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Tumour Review

Resistance to PD1/PDL1 checkpoint inhibition

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**Title**: Resistance to PD1/PDL1 checkpoint inhibition.

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Abstract: For the first time in decades, patients with difficult-to-treat cancers such as melanoma are being offered a glimpse of hope in the form of immunotherapies. By targeting factors that foster the development and maintenance of an immunosuppressive microenvironment within tumors, these therapies release the breaks on the host’s own immune system; allowing cure of disease. Indeed, phase III clinical trials have revealed that therapies such as ipilimumab and pembrolizumab which target the CTLA4 and PD-1 immune checkpoints, respectively, have raised the three-year survival of patients with melanoma to ~70%, and overall survival (>5 years) to ~30%. Despite this unprecedented efficacy, many patients fail to respond, and more concerning, some patients who demonstrate encouraging initial responses to immunotherapy, can acquire resistance over time. There is now an urgent need to identify mechanisms of resistance, to predict outcome and to identify targets for combination therapy. Here, with the aim of guiding future combination trials that target specific resistance mechanisms to immunotherapies, we have summarised and discussed the current understanding of mechanisms promoting resistance to anti-PD1/PDL1 therapies, and how combination strategies which target these pathways might yield better outcomes for patients.

Key words:

• Immunotherapy, Resistance, anti-PD1, Combination Therapies, Checkpoint
Introduction

Cancer immunotherapies that target the immunosuppressive checkpoint receptors cytotoxic T-lymphocyte-associated protein 4 (CTLA4) or programmed death 1 (PD1) and its ligand, programmed death 1 ligand (PDL1) have changed the landscape of anti-cancer immunotherapy [1]. In particular, checkpoint inhibitors targeting PD1 and PDL1 have demonstrated unprecedented clinical efficacy in more than 15 cancer types including melanoma, non-small cell lung cancer (NSCLC), renal cell carcinoma (RCC), bladder carcinoma and Hodgkin’s lymphoma [2]. This success prompted FDA approval of two anti-PD1 monoclonal antibody (mAb) therapies, nivolumab (Opdivo®) and pembrolizumab (Keytruda®) for the treatment of NSCLC, metastatic melanoma, and renal cell carcinoma, or NSCLC, and advanced melanoma, respectively. Nevertheless, primary resistance to anti-PD1 therapies is common, affecting up to 60% of patients in some cancer types [3]. Furthermore, it is now becoming apparent that encouraging initial responses observed amongst some patients can be undone by their development of acquired resistance to anti-PD1 therapies (referred to throughout as acquired resistance) leading to disease relapse [4]. A substantial effort is currently underway to fully elucidate the mechanisms by which anti-PD1/PDL1 therapies exert their efficacy, to understand mechanisms of resistance present within some patients that limit their activity, and to develop a priori combination therapeutic approaches to sensitise resistant patients.

The mechanisms of action and clinical efficacy of anti-PD1 therapies have been reviewed extensively elsewhere [5-7]. By contrast, this review will discuss the current understanding of cellular and molecular pathways that contribute to the development of anti-PD1/PDL1 resistance. This will be done by briefly discussing the current understanding of tumor-induced immune suppression, the effect that anti-PD1 therapy can have upon anti-tumor immune responses, and the particular phenotypes and pathways that have been identified to contribute to resistance. Finally, the potential efficacy of specific combination therapeutic approaches for improving sensitivity to anti-PD1/PDL1 therapies will be discussed.
PD1 within the tumor microenvironment

It is now appreciated that tumors usually contain specialized immunosuppressive microenvironments that enable disease development and progression. These tumor microenvironments are dynamic and regulated by complex interactions between tumor tissue and tumor-associated immune and non-immune cells [8]. In recent years, a number of immunoregulatory pathways have been shown to be essential for maintaining immune suppression and preventing the release of host-generated anti-tumor immune responses. These pathways, known as immune checkpoints, which function under physiological conditions to modulate the duration and amplitude of immune responses, are often hijacked by tumors [9]. The checkpoint regulated by interactions between the PD1 receptor and its primary ligand, PDL1, appears to be of extreme importance within a variety of tumors. Under physiological conditions, the engagement of PD1 expressed by activated CD8+ T-cells with PDL1 functions to limit collateral damage. Following engagement, PD1 transmits inhibitory signals that abrogate T-cell receptor (TCR)-mediated activating signals; preventing further antigen-mediated T-cell activation. Not only does this pathway limit T-cell responsiveness, it can also lead to the development of an exhausted T-cell phenotype; characterized by a hierarchical loss of proliferation and cytolytic activity, followed by defects in cytokine production, and eventually deletion [10].

Many tumors express PD1 ligands which can engage with PD1 expressed by tumor antigen-specific T-cells patrolling the tumor microenvironment. Within a minority of tumors, PDL1 has been reported to be expressed in a constitutive fashion driven by oncogenic (e.g. EGFR and ALK, within NSCLC) or tumor suppressor mutations (PTEN within some glioblastomas) [11-13]. Alternatively, most tumors express PDL1 in a reactive and adaptive fashion in response to inflammatory cytokines such as type I and II interferons (IFNs, primarily IFNγ) and tumor necrosis factor α (TNFα) to prevent immune-mediated assault; a phenomenon now defined as adaptive immune resistance [6, 9]. Of note, some human liver and ovarian tumors have been shown to up-regulate the alternative PD1 ligand, programmed death ligand 2 (PDL2), however, these comprise a
minority of cases, and PDL1 appears to be the dominant PD1 ligand within human cancers [14]. Expression of PD1 by effector lymphocytes, and PDL1 by tumor cells and tumor-associated immune cells, can be indicative of an ongoing immune response. Following the manifestation of clinically apparent disease, however, the steady-state function of tumor-infiltrating lymphocytes (TILs) is clearly insufficient to control disease progression [15]. Therapeutic mAbs targeting either PD1 or PDL1 block ligand/receptor interactions to release T-cells from their exhausted phenotype, and allow for reinvigoration of tumor antigen-specific immunity [9].

Clinical data for anti-PD1 therapy

As previously mentioned, while highly effective, anti-PD1/PDL1 therapies engender little to no benefit for many patients, and patients can be stratified as being sensitive, or as displaying either primary or acquired resistance (Figure 1) [16, 17]. For example, the posterchild for success in anti-PD1 therapy, and also the example for which clinical data are most mature, is melanoma. Treatment with anti-PD1 immunotherapy and BRAF-molecularly targeted therapy has revolutionised the treatment of advanced-stage disease (unresectable stage III or stage IV); the 1 year overall survival (OS) for such patients was 25-30% in 2009 [18], and exceeds 70% for patients treated with single agent anti-PD1 therapies or targeted therapies [19-23]. Phase 3 trials of either of the PD1 inhibitors nivolumab or pembrolizumab, demonstrated a significant improvement in outcomes for patients with advanced melanoma compared with ipilimumab.

In the phase 3 study Keynote 006 [20], the response rate for two different dosing schedules of pembrolizumab was 37% (2-weekly pembrolizumab) and 36% (3-weekly pembrolizumab, the currently approved dosing interval), whereas the response rate for ipilimumab was 13%. Moreover, the 1- and 2-year PFS was 38% and 28% for the pembrolizumab cohort treated every 3 weeks, and 19% and 14% for ipilimumab (HR=0.61, p<0.00001). Pembrolizumab also significantly improved the OS, with a 1- and 2-year OS of 68% and 55% (3 weekly cohort) compared with 59% and 43% for ipilimumab (HR=0.68, p<0.001), despite subsequent anti-PD1/PDL1 based therapy in 31% of
all those who received ipilimumab. Similarly, in the three-arm phase 3 study Checkmate 067 [22, 23], the response rate for ipilimumab, nivolumab and the combination was 19%, 44%, and 58% respectively, and the 1-year progression-free survival was 18%, 42% and 49% (HR=0.55 [nivolumab versus ipilimumab], p<0.00001; HR=0.42 [nivolumab+ipilimumab vs ipilimumab], p<0.00001). Caution should be taken in the interpretation of such data as the latter trial was not powered to allow for comparisons between responses to nivolumab versus nivolumab in combination with ipilimumab.

Long-term survival data for these therapies are now emerging, and in the phase 1 study of pembrolizumab in 655 patients [24], the 3-year survival was 40% for the total population and 45% for patients who received pembrolizumab in the first line setting. The RECIST response in the overall population was 33%, yet only 57% of these responders remained in response at 3 years; the remainder developed acquired resistance. By contrast, 61 patients who had a CR ceased therapy, and with a median follow up of 10 months, 59 (98%) remained in CR [24] . Similarly, in a small phase 2 study of nivolumab combined with ipilimumab versus ipilimumab alone, the combination resulted in a 1- and 2-year OS of 73% and 64% respectively [25, 26] and in the 23 responders who ceased due to toxicity, 17 (74%) remained in response after a minimum follow up of two years.

Resistance to PD1/PDL1 checkpoint inhibition

When considering why some patients are resistant to anti-PD1/PDL1 therapies, it is likely that understanding their mechanisms of action is important. Sensitivity to anti-PD1 therapy requires the existence of tumor antigen-specific T-cells within tumor tissue, functionally suppressed by PD1/PDL1 interactions [27]. By limiting the interaction of PD1 with PDL1, anti-PD1/PDL1 therapies can promote reinvigoration of anti-tumor immunity [28]. As a consequence, such T-cells should be able to mount effector responses with the same vigour as newly-generated effector T-cells; as cytokine production, proliferative capacity, and memory development appear to be equally restored [29]. By implication, the mechanisms underlying the development of either primary or
acquired resistance to anti-PD1/PDL1 therapies must function to counteract the activity of tumor-specific T-cells prior to, or in response to therapy. To that end, we propose that mechanisms promoting either primary or acquired resistance are largely conserved, and that they must affect either, (i) tumor immunogenicity, (ii) antigen presentation and generation of effector T-cells, (iii) the encounter of antigen and PDL1 by tumor-specific T-cells, (iv) the activity and efficacy of tumor-specific immune responses, (v) or the induction of immunological memory (Figure 2). The remainder of this review will explore how these pathways can be disrupted, by drawing upon recent data derived from both pre-clinical mouse models and clinical trials, which have elucidated some mechanisms of resistance to anti-PD1/PDL1 therapies.

**Tumor immunogenicity**

The efficacy of anti-PD1 therapy is dependent upon the existence of tumor antigen-specific T-cells within tumor tissue. This requires that tumors express antigens that differentiate themselves from their non-transformed counterparts. Failing this, poor or absent anti-tumor immunity likely renders anti-PD1 therapy ineffective. Such antigens must theoretically be derived from non-mutated proteins to which T-cell tolerance is incomplete, viral antigens where malignant transformation is promoted by viral infection, or non-synonymous mutations that give rise to novel protein products known as neoantigens. The in-depth profiling of human cancer mutations by deep sequencing has enabled recognition that non-synonymous mutations which generate tumor-specific neoantigens are of extreme importance in directing tumor-specific immunity [30-33]. Importantly, the pool of mutations capable of producing immunogenic neoantigens is limited by their requirement to be non-synonymous and present within exomes, to be efficiently cross-presented by antigen presenting cells (APCs), and unaffected by the immunodominance of other antigens. This implies that poorly immunogenic tumors should be largely resistant to anti-PD1 therapy. This has been recently borne-out by the observation that tumor neoantigen burden strongly correlates with immunogenicity and with sensitivity to anti-PD1 therapy [34]. Human melanoma, RCC, and NSCLC are highly immunogenic, boasting mutational loads in the range of 5 to 10 somatic mutations per mega-base of
Accordingly, these are amongst the most sensitive of tumors to anti-PD1 therapy. By contrast, poorly immunogenic tumors such as those of the pancreas and prostate which tend to have between 0.1 and 1 somatic mutation per mega-base of DNA are largely resistant to anti-PD1 therapy [32, 35]. Additionally, neoantigen-enriched tumors have been shown to contain a greater abundance of CD8$^+$ T-cells and elevated levels of granzyme A (GZMA) and perforin (PRF1) mRNA; consistent with elevated T-cell-mediated cytolytic activity [36]. Although likely affected by a variety of tumor cell-intrinsic factors, the mutational burden, and in turn, the immunogenicity of individual tumors might be influenced during development by the speed at which immunosuppressive pathways are adopted. For example, during early stages of tumor development, a large proportion of neoantigens can be edited-out by a process of immunoediting [37]. The induction of PDL1 at an earlier rather than later stage of tumor development might lead to the outgrowth of tumors with greater immunogenicity. The timing of PDL1 induction within different tumors might be influenced by the particular tissue from which the tumor arises and the relevance of the PD1/PDL1 checkpoint therein. As such, the paucity of PDL1 expression within the prostate might explain a lack of its expression within tumors arising there and in turn, their common primary resistance to anti-PD1 therapy [35]. Amongst patients who initially respond to anti-PD1 therapy, the reinvigoration to anti-tumor immunity might lead to selection of tumor clones with the capacity to subvert T-cell responses. This could occur via a number of pathways including antigen loss, down-regulation of class I MHC via β2M mutation [38, 39], mutations that affect signalling pathway molecules within the JAK-STAT pathway, necessary for responsiveness to IFNs [40, 41], or even outgrowth of tumor cells which induce alternative immunosuppressive pathways. Therefore, mutational burden and immunogenicity may be relevant as either a primary or acquired resistance mechanism.
Antigen presentation and effector T-cell activation

The priming and activation of effector T-cells with specificity for tumor antigen is carried out by APCs such as dendritic cells (DCs). Constitutively patrolling the blood, peripheral tissues, lymph, and secondary lymphoid organs, DCs sample the extracellular environment and phagocytose self- and non-self-antigens [42]. In the presence of activating signals, DCs undergo maturation, which amongst other things, increases the efficiency of antigen processing, motility, and expression of costimulatory machinery necessary for T-cell activation. Maturation is, however, insufficient for T-cell activation; DCs must migrate to secondary lymphoid organs and present MHC to naïve T-cells [42]. Engagement between a DC and T-cell expressing cognate TCR, with appropriate costimulation can lead to T-cell activation [43]. Unsurprisingly, mechanisms have been identified that perturb antigen presentation and T-cell activation resulting in resistance to anti-PD1 therapy. Recently, Spranger et al. identified that mutations in human melanomas which increase the stability and signalling of β-catenin, can reduce expression of chemokine C-C motif ligand 4 (CCL4); important for DC migration [44]. Reduced CCL4 was associated with a general absence of TILs, culminating in resistance to anti-PD1 therapy. Other studies have also linked the inefficient infiltration of DCs into the tumor microenvironment with resistance to anti-PD1 therapy [44]. An recent study showed that mouse models lacking flora of the Bifidobacterium genus within their gut microbiome displayed a significant reduction of intratumoral DCs and extremely poor responses to anti-PD1 therapy [45]. Similar findings have been reported for sensitivity to anti-CTLA4 therapy [46]. Unfortunately, the mechanisms influencing DC infiltration and immune activation in response to the gut microbiome have not been clarified.

In addition to perturbing DC migration, some tumors have been demonstrated to disrupt DC maturation. The cytokine milieu present within some tumors has been demonstrated to affect DC maturation. Although commonly discussed in the context of cancer with respect to its pro-angiogenic properties, vascular endothelial growth factor (VEGF) can also limit DC maturation.
VEGF, a cytokine commonly produced by both tumor and associated immune cells, via stimulation of VEGF receptor 1 (VEGFR1), can suppress DC maturation via a nuclear factor kB (NFkB)-dependent pathway [47]. Similar defects have been noted to occur in the presence of elevated tumor-derived transforming growth factor β (TGFβ) [48]. Additionally, IL-10 produced by immunosuppressive cell types such as regulatory T-cells (Tregs) within some tumors has been shown to affect DC maturation by reducing expression of class II MHC and costimulatory machinery [49]. As well as affecting egress of DCs harbouring tumor antigen from tumor tissue, cytokines such as VEGF and IL-10 can affect antigen presentation and T-cell activation. While immature DCs may still present antigen, defective co-stimulation can render them tolerogenic [38]. Although speculative, tumor microenvironments in which VEGF, TGFβ or IL-10 are elevated, might also promote resistance to anti-PD1 therapies by preventing activation of tumor antigen-specific T-cells. Selection of tumor cells capable of subverting physiological antigen presentation and T-cell activation likely promotes their development by enhancing survival [50].

**PD1/PDL1 interactions**

T-cell activation requires the direct apposition of antigen-specific T-cells with cells displaying cognate antigen; a phenomenon that also allows for ligation of PD1 expressed by T-cells with PDL1 expressed within the tumor microenvironment [51]. By implication, tumor-intrinsic properties that prevent migration of tumor-specific T-cells or PD1 activity within tumor tissue, likely limit the efficacy of anti-PD1 therapy. The immune contexture, including the location, density, and type of cells within the tumor microenvironment has been assessed in a variety of cancer types and shown within melanoma to have relevance to the sensitivity of patients to anti-PD1 therapy [52-54]. Generally, the presence of a lymphocyte-enriched inflammatory infiltrate within colorectal, ovarian, pancreatic, oesophageal, and NSCLC tumors has been associated with better outcomes, and in some cases, response to anti-PD1 therapy [52]. In melanoma, where investigations have been most extensive, the presence of TILs within the invasive margin, but not
necessarily the tumor parenchyma was correlated with sensitivity to anti-PD1 therapy. Analyses of specific lymphocyte subsets present highlighted CD8+ but not necessarily CD4+ T-cells as being correlated with response [15, 53]. Unsurprisingly, the presence of suppressive cell types such as myeloid-derived suppressor cells (MDSCs) within tumor tissue in abundance, and higher ratios of Foxp3+ Tregs to CD8+ T-cells have been associated with resistance [55-57]. Further supporting the importance of lymphocyte subsets within tumor tissue, the presence of a cytotoxic gene signature associated with functional immune responses has been correlated with sensitivity [58]. The prognostic significance of these observations is clear, however, the mechanisms promoting differences in the infiltration of lymphocyte and myeloid cells between tumors is poorly understood.

One reason for poor immune infiltration might be the absence of tumor-specific T-cells; as previously discussed, tumor development would likely benefit from poor immunogenicity or suppression of effective antigen presentation [44]. However, some resistant tumors do contain TILs [59]. For example, in human melanomas, weak in situ expansion of exhausted, tumor-associated CD8+ T-cells has been shown to correlate with poor prognosis, even in patients with robust tumor invasion of lymphocytes [53]. In contrast, in human melanomas, co-localisation of PD1+ TILs with PDL1+ tumor cells has been strongly correlated with response to anti-PD1 therapy; supporting the currently accepted mechanism by which anti-PD1/PDL1 therapies exert their efficacy [15]. Interestingly, however, anti-PD1 therapeutic resistance mechanisms have been described that overcome the reinvigoration provided by anti-PD1 therapy to exhausted tumor-associated lymphocytes. While T-cell exhaustion is the phenomenon targeted by anti-PD1 therapy, it appears that when taken to extreme limits, it can promote resistance to anti-PD1 therapy (Figure 3).

**Severe exhaustion**

Chronic exposure to cognate antigen can trigger up-regulation of PD1 expression, and by interacting with its ligand, PD1 can induce T-cell exhaustion [10]. Importantly, in vitro techniques have revealed that the strength of PD1 signalling – determined by its relative expression level or
ligand abundance – can define the severity of T-cell exhaustion [60]. A consequence of this is that relative PD1 expression level likely affects the sensitivity of exhausted T-cells to anti-PD1 therapy. The presence of PD1$^{\text{high}}$, severely exhausted T-cells within some tumor microenvironments studied in mice, have been associated with resistance to anti-PD1 therapy. Unlike such populations, exhausted T-cells with either PD1$^{\text{low}}$ or PD1$^{\text{intermediate}}$ expression retain their capacity to be reinvigorated by anti-PD1 therapy [61]. It is therefore likely that potent reversal of exhaustion by therapeutic intervention might depend on the proportion of T-cells that display a PD1$^{\text{intermediate}}$ to PD1$^{\text{high}}$ phenotype prior to therapy [10]. The PD1$^{\text{high}}$ phenotype of tumor-associated CD8$^+$ T-cells within resistant tumors was found to partially depend upon tumor-associated Tregs [59]. While the existence of tumor-associated PD1$^{\text{high}}$ CD8$^+$ T-cells was associated with primary resistance to anti-PD1 therapy, it is possible that the development of severe T-cell exhaustion could lead to the development of acquired resistance. Although speculative, the compensatory induction of Tregs into tumor tissue following the anti-PD1-mediated release of adaptive immune resistance, could promote severe exhaustion of tumor-associated CD8$^+$ T-cells and limit the efficacy of anti-PD1 therapy.

**Co-expression of inhibitory receptors**

Associated with the severely exhausted phenotype of some T-cells is over-expression of multiple inhibitory receptors including, T-cell immunoglobulin mucin 3 (TIM3), CLTA4, lymphocyte activation gene 3 (LAG3) and B and T lymphocyte attenuator (BTLA), in addition to PD1. The immunosuppressive capacity of these receptors was initially defined in models of chronic infection such as lymphocytic choriomeningitis virus (LCMV) [62]. While there are similarities between antigen persistence in chronic infection and antigen persistence in cancers, it should be noted that it is not clear whether expression patterns and activity of such receptors behave in the same fashion under both conditions [10]. Recently, Thommen et al. identified that co-expression of PD1, TIM3, LAG3, CLTA4 and BTLA was associated with resistance to anti-PD1 therapy in
NSLC [63]. CD8$^+$ T-cells expressing the five receptors demonstrated severe defects in cytokine production, proliferation and migration. Interestingly, a spectrum of exhaustion was proposed whereby expression of PD1, TIM3, LAG3, and CTLA4 was associated with a mildly exhausted phenotype which was further exaggerated following expression of BTLA [63]. It is not known whether the primary resistance displayed by severely exhausted PD1$^{\text{high}}$ CD8$^+$ T-cells is a product of compensatory signalling mediated through co-expressed inhibitory receptors, or whether elevated PD1 endows exhausted T-cells with an intrinsic defect in sensitivity to anti-PD1 therapy. Recently, Koyama et al. using two mouse models of lung adenocarcinoma, demonstrated that up-regulation of TIM3 on PD1$^+$ lymphocytes was correlated with the induction of acquired resistance to anti-PD1 therapy. Interestingly, it was shown that TIM3 expression was highest amongst T-cells with the greatest amount of PD1 bound. Given that anti-PD1 binding is limited by PD1 expression, this result would imply that in this model TIM3 was expressed upon severely exhausted PD1$^{\text{high}}$ CD8$^+$ T-cells. Interestingly, T-cells from resistant tumors also over-expressed LAG3 and CTLA4, however, BTLA expression appeared variable [64]. The mechanisms that promote the induction of severe T-cell exhaustion within tumor microenvironments have not been completely characterized. Additionally, it is still not known whether reducing PD1 expression amongst severely exhausted, PD1$^{\text{high}}$ CD8$^+$ T-cells would enhance sensitivity to anti-PD1/PDL1 therapies, or whether expression of receptors such as TIM3, BTLA, LAG3 can be independent of PD1. Further characterisation of exhausted T-cells in the context of anti-PD1/PDL1 resistance is therefore required.

**PD1-independent pathways**

Some tumors resistant to anti-PD1 therapy have been identified to simultaneously utilize multiple immunosuppressive pathways, indicating that in terms of function, additional pathways may compensate for the release of immune suppression provided by PD1 blockade. Metabolically limited by the local bioavailability of tryptophan, T-cells undergo proliferation arrest in its absence. Many tumors express the enzyme, indoleamine 2,3-dioxygenase (IDO) via the same IFN inducible
mechanism as PDL1 [65]. Interestingly, expression of IDO by tumor cells has been demonstrated to render experimental mouse models of melanoma resistant to anti-PD1 therapy [66, 67]. While some metabolites are fundamentally necessary to facilitate T-cell activity, others behave in an inhibitory fashion. Under physiological conditions the production of pro-inflammatory stimuli such as cellular stress initiated by hypoxia or ischemia can promote the production of immunosuppressive adenosine. ATP released into the extracellular environment interacts with CD39, to produce AMP, which is further dephosphorylated by CD73 to adenosine. The ligation of adenosine with $A_{2A}$ receptors expressed by lymphocytes such as CD8$^+$ T-cells inhibits their effector functions. Under physiological conditions, this pathway also prevents exaggerated tissue injury as a result of antigen-specific immune responses [68]. The over-expression of CD73 in multiple cancer types has been associated with poor prognosis. Specifically, the absence of estrogen receptor expression has been inversely associated with high levels of CD73 expression, and also with metastatic potential [69, 70]. In experimental mouse models of colon, prostate and breast cancers, Allard et al. demonstrated that activation of adenosine signalling enhanced the expression of PD1 on tumor-associated CD8$^+$ T-cells, promoting a severely exhausted phenotype, and resistance to anti-PD1 therapy [71]. The functional significance of this pathway to PD1 inhibition remains to be characterized in humans, however, these preliminary studies provide compelling evidence supporting the notion that multiple independent immunosuppressive pathways within the tumor microenvironment can promote tumor development and resistance to anti-PD1 therapy. It is likely given the cellular heterogeneity of many tumors, that the activity of multiple immunosuppressive could function as both a primary and acquired resistance mechanism.

In the context of anti-PD1 therapy, the direct subversion of T-cell-mediated effector functions appears to be a dominant feature amongst resistant tumors. The elevation of PD1 expression level, co-expression of multiple inhibitory receptors, and production of immunosuppressive metabolites have each been demonstrated to affect the sensitivity of tumors to anti-PD1 therapy. The selection of tumor cells capable of limiting the exertion of T-cell-mediated
anti-tumor immunity by promoting, an influx of Tregs, expression of PD1-independent inhibitory receptors, or production of immunosuppressive metabolites, could promote either primary or acquired resistance. Additionally, the heterogeneity present within most tumors might allow for the activity of multiple resistance pathways in tandem. The reinvigoration to T-cell effector functions provided by anti-PD1 therapy might lead to the selection of occult clones capable of subverting re-invigorated immunosurveillance; their outgrowth and would likely herald the acquisition of resistance.

T-cell memory

While effector CD8\(^+\) T-cells have been demonstrated to be necessary for the efficacy of anti-PD1/PDL1 therapies, recent evidence suggests that the induction of T-cell memory is also an important consequence of sensitivity. Help by CD4\(^+\) T-cells, licenses DCs with the ability, not only to promote differentiation from a naïve to an effector CD8\(^+\) T-cell phenotype, but to also enable the eventual induction of CD8\(^+\) T-cell memory. This priming step induces rapid expansion and enables production of essential effector molecules [72]. Following the exertion of effector activity and antigen clearance, the contraction and resolution phase ensues. The small number of remaining cells enter into the memory phase where they can be maintained for the life of the host; capable of mounting rapid responses following antigen re-challenge [73]. Differences in their localisation, recall ability, and effector functions allow them to provide overlapping layers of protection against antigen re-encounter [74-77].

Physiologically, activation and expansion of short lived CD4\(^+\) and CD8\(^+\) T-cells gives rise to a KLRG1\(^{hi}\)CD127\(^{lo}\) effector and KLRG1\(^{lo}\)CD127\(^{hi}\) memory precursor populations. Interestingly, chronic exposure to antigen can render the KLRG1\(^{lo}\)CD127\(^{hi}\) memory pre-cursors exhausted. Given that an endpoint of exhaustion can be deletion, this can have significant implications for memory cell development [78]. Exhausted CD8\(^+\) T-cells become addicted to cognate antigen and depend on TCR signalling for survival [79]. Recently Ribas et al. who compared immune cell infiltrates within
tumors pre- and post-treatment with anti-PD1 therapy, showed that CD8$^{+}$ T$^{\text{EM}}$ were the major T-cell subset expanded in sensitive patients [80]. Specifically, patients categorised to have responded poorly to anti-PD1 therapy contained significantly fewer tumor-associated T$^{\text{EM}}$ than responsive patients. This result suggests two things; first, that induction of T-cells with an effector memory phenotype is a mechanism of action important to the efficacy of anti-PD1 therapy, and second, that resistance to anti-PD1 therapy limits memory T-cell induction [80]. Further exploration of resistance mechanisms active in non-responsive patients might allow for alternative therapeutic intervention and sensitisation.

**Progress of strategies to sensitize tumors to PD1 blockade.**

By summarising the current understanding of mechanisms contributing to the development of resistance to anti-PD1 therapy, we have highlighted several common targets for sensitising therapeutic intervention that may allow for effective combination therapeutic strategies to sensitise resistant patients.

The role of immunosuppressive cell types within the tumor microenvironment has been well documented with respect to their ability to suppress anti-tumor immunity. Indeed, elevated Foxp3$^{+}$ Tregs to CD8$^{+}$ T-cell ratios are commonly associated with resistance to anti-PD1 therapy [59]. To reduce this ratio within the tumor microenvironment, a number of therapeutic strategies have demonstrated efficacy in targeting and depleting Tregs. The use of anti-CTLA4 therapy has been shown in several preclinical studies to deplete tumor-associated Tregs via an FcγR-dependant mechanism, and to also enhance the efficacy of tumor-specific T-cell-mediated anti-tumor immunity [81]. Given that the use of anti-CTLA4 in the clinic is associated with an increase in immune-related adverse events, it is possible that its mechanisms of action in humans involves depletion of Tregs, leading to a break down in peripheral tolerance [82]; its combination with anti-PD1 therapy appears to be highly synergistic [22, 83]. Bulliard et al. demonstrated that tumor-associated Tregs could be selectively depleted using anti-OX40 (CD134) mAbs, and that this
resulted in growth reduction of tumors [84]. Encouragingly, the combination of anti-PD1 therapy with agonistic anti-OX40 mAbs has demonstrated synergistic anti-tumor efficacy in a model of ovarian cancer resistant to anti-PD1 therapy alone, and are currently being investigated within the clinic (ClinicalTrials.gov; NCT02205333 and NCT01303705) [85]. Additionally, TIM3 and T-cell Ig and ITIM domain (TIGIT) are commonly over-expressed by tumor-associated Tregs [86, 87]. Therapies which target and block the activity of either receptor have been shown to reduce Treg suppressive activity and to synergise with anti-PD1 therapy to enhance anti-tumor immunity in mouse models of colon cancer and melanoma, respectively [87, 88]. Anti-OX40, anti-TIM3 and anti-TIGIT therapies have not yet been trialed in combination with anti-PD1 therapy in the clinic. By contrast, clinical trials combining anti-CTLA4 and anti-PD1 therapy resulted in an objective response rate of 58% in patients with advanced melanoma; a significant improvement over single agent anti-CTLA4 therapy (19%) and numerically higher than anti-PD1 monotherapy (44%) [25]; representing a feasible method for alleviating Treg-mediated anti-PD1 therapeutic resistance.

The poor immunogenicity of some tumors can limit the development of antigen-specific effector T-cells and consequently sensitivity to anti-PD1 therapy. Therapies that serve to liberate tumor antigen available for uptake by APCs are likely to enhance sensitivity to anti-PD1 therapy for patients with poorly immunogenic tumors [89-92]. Radio- and some chemo-therapeutic regimes can result in immunogenic cell death. Allowing for the release of tumor antigens, the use of such therapies in combination with anti-PD1/PDL1 mAbs might promote effective anti-tumor immunity [93, 94]. Both approaches have recently been proposed to synergise with checkpoint blockade by acting as a type of vaccination to prime adaptive immunity, while anti-PD1/PDL1 therapy amplifies anti-tumor immune responses by overcoming or preventing CD8+ T-cell exhaustion [93, 94]. Encouragingly, clinical trials combining radiotherapy (ClinicalTrials.gov; NCT02617589), or some chemotherapies such as doxorubicin – capable of inducing immunogenic cell death - (ClinicalTrials.gov; NCT02423954), with anti-PD1 therapy are currently underway [89-92]. Given that such regimes are commonly associated with severe side-effects, approaches that overcome
these while offering their therapeutic benefit may prove more attractive. As such, autologous cancer vaccination strategies that prime adaptive immune responses with tumor-specific antigen have been demonstrated to improve sensitivity to anti-PD1 therapy [95]. As an alternative, neoantigen vaccination approaches have been demonstrated in mouse models to effectively increase tumor control. These studies have provided sufficient impetus to warrant investigation in humans. Neoantigen vaccination approaches have the benefit of being tumor-specific, however, they do run the risk of being diluted out by non-mutated peptide. Rationally, the combination of such vaccination strategies with anti-PD1 therapy would likely serve two purposes: first, increasing the proportion of tumor-specific T-cells, and releasing them from tumor-induced immune suppression [32].

An absence of lymphocyte enrichment within human melanomas has been associated with poor response to anti-PD1 therapy. Currently within the clinic, the combination of BRAF with MEK inhibitors (MEKi) have demonstrated considerable synergy in the treatment of metastatic BRAF mutant melanoma [96]. Having been demonstrated to require an intact immune system [97], this therapeutic combination has been shown to increase the proportion of CD8+ and CD4+ lymphocytes within tumor tissue [98]. As such, the combination of BRAF/MEKi with anti-PD1 therapy is currently under investigation within a clinical trial (ClinicalTrials.gov; NCT02130466) [99]. Alternative approaches for enhancing lymphocyte infiltration within tumor tissue have been investigated within pre-clinical models. Recently, as a means of promoting cytotoxic inflammatory responses within a mouse model of melanoma, Bald et al. demonstrated that peri-tumoral injections of immunostimulatory poly(I:C) RNA effectively promotes lymphocyte infiltration and impaired tumor growth. RNA-stimulated IFN-γ secretion was found to promote adaptive immune resistance within the tumor microenvironment by upregulating PDL1 expression. By combining immunostimulatory RNA with anti-PD1 therapy, anti-tumor immunity and tumor rejection were significantly improved. Such therapies, by promoting an IFNγ-rich environment might also serve to increase expression of class I MHC within many tumors, which is commonly down-regulated by
tumors but required for T-cell-mediated anti-tumor responses. While effective \textit{in vivo}, improved delivery mechanisms must be explored, as peri-tumoral injection into human metastases might prove anatomically limiting in some cases [100].

The suppression placed upon APCs within the tumor microenvironment is largely supported by expression of cytokines such as VEGF and TGF-\(\beta\). Encouragingly, the neutralisation of these cytokines using mAbs that bind and limit their bioavailability has been shown to liberate DC effector functions [101]. Indeed, therapeutic anti-VEGF mAbs (bevacizumab) are currently implemented within the clinic in combination with chemotherapeutic regimes to treat some cancers; its efficacy in combination with PD-1 blockade is currently being tested in clinical trial (ClinicalTrials.gov; NCT01454102). In cases where unknown suppressive factors within the microenvironment limit the activity of antigen presenting cells, stimulating effector functions may serve as method for improving anti-tumor immunity. Agonistic mAbs targeting the immunostimulatory molecules, CD40 or CD137 have also been shown to improve the effector functions of DCs. Approaches in which anti-PD1 mAbs have been combined with either anti-CD40 or anti-CD137 mAbs have demonstrated considerable synergy in models of melanoma, breast and colon cancer [102, 103]. Additional approaches to enhance the activity of APCs within the tumor microenvironment include the use of oncolytic viruses. Talimogene laherparepvec (T-VEC) is a herpes simplex virus type 1-derived oncolytic immunotherapy designed to selectively replicate within tumors, to produce granulocyte macrophage colony-stimulating factor (GM-CSF) and to promote lytic tumor cell death. Tumor cell lysis combined with the chemotactic properties of GM-CSF promotes the migration, antigen processing and maturation of DCs within the tumor microenvironment [104]. Indeed, intra-lesional T-VEC has demonstrated durable responses in highly selected patients with injectable metastases in phase III clinical trials of patients with melanoma [105]. Oncolytic viruses have also been demonstrated \textit{in vivo} to prevent resistance to anti-PD1 therapy [106]. Indeed, T-VEC is currently being trialed in combination with anti-PD1 therapy for patients with non-resectable melanoma, and in the phase 1 study of 21 patients with
advanced melanoma, the combination was tolerable with a response rate of 57% 
(ClinicalTrials.gov; NCT02263508) [107]. While it is understood that cytolytic viruses can promote 
epitope spreading of tumor neoantigens [105], it is not clear what effect viral antigens have upon 
their efficacy. Importantly, therapies such as these are also limited by delivery methods. Currently, 
the efficacy of such therapies is dependent on local administration into tumor tissue [104]. 
Investigation of additional delivery methods that might allow for systemic administration and tumor 
specificity might improve its applicability for tumors in areas where local access is limited. 
Together, these studies demonstrate that antigen presentation can be improved within the tumor 
 microenvironment. While trialed individually, therapeutic regimes combining anti-CD40 or anti-
CD137 with anti-PD1 mAbs, while likely to be effective, have not yet been trialed in humans. 
Current trials underway for combination anti-VEGF or viral therapies with anti-PD1 therapy 
represent a possible approach for enhancing antigen presentation within the tumor 
 microenvironment and for enhancing sensitivity to anti-PD1 therapy within the not too distant 
future.

Co-blockade of pathways, which in addition to PD1, promote T-cell exhaustion may 
overcome the resistance to anti-PD1 therapy. Encouragingly, therapeutic mAbs targeting either 
TIM3 or LAG3 in combination with PD1 have demonstrated synergistic outcomes in a variety of 
pre-clinical models. Indeed, the efficacy of anti-LAG3 with anti-PD1 is currently being investigated 
in a clinical trial for a number of solid tumors (ClinicalTrial.gov NCT01968109) [87, 108]. 
Additionally, blockade of immunosuppressive metabolites such as adenosine has also been 
investigated as a means to increase sensitivity to anti-PD1 therapy. Following the demonstration 
that inhibition of adenosine production within the tumor microenvironment could enhance the 
sensitivity of tumors to anti-PD1 and anti-CTLA4 mAb therapy in mouse models [71], clinical trials 
assessing the efficacy of anti-CD73 mAbs in cancer therapy have been initiated. Similarly, 
inhibition of A2A receptors in combination with anti-PD1 mAbs has also demonstrated synergistic 
anti-metastatic effects in pre-clinical models. We propose that the existence of clinically available
A	extsubscript{2}A receptor inhibitors used in the treatment of Parkinson’s disease with a broad safety profile, might allow for rapid progression into clinical trials alongside anti-PD1 mAbs (NCT02655822: clinicaltrials.gov) [109]. A common problem associated with the use of antibodies with targets within the cellular milieu of tumors, is a lack of penetrance. IgG molecules are large and their distribution into tumor tissue can be limited by vasculature, stoma and cell density. To address this, high-affinity, soluble PD1 proteins have been developed to bind to and limit the association of PDL1 expressed within the tumor microenvironment with endogenously expressed PD1 on infiltrating lymphocytes [61]. Preliminary studies have demonstrated that the soluble PD1 protein is able to penetrate further into tissues than conventional therapeutic mAbs, that it avoids depletion of PDL1	extsuperscript{+} lymphocytes, and can increase anti-tumor immunity [110]. Therapies targeting T-cell immune suppression have collectively demonstrated high levels of efficacy. Indeed, mAb-based therapies against CD73, LAG3, TIM3 are all currently under investigation either alone or in combination with anti-PD1 therapy. A	extsubscript{2}A receptor inhibitors are already used within the clinic and given their broad safety spectrum could rationally be trailed in combination with anti-PD1 therapy. The ability to sensitise resistant tumors in which T-cell exhaustion or suppression are limiting factors might therefore be a realistic future therapeutic approach.

Future directions

To date, anti-cancer immunotherapies that target the PD1/PDL1 axis represent the most effective means of treatment for a variety of cancer types; they are well tolerated and can be used as monotherapies, but they are not perfect. Although a large proportion of patients treated with anti-PD1 garner substantial benefits, most do not [3]. Here, we have proposed a model to explain how resistance to anti-PD1 therapy, as it is currently understood, is presented within a variety of tumors. Currently, the field of cancer immunotherapy is in its adolescence; it is almost certain that more will be learnt regarding mechanisms of resistance to anti-PD1 therapy as they are widely adopted within the clinic, however, the development of combination therapies aimed to sensitise patients may take much longer. Indeed, the vast majority of therapeutic combinations discussed here will require
continuing research to identify optimal dosage and timing, and to demonstrate safety and efficacy when used in combination with anti-PD1/PDL1 therapies. If effective, these therapies will allow clinicians to tailor regimes in a patient-specific fashion, based not only upon tumor grade or biomarker status, but also upon the immunophenotype of the tumor itself.

Until such therapies undergo late-stage large-scale trials it is unlikely that biomarkers to predict the efficacy of individual combination therapies will be identified. Importantly, tumor characteristics such as PDL1 expression and TIL abundance are not routinely examined. We recently discussed the strengths and limitations of a pragmatic framework to stratifying patients based upon PDL1 expression and TIL abundance in melanomas where such data has been gathered. This dictated that patients were divided into one of four groups; it accurately predicted that PDL1 expression and TILs were predictive of response to anti-PD1 therapy in treatment of melanoma [27]. Given the importance of an immune permissive microenvironment to predict a long-term response, the immune contexture of pre-treatment biopsies might not accurately predict outcome. It might, therefore, be of benefit to also biopsy tumors under the influence of therapy to see whether it can, in itself influence immune infiltration. It is likely that as the mechanisms by which anti-PD1 therapy functions and mechanisms of resistance are elucidated it is likely that more accurate stratification procedures may be developed; their implementation might inform clinicians as to which combination therapies might be applied to yield the greatest benefit for patients.
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References


12. Wolchok, J.D., Chiarion-Sileni, V., Gonzalez, R., Updated results from a phase III trial of nivolumab (NIVO) combined with ipilimumab (IPI) in treatment-naive patients (pts) with advanced melanoma (MEL) (CheckMate 067), in ASCO Meeting Abstracts 34:9505. 2016.


99. Ribas, A., Hodi, F.S., Lawrence, D.P., Pembrolizumab (pembro) in combination with dabrafenib (D) and trametinib (T) for BRAF-mutant advanced melanoma: Phase 1 KEYNOTE-022 study., in ASCO Meeting Abstracts 34:3014. 2016.
Figure Legends

Figure 1 – Clinical response to anti-PD1/PDL1 therapies

Patients can be stratified based upon response to anti-PD1/PDL1 therapies. Sensitivity is observed amongst ~30% of melanoma patients treated and is characterised by stable disease progression or complete response; in combination with anti-CTLA4 therapy, this is further increased to ~50%. These are patients within whom adaptive immune resistance mechanisms function to subvert tumor-specific anti-tumor immune responses.

By contrast, primary resistance is observed amongst 70% of patients and occurs within patients who garner no benefit from the outset of therapy. This is characterised as disease progression. Some patients who demonstrate encouraging initial responses to anti-PD1 therapy can eventually experience disease progression. These patients have acquired resistance.

Figure 2 – Categories of resistance to anti-PD1 therapy

In order for patients to demonstrate sensitivity to anti-PD1/PDL1 therapies a number of conditions must be met. A. Tumors must express antigens that differentiate them from their non-transformed counterparts, and to be permissive to antigen presenting cells (APCs) that are capable of gathering tumor antigen. B. APCs harbouring tumor antigen must undergo efficient migration and maturation; culminating in presentation to, and activation of, tumor antigen-specific T cells. C. Tumor-specific effector T cells must be capable of carrying out their effector functions in the presence of tumor antigen and unaffected by PD1-independent immunosuppressive factors within the tumor microenvironment. D. Tumors must display cognate antigen in order to be recognised, and also to express PDL1 – otherwise, it would be unlikely for PD1-mediated immune suppression to be active within the tumor microenvironment. E. In order to sustain anti-tumor immune responses long-term, induction of immunological memory must be preserved.

Figure 3 – T cell exhaustion and resistance to anti-PD1/PDL1 therapies

Exhaustion induced by PD1-independent pathways can induce resistance to anti-PD1/PDL1 therapies. A. Tumor-specific T cells that demonstrate a severe exhaustion phenotype, characterised by highly elevated PD1 expression have been demonstrated to be resistant to anti-PD1 therapies. B. The expression of
exhaustion markers such as TIM3, LAG3 and CTLA4 might be capable of facilitating the induction of exhaustion via non-redundant pathways even in the presence of anti-PD1 therapy. C. The expression of immunosuppressive metabolites such as IDO or adenosine have been shown to suppress T cell effector functions even in the presence of anti-PD1 therapy.
Anti-PD1 therapy _does not_ enable the release of tumor-specific immunity

Clinical phenotype ~70% (of melanoma patients)
- Disease progression

Primary Resistance

Sensitivity (adaptive immune resistance)

Anti PD1 therapy releases adaptive anti-tumor Immunity

Clinical phenotype ~30% (of melanoma patients)
- Stable disease progression
- Complete response

Resistance mechanisms?

Anti-PD1 therapy _initially_ releases tumor-specific Immunity but eventually _fails_

Clinical phenotype 7%
- Initial sensitivity
Eventual disease relapse

Acquired Resistance
Highlights:

- Resistance to anti-PD1 therapy affects up to ~70% of patients treated.
- Resistance can be primary or acquired.
- Tumor intrinsic mechanisms that limit the induction of tumor-specific T cells or their efficacy within tumor tissue may promote resistance.
- Logical therapeutic combinations might prove effective to prevent, or to treat resistant tumors.