working in a codependent manner. In this review, we discuss monotherapies and anti-PD-1 and anti-CTLA-4 combination checkpoint blockade, the merits and potential mechanisms associated with combined immunotherapy in treating melanoma.

Checkpoint inhibitor targets

The CTLA-4 pathway

CTLA-4, an immunoglobulin cell surface receptor, is an inhibitor of T cell activation [5,6]. It is primarily expressed on naïve T cells after activation [7] and FoxP3⁺ regulatory T cells (Tregs) [8]. T cell activation is dependent not only TCR binding with an antigen presented via an APC, but also on the presence of a costimulatory second signal, typically through binding of CD28 expressed on the T cell to CD80/86 found on the APC. Absence of this secondary signal may lead the T cell to recognize the presented peptide as a “self-antigen” or to develop tolerance to the antigen. CTLA-4 is a competitive homolog for CD28 that has a higher affinity to CD80 (B7-1), and to a lesser extent CD86 (B7-2) compared with CD28 [9], leading to inhibition of T cell costimulation (Fig. 1A). TCR signaling immediately upregulates cell

surface CTLA-4 expression, reaching peak expression at 2 to 3 days after activation [7,8,10], providing a negative feedback loop upon T cell activation. CTLA-4 within intracellular vesicles is also quickly transported to the immunologic synapse following T cell activation [11]. At the immunologic synapse, CTLA-4 is stabilized by CD80/CD86 binding, allowing it to collect and inhibit CD28 binding.

CTLA-4 limits CD28 downstream signaling, inhibiting PI3K and AKT pathways [12] (Fig. 1). CTLA-4 binding to CD80/86 mediates an intracellular negative feedback pathway, achieved via the tyrosine phosphatase SHP-2 and the serine/threonine phosphatase PP2A (Fig. 1A), dephosphorylating signaling kinases further downstream. In addition, CTLA-4 acts extracellularly to remove CD28 ligands CD80/86 from nearby cells (Fig. 1A) by transendocytosis *in vivo*, including from APCs, further inhibiting T cell activation [13].

Physiologically CTLA-4 is considered to primarily exert a modulatory role in T cell priming in regional secondary lymphoid organs, by inhibiting T cell activation and arresting the production of effector T cells [14]. The critical function of CTLA-4 in maintaining self-tolerance is illustrated by CTLA-4 knockout (KO) mice which have been shown to develop fatal lymphoproliferative disease at 3–4 weeks of age [15,16]. As well as this vital function, CTLA-4 is also thought to dampen T-cell activation in the periphery. This is supported by reported constitutive expression of its ligands CD80/CD86 to varying degrees by APC and also by activated T cells [17]. Therefore, as well as attenuating T cell activity through cell-intrinsic functions, CTLA-4 also serves cell-extrinsic functions mediated primarily through CTLA-4 expressing FoxP3⁺ Tregs [18,19]. Tregs can potentially negatively regulate nearby effector T cells expressing CD80/86 by limiting availability of these ligands for CD28 costimulation. Specific loss of CTLA-4 expression in Tregs in mice has been linked with autoimmunity and excessive T cell activation [20,21].

Anti-CTLA-4 induced tumor rejection in melanoma

In 2011, the first CPI was approved by the Food and Drug Administration (FDA) for use in advanced melanoma, Ipilimumab, a fully human, IgG1κ monoclonal, anti-CTLA-4 antibody which mediates sustained positive responses in advanced melanoma.

The main mechanism of action of Ipilimumab appears to be via direct inhibition of CTLA-4 binding with ligands CD80/CD86, allowing for CD28 costimulation and subsequent T cell activation (Fig. 1B). CD80/86 are primarily expressed in the secondary lymphoid organs leading to the hypothesis that ipilimumab acts to halt T cell activation early in T cell development. The mechanisms of anti-CTLA-4 induced tumor rejection are not fully defined but evidence from preclinical and clinical studies identifies the key targets as the effector T cell compartment and Tregs. Ipilimumab treatment has been linked with expansion of ICOS⁺ CD4⁺ T cells in melanoma as well as other tumors [22–24].

In melanoma, the immunosuppressive effect of tumor cells is in part mediated by recruitment of Tregs [25,26]. These tumor

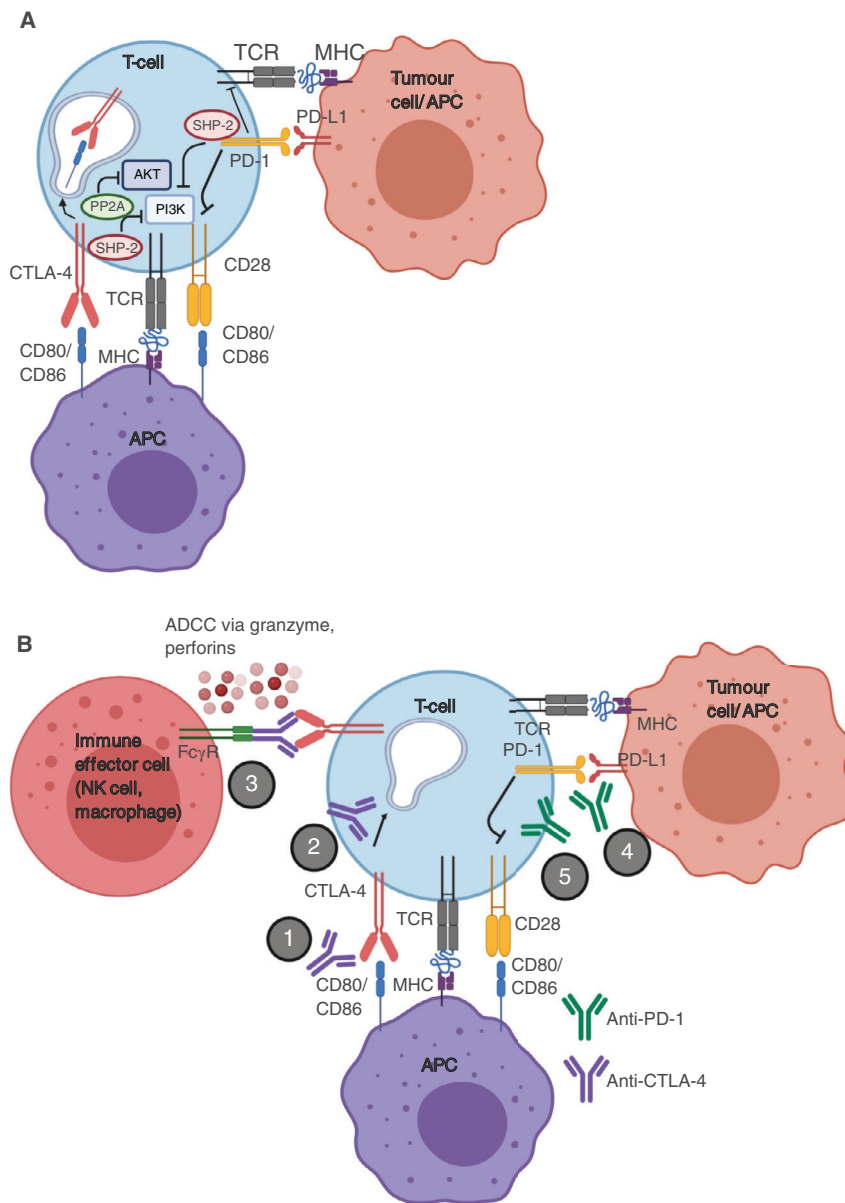


Figure 1. The CTLA-4 and PD-1 checkpoint pathways, and proposed mechanisms of action of anti-CTLA-4 and anti-PD-1 antibodies. (A) The CTLA-4 and PD-1 pathways negatively regulate Tcell activation. Tcell receptor (TCR) engagement with an antigen presented via the major histocompatibility complex (MHC) requires a costimulatory second signal for activation delivered via CD28. CTLA-4 is a competitive CD28 homolog that binds CD28 ligands CD80/86, preventing Tcell activation. CTLA-4 also mediates transendocytosis of CD28 ligands CD80/86. PD-1 engages with its ligand PD-L1 to negatively regulate Tcell activation. CD28 is also a secondary target for PD-1 and a point of convergence of the two pathways. Both CTLA-4 and PD-1 expression are upregulated upon TCR activation. Intracellular signaling for both pathways is mediated via the phosphatase Src homology region-2 containing protein tyrosine phosphatase (SHP-2) inhibiting PI3K downstream signaling. CTLA-4 in addition interacts with the serine/threonine phosphatase PP2A which dephosphorylates AKT, further inhibiting the pathway. (B) (1) Anti-CTLA-4 restores Tcell activation by inhibiting interaction between CTLA-4 and CD80/CD86 on APC. (2) Anti-CTLA-4 may inhibit transendocytosis of CD28 ligands CD80/86 mediated through CTLA-4. (3) The anti-CTLA-4 IgG1 antibody Ipilimumab can engage FcγRs on immune effector cells (NK cells, monocytes/macrophages) via its Fc region, leading to antibody-dependent cellular cytotoxicity (ADCC) and depletion of some high-CTLA-4-expressing Tcell subsets (e.g. Tregs). (4) Anti-PD-1 restores Tcell activation by inhibiting the interaction between PD-1 on T cells and PD-L1 (PD-L1 may be expressed by tumor cells and various immune cells). (5) Anti-PD-1 restores Tcell activation by interaction between PD-1 and CD28 point of convergence of the two pathways. ADCC, antibody-dependent cellular cytotoxicity; APC, antigen-presenting cell; CTLA-4, cytotoxic T lymphocyte antigen-4; FcγRs, Fc gamma receptors; TCR, Tcell receptor; MHC, major histocompatibility complex; NK cells, Natural Killer cells; PD-1, programmed death-1; PD-L1, programmed death-1 ligand; PI3K/Akt, phosphatidylinositol 3-kinase (PI3K) and Akt/Protein Kinase B; SHP-2, phosphatase Src homology region-2 containing protein tyrosine phosphatase; Treg, regulatory T cells.

resident Tregs highly express CTLA-4. Murine tumor models, ex vivo studies and neoadjuvant clinical studies of Ipilimumab have identified a reduction in tumor infiltrating and circulating Tregs after therapy [27,28]. Ipilimumab induced Treg depletion is thought to be achieved by Fc binding to Fc γ receptors on atypical macrophages in the TME leading to antibody-dependent cell-mediated cytotoxicity (ADCC) (Fig. 1B). Evidence for this has been illustrated in mouse models and ex vivo culture studies [29–31]. An effector mechanism is also supported by the finding that germline presence of a high-affinity polymorphism of the Fc receptor CD16a/Fc γ RIIIa (CD16a-V158F) is linked with better responses to Ipilimumab suggesting that Fc-dependent cell removal partly confers the antitumor mechanism [32]. A recent clinical study opposes this by describing that anti-CTLA-4 does not deplete Tregs [33]. Quezada et al. [34] recently reviewed this

and identified that timing of biopsies and sampling bias are more difficult in the context of clinical studies as compared to mouse models, therefore, making interpretation challenging.

The PD-1 pathway

PD-1 is also part of the immunoglobulin superfamily known to contribute to immune homeostatic processes by delivering inhibitory signals upon binding with its ligands, programmed death ligands 1 and 2 (PD-L1/2). Like CTLA-4, PD-1 is thought to be a negative regulator of T cell function, regulating peripheral tolerance and T-cell responses.

PD-1 is expressed more broadly than CTLA-4 and can be found on T cells, B cells, NK cells, and a variety of peripheral tissues. The

Table 1. Comparisons of the cytotoxic T-lymphocyte antigen 4 (CTLA-4) and programmed death-1 (PD-1) signaling and immune pathways

	CTLA-4	PD-1
Ligands	CD80 and CD86	PD-L1 and PD-L2
Expression profile of immune checkpoint	Limited expression Naïve T cells after activation FoxP3+ regulatory T cells	Broad expression T cells, B cells, NK cells Peripheral tissues
Expression profile of ligands	Limited expression T cells and APCs in lymphoid organs Some expression by T cells and APCs in peripheral tissues Note not expressed by tumor cells	Broad expression (particularly PD-L1) T cells, B cells, dendritic cells, macrophages in peripheral tissues Tumor microenvironment including tumor cells and stromal elements
Stimulus for expression	TCR activation upregulates CTLA-4 expression	TCR activation upregulates PD-1 expression BCR activation upregulates PD-1 expression
Timeframe for expression	Expression within 1 h	Expression within 6–12 h
Downstream signaling	Signals via SHP-2 to inhibit PI3K Signals via PP2A to inhibit AKT	Signals via SHP-2 to inhibit ZAP-70 and PI3K
Proposed roles in human physiology of immune modulation	Acting primarily in secondary lymphoid organs for induction of peripheral tolerance	Acting primarily in peripheral tissues for long-term maintenance of peripheral tolerance
Modulation of T-cell responses with checkpoint blockade immunotherapy	Upregulation of CD4+ effector T cells Depletion of FOXP3+ regulatory T cells	Upregulation of activated CD8+ T cells

ligand PD-L1 is broadly expressed by immune cells including T cells, B cells, DCs, and macrophages, and in nonlymphoid tissues including on tumor cells or stromal elements in the TME [35]. The expression of PD-L2 is more restricted but it has also been demonstrated on a range of tumors including melanoma [36]. Expression of PD-1 is upregulated on activation of T cells and B cells [37]. Inflammatory cytokines, such as IFN- γ , are thought to induce expression of PD-L1, and to a lesser degree expression of PD-L2 [38,39]. Therefore, PD-1-mediated modulation of T-cell function is inducible upon IFN- γ production typically in the context of cytolytic and effector T-cell activities.

After PD-1 engages with its ligands, a negative signal is transmitted via the tyrosine phosphatase SHP2 to halt T-cell activity (Fig. 1A). Enlisting SHP2 directly downregulates TCR signaling through dephosphorylation of proximal signaling components ZAP-70 and PI3K [40].

Physiologically, due to its negative costimulatory effects PD-1 is needed for achievement of tolerance in peripheral tissues as supported by the autoimmune anomalies that develop upon genetic deletion of *Pdcd1* (that encodes PD-1) including a lupus-like syndrome and autoimmune dilated cardiomyopathy [12,41]. PD-1 expression is linked with a “exhausted” T-cell phenotype in the context of prolonged antigen exposure. This occurs in the setting of cancer or chronic viral infection whereby prolonged T cell stimulation results in a gradual decrease in the effector function of CD8+ cells, an outcome referred to as exhaustion. It should be noted that although PD-1 is a marker of exhaustion, its expression alone does not define an exhausted T cell phenotype (typically PD-1+LAG-3+TIM3+). PD-1 is a marker for T cell activation, an exhausted T cell phenotype is a subset of this, and these exhausted cells still retain some effector activity [17].

PD-1 expression takes longer to achieve than CTLA-4 (6–12 h for PD-1 as opposed to 1 h for CTLA-4 [14,42]) (Table 1). The diverging expression patterns of CD80/86 and PD-L1/PD-L2 are also pivotal distinctions between CTLA-4 and PD-1, and lead to the theory that CTLA-4 functions early for induction of tolerance and PD-1 function is delayed to achieve sustained maintenance of tolerance [43]. Intracellularly, both PD-1 and CTLA-4 signal via SHP2 and converge to act to inhibit downstream PI3K signaling. Another point of convergence is that CD28, for which CTLA-4 is a homolog, is also a secondary target for PD-1-mediated desphosphorylation leading to further inhibition of costimulation [44].

Anti-PD-1 induced tumor rejection in melanoma

Anti-PD-1 antibodies Nivolumab (a fully human, monoclonal, anti-PD-1 IgG4 antibody), and Pembrolizumab (a humanized, engineered, monoclonal, anti-PD-1 IgG4 antibody) were licensed in advanced melanoma by the FDA in 2014 and by the European Medicines Agency (EMA) in 2015 [45]. PD-1 is considered to halt cell activity in the effector phase in tissues and tumors, this is in contrast with the role of CTLA-4 which is thought to primarily modulate immune functions in the early phase of T cell activation. Preclinical and clinical models suggest that the primary mechanism of action of anti-PD-1 therapy is to reduce the number of phenotypically “exhausted” CD8+ cytotoxic cells [46,47].

In mouse melanoma models [48], tumor growth was halted in PD-1 KO mice or with anti-PD-1 antibodies. PD-L1 expression is often upregulated in tumors and may be prognostic in melanoma [49,50]. However, PD-L1 positivity is not a prerequisite to successful anti-PD-1 therapy. In addition to directly modulating T cell activation, expression of PD-L1 on macrophages has also been

linked with increased eviction of T cells from the TME, suggesting a role for the PD-1/PD-L1 axis in influencing T cell migration. T cells positive for PD-1 are also considered more likely to be tumor antigen-specific compared with T cells halted in the priming stage by CTLA-4. Therefore, monoclonal antibodies able to interfere with the T cell immunoinhibitory functions of PD-1 would in theory be more likely to activate tumor antigen-specific T cell responses in cancer patients [17].

In melanoma, anti-PD-1 therapy is believed to induce tumor rejection predominantly by reactivating CD8⁺ T cells previously in a “exhausted” state. Sequential blood samples of patients on anti-PD-1 therapy demonstrate increase of PD-1⁺ CD8⁺ T cells [47]. Wei et al. [24] also describe expansion of an “exhausted-like” CD8⁺ phenotype in murine melanoma models in response to anti-PD-1 therapy and this negatively correlated with tumor growth. It appears that an anti-PD-1 treatment response is dependent on a T cell response within the TME, with subsequent PD-L1 expression on tumor cells as a consequence of IFN- γ secretion upon T-cell activation.

This is a rapidly evolving field and several other inhibitory checkpoint molecules are being investigated, including T cell immunoglobulin 3 (TIM3), lymphocyte activation gene 3 (LAG3), T cell immunoglobulin and ITIM domain TIGIT, and programmed death-1 homologue (PD-1H, also known as VISTA). A number of clinical and preclinical studies are currently investigating targeting these checkpoint molecules either alone or in combination with existing therapies [31,51,52].

Checkpoint inhibitors in the clinic: From monotherapy to combination therapy

The anti-CTLA-4 antibody Ipilimumab together with anti-PD-1 agents Nivolumab and Pembrolizumab are licensed for use as monotherapy for the treatment of advanced metastatic melanoma, and more recently as adjuvant therapy in resected disease. Combination therapy with Ipilimumab and Nivolumab is licensed for metastatic disease. Nivolumab and Pembrolizumab have shown superior long-term efficacy and safety profiles versus the anti-CTLA-4 agent Ipilimumab in head-to-head phase 3 trials in advanced melanoma [53,54]. The key evidence for the clinical use of combination CPI therapy comes from the landmark CheckMate 067 clinical trial (ClinicalTrials.gov NCT01844505). In 945 patients with stage III or IV melanoma, this study directly evaluated combination therapy with Nivolumab plus Ipilimumab in comparison with monotherapy with Nivolumab or with Ipilimumab. The median overall survival (OS) was greater than 60 months, at a minimum follow-up time of 60 months (median not yet achieved), for the Nivolumab-plus-Ipilimumab cohort, as compared with 36.9 months for the Nivolumab cohort and 19.9 months for the Ipilimumab cohort. The 5-year OS rates were 52, 44, and 26%, respectively. Although OS appears improved with combination therapy, the trial was not powered to show a statistical difference between the two Nivolumab containing groups (the HR) for Nivolumab-plus-Ipilimumab versus single agent

Nivolumab is not significant (HR 0.83 95% CI, 0.67 to 1.03)). The incidence of adverse events was increased in combination therapy in this trial, and highlights challenges in predicting patient response [53].

Combination therapy of Ipilimumab and Pembrolizumab is not currently licensed in clinical practice. An open label, phase 1b trial (KEYNOTE-029) has demonstrated feasibility of this combination therapy in advanced melanoma and further studies are pending [55].

Potential mechanisms and merits of anti-PD-1 and anti-CTLA-4 checkpoint blockade antibody combination treatment

CTLA-4 and PD-1 are coinhibitory molecules, however, evidence indicates that they function to inhibit T cell activation via distinct nonredundant mechanisms, potentially functioning at separate locations and time points in T cell evolution (Fig. 2A-C). Combination anti-CTLA-4 and anti-PD-1 therapy may confer enhanced clinical outcomes as compared to monotherapy. However, we do not know if combination therapies operate in a complementary fashion and several studies have explored whether these molecules may even function in a synergistic manner (Fig. 2A-D).

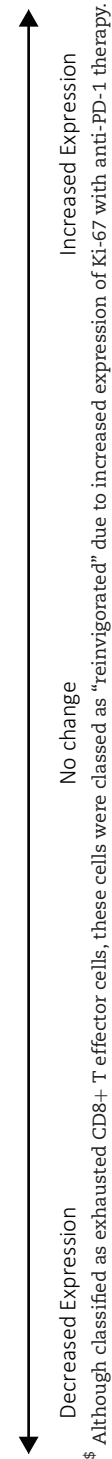
As well as distinct cellular mechanisms underlying the CTLA-4/PD-1 pathways, the expression profiles of CTLA-4/PD-1 and their ligands implies that they can act at distinct times in T cell evolution, that is, at the secondary lymphoid organs (CTLA-4) versus in the TME (PD-1). This is supported by the differential timeframes over which CTLA-4 and PD-1 are expressed (6–12 h for PD-1 in contrast with 1 h for CTLA-4 upon T cell activation [14,42]). However, we know that there is a degree of convergence with regards to the cell-intrinsic mechanisms of these pathways: both pathways converge on the PI3K/AKT pathway (Fig. 2D) and PD-1 inhibits CD28 for which CTLA-4 is a competitive homolog, leading to overlap and enhancement of T cell activity. The underlying mechanisms of combination therapy have been studied in both animal models and in the clinical setting. As yet there is no clear consensus as to the immunological signatures and their benefits seen in the setting of combination CPI, however, future studies can build on existing insights on emerging immune signatures associated with response (findings from animal models and patient studies summarized in Table 2).

Preclinical findings

Several murine models demonstrate evidence of synergistic effects of combination therapy, particularly focusing on the function of T cells in the TME. An *in vivo* rodent model has shown combination therapy to be associated with increased numbers of IL-2 secreting and proliferating CD8⁺ T cells in the TME. Interestingly, the results of this investigation suggest that this is a local response and not via of T-cell recruitment from the periphery. These changes are correlated with tumour regression [56].

Table 2. Evidence for potential immunological changes reported with anti-PD-1 or anti-CTLA-4 antibody monotherapy versus combination therapy of anti-PD-1 and anti-CTLA-4 in direct comparison studies

	CD4 ⁺ effector T cells		CD8 ⁺ effector T cells		Regulatory T cells		B cells	
	All CD4 ⁺ effector T cells (CD4 ⁺ FoxP3 ⁻)	Th1-like (PD1 ⁺ ICOS ^{int} TBET ⁺)	All CD8 ⁺ T cells	Exhausted (PD1 ^{high} Lag3 ⁺ Tim3 ⁺ (57), murine studies, TBET ⁺ EOMES ⁺ in human)	Activated (PD1 ⁺ Lag3 ^{int} Tim3 ^{int} in murine studies, TBET ⁺ EOMES ⁺ in human)	All T _{regs} (CD4 ⁺ FoxP3 ⁻)	All CD19 ⁺ B cells	CD21 ^{low} CD38 ^{hi}
Anti-CTLA-4 monotherapy	Preclinical murine (58)	↑	↑			↓		
	Tumor model (Wei et al. (57))		↑			↓		
	Clinical PBMCs (Spranger et al. (56))	↑				=		
	Clinical PBMCs (Wei et al. (55))	↑				=		
Anti-PD-1 monotherapy	Preclinical murine (58)	=	↑	↑	=	↓	=	=
	Tumor model (Curran et al. (58))			↑		=		
	Clinical PBMCs (Wei et al. (57))		=		↑	↓		
	Clinical PBMCs (Spranger et al. (56))					=		
Anti-CTLA-4 & anti-PD-1 combination	Preclinical murine (58)	=	=	↑	↑	=	=	=
	Tumor model (Curran et al. (58))	↑	↑↑	↑↑		↓		
	Clinical PBMCs (Wei et al. (57))		↑↑	↑		↓↓		
	Clinical PBMCs (Spranger et al. (56))			↑		=		
Consensus		=/↑	↑	=	↑	↓	↑	↑



Decreased Expression No change Increased Expression
 \$ Although classified as exhausted CD8⁺ T effector cells, these cells were classed as “reinvigorated” due to increased expression of Ki-67 with anti-PD-1 therapy.

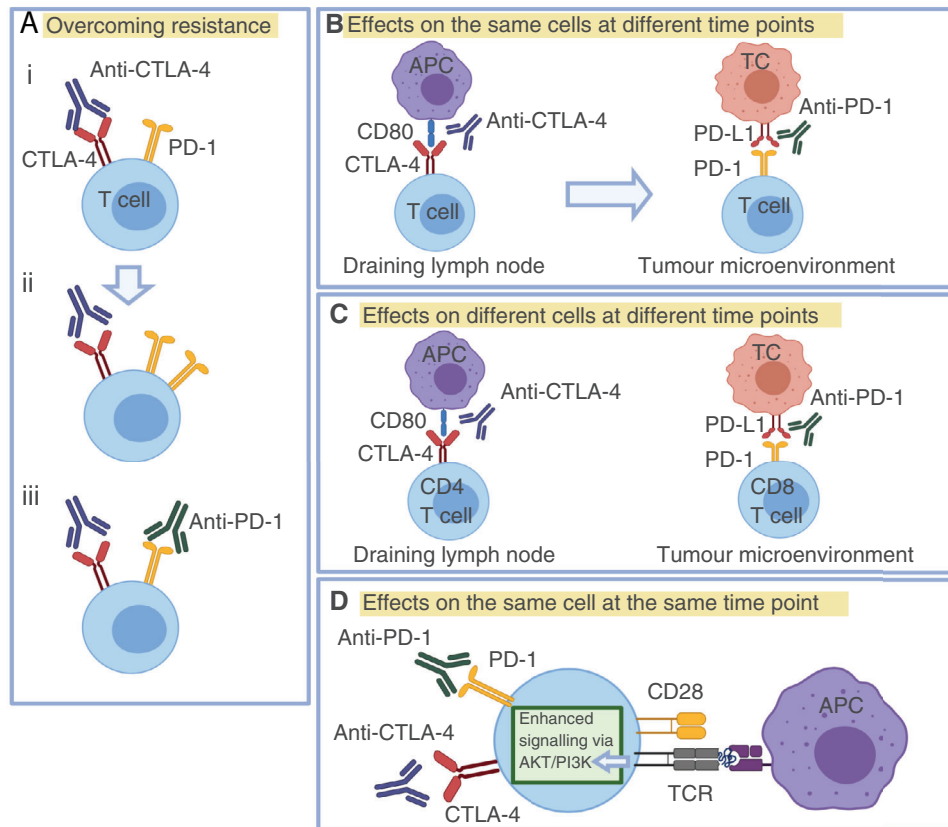


Figure 2. Potential effects of anti-CTLA-4 and anti-PD-1 combination therapy on T cells. (A) Overcoming CTLA-4 blockade-associated upregulation of PD-1: (i–ii) Anti-CTLA-4 monotherapy may upregulate PD-1 expression. This escape mechanism which allows PD-1 signaling can limit further expansion of T-cell. (iii) Simultaneous blockade of anti-CTLA-4 and anti-PD-1 overcomes this and limits PD-1 expressing T cells and exhausted T phenotypes. (B) Anti-CTLA-4 and anti-PD-1 may act on the same T cell at different time points, initially during the priming phase in the lymph node (anti-CTLA-4) and subsequently in the tumor microenvironment (anti-PD-1) to illicit an effector response. Potentially this could allow sustained costimulation over a longer timeframe. (C) Anti-CTLA-4 and anti-PD-1 may act on different T cell populations at different anatomical locations; for example, anti-CTLA-4 may enhance CD4⁺ T cell populations in the draining lymph while anti-PD-1 may enhance tumor-infiltrating CD8⁺ T cells. (D) Anti-CTLA-4 and anti-PD-1 may act simultaneously on the same T cell either at the site of priming (lymph node) or in the tumor microenvironment; this may enhance costimulation beyond that achieved with monotherapy, thus, further promoting T cell activation. Since CTLA-4 and PD-1 intracellular signaling converges on the PI3K/AKT pathways, this response may be amplified with simultaneous dual checkpoint blockade. APC, antigen-presenting cell; CTLA-4, cytotoxic T lymphocyte antigen-4; PD-1, programmed death-1; PD-L1, programmed death-1 ligand; PI3K/Akt, phosphatidylinositol 3-kinase (PI3K) and Akt/Protein Kinase B; TC, tumor cell.

Similarly, in a syngeneic murine C57BL/6 model of MC38 colon adenocarcinoma, known to be immunoresponsive to checkpoint treatment, Wei et al. [57] phenotyped tumor-derived T cells by mass cytometry in the setting of both monotherapy (anti-CTLA-4 or anti-PD-1) and combination CPI. They showed that combination therapy to be the superior treatment, accompanied by a distinct T-cell phenotype. Combination CPI considerably increased the frequency of activated effector CD8⁺ cells (PD-1⁺ Lag3^{int} Tim3⁺), simultaneously decreasing the frequency of exhausted effector CD8⁺ cells (PD-1^{high} Lag3⁺ Tim3⁺). Anti-PD-1 monotherapy, however, had the reverse effect: it led to increase of exhausted CD8⁺ T cells, with no impact on activated CD8⁺ T cells. These data show that combined immunotherapy may prevent or reverse exhaustion of CD8⁺ cells. Of note, it was suggested that there is no difference in the proliferation of phenotypically exhausted CD8⁺ T cells between treatment groups (as assessed by short-term iodo-deoxyuridine (IdU) incorporation). This may

mean that the observed decrease in frequency of exhausted T cells in response to combination therapy is not due to an altered proliferation of these cells. The CD4⁺ effector T-cell population was also positively modulated by combination therapy in this study. T cells of a Th1-like CD4⁺ effector phenotype expanded in frequency following anti-CTLA-4 monotherapy but not anti-PD-1 monotherapy. Contrastingly, combination therapy caused a further increase in CD4⁺ effector T-cell frequency compared with anti-CTLA-4 monotherapy. This additive increase of the Th1-like CD4⁺ effector cell compartment in the context of combination therapy suggests a potential interdependence of the two pathways. One mechanistic hypothesis is that anti-CTLA-4 monotherapy may upregulate PD-1 expression limiting further expansion of CD4⁺ cells (Fig. 2A). Modulation of the CD4⁺ T cells by anti-PD-1 therapy would therefore be dependent on initial enlisting of anti-CTLA-4. Combination therapy also led to decreased Treg frequency, beyond the suppression achieved by each monotherapy.

Combination blockade also synergistically increased T cell effector functions in a B16 murine model of melanoma [58]. Single checkpoint inhibition using anti-CTLA-4 or anti-PD-1 was accompanied by increased effector CD8⁺ T-cell infiltration in the TME. However, this expansion was limited due to a compensatory increase of CTLA-4 and PD-1 expression. By contrast, simultaneous inhibition of both checkpoint pathways allowed effector T cells to continue to proliferate in the TME and led to preferential upregulation of effector T cells compared with inflammation-promoting Tregs. The same study also demonstrated increased synthesis of proinflammatory cytokines IFN- γ and TNF- α from effector CD8⁺ T cells within tumors in the context of combination therapy [58]. Their findings suggest that combination therapy may be twice as effective as monotherapy in achieving rejection of B16 melanoma and the authors associate this with expansion of the effector T cell compartment.

Insights from treating patients with CPI

The effects of combined anti-CTLA-4 plus anti-PD-1 treatments have been studied in peripheral blood samples from melanoma patients, specifically analyzing T cell populations in samples from patients managed with monotherapy Ipilimumab, Nivolumab, or Pembrolizumab, or combination of Ipilimumab and Nivolumab. Combined immunotherapy enabled an expansion of circulating PD-1⁺ and PD-1⁻ CD8⁺T cells expressing the proliferation and cell cycling marker Ki-67, compared with samples from individuals that were treated with anti-PD-1 or anti-CTLA-4 monotherapies, where Ki-67 was detected only in the activated PD-1⁺ CD8⁺ T cell subsets. As a result, CD8⁺ T-cell frequency was increased in the cohort who received combined immunotherapy compared to a group that received anti-PD-1 mAb alone. Terminally differentiated metacluster 1 CD8⁺ T-cell (TBET⁺ EOMES⁺ CD8) levels in the blood were significantly higher following combined immunotherapy as opposed to anti-CTLA- or anti-PD-1 therapies alone [57].

Combination anti-CTLA-4 plus anti-PD-1 therapy also gives rise to unique transcriptional effects compared with monotherapy. Das et al. analyzed blood from 45 patients undergoing monotherapy with anti-PD-1 or anti-CTLA-4 versus combination CPI. They initially used a genome wide strategy to analyze gene expression profiles in peripheral T cells and monocytes before and 3 weeks following either monotherapy (with Ipilimumab or Nivolumab) or combination (Ipilimumab and Nivolumab). They reported that changes in peripheral T cells were more marked compared with changes in monocytes [59]. Combination blockade also led to nonoverlapping changes in gene expression compared with monotherapy. For example, only combination therapy upregulated gene expression of IL-8 and HLA-DR. Anti-CTLA-4 and combination therapy (but not anti-PD-1) both induced Ki-67, a marker of proliferation. Mass cytometry of peripheral blood T cells identified that Ki-67⁺ T cells in the setting of anti-CTLA-4 and combination therapy have a transitional cell memory phenotype (CD45RO⁺, CCR7⁺CD27⁺CD28⁺CD95⁺). This is in line with preclinical studies demonstrating CTLA-4-mediated

reduction of proliferation and increased memory after CTLA-4 inhibition in mice [5,60]. Analysis of differentially expressed coding transcripts showed that the main pathway expressed in the context of anti-CTLA-4 therapy and combination therapy was cell cycle/proliferation. The upregulation of these pathways was more marked in the setting of combination therapy. In contrast to anti-CTLA-4 or the combination therapy, genes modified by anti-PD-1 therapy did not form a proliferation signature and instead showed enrichment for genes involved in cytolytic activities and control of effector T and NK cell function. This indicates that each monotherapy and combination therapies lead to differential outcomes in modifying human T cells in vivo [59].

As well as distinct changes in circulating T cells, Ipilimumab, Nivolumab, and combination therapy may also cause disparate changes in systemic cytokine levels. Specifically, soluble IL-2R is upregulated after combination therapy. Furthermore, IL-1 α levels increased after anti-PD-1 and combination therapy with anti-CTLA-4 and anti-PD-1. Levels of CXCL10, an important immune cell chemoattractant that can exert angiostatic and immune cell-activating effects in the TME, were enhanced after anti-PD-1, anti-CTLA-4, and combination treatment [59]. These findings illustrate the distinct changes of peripheral blood cytokine levels seen with each type of immune checkpoint therapy.

Although resistance to CPI monotherapy is not fully elucidated, it is clear that the enhanced efficacy observed with combination may be through overcoming some key resistance mechanisms. For example, a compensatory increase T-cell associated checkpoints has been demonstrated with CPI monotherapy [58]. In line with this, PD-L1 expression on peripheral blood CD4⁺ and CD8⁺ T cells may predict resistance to anti-CTLA-4 therapy, suggesting a need for combination therapy [61]. PD-L1 expression in tumors has been studied as a possible biomarker for patient selection for CPI. Within the CHECKMATE 067 study, PD-L1 is shown to act as an imperfect biomarker, with receiver operating characteristics (ROC) curve analyses demonstrating that PD-L1 enriches only marginally the prediction compared to random assignment, arguing for limited utility in this setting [62,63]. However, within the same study, an underpowered subgroup analysis revealed that the progression-free survival (PFS) of the PD-L1 positive population treated with Nivolumab monotherapy is the same as the PFS for that of combination therapy with Nivolumab and Ipilimumab at 14 months. This suggested that there may be utility in assessing PD-L1 positivity as a biomarker for patient stratification and may identify a subgroup of patients that may not gain added merit from combination CPI.

Levels of MHC class I proteins expressed in melanoma may confer different sensitivities to anti-CTLA-4 and anti-PD-1 blockade, with the downregulation of MHC class I being a mechanism of primary resistance to anti-CTLA-4 blockade, but not to anti-PD-1 blockade, associated with progressive disease and a lack of clinical response [64]. This association was not found shown to effect responses in combination therapy, further bolstering the idea that different mechanisms of action in these agents act complementary to generate better antitumor immune responses. Identifying patients with low levels of MHC class I molecules on

biopsy at presentation may act as a selector for patients who may be able to benefit from the durable responses of combination therapy, thereby, minimizing the risk of disease progression associated with monotherapy.

While CPIs are designed to upregulate T cell effector functions, there is increasing attention on the contribution of B-cell responses to patient outcomes and the development of immune-related adverse events (irAE). CPI may modulate the B cell phenotype in the periphery and the melanoma TME; however, correlation with clinical response is variable. One study has demonstrated a distinct B cell phenotype in the context of combination therapy compared with monotherapy. It was reported that in 23 patients after one cycle of combination therapy with Ipilimumab and Nivolumab, there was an overall decline in the absolute numbers of circulating CD19⁺ B cells but increased plasmablast and CD21^{low} B cell subset counts [65]. This finding was not observed in 16 patients who received monotherapy with either Ipilimumab or Nivolumab. PD-1 expression was higher on the CD21^{low} B cells than on other B-cell populations, suggesting that these cells may be specifically modulated by anti-PD-1 therapy. This investigation did not demonstrate an association between changes in B cell response in combination therapy and clinical response. However, the extrapolation of these findings may be limited due to the limited sample size and the fact that ontreatment peripheral blood samples were only taken at an early time point after commencement of CPI and may not therefore be representative of the full- or long-term response.

New data have recently come to light identifying a group of patients who not only fail to respond to immunotherapy but who also experience acceleration of disease progression in the context of checkpoint inhibition. This is referred to as “hyperprogression” and has been described in a range of tumors including select cases of melanoma. While not fully defined, hyperprogression is referred to by some studies as a >50% increase in tumor volume at first assessment after treatment as compared with baseline [66–68]. The vast majority of cases of hyperprogression received CPI monotherapy alone. However, there are reported cases of patients developing hyperprogressive disease following combination CPI with anti-CTLA-4 and anti-PD-1. The mechanisms underlying this are as yet poorly understood, but are likely to be distinct based on tumor type, modulation of TME by previous therapy and the patient’s own immune system. One mechanism hypothesized is that previous treatment with chemotherapy may select resistant cancer cell clones which are subsequently released to proliferate following CPI [68]. Modulation of immune subsets is also likely to play a role. Kamada et al. demonstrated an increase in intratumoral Tregs on treatment in patients with gastric cancer who developed hyperprogressive disease [69]. Further mechanistic insights are needed before it can be established whether combination CPI is protective or deleterious with regards to this relatively rare clinical phenomenon.

In summary, preclinical and clinical studies suggest that combination anti-PD-1 and anti-CTLA-4 therapy may lead to a distinct immune profile facilitating enhanced tumor rejection. This includes upregulation of effector T cell CD8⁺ and CD4⁺ pop-

ulations as well as downregulation of inhibitory T cell populations, namely CD8⁺ exhausted T cells and Tregs. Beyond this, combination CPI may lead to distinct cytokine and transcriptional profiles. The mechanisms of enhanced efficacy underlying this are not fully elucidated but likely relate to overcoming resistance mechanisms of compensatory increase in checkpoint molecules upon CPI monotherapy.

Challenges of combining anti-PD-1 and anti-CTLA-4 checkpoint blockade antibodies

Checkpoint inhibition can reduce normal immune self-tolerance and lead to the onset of irAEs mimicking autoimmunity. irAEs contrast with the classical immunosuppression of cytotoxic chemotherapy.

Grading the severity of irAEs is according to the Common Terminology Criteria for Adverse Events (CTCAE), where grade 1 is a mild event and grade 5 a fatal event [70]. Combining CPI not only improves efficacy in melanoma but also toxicity and this has limited the utility of combination therapy for selected patients. The CHECKMATE 067 trial reported rates of 59, 23, and 28% of patients suffering with Grade 3 or 4 treatment-related adverse events in the Nivolumab-plus-Ipilimumab, Nivolumab, and Ipilimumab groups, respectively in the CHECKMATE 067 trial [71]. It is not known if the mechanisms of irAE in the context of combination therapy are distinct from those in monotherapy.

irAEs frequently lead to an interruption or discontinuation, of the CPI therapy. In the CHECKMATE 067 study, 42% of patients on combination therapy discontinued their treatment due to irAEs, compared with 13% for Nivolumab and 15% for Ipilimumab monotherapy [71].

Ex vivo peripheral blood studies focused on autoimmunity have demonstrated that T cell exhaustion correlates with a state of low autoimmune disease activity [72]. CPI therapy is linked with a change in the phenotype of T cells from an exhausted (PD-1^{high} Lag3⁺ Tim3⁺) phenotype to an active effector phenotype (PD-1⁺ Lag3^{int} Tim3⁺) which can underly this generalized activation that can lead to irAEs [45,73,74]. Recent studies show modifications in circulating T cell repertoires in Ipilimumab-treated patients precede the onset of irAEs [75,76]. Further research has pointed to distinct B cell signatures. Combination Ipilimumab plus Nivolumab but not monotherapy has been associated with a decline in overall B cell numbers in the peripheral blood but a relative increase in CD21^{lo} and plasmablast B cell subsets. Changes in B cells following combination CPI correlated with frequency and severity of irAEs. Patients showing altered B-cell responses with combination treatments had an increased risk of multiorgan immunotoxicity and these modifications in B cells were observed around 3 weeks prior to the onset of toxicity [65].

Analysis of inflammatory markers may provide an important mechanism to monitor patients to identify those at risk of developing irAEs and give insight in to the mechanisms of irAE in combination therapy. For example, one group assessed 65 cytokines in 98 melanoma patients managed with combination

anti-PD-1 and anti-CTLA-4 therapy or single agent anti-PD-1. A combination of 11 cytokines at pretreatment sampling and early on treatment were associated with severe irAEs in the combination cohort. These cytokines were compiled into a single CYTOX score: IL-1 α , IL-2, IFN- α 2, G-CSF, GM-CSF, Fractalkine, FGF-2, IL-12p70, IL-1B, IL-1RA, IL-13. This CYTOX score was further validated in an independent group of melanoma patients managed with combination therapy [77,78]. The authors highlight that these cytokines have proinflammatory activities, including promoting immune cell recruitment, proliferation, survival, differentiation and effector functions, and many of these cytokines (i.e. IL-1 α , IL-1b, IL-2, IFN- α 2, and IL-12p70) have been implicated in autoimmune diseases. irAE continue to limit the utility of combination CPI and lead to high rates of treatment discontinuation. The identification of biomarkers to predict treatment outcome and irAE is highly desirable.

Predictive biomarkers are desirable in the context of variable patient outcomes in combination anti-PD-1 and anti-CTLA-4 therapy, but would be very useful in stratifying patients between monotherapy and combination immunotherapy [79]. Despite our improved understanding of the biology of melanoma, our ability to recognize particular molecular signatures, and the dramatic improvement of outcomes associated with targeted and immune therapies, there is a lack of a consensus on biomarker-directed treatment strategies, beyond the assessment of baseline BRAF mutational status [80,81]. In current European and American guidelines, testing for actionable mutations (BRAF, NRAS, and *c-Kit*) is recommended in patients with resectable or unresectable stage III and stage IV melanoma [62], and this can direct selection of small molecule inhibitors where the presence or absence of an oncogenic marker determines eligibility for such therapies.

In the context of immunotherapy, current biomarker strategies have emerged that examine the inflamed T-cell phenotype and tumor foreignness (tumor mutational burden and neoantigens) as approaches associated with clinical outcomes [82].

Direct assessment of the expression of the PD-1/PD-L1 on tumors would appear to be a logical choice as a predictive biomarker for treatment response for anti PD-L/PD-L1 therapies. In the setting of both melanoma and non-small cell lung cancer, improved PFS and OS has been demonstrated for PD-L1 positive cases compared to a PD-L1 negative subgroup in anti-PD-L1 monotherapy [83,84]. However, in the setting of melanoma, PD-L1 concentrations did not appear to be reliable in predicting treatment response to Nivolumab, or in the setting of combination therapy with Nivolumab and Ipilimumab [53,85]. The variability in the evidence related to PD-L1 expression to predict immune response may lie in the lack of standardization across studies in determining a significant PD-L1 threshold [71,79]. Furthermore, PD-L1 is not a static biomarker, and its expression appears dynamic, likely reflecting the complex signaling interactions between tumor and immune cells during treatment. Therefore, expression may vary with timing of the biopsy [86].

Tumors with a large number of somatic mutations are linked with improved outcomes among patients treated with anti-CTLA-

4 or anti-PD-1 monotherapy [87]. This may be due to an increased number of neopeptide antigens presented in the context of MHC class I presentation, thereby, stimulating an antitumor response. Further studies are required to assess this in the context of combination therapy. Other genetic markers that result in an increased tumor mutational burden, including MicroSatellite Instability High (MSI-H) and Mismatch Repair deficiency (dMMR) (MSI/dMMR), have been demonstrated to hold predictive value in the response to immunotherapy [88,89].

Clinical parameters, including tumor staging and patient performance status, remain important for assessing patients for CPI treatment and also for selecting patients for combination therapy over monotherapy. Patients with asymptomatic brain metastases treated with Nivolumab and Ipilimumab combination therapy demonstrated higher intracranial tumor regression, leading to increased PFS with combination than with monotherapy [90,91]. Furthermore, studies have demonstrated an PFS/OS HR of 0.69/0.73 favoring combination therapy in individuals with an LDH above >2x the upper limit of normal [63]. The majority of research focused on biomarkers is primarily within the setting of monotherapy rather than combination therapy. However, it is increasingly apparent that in order to appreciate the dynamic and multiple interactions that occur within the TME, composite rather singular biomarkers are desirable [92].

Conclusion

The therapeutic landscape for advanced melanoma has been revolutionized by CPIs with enhanced median and long-term survival compared to typical cytotoxic chemotherapy [93]. Combination anti-CTLA-4 and anti-PD-1 therapy offers potential for superior efficacy and this may be attributed to each agent functioning in a complementary or perhaps even synergistic manner. It is evident that combination CPI can lead to a distinct immunological profile, modifying T and B cell populations with distinct cytokine and transcriptional effects. Further mechanistic studies are needed to enable us to predict patient responses and toxicity.

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Abbreviations: ADCC: antibody-dependent cell-mediated cytotoxicity · CPI: checkpoint inhibition · HR: hazard ratio · irAE: immune-related adverse events · LAG3: lymphocyte activation gene 3 · OS: overall survival · PFS: progression-free survival · TIM3: T-cell immunoglobulin 3 · Treg: regulatory T-cell

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