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PD-1/PDL-1 BLOCKADE IN CANCER IMMUNOTHERAPY

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MRes Biomolecular Technology

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ABSTRACT

Molecules that downregulate the immune response are key during cancer treatment because they pose a mechanism to avoid immunotherapy, also called immune checkpoints. In that regard, the PD-1/PD-L1 pathway has been thoroughly studied to deepen our understanding of the function of these immune checkpoint molecules. This signalling pathway involves the interaction of programmed cell death (PD-1), which is a surface protein found mainly in T and B cells, and programmed cell death ligand 1 (PD-L1) found in antigen-presenting cells. However, there is clear evidence demonstrating the overexpression of PD-L1 in some cancer cells, and when this protein interacts with PD-1, it leads to T-cell inactivation and thus helps cancer cells escape clearance by the immune system.

In this literature review, the knowledge around the components of the PD-1/PD-L1 pathway and how these mediate immune evasion in the tumour microenvironment will be summarized. Given the importance of this signalling pathway, the potential for developing cancer therapeutics targeting this biological process started to grow. The predominant PD-1/PD-L1 inhibitors in the market are monoclonal antibodies, but there are other peptides and non-peptide molecules in research that seem promising in terms of their effectiveness and produce few immune adverse effects. To test the activity of such inhibitors, an array of assays can be used to characterize the molecules in different contexts, from a physicochemical to a cellular and organ level. Despite the favourable results, not all patients respond to PD-1/PD-L1 inhibitors and the reason for that, although not fully understood, is still an ongoing challenge. Overall, the complexity of the immune regulation mediated by PD-1 and PD-L1 is not completely unveiled but is an active area of cancer immunotherapy research.

INTRODUCTION

An effective immune response, in any context (i.e., infectious diseases, cancer, etc.), requires the cooperation of cells and active molecules from the innate and adaptive immune system. Overall, the stages in a defensive reaction can be summarised as (i) recognition, (ii) processing, and (iii) reaction (Kim & Cho, 2022). The first step typically involves T cell antigen-mediated activation through peptide–MHC engagement in antigen-presenting cells (APCs) with T cell receptor (TCR). This stimulus is reinforced by positive costimulatory signals such as interactions between CD28 on T cells and CD80 (also known as B7.1) and/or CD86 (also known as B7.2) on APCs. On the counterpart, the T cell inhibitory signal can be promoted by cytotoxic T lymphocyte antigen 4 (CTLA-4; also known as CD152) which directly competes with CD28 for the ligands CD80 and CD86 (Kim & Cho, 2022; Sharpe & Pauken, 2018) (see **Figure 1**).

The immune system must find a balance between protecting the body from pathogenic agents while sparing healthy cells and maintaining self-tolerance. Thus, multiple checks on immune responses are set during lymphocyte development in central lymphoid organs (central tolerance) and the periphery (peripheral tolerance) (Sharpe & Pauken, 2018). Another way to interpret these processes is by picturing them as “brakes” to “slow down” overactive immune responses, especially against self-antigens to prevent autoimmunity. This is achieved through a class of negative immune modulatory molecules (called immune checkpoints) that halt T cell effector functions (Ganesan et al., 2019). During the last decade, the potential of immune checkpoints as therapeutic targets has shaped cancer immunotherapy and the progress in the field has been exponential (Upadhaya et al., 2022). In particular, the PD-1/PD-L1 signalling pathway has proved a popular target for the development of immune checkpoint inhibitors, the reasons for this will be discussed below.

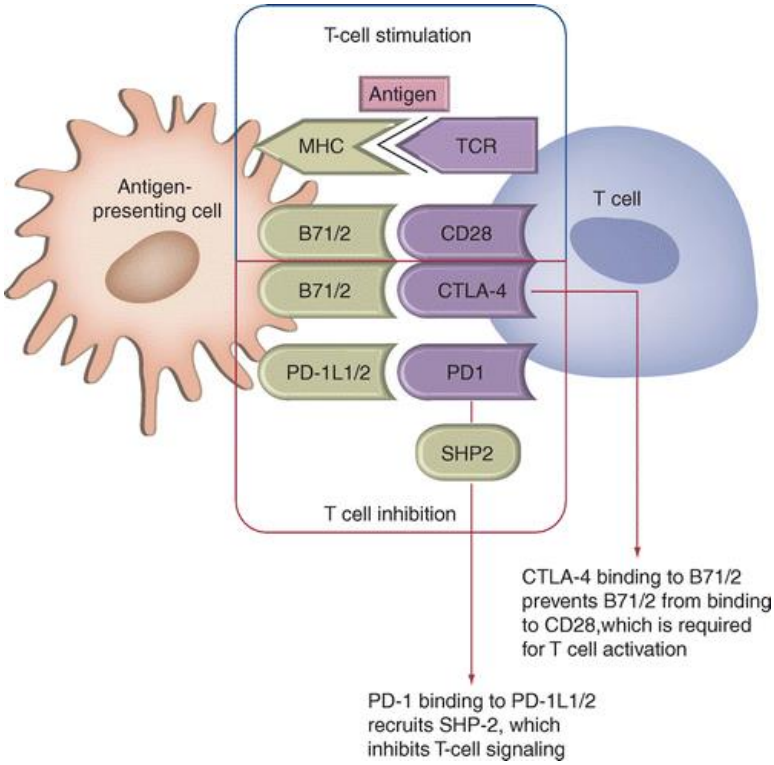


Figure 1. The main function of cytotoxic T lymphocyte antigen 4 (CTLA-4) and programmed cell death protein 1 (PD-1): receptors and ligands involved in T cell stimulation or inhibition through antigen-presenting cells. Figure from Heng, 2011.

PD-1/PDL-1 PATHWAY

As mentioned above for the CTLA-4 signalling pathway, there are other molecules considered immune checkpoints (the difference will be discussed below). For instance, programmed cell death protein 1 (PD-1, also known as PDCD1 or CD279) is considered an immune checkpoint molecule because it counters positive signals mediated by the TCR and CD28, by engaging its ligands (PD-L1 and PD-L2) promoting tyrosine phosphorylation of the PD-1 cytoplasmic domain and recruitment of phosphatases (**Figure 2B**) (Freeman, 2008). As seen in **Figure 1**, distinct types of immune cells bear PD-1 or PD-L1/PD-L2. However, these ligands are also found on the surface of some cancer cell types, preventing their clearance by the immune system (Furuse et al., 2020; Xing et al., 2018; Xue et al., 2019).

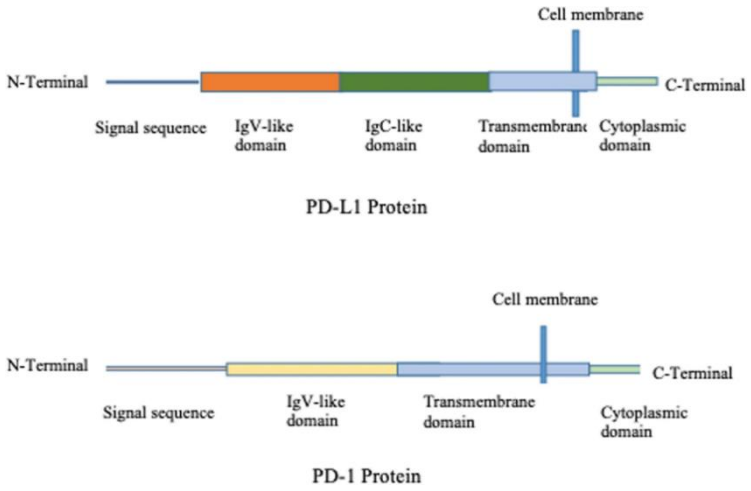
Overall, the PD-1/PD-L1 pathway is involved not only in regulating initial T cell activation but also plays a role in T cell tolerance and fine-tuning of T cell fate and functions (Sharpe & Pauken, 2018). As expected, if one of the components in the PD-1/PD-L1 interaction is suppressed or

deficient, autoimmunity problems arise as proven by knock-out mice experiments. In the case of PD-1-deficient mice, the developed autoimmune disease depended on the genetic nature of the mice (Ghosh et al., 2021). Additionally, upon PD-1 silencing T cells' anti-tumour function becomes impaired by inhibiting their proliferation activity and differentiation of T cells (Wei et al., 2019). However, there is also some evidence showing that with the CRISPR-Cas9 novel technique, the activity of knocking out PD-1 in cytotoxic T lymphocytes proved to increase cytokines secretion and enhanced cancerous cell death (Zhao et al., 2017).

PROGRAMMED CELL DEATH 1 (PD-1)

PD-1 was first described in 1992 as it is expressed while inducing apoptosis in T-cell hybridoma (Ishida et al., 1992). Following that, several studies tried to unveil its role in the immune response. Now it is known that PD-1 belongs to the CD28/CTLA-4 family of co-receptors characterized by an immunoglobulin (Ig) variable-type (V-type) amino-terminal extracellular domain, a transmembrane region, and a cytoplasmic tail with an immunoreceptor tyrosine-based inhibitory motif and an immunoreceptor tyrosine-based switch motif (**Figure 2A**) (Pascolutti et al., 2016). PD-1 is expressed in T cells, B cells, Natural Killer T (NKT) cells, some myeloid and APC populations, and innate lymphoid cells (ILC) progenitors. PD-1 expression is regulated by transcription factors like the nuclear factor of activated T cells (NFAT) and forkhead box protein O1 (FOXO1) upon stimulation with cytokines and factors that stimulate B-cell receptor (BCR) or TCR signalling (e. g. Concanavalin A, phorbol 12-myristate-13-acetate (PMA)/ionomycin, and anti-IgM antibody) (Qin et al., 2019; Sharpe & Pauken, 2018).

A)



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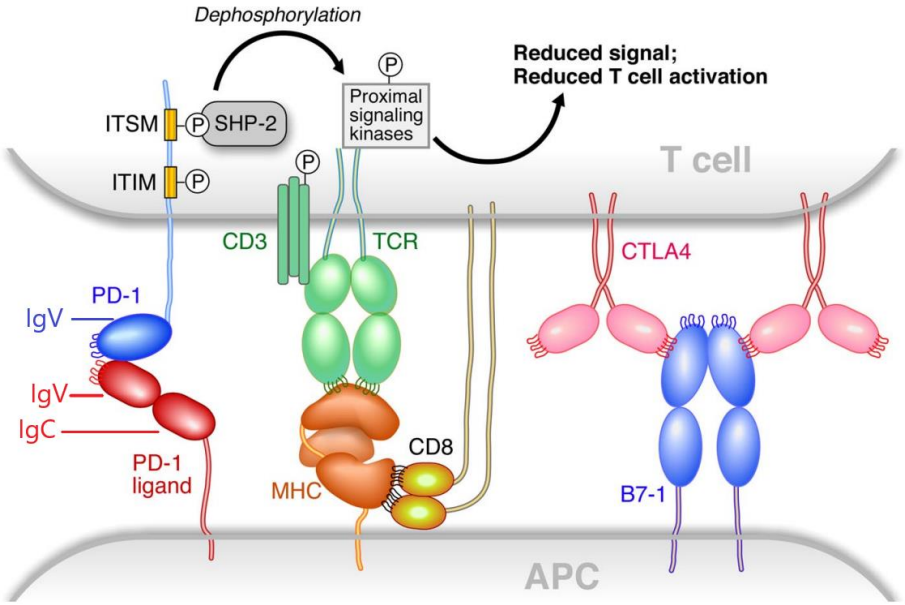

Figure 2. PD-1 and PD-L1 modelled protein interaction. (A) Protein structures of PD-L1 and PD-1 are both transmembrane proteins with four (Ig variable-like –IgV–, Ig constant-like –IgC–, transmembrane and cytoplasmic) and three (IgV, IgC, transmembrane and cytoplasmic) domains, respectively. (B) These proteins interact with each other through their conserved IgV. Protein loops at the end of IgV in PD-1/PD-L1 are depicted as forming a surface that is like the antigen-binding surface of antibodies and T-cell receptors based on the crystal structures. Since structures are modelled on crystal determinations, it is noteworthy, the difference in the angle of the interaction and the stoichiometry between CTLA-4/B7-1, as compared to PD-1/PD-L1. Additionally, the mechanism of the inhibitory signal produced by PD-1/PD-L1 interaction is presented in brief. Figure adapted from: Akinleye & Rasool, 2019; Freeman, 2008.

PD-1 is expressed in T cells during its activation, but recently, evidence about the expression of PD-1 on some cancer cells has been published, particularly for liver cancer, Non-Small Lung

Cancer (NSCLC), and melanoma cells (Takeuchi et al., 2022). It has been suggested that intrinsic PD-1 in melanoma cells has a protumour effect independently of adaptive immunity through the activation of effectors of the mammalian target of rapamycin (mTOR) signalling pathway downstream of the melanoma-PD-1 receptor (Kleffel et al., 2015). Conversely, the research of Wang and colleagues (2020) showed that intrinsic PD-1 suppresses tumour growth in the absence of adaptive immunity through inhibition of the AKT and ERK1/2 signalling pathways in NSCLC. Based on the above evidence, tumour cell-intrinsic PD-1 might act as an antagonist depending on different tumour types and/or selective signalling pathways. Nonetheless, the precise molecular and cellular mechanisms that mediate this "paradox" of the PD-1 axis blockade are yet to be determined (Wang et al., 2020).

PROGRAMMED CELL DEATH 1 LIGAND 1 (PD-L1)

Two ligands have been described for PD-1, called programmed cell death 1 ligand 1 (PD-L1 also known as CD274 and B7-H1) and programmed cell death 1 ligand 2 (PD-L2 also known as CD273 and B7-DC). As PD-1, its ligands are also transmembrane proteins considered 'coinhibitory' receptors that regulate effector T cell function (Sharpe & Pauken, 2018). Both ligands are type 1 transmembrane glycoproteins that contain IgC and IgV domains (**Figure 2A**) (Ghosh et al., 2021; Pascolutti et al., 2016). However, their biological functions and expression patterns differ. While PD-L1 is widely expressed in both hematopoietic cells (including T cells, B cells, Dendritic cells (DC), and other myeloid cells) and non-hematopoietic cells (including vascular endothelial cells and pancreatic islets); PD-L2 expression is restricted to activated macrophages and DC cells (Pascolutti et al., 2016; Q. Wang et al., 2021).

PD-L1 has been studied for its expression in several cancerous cells associated with evasion from immune attacks. For example, PD-L1 overexpression has been found in breast cancer, lung cancer, bladder cancer, gastric cancer, lymphoma, glioblastoma and melanoma (Ganesan et al., 2019; X. Wang et al., 2016). Consistently, a high expression of PD-L1 in tumours is strongly associated with poor prognosis in several different cancers (Furuse et al., 2020; Sakuishi et al., 2010; Xue et al., 2019). Genetic aberration, gene duplication, transcription control, and post-transcriptional modulation are the main sources for PD-L1 up-regulation (Ghosh et al., 2021).

Cancer immune evasion in general is thought to be mediated by PD-L1, although PD-L2 may also play a role in some cases (Pascolutti et al., 2016). Regarding the difference in effector functions, it has been shown that PD-L2 can interact with other membrane receptors such as repulsive guidance molecule family member 2 (RGM-2) in lung macrophages to maintain respiratory tolerance (Xiao et al., 2014). In addition, PD-L1 and PD-L2 can interact with B7-1 (CD-80), typically a CD28 and CTLA-4 ligand, on activated T-cells to produce the inhibitory immune signal (Ghosh et al., 2021).

PD-1/PD-L1 MEDIATED MECHANISM OF TUMOUR EVASION

To kill tumour cells during cancer, T cell activation involves recognition of tumour antigens presented by major histocompatibility complexes (MHCs) on APCs and further proliferation. However, excessive activation of T cells can impose severe organ damage. Hence, immune checkpoints like PD-1 and its ligand PD-L1 are also needed to avert self-damage. Nonetheless, this mechanism has been exploited by tumour cells to resist the apoptosis induced by immune cells (Lei et al., 2020). PD-L1 and PD-1 suppress anti-tumour immunity and promote tumour progression by inducing T-cell apoptosis (Ostrand-Rosenberg et al., 2014). This mechanism has been validated in primary gastric adenocarcinoma models, where CD3+ T cells stimulated cancer cells to up-regulate PD-L1 by an IFN-γ-dependent mechanism upon PD-1/PD-L1 interaction, which was associated with an increase in CD3+T cells apoptosis (Chiu et al., 2018). In addition, other cancer cells have demonstrated the same pattern, and the administration of blocking agents has reversed the phenomenon (Dong et al., 2002; Shi et al., 2011).

Inhibition of the effector function of T cells has also been proposed as one of the central mechanisms for tumour surveillance. Upon binding of PD-1 and PD-L1, there is inhibition of tumour infiltrating CD4+/CD8+ T cells (CD4+/CD8+ TILs), the quantity of cytokines such as Tumour Necrosis Factor (TNF), Interferon-gamma (IFN-ɣ) and Interleukin-2 (IL-2) decreases and cancer cells can escape the immunoreaction (Liu et al., 2021). This is supported by the transcriptomic studies of Shimizu and colleagues (2020) showing that PD-1 preferentially inhibits the upregulation of genes related to the effector function of T cells such as cytokine production, which in turn depends on the TCR signal strength (Shimizu et al., 2020). In summary, the PD-

1/PD-L1 signal transduction pathway strengthens the tumour's cellular immune tolerance through different mechanisms.

At the receptor level, attenuation of T cell activation has been described as phosphorylation of the intracellular tyrosines of PD-1 upon binding between PD-1 and either PD-L1 or PD-L2. This tag is recognized by Src homology-2 (SH2) domain-containing protein tyrosine phosphatase 2 (SHP-2) and the homologous Src homology-2 (SH2) domain-containing protein tyrosine phosphatase 1 (SHP-1) which promote dephosphorylation of both TCR and other costimulatory signalling components (Marasco et al., 2020). Similarly, with TCR signalling SHP-2 and SHP-1 can promote downstream inhibitory signaling (see **Figure 3**) (Freeman, 2008; Q. Wang et al., 2021). Consequently, not only the cytokine production decreases but a depletion of T cell proliferation is also seen (Q. Wang et al., 2021). Positive feedback enhances T cell inhibition upon TCR signalling by increasing the PD-1 expression on the T cell surface, overall blunting the antitumour function of T cells (Lei et al., 2020). Additionally, PD-L1 expression on tumour cells is upregulated by the release of IFN-ɣ from CD8+ T cells, which in turn is stimulated by repeated antigen presentation (Chen et al., 2019).

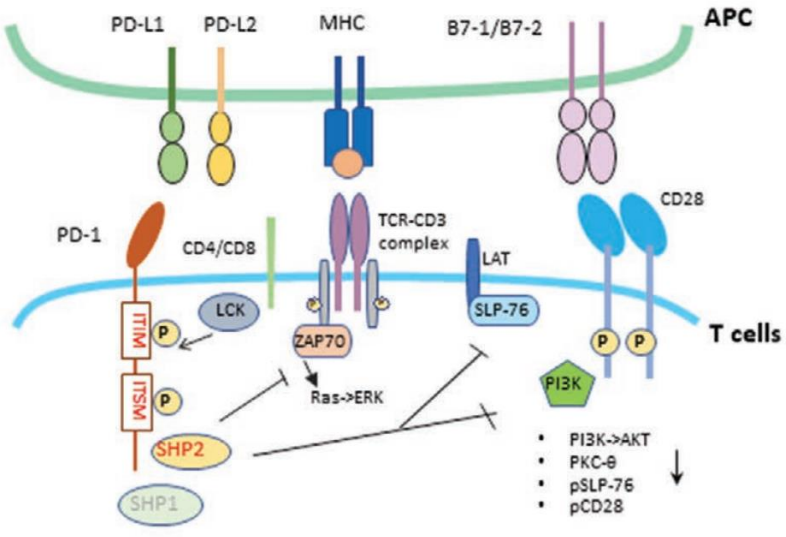

Figure 3. PD-1 signalling pathway. Upon PD-1/PD-L1 interaction, the recruited Src-family kinases, Lck and Fyn phosphorylate ITIM and ITSM tyrosines of the cytoplasmic tail of PD-1 (pPD-1). This phosphorylation is further recognized by SHP-2 and SHP-1 to dephosphorylate costimulatory signalling

components such as ZAP70, SLP-76, protein kinase C (PKC-θ), phosphatidylinositol-3-kinase (PI3K), and the Ras signalling pathway. Figure adapted from: Q. Wang et al., 2021.

T CELL EXHAUSTION

Although PD-1 expression is thoroughly controlled, as exhibited by the limited expression in naïve T-cells (Ghosh et al., 2021), during persistent antigen stimulation, like in chronic diseases and cancer, PD-1 expression is maintained leading to a progressive loss of T-cell functions, also known as T cell exhaustion (Simon & Labarriere, 2018; Q. Wang et al., 2021). In general, cytotoxicity, proliferation, and cytokine secretion in response to antigen stimulation are the main activities compromised in exhausted T cells (Sakuishi et al., 2010). However, PD-1 expression is not always associated with an exhausted phenotype and reversal of T cell exhaustion is not always reached when either blocking PD-1/PD-L1 interaction or through genetic ablation of PD-1. Therefore, evidence suggest that PD-1 might not be the dominant driver of T cell exhaustion (He & Xu, 2020; Sakuishi et al., 2010).

Consequently, regarding the PD-1/PD-L1 pathway, context is everything because factors such as T cell differentiation state, antigen burden, inflammation levels, metabolic state, and location, amongst others can impact the effector activity of PD-1 engagement (Kumar & Chamoto, 2021; Sharpe & Pauken, 2018; C. Wang et al., 2011). In addition to PD-1, other coinhibitory receptors, such as TIM3 and LAG3, contribute to T cell exhaustion (He & Xu, 2020). For instance, the research of Sakuishi and colleagues (2010) showed that in the tumour microenvironment (TME), PD-1+TIM3+ T cells show higher levels of exhaustion than PD-1+ TIM3- T cells in mice bearing solid tumours. Furthermore, a reduction in tumour growth by targeting TIM3 and PD-1 pathways using antibodies against TIM3 and PD-1 at the same time proved to be more effective than targeting just PD-1 pathway (Sakuishi et al., 2010). In conclusion, PD-1 and other extracellular receptors might function synergistically to induce and reinforce T cell exhaustion during long-term antigen exposure (He & Xu, 2020).

PD-1/PDL-1 PATHWAY COMPARED TO CTLA-4

As the knowledge around PD-1/PD-L1 pathway developed, the relevance on cancer immunotherapy was evident. Currently, several checkpoint inhibitors which block CTLA-4, PD-1 or PD-L1 have been proposed as cancer drugs (Lin et al., 2020; J. Liu et al., 2021; Zheng et al., 2019). Despite all being categorized as "immune checkpoints" it is worth mentioning that at a cellular level, they work different. PD-1/PD-L1 major physiological function appears to contribute to the negative feedback control of tissue inflammation and prevent the spread of inflammation (Francisco et al., 2010; Han, 2015). On the other hand, the leading role of CTLA-4 is to regulate T-cell responses to self-antigens (Han, 2015). Overall, those roles have been validated by research using mice lines with gene knockouts (KO). While both CTLA-4 KO and PD-1 KO mice are prone to autoimmune diseases, only CTLA-4 KO mice spontaneously develop massively infiltrating T cells to normal organs within weeks (Chambers et al., 1997; Nishimura et al., 1999). Regarding tumour immunology, another difference between CTLA-4 and PD-1 is that at preliminary stages CTLA-4 regulates T-cell response in the lymph nodes, while PD-1 limits T-cell activity in the TME at later stages of tumour growth (Dermani et al., 2019; Krummel & Allison, 1995).

IMMUNE CHECKPOINT THERAPEUTICS: PD-1/PD-L1 PATHWAY INHIBITORS

As mentioned before, during chronic infections and cancer, high and sustained expression of PD-1 and its ligands is common (Lee et al., 2015). The essential role of PD-1/PD-L1 in promoting a negative immune response was seized by Tasuku Honjo and James Allison to propose a line of research in cancer therapy through the blockade of this interaction, such achievement merited the Nobel Prize in 2018 (Huang & Chang, 2019). Nonetheless, before that, in 2011, Ipilimumab, the first antibody blocking an immune checkpoint (CTLA-4) was authorized. Since then, the development of other immune checkpoint inhibitors (ICI) flourished as exemplified by the launch of monoclonal antibodies targeting PD-1 (Pembrolizumab and Nivolumab) and PD-L1 (Atezolizumab and Durvalumab) a few years later. Overall, the cancer treatment breakthrough in the last decade has been driven largely by T cell targeted immunomodulators blocking the immune checkpoints CTLA-4 and PD-1 or PD-L1 (Robert, 2020).

Currently, therapies targeting PD-1/PD-L1 pathway (also called anti-PD therapy) are an active area of research either to treat cancer or viral infections (Sharpe & Pauken, 2018). Essentially, immunotherapy against the interaction between PD-1 and PD-L1 reinvigorates T cells, previously inactive due to the PD-1/PD-L1 signalling inhibition. Particularly for cancer, PD-1/PD-L1 blockade significantly enhanced antitumour effects in different solid tumours including melanoma, NSCLC, urothelial carcinoma, renal cell carcinoma, head and neck squamous cell carcinoma, and microsatellite instability–high colorectal cancer (Ai et al., 2020; Dermani et al., 2019; Guzik et al., 2019; Lei et al., 2020). On the other hand, although effective as exhibited by a durable anti-tumour activity, anti-CTLA-4 therapy tend to produce immune-related adverse events (IAEs) upon administration for different cancers. In that regard, PD-1/PD-L1 inhibitors outperform classical anti-CTLA-4 antibody therapy proving to be promising immunotherapeutic agents that can achieve satisfactory efficacy for different tumour types, different drug combinations and different treatment regimens and routes (Han, 2015; J. Liu et al., 2021).

Interestingly, anti-PD-1 drugs have been approved for the highly mutated cancers linked to a mismatch DNA repair deficiency (microsatellite instability) representing an unprecedent case for cancer treatment based not on the tumour type but on a biological oncologic mechanism. The evidence behind that decision suggests that highly mutated tumours have the potential of producing multiple neoantigens which seems favourable for ICI therapy response (Twomey & Zhang, 2021). This, in combination with the low reactivity of immune non-infiltration tumours to ICI therapy, led to the hypothesis that antigen recognition and migration of T cells to the tumour are key factors influencing response to ICI (Kim & Cho, 2022). However, as any other immunotherapy, inhibiting the PD-1/PD-L1 pathway can produce IAEs exhibited as immune-mediated inflammation of diverse organs or tissues due to the alteration of the physiological brake of immune activation (Robert, 2020).

There are many anti-PD-1/PD-L1 potential therapies relying on the physical blockade of the interaction between PD-1 and PD-L1 in research, they involve the use of antibodies and small molecules, either of peptide nature or not, which employ different mechanisms of action (summarised in **Figure 4**) (Liu et al., 2021). In the following pages, this review will focus on

reporting the information available about the characteristics and mechanism of action of those therapeutics.

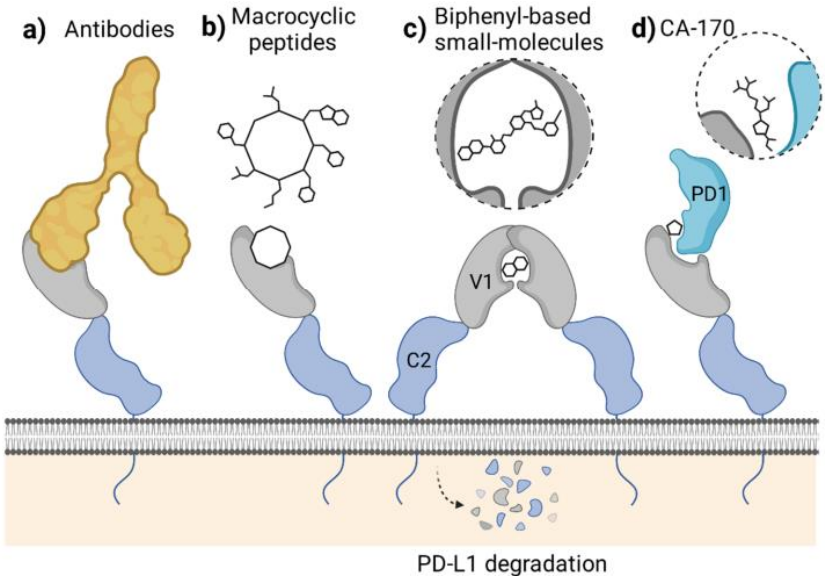

Figure 4. Mechanisms of PD-1/PD-L1 blockade attributed to different classes of molecules targeting the extracellular domain of PD-L1. Mechanisms such as antagonism of anti-PD-L1 antibody (a), macrocyclic peptides (b), small-molecule-induced PD-L1 dimerization and degradation (c), and formation of a defective ternary complex between CA-170, PD-1, and PD-L1 (d). Figure from Surmiak et al., 2021.

MONOCLONAL ANTIBODIES

Anti-PD antibodies have become some of the most widely prescribed anticancer therapies and function by binding to either PD-1 or PD-L1 (**Figure 4a**) (Robert, 2020). In general, monoclonal antibodies (mAb), at the right doses, can significantly reduce the solid tumour size, as well as inhibit advanced tumours and metastases, thereby improving the overall survival in patients (Brahmer et al., 2012; Horn et al., 2018; Rizvi et al., 2015). In general, adverse effects of these therapeutics include but are not limited to fatigue/weakness, nausea, cough, dyspnoea, decreased appetite and infection and even infusion-related reactions (Ai et al., 2020; Brahmer et al., 2012). **Table 1** summarizes the mAb approved by the FDA (Food and Drug Administration) to treat distinct types of cancers and their mechanism of action. The therapeutics available either target PD-1 or PD-L1 by binding to different antigenic epitopes. In general, mAb have IC_{50} values around 0.2 nM when studied with biochemical methods such as homogenous time-resolved

fluorescence (HTRF) assays (Surmiak et al., 2021). Because of their high specificity and affinity for their targets, mAb have been a central therapeutic method for decades (Costa & Vale, 2022).

Despite that, multiple alternatives to the use of mAb are sought by researchers because mAb are expensive, complex to produce, and difficult to store and transport (Liu et al., 2021). Although PD-1/PD-L1 antibodies have shown activity in more than 20 tumour types, only a subset of patients will respond to their treatment (Raghavan et al., 2021). Common mechanisms for drug resistance of different PD-1 or PD-L1 inhibitors will be reviewed later, but to exemplify one of the reasons for the failure of anti-PD-1/PD-L1 therapeutics in cancer it is relevant the activity of macrophages in the TME. Evidence suggests that macrophages in the TME capture and remove the PD-1 antibody bound on $CD8^+$ T cells through FcγRIIB/III interactions making the PD-1 available to interact with its ligan and then allow tumour growth (Kim & Cho, 2022; Raghavan et al., 2021).

Table 1. Monoclonal antibodies approved by the FDA to treat several types of cancers.

Target	Therapeutic agent	Class	Treatment	Mechanism of action	Reference
PD-1	Nivolumab (MDX-1106 or BMS-936558 or OPDIVO)	Human IgG4	Melanoma NSCLC Head and neck cancer Renal-cell carcinoma	Blocks the interaction between the PD-1 receptor and its two known ligands by binding to the PD-1 receptor, thereby releasing the immune response inhibition mediated by PD-1 pathway, including anti-tumour immune response.	(Ai et al., 2020; Dermani et al., 2019; Twomey & Zhang, 2021)
	Pembrolizumab (MK3475 or KEYTRUDA)	Humanized IgG4	Melanoma Breast cancer NSCLC	Blocks the PD-1/PD-L1 pathway by binding to the PD-1 receptor, leading to a physiological shift to immune reactivity and anti-tumour effect.	(Ai et al., 2020; Dermani et al., 2019; Ghosh et al., 2021; Twomey & Zhang, 2021)
	Cemiplimab	Human IgG4	Metastatic cutaneous squamous cell carcinoma	Binds to PD-1 receptor and blocks its interaction with PD-L1, thus, Cemiplimab up-regulates cytotoxic T cells and enhances the antitumor activity of the immune system.	(Ai et al., 2020; Ghosh et al., 2021; Twomey & Zhang, 2021)
	Dostarlimab (TSR-042 or Jemperli)	Humanized IgG4	Recurrent or advanced endometrial cancer Mismatch repair deficient	Binds to PD-1 on T cells and blocks interactions with its ligands PD-L1 and PD-L2, activating immune responses.	(Costa & Vale, 2022)
	Retifanlimab (POD1UM-201 or ZYNYZ)	Humanized IgG4κ	Metastatic or recurrent locally advanced Merkel Cell Carcinoma	Binds to PD-1 and prevents the interaction between PD-1 and its ligands, aiming to sustain/restore T-cell antitumor function.	(Rao et al., 2022)
PD-L1	Atezolizumab (MPDL-3280A or TECENTRIQ)	Human IgG1	Urothelial carcinoma	By specifically binding to PD-L1, Atezolizumab blocks the interaction of PD-L1 with PD-1 and CD80 receptors (B7-1). However, PD-1 can still interact with its alternative ligand, PD-L2, while eliminating the inhibitory effect on cytotoxic T cells.	(Ai et al., 2020; Dermani et al., 2019; Ghosh et al., 2021; Twomey & Zhang, 2021)
	Durvalumab (MEDI4736)	Humanized IgG1	Urothelial carcinoma NSCLC	By binding with PD-L1 blocks the binding with PD-1 or CD80 so that T cells can recognize and kill tumour cells.	(Ai et al., 2020; Dermani et al., 2019; Ghosh et al., 2021; Twomey & Zhang, 2021)
	Avelumab (MSB0010718C)	Humanized IgG1	Ovarian cancer Breast cancer NSCLC	Inhibits PD-1/PD-L1 interactions while maintaining the integrity of the PD-1/PD-L2 pathway. On top of enhancing immune activation, due to its inherent Fc domain, Avelumab can induce NK-mediated antibody-dependent cytotoxicity (ADCC) *in vitro*.	(Ai et al., 2020; Dermani et al., 2019; Ghosh et al., 2021; Twomey & Zhang, 2021)

SMALL MOLECULES

Pharmacokinetic limitations of mAb therapies, such as poor drug diffusion and intravenous administration (lack of oral bioavailability), have led scientist to develop other cancer drugs. Additionally, moving towards small molecule inhibitors generally improves oral bioavailability and tumour penetration, which can result in a higher response rate (Ryman & Meibohm, 2017). Finally, manufacturing costs tend to be less expensive, and it is likely that small molecules are more stable (Ai et al., 2020). Inhibitors of the PD-1/PD-L1 pathway come in different formats but in general aim to reduce the drug half-life to fit flexible treatment schemes, thereby avoiding immune adverse effects. Nevertheless, so far, no small molecule inhibitor has achieved the same inhibitory activity of the PD-1/PD-L1 axis as mAb have (X. Wu et al., 2021). In the subsequent sections information on various non-antibody PD-1/PD-L1 ICI will be discussed.

PEPTIDES

Immune checkpoint peptides and peptidomimetics have been called the "binding bridge" between antibodies and small-molecular inhibitors. Particularly, the first multipeptide human PD-1 (hPD-1) inhibitor, AUNP-12, set ground for further macromolecules. AUNP-12 is a 29-amino acid branching peptide that proved reduction in tumour cell growth and metastasis with an animal model, and only a few adverse reactions were observed (Guzik et al., 2019; C. Liu et al., 2021). On the other hand, macrocyclic peptides, similarly to antibodies, can restore the function of T cells by directly binding to PD-L1 (**Figure 4b**) (Magiera-Mularz et al., 2017). Overall, macrocyclic peptides have also produced promising PD-1/PD-L1 pathway inhibition results as seen from IC_{50} (half-maximal inhibitory concentration) values around 10 nM from homogenous time-resolved fluorescence (HTRF) assay and EC_{50} (half-maximal effective concentration) between 300-500 nM from Immune Checkpoint Blockade (ICB) assays (J. Liu et al., 2021; Surmiak et al., 2021; X. Wu et al., 2021).

The case of inhibitor CA-170 is interesting for several reasons, one of them is that CA-170 does not produce obvious adverse reactions at high concentration at which mAb normally do but activate T cells in a comparable way. Additionally, its structure has not been disclosed despite advancing to clinic-stage research. CA-170 is presumed to be a class of 1,2,4-oxadiazole or 1,3,4-

oxadiazole, and the substituted side chain in such compounds is an amino acid residue base. It is a dual inhibitor of oral anti-V-domain immunoglobulin suppressor of T cell activation (VISTA) and PD-L1 (Surmiak et al., 2021; Q. Wu et al., 2021). Preclinical data indicated that CA-170 could significantly increase the proliferation and activation of T cells in the TME in an immunocompetent mouse tumour model (Sasikumar et al., 2021). Despite the controversial data suggesting that CA-170 did not bind to PD-L1 but still had a positive effect on T cell activation (Musielak et al., 2019), it has been shown that even though CA-170 binds to PD-L1 in the cellular context, there is no dissociation of the PD-1:PD-L1 complex, instead a defective ternary complex has been proposed as the mechanism of action of CA-170 (**Figure 4d**) (Sasikumar et al., 2021).

Other strategies include inhibiting the sustained, tonic, intracellular PD-1 signalling that continues to mediate T cell suppression even after immune checkpoint blockade therapy targeting PD-1. Fernandez and colleagues (2020) developed a hetero-bispecific diabody that binds to the CD45 and PD-1 extracellular domains to compel cis-ligation and favour the dephosphorylation of tyrosine residues in thePD-1 cytoplasmic tail. Impairing this segment of the signalling cascade reduced tumour burden *in vivo* in murine models of small cell lung cancer and colon adenocarcinoma, but it might pose a critical threat to immune homeostasis (Fernandes et al., 2020).

NON-PEPTIDE INHIBITORS

The disclosure of the Bristol-Myers Squibb (BMS) inhibitors provided a favourable starting point for the development of other non-peptide small molecule inhibitors (J. Liu et al., 2021). BMS inhibitors are grouped into the biphenyl-based small molecules group because they share a core group including a biphenyl moiety or a 2,3-dihydro-1,4-benzodioxine substituted phenyl moiety. The core group is linked to a central aryl group via a benzyl ether bond, and on the other extreme the compounds possess a tail group, which may form polar interactions with the PD-L1 protein to further stabilize it into a homodimer. The hydrophobic pocket formed by dimeric PD-L1, where the inhibitor resides upon interaction, is of great interest for drug discovery due to its degree of flexibility, in terms of the inhibitor binding site (X. Wu et al., 2021).

As introduced above, the proposed mechanism for suppressing the activation of PD-1 by biphenyl-based compounds is PD-L1 dimerization (J. Liu et al., 2021). While some compounds reduce the availability of PD-L1 in the cell surface by internalising and degrading PD-L1 (**Figure 4c**) (Lai et al., 2022; T. Wang et al., 2022), other compounds have shown a different mechanism in which newly synthesised PD-L1 molecules are retained and dimerized in the endoplasmic reticulum, aside from dimerization happening in cell surface (Chai et al., 2022). In terms of biochemical activity, HTRF results show that BMS inhibitors display a wide range of inhibition of the PD-1/PD-L1 interaction. Based on the former technique, the IC_{50} has been reported for BMS-8 (146 nM), BMS-202 (18 nM), BMS-200 (80 nM), BMS-1166 (1.4 nM) and BMS-1001 (2.25 nM) (Lin et al., 2020; Surmiak et al., 2021).

As part of the research line, the biological activity of BMS inhibitors has been studied with metabolic activity assay in modified Jurkat T cells. The results demonstrated that BMS-1166 and BMS-1001 displayed low toxicity with EC_{50} values of 40.5 and 33.4 µM, respectively (Skalniak et al., 2017). Conversely, Surmiak and colleagues (2021) reported an EC_{50} value of 1574 nM for BMS-1166 using the same cell-based assay. Nonetheless, differences in the employed parameters to calculate the EC_{50} could account for a certain degree of variability (Surmiak et al., 2021). As opposed to BMS-1166 and BMS-1001, compounds BMS-8 and BMS-200 were more toxic; which points out the relevance of cell toxicity assays during inhibitor development (Skalniak et al., 2017). Finally, even though small inhibitors targeting immune checkpoints have proven potential for cancer immunotherapy, efficacy and safety remains a concern for clinical testing in large numbers of cancer patients (Lai et al., 2022).

COMBINATION THERAPIES

Looking for a synergistic effect and to overcome resistance to anti-PD therapy, combination therapies led to a series of clinical trials. Particularly, dual blockade of CTLA-4 and PD-1 showed to increase the overall survival of patients. Nevertheless, this approach turned out to produce severe systemic toxicity (Chae et al., 2018). At least until 2020, the anti-PD therapy clinical trial landscape seemed broad and bright; with around 80% of three thousand clinical trials involving combination therapy. Anti-PD therapy has been combined with 253 distinct drug-target groups

including immuno-oncology therapies (e.g., CTLA-4, oncolytic viruses, cancer vaccines, other immune inhibitory molecules), targeted therapies (e.g., VEGF), chemotherapy and radiotherapy (Vesely et al., 2022). Rather than choosing a mix of immune components that inhibit cancer progression, careful and informed decisions are a priority. For instance, while localized radiation can enhance the activity of anti-PD therapy by inducing inflammation, for patients with significant infiltration in the tumour this is contraindicated since this may impair ongoing immune responses (Han, 2015).

ANTI-PD-1 CANCER IMMUNOTHERAPY RESISTANCE

Unfortunately, the percentage of patients experiencing a long-term and sustained positive effect varies between 10-30% upon PD-1/PD-L1 inhibitors treatment (J. Liu et al., 2021). Raghavan and colleagues have summarized into three paths for tumour immunity escape after the PD-1 or PD-L1 blockade. First, the inherent low immunogenicity of the tumour should be considered. Secondly, due to impaired T-cell trafficking and retention of T cells in the tumour, immune response might be truncated (also known as T cell exclusion in **Figure 5**). Finally, inhibitory factors expressed in the TME could prevent T-cell activation (Raghavan et al., 2021).

As seen in **Figure 5**, the low immunogenicity of some tumours can be explained by defects in antigen-presenting and impairment of the interferon signalling pathway. Low levels of PD-L1 and/or disperse expression patterns in tumour tissues (i.e., an expression often only adjacent to the IFN-γ–producing TILs) directly promotes resistance to the therapy (Vesely et al., 2022). Additionally, it is plausible that a high and sustained cancer-antigen exposition could revert the effector functions of reinvigorated T cells after PD-1 blockade since this has been observed in a model of chronic lymphocytic choriomeningitis virus infection. In this model, even upon antigen clearance, cells failed to become T memory cells (Pauken et al., 2016). In addition to this, other coinhibitory signals in TME can boost the resistance mechanism (**Figure 5**). For instance, overexpression of receptors such as LAG-3, VISTA/PD-1H and TIM-3 has been associated with resistance to anti-PD-1 therapy (Hernandez-Martinez et al., 2018).

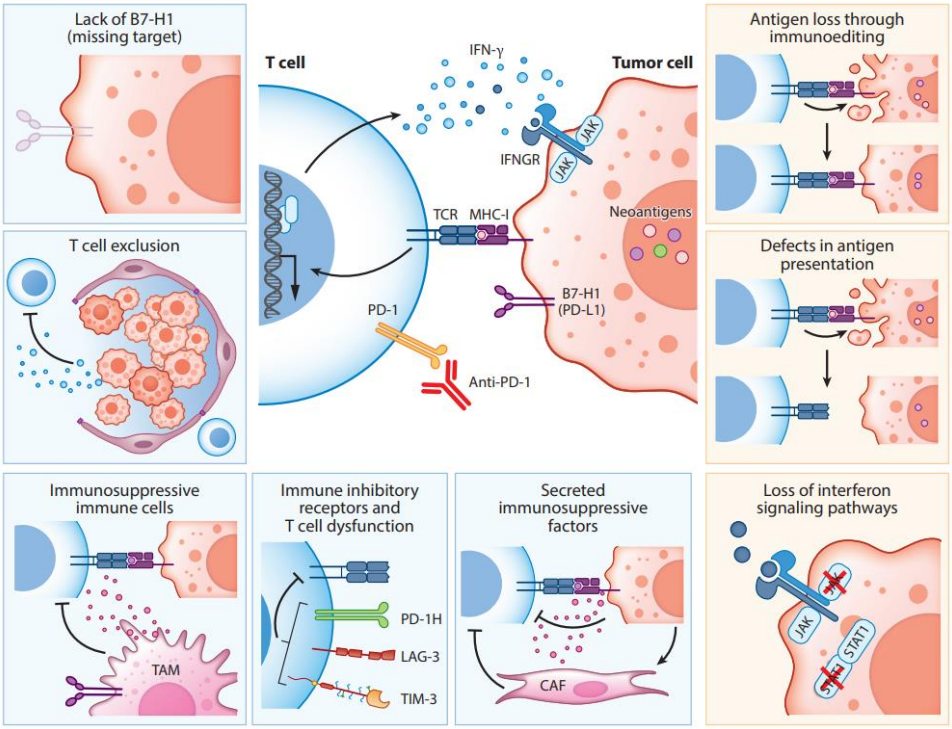

Figure 5. Mechanisms of immune-mediated resistance to anti-PD therapy. T cells are represented in blue whilst cancer cells are colored red. In the center, the expected effective antitumor immune response by a T cell, during anti-PD therapy, is depicted. Diagrams on the outer layer show mechanisms through which cancer cells evade immune attack. Figure adapted from Vesely et al., 2022.

In addition, several articles have highlighted the segregation between microbiomes from responders and non-responders to anti-PD therapy in different cancer cells (Najafi et al., 2022; Routy et al., 2018) and an improvement in the responsiveness to anti-PD therapy has been reported after faecal microbiota transplant (Davar et al., 2021). Therefore, further research on the mechanisms by which microbiota regulates the resistance to therapy is needed. Broadening the landscape for tumour immunotherapy resistance, epigenetics has drawn researchers' attention. Rather than global epigenetic modifications driving resistance to anti-PD therapy, it is likely that specific epigenetic modifications are responsible (Vesely et al., 2022). For instance, it has been shown that exhausted T cells from a viral infection, which can relate to T cells in the TME, display an epigenetic profile different from that of active T cells or memory T cells (Pauken et al., 2016). In addition, T-cell differentiation and immune signalling-protein expression have been associated with epigenetic regulatory mechanisms (Vesely et al., 2022).

The complexity of cancer becomes evident by the fact that TMEs of advanced cancers are very heterogeneous. Heterogeneity in the resistance mechanisms of tumours encompasses variability between individuals and across time (Vesely et al., 2022). However, some strategies have been proposed to avert the resistance to anti-PD therapy. For instance, by administrating FcR-blocking antibodies (targeting TME macrophages) in combination with PD-1 mAb to tumour-bearing mice, complete tumour regression was achieved (Raghavan et al., 2021). Nonetheless, with the current technology progress shifts towards a holistic view of the TME. For example, Ciccolini and colleagues (2020) worked on an immune algorithm aiming to predict anti-PD-1/PD-L1 primary and adaptive resistance to classify patients before ICI treatment (Ciccolini et al., 2020). On the other hand, imaging strategies such as the multiplexed ion beam imaging by time-of-flight (MIBI-TOF) allowed Keren and colleagues (2018) to quantify *in situ* protein functional networks (including PD-1 and PD-L1) to describe tumour organization. Overall, through these advanced technologies, patients may eventually receive more effective and targeted cancer treatments (Keren et al., 2018).

IMMUNE CHECKPOINT BLOCKADE ASSAYS

The performance of the variety of ICI mentioned above can be tested through different assays that aim to decipher the molecule properties at various levels. Common methods employed by PD-1/PD-L1 pathway inhibitors are condensed in **Figure 6**. During this review, only a couple of them will be addressed following the author's interest. Biochemical and physicochemical analyses are useful for assessing small molecule binding profiles and for the optimization of multiple screening of potential inhibitors. On the other hand, *in vitro* cell-based assays and *in vivo* tumour xenograft models give information on the functional properties of the tested molecules (C. Liu et al., 2021). Nonetheless, each method requires acknowledging its limitations and controlling as many factors as possible. For instance, when defining the right model for the *in vivo* evaluation of the bioactivity of an anti-PD-1/PD-L1 drug prospect, it must be considered the interspecies specificity towards the molecular target, since the mouse and human PD-L1 analogues differ in their druggability profiles, despite the similarity in the structure and sequence (Surmiak et al., 2021).

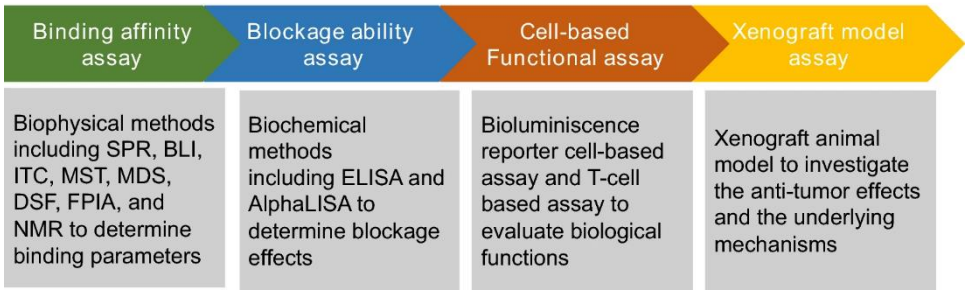

Figure 6. The screening workflow of PD-1/PD-L1 inhibitors. The characterization of PD-1/PD-L1 inhibitors can be achieved through a series of assays including binding affinity assay, blockage ability assay, cell-based functional assay, and xenograft model assay. Figure adapted from C. Liu et al., 2021.

BIOCHEMICAL ASSAYS

HTRF is a popular technique for the initial screening of putative hits against PD-1/PD-L1 systems. Essentially, it measures fluorescent intensity resulting from fluorescence resonance energy transfer (FRET) when two fluorophore-labelled proteins are in proximity (<8-9 nm). The presence of binding agents interfering with the interaction of a protein complex (PD-1 and PD-L1 in this case) results in the loss of fluorescence (Guzik et al., 2019; Surmiak et al., 2021). The resultant parameter that describes the compound potency, IC_{50}, is highly dependent on both the target protein and ligand concentration as well as the process environment. Therefore, there is a general trend to move towards other, more accurate and comparable, parameters to determine the affinity of the potential inhibitors, such as apparent Ki or inhibitory constant. Additionally, with this technique, no conclusions about the compound's mechanism of action can be established (Surmiak et al., 2021).

Furthermore, a better understanding of the complexity inside and around the cells upon PD-1/PD-L1 inhibitor treatment can be achieved by other types of assays. Because of post-translational modifications of the receptors such as glycosylation, molecular crowding effect, compound solubility problems (due to compound hydrophobicity), along with unspecific toxicity (due to limited tolerance to DMSO), the concentrations required for inhibitor-bioactivity in cell-based assays increase (Surmiak et al., 2021).

CELL-BASED ASSAYS

In a cellular context, mouse splenocyte proliferation assays (MSPA) have been widely used. This assay takes advantage of the PD-L1 mediated inhibition of the proliferation of splenocytes stimulated with anti-CD3/CD28 via interacting with PD-1 expressed by splenocytes. Thus, a rescue of the splenocyte proliferation is expected when treated with compounds that could effectively disrupt the PD-1/PD-L1 interaction (Jiao et al., 2018). However, it does not have to be hPD-1/hPD-L1 specific due to its complex nature and can lead to contrasting results (C. Wu et al., 2021). Additionally, the immune checkpoint blockade (ICB) of the PD-1/PD-L1 interaction can be assessed by co-culturing cells expressing PD-1 and PD-L1 separately. High-throughput screening has driven the commercialisation of this type of assay and has proven to be a labour- and time-efficient tool (C. Liu et al., 2021).

For instance, Promega (USA) has a PD-1/PD-L1 Blockade Bioassay consisting of artificial antigen-presenting CHO-K1 cells overexpressing PD-L1 and a TCR Activator protein (CHO/TCRAct/PD-L1 cells), and Jurkat T cells overexpressing PD-1 and luciferase gene controlled by NFAT Response Element (Jurkat Effector Cells, Jurkat-ECs). Hence, when cells interact with each other, TCR Activator binds to TCR, activating Jurkat-ECs which leads to the activation of the NFAT transcription factor. Then, a luciferase gene, controlled by NFAT, becomes transcribed and translated. When the luciferase substrate is added, the resultant luminescent signal is the readout in the experiment. However, PD-1 in Jurkat T cells can still bind to PD-L1 in CHO-K1 cells; only upon ICI treatment, the inhibitory activity of PD-1/PD-L1 is blocked (i.e., Jurkat-ECs activation is restored) (Surmiak et al., 2021). The activity of luciferase is an indicator of cell activation but cannot be associated with antigen-specific or multiparametric interactions, neither can evaluate the functions of PD-1/PD-L1 inhibitors on the signalling transduction-related proteins by themselves (C. Liu et al., 2021).

PERSPECTIVES

Currently, the broad role of the PD-1/PD-L1 axis on immune homeostasis is well established. However, there are some open questions around what has been named as the PD-1 interactome, which involves all the molecules that interact with PD-1, or even the implications on effector functions in different subsets of immune cells. Regarding therapeutics, anti-PD therapy has the advantage of modulating immune responses at the tumour site, targeting tumour-induced immune defects, and also reinvigorating ongoing immune responses. Additionally, it has proven to be very versatile since numerous experimental studies show clinical efficacy in a wide range of solid tumours. Although there are still many challenges in the development of PD-1/PD-L1 inhibitors, the ICI landscape keeps evolving and continuously looks to overcome the immunotherapy resistance mechanisms, for instance, by employing combinatory therapies. To understand the reason for anti-PD therapy resistance an integrated approach should be taken by considering cell metabolism, epigenetics, cell signalling pathways and human physiology. As we discussed here, different assays can be used to investigate the activity of potential inhibitors, and it is encouraged to complement biochemical results with relevant physiological models while acknowledging limitations at each step. Finally, it seems that scientists in the field have directed their efforts to unveil cancer biomarkers and develop personalized immunotherapy, and in doing so, PD-1/PD-L1 axis represents a cornerstone.

ACKNOWLEDGEMENTS

The author would like to thank PhD James Chamberlain from Charnwood Molecular Ltd., and Professor Stephen Harding the Head of the National Centre for Macromolecular Hydrodynamics (NCMH) University of Nottingham, for their assist during the period of this work.

REFERENCES

Ai, L., Chen, J., Yan, H., He, Q., Luo, P., Xu, Z., & Yang, X. (2020). Research status and outlook of pd-1/pd-l1 inhibitors for cancer therapy. In *Drug Design, Development and Therapy* (Vol. 14, pp. 3625–3649). Dove Medical Press Ltd. https://doi.org/10.2147/DDDT.S267433

Akinleye, A., & Rasool, Z. (2019). Immune checkpoint inhibitors of PD-L1 as cancer therapeutics. *Journal of Hematology & Oncology*, *12*(1), 92. https://doi.org/10.1186/s13045-019-0779-5

Brahmer, J. R., Tykodi, S. S., Chow, L. Q. M., Hwu, W.-J., Topalian, S. L., Hwu, P., Drake, C. G., Camacho, L. H., Kauh, J., Odunsi, K., Pitot, H. C., Hamid, O., Bhatia, S., Martins, R., Eaton, K., Chen, S., Salay, T. M., Alaparthy, S., Grosso, J. F., … Wigginton, J. M. (2012). Safety and Activity of Anti–PD-L1 Antibody in Patients with Advanced Cancer. *New England Journal of Medicine*, *366*(26), 2455–2465. https://doi.org/10.1056/nejmoa1200694

Chae, Y. K., Arya, A., Iams, W., Cruz, M. R., Chandra, S., Choi, J., & Giles, F. (2018). Current landscape and future of dual anti-CTLA4 and PD-1/PD-L1 blockade immunotherapy in cancer; lessons learned from clinical trials with melanoma and non-small cell lung cancer (NSCLC). *Journal for ImmunoTherapy of Cancer*, *6*(1). https://doi.org/10.1186/s40425-018-0349-3

Chai, I., Kornyeyev, D., Hsieh, E., Magombedze, G., Stapleton, L., Hung, M., Kwon, H. J., Stefanutti, E., Belzile, J. P., Czerwieniec, G., Wang, A. Y., Morar, M., & Lad, L. (2022). Effects of small molecule-induced dimerization on the programmed death ligand 1 protein life cycle. *Scientific Reports*, *12*(1). https://doi.org/10.1038/s41598-022-25417-6

Chambers, C. A., Sullivan, T. J., & Allison, J. P. (1997). Lymphoproliferation in CTLA-4–Deficient Mice Is Mediated by Costimulation-Dependent Activation of CD4+ T Cells. *Immunity*, *7*(6), 885–895. https://doi.org/https://doi.org/10.1016/S1074-7613(00)80406-9

Chen, S., Crabill, G. A., Pritchard, T. S., McMiller, T. L., Wei, P., Pardoll, D. M., Pan, F., & Topalian, S. L. (2019). Mechanisms regulating PD-L1 expression on tumor and immune cells. *Journal for ImmunoTherapy of Cancer*, *7*(1). https://doi.org/10.1186/s40425-019-0770-2

CHIU, Y.-M., TSAI, C.-L., JUNG-TA, K. A. O., HSIEH, C.-T., SHIEH, D.-C., YI-JU, L. E. E., TSAY, G. J., CHENG, K.-S., & YI-YING, W. U. (2018). PD-1 and PD-L1 Up-regulation Promotes T-cell Apoptosis in Gastric Adenocarcinoma. *Anticancer Research*, *38*(4), 2069. http://ar.iiarjournals.org/content/38/4/2069.abstract

Ciccolini, J., Benzekry, S., & Barlesi, F. (2020). Deciphering the response and resistance to immune-checkpoint inhibitors in lung cancer with artificial intelligence-based analysis: when PIONeeR meets QUANTIC. *British Journal of Cancer*, *123*(3), 337–338. https://doi.org/10.1038/s41416-020-0918-3

Costa, B., & Vale, N. (2022). Dostarlimab: A Review. In *Biomolecules* (Vol. 12, Issue 8). MDPI. https://doi.org/10.3390/biom12081031

Davar, D., Dzutsev, A. K., McCulloch, J. A., Rodrigues, R. R., Chauvin, J.-M., Morrison, R. M., Deblasio, R. N., Menna, C., Ding, Q., Pagliano, O., Zidi, B., Zhang, S., Badger, J. H., Vetizou, M., Cole, A. M., Fernandes, M. R., Prescott, S., Costa, R. G. F., Balaji, A. K., … Zarour, H. M. (2021). Fecal microbiota

transplant overcomes resistance to anti–PD-1 therapy in melanoma patients. *Science*, *371*(6529), 595–602. https://doi.org/10.1126/science.abf3363

Dermani, F. K., Samadi, P., Rahmani, G., Kohlan, A. K., & Najafi, R. (2019). PD-1/PD-L1 immune checkpoint: Potential target for cancer therapy. *Journal of Cellular Physiology*, *234*(2), 1313–1325. https://doi.org/https://doi.org/10.1002/jcp.27172

Dong, H., Strome, S. E., Salomao, D. R., Tamura, H., Hirano, F., Flies, D. B., Roche, P. C., Lu, J., Zhu, G., Tamada, K., Lennon, V. A., Celis, E., & Chen, L. (2002). Tumor-associated B7-H1 promotes T-cell apoptosis: A potential mechanism of immune evasion. *Nature Medicine*, *8*(8), 793–800. https://doi.org/10.1038/nm730

Fernandes, R. A., Su, L., Nishiga, Y., Ren, J., Bhuiyan, A. M., Cheng, N., Kuo, C. J., Picton, L. K., Ohtsuki, S., Majzner, R. G., Rietberg, S. P., Mackall, C. L., Yin, Q., Ali, L. R., Yang, X., Savvides, C. S., Sage, J., Dougan, M., & Garcia, K. C. (2020). Immune receptor inhibition through enforced phosphatase recruitment. *Nature*, *586*(7831), 779—784. https://doi.org/10.1038/s41586-020-2851-2

Francisco, L. M., Sage, P. T., & Sharpe, A. H. (2010). The PD-1 pathway in tolerance and autoimmunity. In *Immunological Reviews* (Vol. 236, Issue 1, pp. 219–242). https://doi.org/10.1111/j.1600-065X.2010.00923.x

Freeman, G. J. (2008). *Structures of PD-1 with its ligands: Sideways and dancing cheek to cheek*. www.pnas.orgcgidoi10.1073pnas.0805459105

Furuse, M., Kuwabara, H., Ikeda, N., Hattori, Y., Ichikawa, T., Kagawa, N., Kikuta, K., Tamai, S., Nakada, M., Wakabayashi, T., Wanibuchi, M., Kuroiwa, T., Hirose, Y., & Miyatake, S.-I. (2020). PD-L1 and PD-L2 expression in the tumor microenvironment including peritumoral tissue in primary central nervous system lymphoma. *BMC Cancer*, *20*(1), 277. https://doi.org/10.1186/s12885-020-06755-y

Ganesan, A., Ahmed, M., Okoye, I., Arutyunova, E., Babu, D., Turnbull, W. L., Kundu, J. K., Shields, J., Agopsowicz, K. C., Xu, L., Tabana, Y., Srivastava, N., Zhang, G., Moon, T. C., Belovodskiy, A., Hena, M., Kandadai, A. S., Hosseini, S. N., Hitt, M., … Barakat, K. (2019). Comprehensive in vitro characterization of PD-L1 small molecule inhibitors. *Scientific Reports*, *9*(1), 12392. https://doi.org/10.1038/s41598-019-48826-6

Ghosh, C., Luong, G., & Sun, Y. (2021). A snapshot of the PD-1/PD-L1 pathway. *J Cancer*, *12*, 2735–2746. https://doi.org/10.7150/jca.57334

Guzik, K., Tomala, M., Muszak, D., Konieczny, M., Hec, A., Błaszkiewicz, U., Pustuła, M., Butera, R., Dömling, A., & Holak, T. A. (2019). Development of the inhibitors that target the PD-1/PD-L1 interaction—a brief look at progress on small molecules, peptides and macrocycles. In *Molecules* (Vol. 24, Issue 11). MDPI AG. https://doi.org/10.3390/molecules24112071

Han, L. C. A. N. D. X. (2015). Anti–PD-1/PD-L1 therapy of human cancer: past, present, and future. *The Journal of Clinical Investigation*, *125*(9), 3384–3391. https://doi.org/10.1172/JCI80011

He, X., & Xu, C. (2020). PD-1: A Driver or Passenger of T Cell Exhaustion? In *Molecular Cell* (Vol. 77, Issue 5, pp. 930–931). Cell Press. https://doi.org/10.1016/j.molcel.2020.02.013

Heng, D. Y. C. (2011). Next-generation immunotherapy: CTLA-4 and PD-1 inhibitors. In *Targeted Therapies for Renal Cell Carcinoma* (pp. 58–66). Future Medicine Ltd. https://doi.org/doi:10.2217/ebo.11.45

Hernandez-Martinez, J. M., Vergara, E., Zatarain-Barrón, Z. L., Barrón-Barrón, F., & Arrieta, O. (2018). Vista/PD-1H: A potential target for non-small cell lung cancer immunotherapy. In *Journal of Thoracic Disease* (Vol. 10, Issue 12, pp. 6378–6382). AME Publishing Company. https://doi.org/10.21037/jtd.2018.11.39

Horn, L., Gettinger, S. N., Gordon, M. S., Herbst, R. S., Gandhi, L., Felip, E., Sequist, L. V, Spigel, D. R., Antonia, S. J., Balmanoukian, A., Cassier, P. A., Liu, B., Kowanetz, M., O'Hear, C., Fassò, M., Grossman, W., Sandler, A., & Soria, J.-C. (2018). Safety and clinical activity of atezolizumab monotherapy in metastatic non-small-cell lung cancer: final results from a phase I study. *European Journal of Cancer*, *101*, 201–209. https://doi.org/https://doi.org/10.1016/j.ejca.2018.06.031

Huang, P. W., & Chang, J. W. C. (2019). Immune checkpoint inhibitors win the 2018 Nobel Prize. In *Biomedical Journal* (Vol. 42, Issue 5, pp. 299–306). Elsevier B.V. https://doi.org/10.1016/j.bj.2019.09.002

Ishida, Y., Agata, Y., Shibahara, K., & Honjo, T. (1992). Induced expression of PD-1, a novel member of the immunoglobulin gene superfamily, upon programmed cell death. *The EMBO Journal*, *11*(11), 3887–3895. https://doi.org/https://doi.org/10.1002/j.1460-2075.1992.tb05481.x

Jiao, P., Geng, Q., Jin, P., Su, G., Teng, H., Dong, J., & Yan, B. (2018). Small Molecules as PD-1/PD-L1 Pathway Modulators for Cancer Immunotherapy. *Current Pharmaceutical Design*, *24*(41), 4911–4920. https://doi.org/10.2174/1381612824666181112114958

Keren, L., Bosse, M., Marquez, D., Angoshtari, R., Jain, S., Varma, S., Yang, S. R., Kurian, A., Van Valen, D., West, R., Bendall, S. C., & Angelo, M. (2018). A Structured Tumor-Immune Microenvironment in Triple Negative Breast Cancer Revealed by Multiplexed Ion Beam Imaging. *Cell*, *174*(6), 1373-1387.e19. https://doi.org/10.1016/j.cell.2018.08.039

Kim, S. K., & Cho, S. W. (2022). The Evasion Mechanisms of Cancer Immunity and Drug Intervention in the Tumor Microenvironment. *Frontiers in Pharmacology*, *13*. https://doi.org/10.3389/fphar.2022.868695

Kleffel, S., Posch, C., Barthel, S. R., Mueller, H., Schlapbach, C., Guenova, E., Elco, C. P., Lee, N., Juneja, V. R., Zhan, Q., Lian, C. G., Thomi, R., Hoetzenecker, W., Cozzio, A., Dummer, R., Mihm, M. C., Flaherty, K. T., Frank, M. H., Murphy, G. F., … Schatton, T. (2015). Melanoma Cell-Intrinsic PD-1 Receptor Functions Promote Tumor Growth. *Cell*, *162*(6), 1242–1256. https://doi.org/https://doi.org/10.1016/j.cell.2015.08.052

Krummel, M. F., & Allison, J. P. (1995). CD28 and CTLA-4 have opposing effects on the response of T cells to stimulation. *Journal of Experimental Medicine*, *182*(2), 459–465. https://doi.org/10.1084/jem.182.2.459

Kumar, A., & Chamoto, K. (2021). Immune metabolism in PD-1 blockade-based cancer immunotherapy. In *International Immunology* (Vol. 33, Issue 1, pp. 17–26). Oxford University Press. https://doi.org/10.1093/intimm/dxaa046

Lai, F., Ji, M., Huang, L., Wang, Y., Xue, N., Du, T., Dong, K., Yao, X., Jin, J., Feng, Z., & Chen, X. (2022). YPD-30, a prodrug of YPD-29B, is an oral small-molecule inhibitor targeting PD-L1 for the treatment of human cancer. *Acta Pharmaceutica Sinica B*, *12*(6), 2845–2858. https://doi.org/https://doi.org/10.1016/j.apsb.2022.02.031

Lee, J., Ahn, E., Kissick, H. T., & Ahmed, R. (2015). Reinvigorating exhausted T cells by blockade of the PD-1 pathway. *Forum on Immunopathological Diseases and Therapeutics*, *6*(1–2), 7–18. https://doi.org/10.1615/ForumImmunDisTher.2015014188

Lei, Q., Wang, D., Sun, K., Wang, L., & Zhang, Y. (2020). Resistance Mechanisms of Anti-PD1/PDL1 Therapy in Solid Tumors. In *Frontiers in Cell and Developmental Biology* (Vol. 8). Frontiers Media S.A. https://doi.org/10.3389/fcell.2020.00672

Lin, X., Lu, X., Luo, G., & Xiang, H. (2020). Progress in PD-1/PD-L1 pathway inhibitors: From biomacromolecules to small molecules. *European Journal of Medicinal Chemistry*, *186*, 111876. https://doi.org/https://doi.org/10.1016/j.ejmech.2019.111876

Liu, C., Seeram, N. P., & Ma, H. (2021). Small molecule inhibitors against PD-1/PD-L1 immune checkpoints and current methodologies for their development: a review. *Cancer Cell International*, *21*(1), 239. https://doi.org/10.1186/s12935-021-01946-4

Liu, J., Chen, Z., Li, Y., Zhao, W., Wu, J. B., & Zhang, Z. (2021). PD-1/PD-L1 Checkpoint Inhibitors in Tumor Immunotherapy. In *Frontiers in Pharmacology* (Vol. 12). Frontiers Media S.A. https://doi.org/10.3389/fphar.2021.731798

Magiera-Mularz, K., Skalniak, L., Zak, K. M., Musielak, B., Rudzinska-Szostak, E., Berlicki, Ł., Kocik, J., Grudnik, P., Sala, D., Zarganes-Tzitzikas, T., Shaabani, S., Dömling, A., Dubin, G., & Holak, T. A. (2017). Bioactive Macrocyclic Inhibitors of the PD-1/PD-L1 Immune Checkpoint. *Angewandte Chemie - International Edition*, *56*(44), 13732–13735. https://doi.org/10.1002/anie.201707707

Marasco, M., Berteotti, A., Weyershaeuser, J., Thorausch, N., Sikorska, J., Krausze, J., Brandt, H. J., Kirkpatrick, J., Rios, P., Schamel, W. W., Köhn, M., & Carlomagno, T. (2020). *Molecular mechanism of SHP2 activation by PD-1 stimulation*.

Musielak, B., Kocik, J., Skalniak, L., Magiera-Mularz, K., Sala, D., Czub, M., Stec, M., Siedlar, M., Holak, T. A., & Plewka, J. (2019). CA-170 - A Potent Small-Molecule PD-L1 Inhibitor or Not? *Molecules (Basel, Switzerland)*, *24*(15). https://doi.org/10.3390/molecules24152804

Najafi, S., Majidpoor, J., & Mortezaee, K. (2022). The impact of microbiota on PD-1/PD-L1 inhibitor therapy outcomes: A focus on solid tumors. *Life Sciences*, *310*, 121138. https://doi.org/https://doi.org/10.1016/j.lfs.2022.121138

Nishimura, H., Nose, M., Hiai, H., Minato, N., & Honjo, T. (1999). Development of Lupus-like Autoimmune Diseases by Disruption of the PD-1 Gene Encoding an ITIM Motif-Carrying Immunoreceptor. *Immunity*, *11*(2), 141–151. https://doi.org/https://doi.org/10.1016/S1074-7613(00)80089-8

Ostrand-Rosenberg, S., Horn, L. A., & Haile, S. T. (2014). The Programmed Death-1 Immune-Suppressive Pathway: Barrier to Antitumor Immunity. *The Journal of Immunology*, *193*(8), 3835–3841. https://doi.org/10.4049/jimmunol.1401572

Pascolutti, R., Sun, X., Kao, J., Maute, R. L., Ring, A. M., Bowman, G. R., & Kruse, A. C. (2016). Structure and Dynamics of PD-L1 and an Ultra-High-Affinity PD-1 Receptor Mutant. *Structure*, *24*(10), 1719–1728. https://doi.org/https://doi.org/10.1016/j.str.2016.06.026

Pauken, K. E., Sammons, M. A., Odorizzi, P. M., Manne, S., Godec, J., Khan, O., Drake, A. M., Chen, Z., Sen, D. R., Kurachi, M., Barnitz, R. A., Bartman, C., Bengsch, B., Huang, A. C., Schenkel, J. M., Vahedi, G., Haining, W. N., Berger, S. L., & Wherry, E. J. (2016). Epigenetic stability of exhausted T cells limits durability of reinvigoration by PD-1 blockade. *Science*, *354*(6316), 1160–1165. https://doi.org/10.1126/science.aaf2807

Qin, W., Hu, L., Zhang, X., Jiang, S., Li, J., Zhang, Z., & Wang, X. (2019). The Diverse Function of PD-1/PD-L Pathway Beyond Cancer. *Frontiers in Immunology*, *10*. https://doi.org/10.3389/fimmu.2019.02298

Raghavan, S., Tovbis-Shifrin, N., Kochel, C., Sawant, A., Mello, M., Sathe, M., Blumenschein, W., Muise, E. S., Chackerian, A., Pinheiro, E. M., Rosahl, T. W., Luche, H., & de Waal Malefyt, R. (2021). Conditional Deletion of Pdcd1 Identifies the Cell-Intrinsic Action of PD-1 on Functional CD8 T Cell Subsets for Antitumor Efficacy. *Frontiers in Immunology*, *12*. https://doi.org/10.3389/fimmu.2021.752348

Rao, S., Anandappa, G., Capdevila, J., Dahan, L., Evesque, L., Kim, S., Saunders, M. P., Gilbert, D. C., Jensen, L. H., Samalin, E., Spindler, K.-L., Tamberi, S., Demols, A., Guren, M. G., Arnold, D., Fakih, M., Kayyal, T., Cornfeld, M., Tian, C., … Spano, J.-P. (2022). A phase II study of retifanlimab (INCMGA00012) in patients with squamous carcinoma of the anal canal who have progressed following platinum-based chemotherapy (POD1UM-202)☆. *ESMO Open*, *7*(4), 100529. https://doi.org/https://doi.org/10.1016/j.esmoop.2022.100529

Rizvi, N. A., Mazières, J., Planchard, D., Stinchcombe, T. E., Dy, G. K., Antonia, S. J., Horn, L., Lena, H., Minenza, E., Mennecier, B., Otterson, G. A., Campos, L. T., Gandara, D. R., Levy, B. P., Nair, S. G., Zalcman, G., Wolf, J., Souquet, P. J., Baldini, E., … Ramalingam, S. S. (2015). Activity and safety of nivolumab, an anti-PD-1 immune checkpoint inhibitor, for patients with advanced, refractory squamous non-small-cell lung cancer (CheckMate 063): A phase 2, single-arm trial. *The Lancet Oncology*, *16*(3), 257–265. https://doi.org/10.1016/S1470-2045(15)70054-9

Robert, C. (2020). A decade of immune-checkpoint inhibitors in cancer therapy. *Nature Communications*, *11*(1), 3801. https://doi.org/10.1038/s41467-020-17670-y

Routy, B., Le Chatelier, E., Derosa, L., Duong, C. P. M., Alou, M. T., Daillère, R., Fluckiger, A., Messaoudene, M., Rauber, C., Roberti, M. P., Fidelle, M., Flament, C., Poirier-Colame, V., Opolon, P., Klein, C., Iribarren, K., Mondragón, L., Jacquelot, N., Qu, B., … Zitvogel, L. (2018). Gut microbiome influences efficacy of PD-1–based immunotherapy against epithelial tumors. *Science*, *359*(6371), 91–97. https://doi.org/10.1126/science.aan3706

Ryman, J. T., & Meibohm, B. (2017). Pharmacokinetics of monoclonal antibodies. *CPT: Pharmacometrics and Systems Pharmacology*, *6*(9), 576–588. https://doi.org/10.1002/psp4.12224

Sakuishi, K., Apetoh, L., Sullivan, J. M., Blazar, B. R., Kuchroo, V. K., & Anderson, A. C. (2010). Targeting Tim-3 and PD-1 pathways to reverse T cell exhaustion and restore anti-tumor immunity. *Journal of Experimental Medicine*, *207*(10), 2187–2194. https://doi.org/10.1084/jem.20100643

Sasikumar, P. G., Sudarshan, N. S., Adurthi, S., Ramachandra, R. K., Samiulla, D. S., Lakshminarasimhan, A., Ramanathan, A., Chandrasekhar, T., Dhudashiya, A. A., Talapati, S. R., Gowda, N., Palakolanu, S., Mani, J., Srinivasrao, B., Joseph, D., Kumar, N., Nair, R., Atreya, H. S., Gowda, N., & Ramachandra, M. (2021). PD-1 derived CA-170 is an oral immune checkpoint inhibitor that exhibits preclinical anti-tumor efficacy. *Communications Biology*, *4*(1), 699. https://doi.org/10.1038/s42003-021-02191-1

Sharpe, A. H., & Pauken, K. E. (2018). The diverse functions of the PD1 inhibitory pathway. *Nature Reviews Immunology*, *18*(3), 153–167. https://doi.org/10.1038/nri.2017.108

Shi, F., Shi, M., Zeng, Z., Qi, R.-Z., Liu, Z.-W., Zhang, J.-Y., Yang, Y.-P., Tien, P., & Wang, F.-S. (2011). PD-1 and PD-L1 upregulation promotes CD8+ T-cell apoptosis and postoperative recurrence in hepatocellular carcinoma patients. *International Journal of Cancer*, *128*(4), 887–896. https://doi.org/https://doi.org/10.1002/ijc.25397

Shimizu, K., Sugiura, D., Okazaki, I. mi, Maruhashi, T., Takegami, Y., Cheng, C., Ozaki, S., & Okazaki, T. (2020). PD-1 Imposes Qualitative Control of Cellular Transcriptomes in Response to T Cell Activation. *Molecular Cell*, *77*(5), 937-950.e6. https://doi.org/10.1016/j.molcel.2019.12.012

Simon, S., & Labarriere, N. (2018). PD-1 expression on tumor-specific T cells: Friend or foe for immunotherapy? *OncoImmunology*, *7*(1), e1364828. https://doi.org/10.1080/2162402X.2017.1364828

Skalniak, L., Zak, K. M., Guzik, K., Magiera, K., Musielak, B., Pachota, M., Szelazek, B., Kocik, J., Grudnik, P., Tomala, M., Krzanik, S., Pyrc, K., Dömling, A., Dubin, G., & Holak, T. A. (2017). Small-molecule inhibitors of PD-1/PD-L1 immune checkpoint alleviate the PD-L1-induced exhaustion of T-cells. In *Oncotarget* (Vol. 8, Issue 42). www.impactjournals.com/oncotarget/

Surmiak, E., Magiera-Mularz, K., Musielak, B., Muszak, D., Kocik-Krol, J., Kitel, R., Plewka, J., Holak, T. A., & Skalniak, L. (2021). PD-L1 inhibitors: Different classes, activities, and mechanisms of action. *International Journal of Molecular Sciences*, *22*(21). https://doi.org/10.3390/ijms222111797

Takeuchi, Y., Inoue, S., & Odaka, A. (2022). Expression of programmed cell death-1 on neuroblastoma cells in TH-MYCN transgenic mice. *Pediatric Surgery International*, *39*(1), 6. https://doi.org/10.1007/s00383-022-05292-y

Twomey, J. D., & Zhang, B. (2021). Cancer Immunotherapy Update: FDA-Approved Checkpoint Inhibitors and Companion Diagnostics. *The AAPS Journal*, *23*(2), 39. https://doi.org/10.1208/s12248-021-00574-0

Upadhaya, S., Neftelinov, S. T., Hodge, J., & Campbell, J. (2022). Challenges and opportunities in the PD1/PDL1 inhibitor clinical trial landscape. In *Nature Reviews Drug Discovery* (Vol. 21, Issue 7, pp. 482–483). Nature Research. https://doi.org/10.1038/d41573-022-00030-4

Vesely, M. D., Zhang, T., & Chen, L. (2022). Resistance Mechanisms to Anti-PD Cancer Immunotherapy. *Annual Review of Immunology*, *40*(1), 45–74. https://doi.org/10.1146/annurev-immunol-070621-030155

Wang, C., Hillsamer, P., & Kim, C. H. (2011). Phenotype, effector function, and tissue localization of PD-1-expressing human follicular helper T cell subsets. *BMC Immunology*, *12*(1), 53. https://doi.org/10.1186/1471-2172-12-53

Wang, Q., Bardhan, K., Boussiotis, V. A., & Patsoukis, N. (2021). The PD-1 Interactome. In *Advanced Biology* (Vol. 5, Issue 9). John Wiley and Sons Inc. https://doi.org/10.1002/adbi.202100758

Wang, T., Cai, S., Cheng, Y., Zhang, W., Wang, M., Sun, H., Guo, B., Li, Z., Xiao, Y., & Jiang, S. (2022). Discovery of Small-Molecule Inhibitors of the PD-1/PD-L1 Axis That Promote PD-L1 Internalization and Degradation. *Journal of Medicinal Chemistry*, *65*(5), 3879–3893. https://doi.org/10.1021/acs.jmedchem.1c01682

Wang, X., Teng, F., Kong, L., & Yu, J. (2016). PD-L1 expression in human cancers and its association with clinical outcomes. In *OncoTargets and Therapy* (Vol. 9, pp. 5023–5039). Dove Medical Press Ltd. https://doi.org/10.2147/OTT.S105862

Wei, J., Luo, C., Wang, Y., Guo, Y., Dai, H., Tong, C., Ti, D., Wu, Z., & Han, W. (2019). PD-1 silencing impairs the anti-tumor function of chimeric antigen receptor modified T cells by inhibiting proliferation activity. *Journal for ImmunoTherapy of Cancer*, *7*(1), 209. https://doi.org/10.1186/s40425-019-0685-y

Wu, C., Cao, X., & Zhang, X. (2021). VISTA inhibitors in cancer immunotherapy: a short perspective on recent progresses. In *RSC Medicinal Chemistry* (Vol. 12, Issue 10, pp. 1672–1679). Royal Society of Chemistry. https://doi.org/10.1039/d1md00185j

Wu, Q., Jiang, L., Li, S. cheng, He, Q. jun, Yang, B., & Cao, J. (2021). Small molecule inhibitors targeting the PD-1/PD-L1 signaling pathway. In *Acta Pharmacologica Sinica* (Vol. 42, Issue 1). Springer Nature. https://doi.org/10.1038/s41401-020-0366-x

Wu, X., Meng, Y., Liu, L., Gong, G., Zhang, H., Hou, Y., Liu, C., Wu, D., & Qin, M. (2021). Insights into non-peptide small-molecule inhibitors of the PD-1/PD-L1 interaction: Development and perspective. *Bioorganic & Medicinal Chemistry*, *33*, 116038. https://doi.org/https://doi.org/10.1016/j.bmc.2021.116038

Xiao, Y., Yu, S., Zhu, B., Bedoret, D., Bu, X., Duke-Cohan, L. M. F., Umetsu, D. T., Sharpe, A. H., DeKruyff, R. H., & Freeman, G. J. (2014). RGMb is a novel binding partner for PD-l2 and its engagement with PD-l2 promotes respiratory tolerance. *Journal of Experimental Medicine*, *211*(5), 943–959. https://doi.org/10.1084/jem.20130790

Xing, X., Guo, J., Ding, G., Li, B., Dong, B., Feng, Q., Li, S., Zhang, J., Ying, X., Cheng, X., Guo, T., Du, H., Hu, Y., Zhou, T., Wang, X., Li, L., Li, Q., Xie, M., Li, L., … Ji, J. (2018). Analysis of PD1, PDL1, PDL2 expression and T cells infiltration in 1014 gastric cancer patients. *OncoImmunology*, *7*(3), e1356144. https://doi.org/10.1080/2162402X.2017.1356144

Xue, C., Zhu, D., Chen, L., Xu, Y., Xu, B., Zhang, D., & Jiang, J. (2019). Expression and prognostic value of PD-L1 and PD-L2 in ovarian cancer. *Translational Cancer Research*, *8*(1), 111–119. https://doi.org/10.21037/tcr.2019.01.09

Zhao, Z., Shi, L., Zhang, W., Han, J., Zhang, S., Fu, Z., & Cai, J. (2017). *CRISPR knock out of programmed cell death protein 1 enhances anti-tumor activity of cytotoxic T lymphocytes*. www.impactjournals.com/oncotarget

Zheng, Y., Fang, Y. C., & Li, J. (2019). PD-L1 expression levels on tumor cells affect their immunosuppressive activity. *Oncology Letters*, *18*(5), 5399–5407. https://doi.org/10.3892/ol.2019.10903

EVALUATION OF PD-1/PD-L1 IMMUNE CHECKPOINT INHIBITORS IN A LUCIFERASE-REPORTER CELL-BASED ASSAY

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ABSTRACT

Interaction between programmed cell death 1 (PD-1) and programmed cell death-ligand 1 (PD-L1) represents an important immune checkpoint that prevents overactive immune responses to self-antigens. However, this mechanism has been hijacked by cancer cells to avoid the immune response. Since the discovery of this mechanism, promising immunotherapies targeting either PD-1 or PD-L1 have been developed, eventually leading to the approval of a series of monoclonal antibodies to treat distinct types of cancer. Nonetheless, many small molecule inhibitors (SMI) are in development because of their advantages over antibodies. Here, a commercially available anti-immune checkpoint cell-based assay from InvivoGen (PD-1/PD-L1 Bio-IC™) was used to test the activity of antibodies and SMI targeting the PD-1/PD-L1 complex. This system takes advantage of a luciferase reporter gene controlled under NFAT response elements, which enables monitoring of T cell activation when the PD-1/PD-L1 axis is blocked. Although most of the inhibitors showed a dose-dependent change in luminescence, antibodies produced a larger assay window compared to SMI. Antibodies showed no impact on cell viability measured with an ATP-based assay, although most SMI severely impacted cell viability at concentrations above 10 µM. Despite efforts to improve the small assay window obtained when screening SMI through use of different target to effector cell ratios or changing the content of serum proteins in the media, the related experiments failed to identify improved assay conditions. Factors such as different mechanisms of action between antibodies and small molecules, and the strong inhibition carried by the overexpression of PD-1 and PD-L1 might account for the small assay window. Despite this, we have validated this cell assay system for screening small molecule and antibody inhibitors of PD-1/PD-L1.

ACKNOWLEDGEMENTS

The work for this report was undertaken using the laboratories and facilities at Charnwood Molecular Ltd. I am incredibly grateful for the opportunity to work at Charnwood Molecular, and for the help of their staff during the placement. In particular, I would like to thank Dr. James Chamberlain and Dr. Tessa McLaren for their support and instruction, as well as assistance in the interpretation and explanation of results. Additionally, I would like to thank Dr. Stephen Harding for his guidance in the development of this project.

INTRODUCTION

T cell-targeted immunomodulators blocking immune checkpoints (IC) have been a breakthrough in cancer treatment during the last decade (Robert, 2020). Immune checkpoints are molecules that halt T cell effector function, naturally preventing autoimmunity and maintaining immune homeostasis (Ganesan et al., 2019; Marin-Acevedo et al., 2018). Programmed cell death 1 (PD-1, also known as PDCD1 or CD279) is one of the most studied IC molecules; it counters positive signals mediated by the T-cell receptor (TCR) and CD28, by engaging its ligand programmed cell death 1 ligand 1 (PD-L1) (Freeman, 2008). PD-1 interaction with PD-L1 promotes tyrosine phosphorylation of the PD-1 cytoplasmic domain and recruitment of phosphatases that in turn translates into a signalling cascade that interferes with Tumour Necrosis Factor (TNF), Interferon-gamma (IFN-ɣ) and Interleukin-2 (IL-2) secretion (C. Liu et al., 2021; Shimizu et al., 2020). Amongst others, these signals inhibit T-cell proliferation and differentiation (Wei et al., 2019; Xie et al., 2020).

The PD-1/PD-L1 axis can be found in different immune cell types, for instance in T cells and antigen-presenting cells (APC) respectively. However, PD-L1 has also been found overexpressed on the surface of some cancer cell types, which in turn prevents their clearance by the immune system, and eventually a poor prognosis in several different cancers (Furuse et al., 2020; Sakuishi et al., 2010; Xing et al., 2018; Xue et al., 2019). Currently, therapies targeting the PD-1/PD-L1 pathway (also called anti-PD therapy) focus on blocking the interaction between PD-1 and PD-L1, ultimately reinvigorating T cells, previously inactive due to the PD-1/PD-L1 signalling inhibition (Akinleye & Rasool, 2019; J. Lee et al., 2015).

So far, only eight antibodies, either targeting PD-1 or PD-L1, have been approved by the Food and Drug Administration (FDA) to treat distinct types of cancer (Ai et al., 2020; Costa & Vale, 2022; Rao et al., 2022). Antibody-mediated PD-1/PD-L1 blockade significantly enhanced antitumour effects in different solid tumours including melanoma, NSCLC, urothelial carcinoma, renal cell carcinoma, head and neck squamous cell carcinoma, and microsatellite instability-high colorectal cancer (Ai et al., 2020; Dermani et al., 2019; Guzik et al., 2019; Lei et al., 2020). Until now, in the anti-PD therapeutics area, much effort has been put into developing small molecule

inhibitors that achieve the potency of monoclonal antibodies (mAb) targeting either PD-1 or PD-L1.

However, there has been increasing interest in small molecule inhibitors (SMI) due to their potential for oral bioavailability, reduced production costs, better management of any emergent adverse events, etc (Ai et al., 2020). Most of these therapeutics in development rely on the physical blockade of the interaction between PD-1 and PD-L1 but preliminary data suggest they employ different mechanisms of action (see **Figure 1**) (J. Liu et al., 2021; Surmiak et al., 2021). Computational modelling and spectroscopic techniques seem to concur that some biphenyl-based PD-L1 inhibitors (which is a SMI) promote dimerization of two PD-L1 receptors (Shi et al., 2019; Skalniak et al., 2017). Nevertheless, the dimerization mechanism and further processing are not fully understood. For a specific small molecule inhibitor, Chen and colleagues (2020) found that interaction between the inhibitor and PD-L1 results in impaired PD-L1 glycosylation that prevents the transport of newly synthesized PD-L1 from the endoplasmic reticulum to Golgi (Chen et al., 2020).

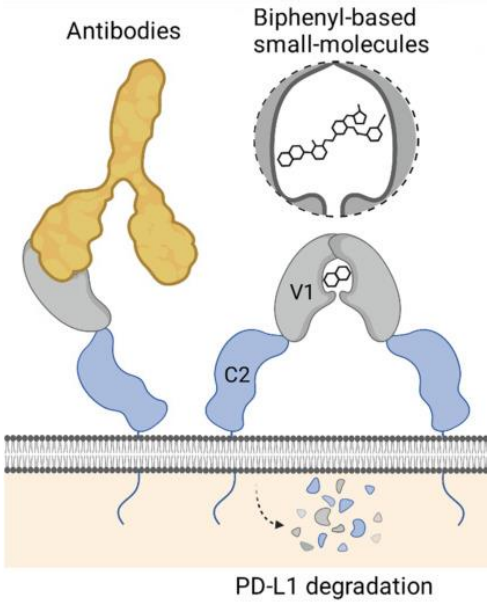

Figure 1. Mechanisms of PD-1/PD-L1 blockade attributed to antibodies and biphenyl-based small-molecule inhibitors targeting the extracellular domain of PD-L1. Figure modified from Surmiak et al., 2021.

The performance of the variety of anti-PD molecules can be tested through different assays that aim to decipher the molecule properties at various levels. Even though biochemical and biophysical analyses are useful for the initial screening of potential inhibitors through their binding profiles, in vitro cell-based assays and in vivo tumour xenograft models give information on the functional properties of the tested molecules in a more physiologically relevant environment (C. Liu et al., 2021). Here, we have employed a commercially available cell-based assay to evaluate the activity of anti-PD molecules towards the inhibition of the PD-1/PD-L1 interaction (PD-1/PD-L1 Bio-IC™). This assay is comprised of two cell lines, one is a T cell reporter line derived from an immortalised human T lymphocyte (Jurkat) expressing a specific TCR (later referred to as Jurkat-Lucia) and overexpression co-stimulatory molecule CD28 along with PD-1. The other is an antigen-presenting cell (APC) derived from a human B lymphocyte cell line (Raji) expressing a specific [HLA::peptide] complex and overexpressing PD-L1.

Interaction between these cell lines mimics the immune synapse between T cells and APCs through the interaction of cell surface molecules capable of activating or inhibiting T cells as represented in **Figure 2**. Signal 1 is an activation signal delivered upon recognition of a specific antigenic peptide complexed with HLA molecules ([HLA::peptide]) on Raji-PDL1 cells by the TCR on Jurkat-Lucia™ cells. The above-mentioned has a co-stimulation signal (signal 2) operated by the interaction of CD80/86 and CD28 molecules at the surface of the APC and T cells, respectively. In addition, the interaction between PD-1 and PD-L1 on the surface of these cell lines inhibits activation stimulated by signals 1 and 2, however, this interaction can be inhibited by the addition of anti-PD-1 or anti-PD-L1 antibodies, allowing activation of the T cell reporter line.

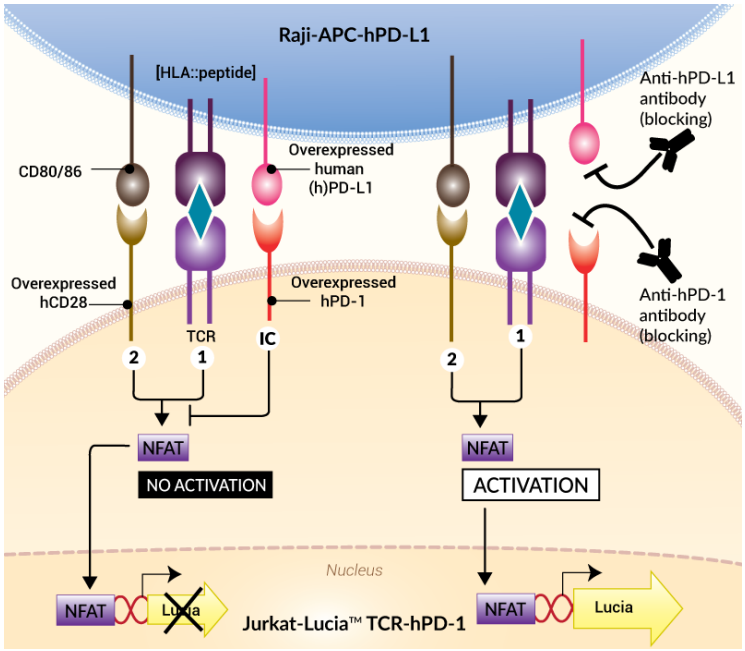

Figure 2. Principle of PD-1/PD-L1 Bio-IC™ cellular assay. Figure from InvivoGen https://www.invivogen.com/hpd1-bioassay

The outcome of the interaction (T cell activation or inhibition) between both cell lines can be assessed by the activity of the Lucia luciferase reporter gene. This gene is under the control of an ISG54 minimal promoter fused to six NFAT response elements. Therefore, if the stimulatory signals allow the expression of the luciferase gene, the protein will be secreted, and the bioluminescent signal can be measured using the coelenterazine-based detection reagent (QUANTI-Luc™ Gold). On the contrary, inhibitory signals will result in a decrease in the luminescent signal. As suggested by the manufacturer, this kit is suitable for screening antibody-, Fc-fusion protein-, and small molecule-based inhibitors of the PD-1/PD-L1 axis.

Charnwood Molecular are currently in the process of developing SMI and require a cell-based assay that evaluates the bioactivity of PD-1/PD-L1 inhibitors for use alongside biophysical and biochemical methods (Surface Plasmon Resonance and Homogeneous Time Resolved Fluorescence, data not shown). Therefore, this work aimed to evaluate the capabilities of the PD-1/PD-L1 Bio-IC™ assay for assessing the rescue of T cell effector properties based on a change in luminescence when anti-PD compounds are administered. Thus, the blockade of the PD-1/PD-L1 interaction is seen as an increase in the luminescence signal which in turn is interpreted as

reinvigoration of T cell effector properties (i.e., cytotoxicity, proliferation, and cytokine secretion) due to the expression of genes controlled by NFAT transcription factors (J. U. Lee et al., 2018; J. Liu et al., 2021; Sakuishi et al., 2010).

Before testing novel compounds, an optimisation of assay conditions was performed with commercially available molecules, known to be effective at inhibiting the interaction between PD-1 and PD-L1. Therefore, mAbs (Atezolizumab, Pembrolizumab, Nivolumab and Durvalumab) and small molecule inhibitors (BMS-8, BMS-1001, BMS-202 and Inhibitor 9) were used to validate this cell-based assay. Additionally, cytotoxicity profiles of the compounds were also tested in Jurkat cells. To improve the assay window, a series of experiments were performed involving different incubation periods, target to effector cell ratios and serum concentrations in the cell media. Additionally, due to evidence showing that some cancer cell lines also express PD-L1 (Xu et al., 2019; Zheng et al., 2019), the expression of PD-L1 was evaluated in a number of different cell lines using flow cytometry. This was performed with the aim of testing the activation of the reporter cell line upon interaction with another PD-L1-expressing cell line.

METHODS

Cell lines and reagents

The kit PD-1/PD-L1 Bio-IC™ containing Jurkat cell line engineered to express a specific T cell receptor, human PD-1, human CD28 and NFAT-inducible Luciferase (Jurkat-Lucia™ TCR-hPD-1 Cells) and the APC cell line expressing a specific HLA:peptide complex and PD-L1 (Raji-APC-hPD-L1 cells) were purchased from InvivoGen. Both cell lines were routinely cultured in IMDM cell culture medium supplemented with 10% heat-inactivated foetal bovine serum (HI-FBS) (Thermo Fisher Scientific). Cells were kept in a humid incubator at 37 °C and 5% CO_2. To maintain selection pressure in the Jurkat-Lucia cell line, antibiotics were added every other passage (10 µg/ml of Blasticidin, 100 µg/ml of Zeocin™, 100 µg/ml Hygromycin, and 250 µg/ml of G418 (Geneticin)). Raji-PDL1 cells were cultured with 10 µg/ml of Blasticidin and 250 µg/ml of G418 (Geneticin) every other passage. For the detection of secreted Lucia luciferase, a coelenterazine-containing luciferase detection reagent was used (QUANTI-Luc™ Gold, InvivoGen).

PD-1/PD-L1 immune checkpoint inhibitor screening assay: T cell activation controls

Jurkat-Lucia cells (effector cells) were co-cultured into 96-well plates (Cellstar) at 2×10^5 cells/well with 1×10^5 cells/well of the Raji-PDL1 cell line (target cells) as suggested by the manufacturer. Depending on the experiment, for stimulating activation in Jurkat-Lucia lymphocytes either 2 µL of T cell TransAct (Miltenbiotec) or a combination of phorbol 12-myristate 13-acetate (PMA, Cayman chemical) and Ionomycin (Enzo) at 1 µM and 3 µM respectively, was used. During the incubation time, cells were kept at 37 °C and 5% CO_2. To quantify the amount of secreted luciferase, 20 µL of supernatant of each well were retrieved and transferred to a white 96-well plate (Greiner Bio One). 50 µL of QUANTI-Luc™ Gold reagent was added to each well and luminescence was measured immediately using an EnSpire™ Multimode Plate Reader (PerkinElmer, Inc.).

Antibody and Small Molecule Inhibitor titration using the Anti-PD-1/PD-L1 Cell-based Assay

Jurkat-Lucia cells were co-cultured with Raji-PDL1 cells as mentioned above. The anti-PD-1 antibodies employed in this work were Nivolumab (Insight Biotechnology) and Pembrolizumab (Insight Biotechnology). The anti-PD-L1 antibodies used here were Atezolizumab (Tebu-bio) and Nivolumab (Insight Biotechnology). The initial antibody concentrations were set at 100 µg/mL with a half-log serial dilution. For SMI, cell density was reduced (2×10^4 with 1×10^4 cells/well, respectively). For this work, well-validated SMI that interact with PD-L1, such as BMS-8 (Cayman), BMS-1001 (MCE), BMS-202 (MCE) and Inhibitor-9 (MCE), were utilised. The initial compound concentrations were set at 100 µM with a third-log serial dilution using an HP D300e Digital Dispenser (HP). After the incubation time (24 hours for antibodies and 48 hours for SMI), luciferase reporter gene expression was determined by measuring the amount of luminescence using QUANTI-Luc™ Gold (InvivoGen) and Envision plate reader (PerkinElmer) as mentioned above.

Evaluation of cytotoxicity profiles of tested molecules in Jurkat-Lucia cells

Jurkat-Lucia cells were cultured into white 96-well plates at 2×10^5 cells/well (for mAb experiments) or 2×10^4 cells/well (for SMI) and incubated for 24 hours with the respective treatments. Following the incubation period, the CellTiter-Glo® Luminescent Cell Viability Assay (Promega) was performed following the manufacturer's protocol.

Target to effector cell ratio in the Anti-PD-1/PD-L1 Cell-based Assay

Jurkat-Lucia cells were co-cultured into a 96-well plate at either 4×10^4, 2×10^4 or 1×10^4 cells/well with either 1×10^4, 2×10^4 or 4×10^4 cells/well of the Raji-PDL1 cell line. Hence, the target to effector (Raji-PDL1 to Jurkat-Lucia) were 1:2, 1:1, 1:4, 2:1 and 4:1. Using the usina HP D300e Digital Dispenser (HP) Inhibitor-9 (final concentration 20 µM) was added to the desired wells, in the same protocol PMA (final concentration 1 µM) and Ionomycin (final concentration 3 µM) were added to the desired wells. The plate was incubated at 37°C and 5% CO_2 and the luminescence was measured at different time points as described before.

Impact of serum concentration in the media on the Anti-PD-1/PD-L1 Cell-based Assay

Both cell lines were cultured in the conditions mentioned above but on the day of the experiment, cells were counted and washed with phosphate buffered saline (Gibco) once and resuspended with media with no HI-FBS, 1% HI-FBS or 10% HI-FBS. $2x10^5$ Jurkat-Lucia cells were seeded in a 96-well plate and then $1x10^5$ Raji-PDL1 cells were added. Using the HP D300e Digital Dispenser (HP) SMI studied here were dispensed (final concentration of 31.62 µM) and the percentage of DMSO was normalized across wells. Alternatively, Atezolizumab (final concentration of 34.5 nM) was dispensed to other wells. The plate was incubated at 37°C and 5% CO_2 for 6 hours. After that, luminescence was measured as already described.

Flow cytometry analysis of PD-L1

SNU-398, MDA-MB-468, A549, HeLa, SH-SY5Y, DLD-1, HT-29, PC-3 and MCF7 cell lines were routinely cultured in DMEM cell culture medium supplemented with 10% foetal bovine serum

(FBS) (Thermo Fisher Scientific). Cells were kept in an incubator at 37 °C and 5% CO_2. Jurkat-Lucia and Raji-PDL1 cells were maintained as mentioned before. Adherent cells were cultured for 24 hours at a density of $4x10^4$ cells in a flat-bottom 96-well plate. After that, cells were harvested for flow cytometry using Dissociation Solution (Sigma-Aldrich). Harvested cells were washed with PBS and then labelled with APC Anti-human CD274 (PD-L1) (Biolegend) for 30 min at 4°C. Flow cytometry analysis was performed on a Attune NxT Flow Cytometer (Thermofisher). The percentage of positive cells was gated against unlabelled cells (0% positive) to measure the expression level of PD-L1. GraphPad Prism was used for data analysis and graphical representation.

PD-1/PD-L1 immune checkpoint inhibitor screening assay with PC-3 cells

PC-3 cells were cultured into 96-well plates (Cellstar) at $1×10^4$ cells/well and were incubated for 4 hours at 37°C and 5% CO_2. After that, Jurkat-Lucia cells were added to the plate at $2×10^4$ cells/well. For stimulating T cell activation in Jurkat-Lucia cells either 2 µL of T cell TransAct (Miltenbiotec) or a combination of Anti-Hu CD3 (Invitrogen) and Anti-Hu CD28 (Invitrogen) both at a concentration of 1 µg/mL. After the incubation time, luciferase reporter gene expression was determined as mentioned before.

Data visualisation

Graphs were constructed using GraphPad Prism v7.03 or above (GraphPad Sofware Inc., La Jolla, CA) and details about the experimental replicates can be found in figure legends.

RESULTS

PD-1/PD-L1 immune checkpoint inhibitor screening assay: T-cell activation and inhibition controls

In order to test if PD-L1 expressed by the Raji cell line could induce inhibition of Jurkat-Lucia T-cell activation we first look at molecules that stimulate T-cell activation. T cell TransAct, a commercially available agonist of CD3 and CD28, was used since it has proved optimal T cell activation in the literature (Casati et al., 2013). The amount of luciferase secreted was detected by the reagent QUANTI-Luc™ Gold (InvivoGen) confirmed that only the Jurkat-Lucia cell line is responsible for the luminescence signal detected as opposed to the Raji cell line (**Figure 3**). The increase in the luminescent signal when T cell TransAct is added to the Jurkat-Lucia cells suggests that the T cell activation pathway was stimulated compared to the background levels of luminescence by the Jurkat-Lucia cells alone. Despite the expected inhibitory effect of the PD-1/PD-L1 interaction, incubation of Raji-PDL1 cells with Jurkat-Lucia cells resulted in an increase in luminescence signal compared to Jurkat-PD1 cells alone. Since the treatment expected to give the highest signal (T cell TransAct) was lower to the inhibition led by the interaction between PD-1 in Jurkat-Lucia cells and PD-L1 in Raji cells, the following experiments looked for other methods to stimulate Jurkat-Lucia cells.

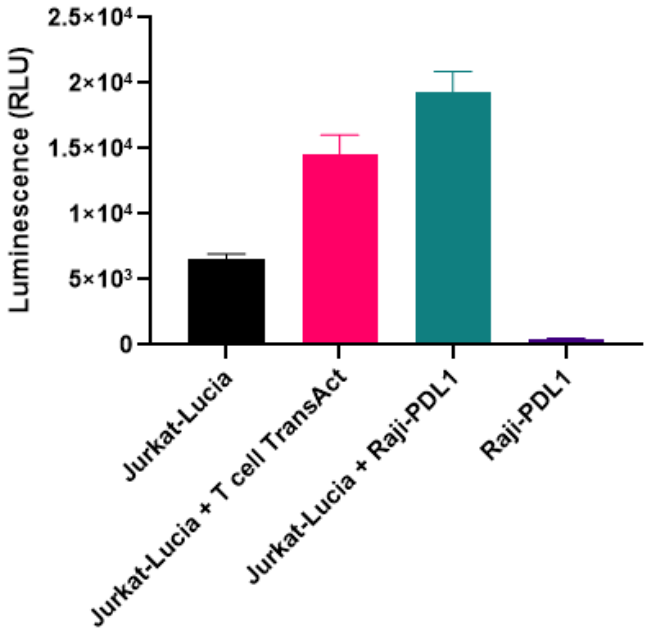

Figure 3. Jurkat-Lucia luciferase activity after 24 hours incubation with T cell TransAct or Raji-PDL1 cells. Data represented as mean ± SD of four technical replicates.

It is well established that a combination of PMA and Ionomycin (in this work referred to as PMA/Ionomycin) is a potent activator of T cells (Han et al., 2013). **Figure 4** depicts the activation capability of a solution with PMA (1 µM) and Ionomycin (3 µM) compared to T cell TransAct. For both treatments, Jurkat activation increased with time. Nevertheless, at 21 hours, the luminescence produced by T cell TransAct is 3 times that of the Jurkat-Lucia cells without treatment (assay window), as opposed to 88 times greater for the treatment with PMA/Ionomycin (**Figure 5**). This change in the assay window was similar after 25 hours of incubation. Consequently, PMA/Ionomycin was considered as a positive control in some following experiments, notwithstanding the exceedingly high luminescence signal due to T cell activation has to be acknowledged.

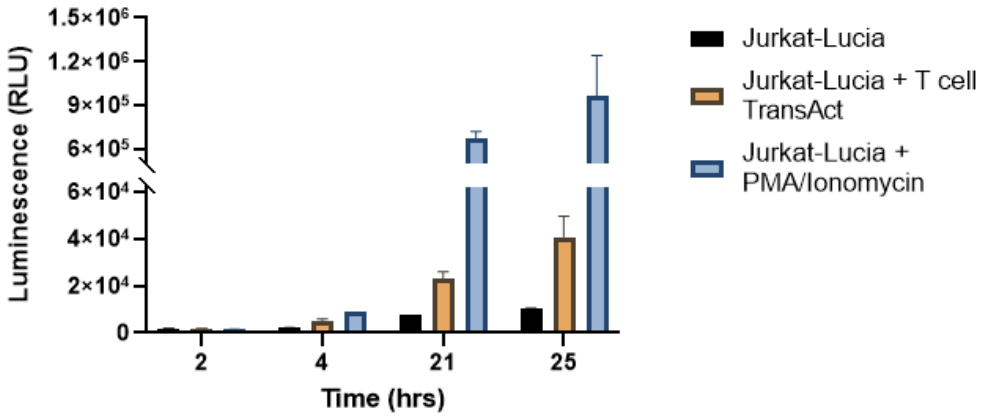

Figure 4. Comparison of the Jurkat-Lucia luciferase activity upon stimulation by T cell TransAct and PMA/Ionomycin. Data represented as mean ± SD of technical duplicates plotted.

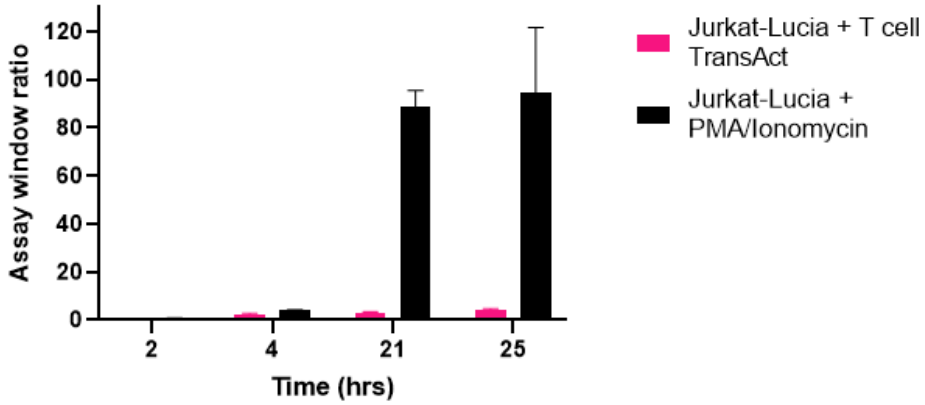

Figure 5. Comparison of the Jurkat-PD1 luciferase assay window (ratio of the luminescence between the activation method and Jurkat-Lucia cells alone) upon stimulation by T-cell TransAct or PMA/Ionomycin. Data represented as mean ± SD of technical duplicates plotted.

To investigate the PD-L1 mediated inhibition of T cell activation, both cell lines of the PD-1/PD-L1 Bio-IC™ kit were combined to see the possible blockade of the inhibition when treated with a commercially available mAb (Atezolizumab, light blue bar in **Figure 6**). In agreement with previous experiments, the luminescent signal produced by interacting Jurkat-Lucia and Raji-PDL1 cells is higher than that of Jurkat-Lucia cells on their own. In addition, T cell TransAct failed to activate Jurkat cells since it showed a smaller response compared to the wells with Jurkat-Lucia and Raji-PDL1 cells. Atezolizumab (anti-PD-L1 antibody) showed the highest activation of Jurkat-

Lucia cells in the presence of Raji-PDL1 cells indicating more luciferase secretion due to the blocking of the interaction between PD-1 and PD-L1. This antibody was also incubated with Jurkat-Lucia cells alone to investigate if the presence of the antibody by itself stimulates T cell activation, but it did not.

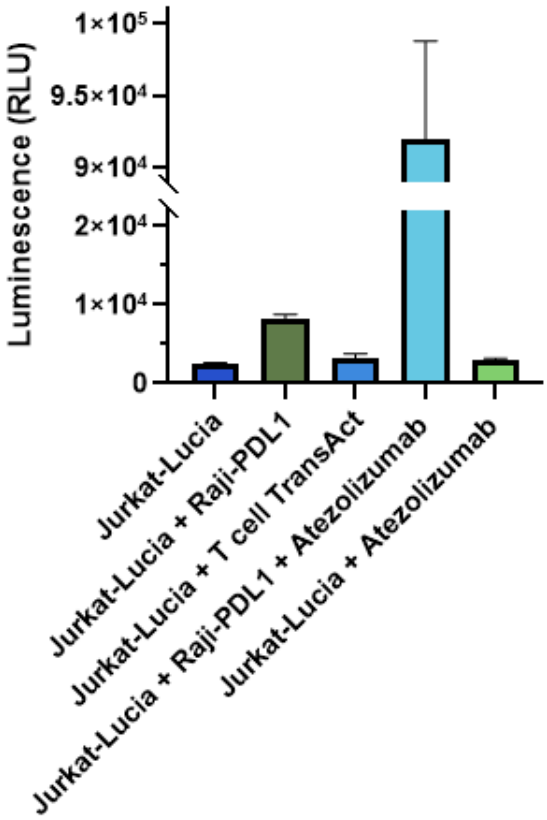

Figure 6. Comparison of the Jurkat-Lucia luciferase activity upon stimulation by T cell TransAct or Atezolizumab after a 4-hour incubation. Data represented as mean ± SD of four technical replicates plotted.

Even though PMA/Ionomycin substantially increases T cell activation at the concentration employed, it also has a detrimental effect on cell viability (Appendix Figure S1). Therefore, an appropriate positive control for the activation of Jurkat-Lucia cells was not identified. Nonetheless, the expression of PD-L1 in Raji cells is enough to counteract the activation signals that happen when Jurkat-Lucia and Raji-PDL1 cells interact together. Thereby, when adding a PD-

1/PD-L1 blockade agent such as Atezolizumab, the activation signals of the Jurkat-Lucia cells translate into an increase in T cell activation (**Figure 6**).

Antibody titration using the Anti-PD-1/PD-L1 Cell-based Assay

Raji-PDL1 and Jurkat-Lucia cells were incubated with serial dilutions of Anti-PD-1 (Nivolumab and Pembrolizumab) or Anti-PD-L1 (Atezolizumab and Durvalumab) antibodies for 24 hours to assess the capabilities in the assay. NFAT activation, reflecting the disruption of PD-1/PD-L1 inhibitory interaction, was evaluated by determining Lucia luciferase activity in the supernatant using the coelenterazine reagent. As shown in **Figure 7**, all antibodies tested display a dose-dependent change in luminescence, indicating that they block the PD-1/PDL-1 interaction and enable T cell activation. IC_{50} values calculated based on a non-linear regression showed that Atezolizumab and Durvalumab have similar values which are approximately 10 times lower than Nivolumab and Pembrolizumab (**Table 1**). Nivolumab did not achieve a plateau at the highest concentrations, however, this was constrained to the same value as Pembrolizumab when performing the non-linear regression analysis.

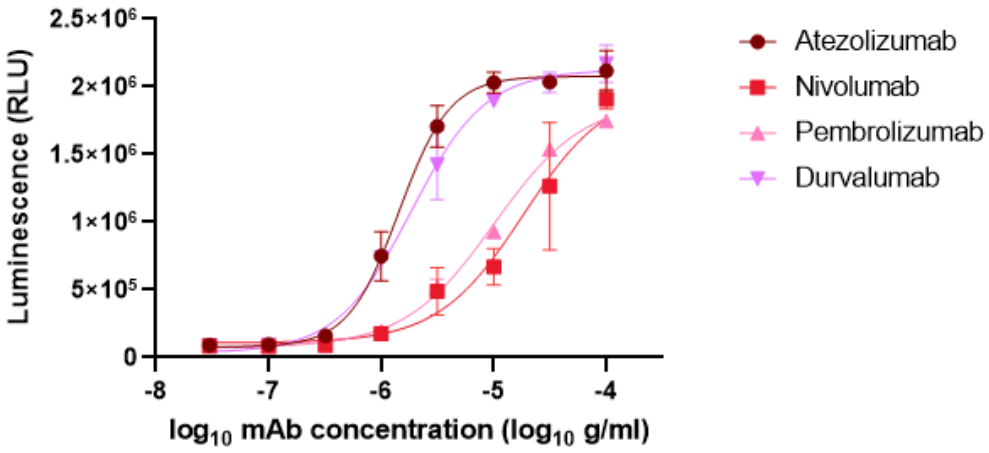

Figure 7. Dose-response curve for different Anti-hPD-1 or Anti-hPD-L1 antibodies after 24-hour incubation with antibody serial dilutions. Each point represents the mean ± SD of technical duplicates. Curves were fitted using a non-linear regression function and the IC_{50} value was determined by interpolation.

Table 1. IC_{50} values and assay windows calculated for the different monoclonal antibodies tested in the Anti-PD-1/PD-L1 Cell-based Assay after a 24-hour incubation.

	Atezolizumab	Nivolumab	Pembrolizumab	Durvalumab
IC_{50} (µg /mL)	1.441	18.8*	10.13	1.849
Assay window	25.38	N/A	24.04	33.40

N/A refers to non-available data.
*Curve fit constrained to $2x10^6$ during non-linear regression analysis

The effect of these antibodies on Jurkat-Lucia cells alone was tested, and results indicate that no T cell activation was promoted for any mAb (Appendix Figure S2). In summary, mAb available in the market such as Atezolizumab, Pembrolizumab, Nivolumab and Durvalumab showed a dose-dependent response in the PD-1/PD-L1 Bio-IC™ kit using a 1:2 target to effect cell ratio. Additionally, the impact of these antibodies on cell health was investigated using the CellTiter-Glo® Luminescent Cell Viability Assay. As shown in **Figure 8**, the addition of mAb did not alter the cell viability profile compared to Jurkat-Lucia controls.

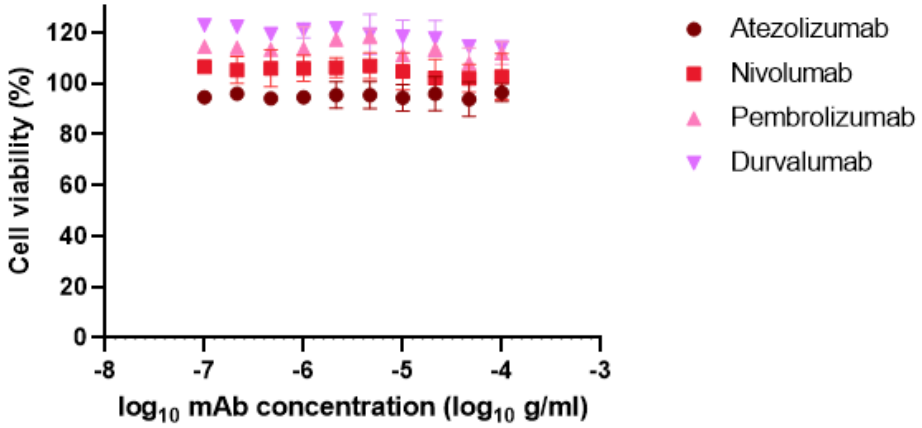

Figure 8. Cytotoxicity profile of Jurkat-Lucia cells after a 24-hour incubation with different Anti-hPD-1 or Anti-hPD-L1 antibodies. Cell viability % was calculated considering 100% equals the average luminescence signal of the control (i.e., Jurkat-Lucia cells with PBS). Data represented as mean ± SD of technical duplicates.

Small molecule PD-L1 inhibitors titration using the Anti-PD-1/PD-L1 Cell-based Assay

The following experiments aimed to evaluate the efficacy of different small molecule PD-L1 inhibitors available in the market (BMS-8, BMS-1001, BMS-202 and Inhibitor-9) to inhibit the interaction between PD-1 in Jurkat-Lucia™ TCR-hPD-1 cells and PD-L1 in Raji-APC-hPD-L1 cells. The activity of these tool compounds was assessed at 24, 48 and 72 hours, but the 48-incubation yielded better curve fits (data not shown), therefore this timepoint was chosen for these set of experiments. Consequently, the cell density was also reduced whilst maintaining the target to effector cell ratio to ensure that cell co-culture did not become overly dense. **Figure 9** depicts a range of activities displayed by the compounds studied here. Compounds BMS-202, BMS-8 and Inhibitor-9 all display a dose-dependent increase in luminescence, and thus T cell activation. However, the assay windows for these compounds were smaller than those mentioned before for antibodies (**Table 2**). Contrastingly, BMS-1001 showed to be inactive in this experiment but intriguingly BMS-8 showed an increase in response with concentrations above 10 µM.

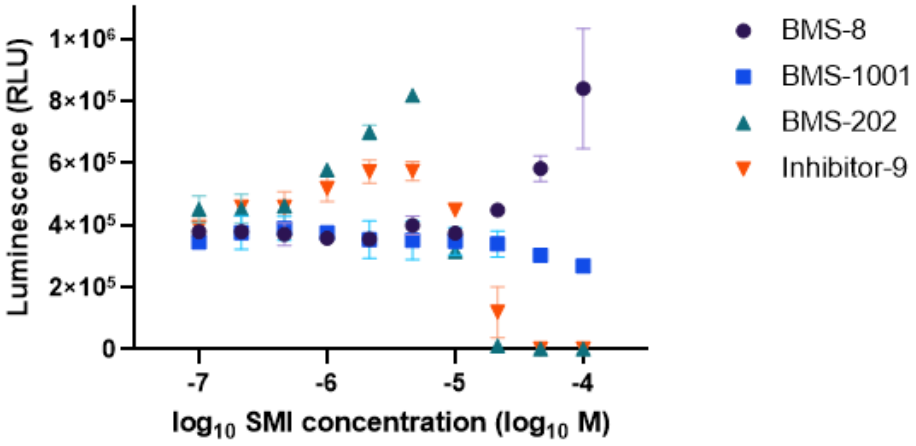

Figure 9. Jurkat-Lucia luciferase activity for different PD-L1 small molecule inhibitors after a 48-hour incubation. Each point represents the mean ± SD of technical duplicates.

The drastic drop-off in the signal at the highest concentrations of BMS-202 and Inhibitor 9 (above 21.5 µM) in **Figure 9** was a consistent phenomenon, which drove future experiments assessing

the impact on cell viability. Despite evidence showing that some anti-PD compounds are strongly active in biochemical assays, they also promote acute cytotoxicity that compromises their immunological activity (Ganesan et al., 2019). In **Figure 9**, the apparent lack of signal for BMS-202 and Inhibitor 9 is due to less than 1% viable cells in those wells at the highest concentrations (see **Figure 10**). Non-linear regression analysis of cell viability data found that BMS-202 reduces the cell viability by half (IC_{50}) at 9.863 µM whilst the IC_{50} of Inhibitor 9 is 16.25 µM.

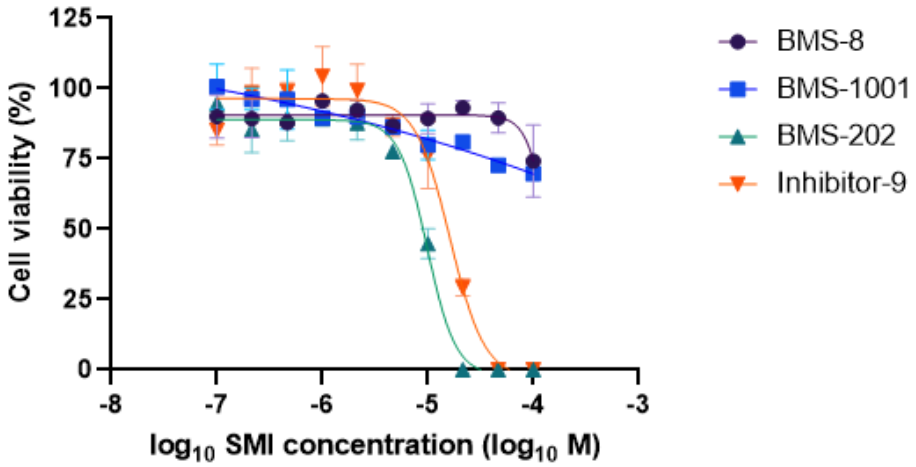

Figure 10. Cytotoxicity profile of Jurkat-Lucia cells after a 24-hour incubation with different small molecule inhibitors. Cell viability % was calculated considering 100% equals the average luminescence signal of the control (i.e., Jurkat-Lucia cells with DMSO). Data represented as mean ± SD of technical duplicates.

Consequently, the points with less than 75% of cell viability seen in **Figure 10** were removed and a nonlinear regression analysis was applied to determine the potential IC_{50} (**Figure 11**). Given that, full sigmoidal dose-response curves were not obtained, and the IC_{50} values displayed in **Table 2** should be considered estimates. Ultimately, this cell-based assay was able to detect the inhibitory activity of BMS-8, BMS-202 and Inhibitor 9 but the range in luminescence responses for similar small molecules is expected to be limited.

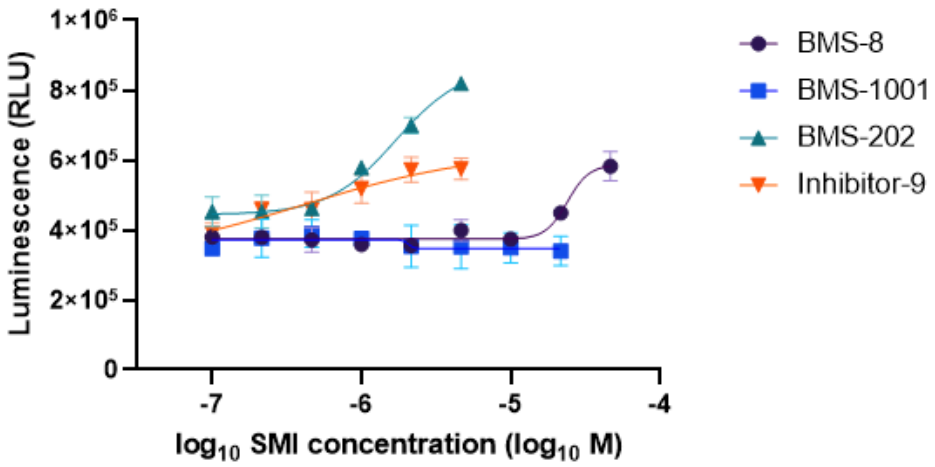

Figure 11. Dose-response curve for different PD-L1 small molecule inhibitors after a 48-hour incubation. Each point represents the mean ± SD of technical duplicates. Where possible, curves were fitted using a non-linear regression function and the IC_{50} value was determined by interpolation.

Table 2. IC_{50} values and assay windows calculated for the different small molecule inhibitors (tool compounds targeting PD-L1) tested in the Anti-PD-1/PD-L1 Cell-based Assay after a 48-hour incubation.

	BMS-8	**BMS-202**	**Inhibitor 9**
IC_{50} (µM)	23.84	1.703	0.4046
Assay window	1.57	1.96	2.03

Target to effector cell ratio in the Anti-PD-1/PD-L1 Cell-based Assay

To improve the assay window, an experiment with different ratios between Raji-PDL1 (target cells) and Jurkat-Lucia (effector cells) was carried out. All the wells with Inhibitor 9 (final concentration 20 µM) produced a higher luminescence signal compared to wells with Jurkat-Lucia and Raji-PDL1 cells, thereby the assay windows are positive (**Table 3**). Generally, over time the assay window decreases. Hence a timepoint between 24 and 48 hours seems suitable to measure changes in this cell-based assay. Nevertheless, there is not a significant difference between the cell ratios studied, which led us to keep the target to effector cell ratio as used before.

Table 3. Comparison of the calculated assay window (average signal from Jurkat-Lucia + Raji-PDL1 + Inhibitor 9 over the average signal from Jurkat-Lucia + Raji-PDL1) at different time points.

Target to effector cell ratio	5 h	24 h	48 h	72 h
1:2	1.577	1.752	1.600	1.446
1:1	1.454	1.689	1.715	1.545
1:4	1.627	1.707	1.652	1.668
2:1	1.615	1.452	1.411	1.411
4:1	1.919	1.436	1.148	1.159

Impact of serum concentration on the Anti-PD-1/PD-L1 Cell-based Assay

Potential binding of SMI to plasma proteins can reduce their effective concentration and therefore efficacy. To assess this impact on the bioassay, three different serum concentrations were employed when co-culturing cells. As seen in **Figure 12**, for each group, either with tool compound or antibody, the luminescence signal is similar regardless of the percentage of HI-FBS, suggesting that these small molecules don't interact strongly with serum proteins. A slight decrease in signal paired with the decrease in serum proteins is seen, yet negligible. Therefore, the protocol for culturing and screening inhibitors remained with 10% of HI-FBS.

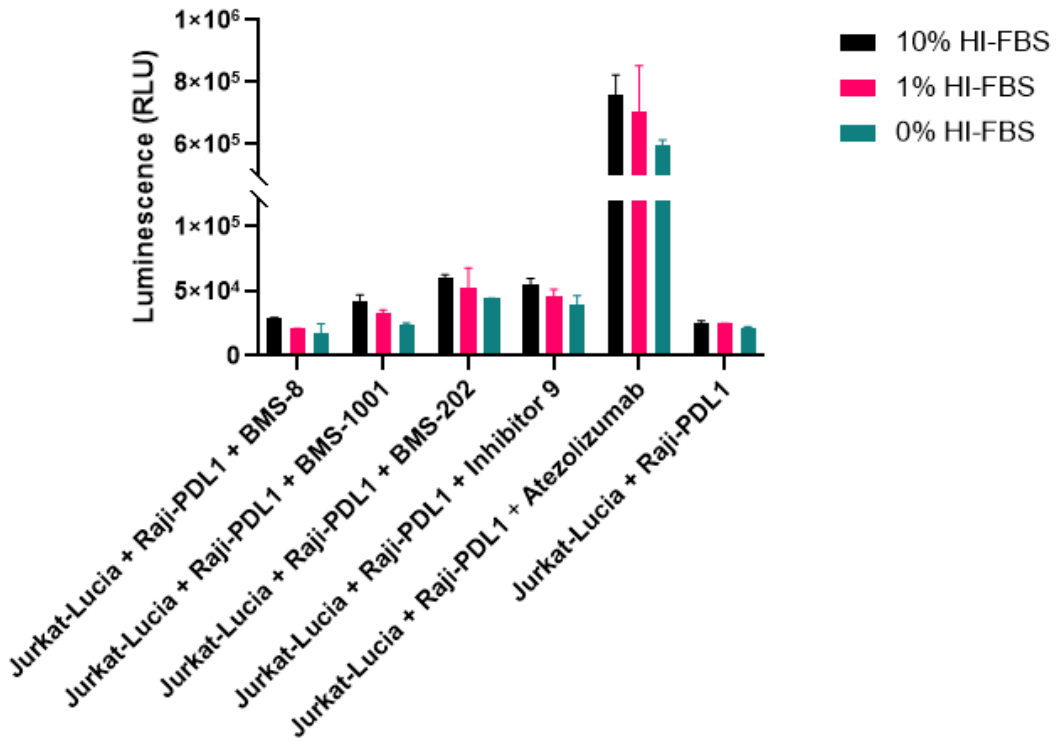

Figure 12. Comparison of the Jurkat-Lucia luciferase activity upon stimulation with different PD-L1 inhibitors (BMS-8, BMS-1001, BMS-202 and Inhibitor-9 at 31.62 µM), or with Atezolizumab (34.5 nM), and without any additional molecule after a 6-hour incubation. Data represented as mean ± SD of technical duplicates.

Screening for PD-L1 expression in cancer cell lines

As shown with the SMI titration, PD-L1 overexpression in Raji cells from the PD-1/PD-L1 Bio-IC™ kit might exert potent inhibitory signals that overshadow the activity of small molecules. Consequently, the hypothesis that a low level of expression of PD-L1 could be enough to both convey inhibitory signals of the T cell activation and can be blocked by the anti-PD compounds was tested in this section. There is evidence showing an increased PD-L1 expression in different cancer cell types (Cha et al., 2019; Ganesan et al., 2019; X. Wang et al., 2016) and immortalized cancer cell lines (Xu et al., 2019; Zheng et al., 2019a). Therefore, with cancer cell lines available in the laboratory, the hypothesis mentioned above was tested first by measuring the percentage of PD-L1 positive cells using an APC Anti-human CD274 (PD-L1) in flow cytometry.

Unlabelled Jurkat-Lucia and Raji-PDL1 cells were used as a reference to set flow cytometry gating (Appendix Figure S3 and S4). When live cells were stained with an APC-labelled anti-PD-L1 antibody almost 100% of Raji-PDL1 cells were positive for APC signal (**Figure 13**). Per cell, PC-3 cells have lower levels of PD-L1 expression compared to Raji-PDL1 cells. However, PC-3 cells had the highest percentage of cells expressing PD-L1 amongst the cell lines studied here.

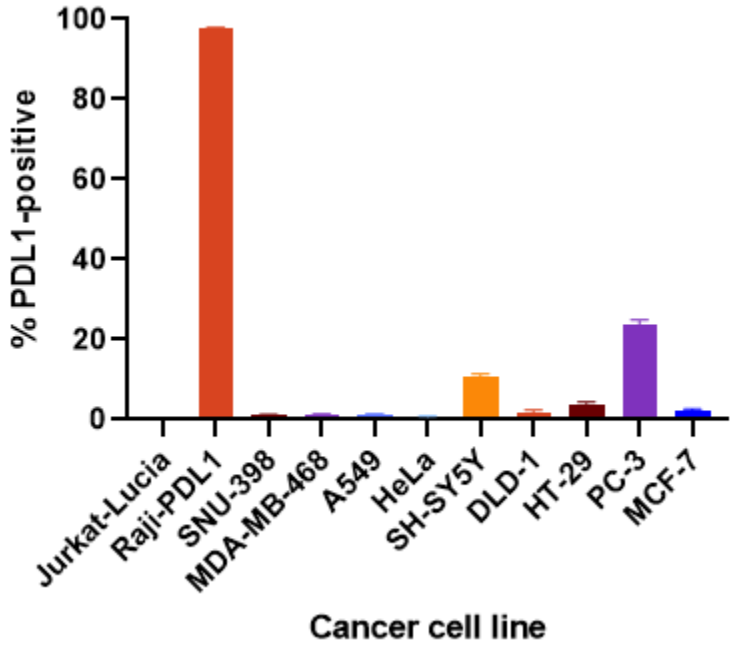

Figure 13. Percentage of PD-L1-positive cells by flow cytometry setting the negative gate to the expression of unlabelled cells. Data represented as mean ± SD of three technical replicates.

PD-1/PD-L1 immune checkpoint inhibitor screening assay with PC-3 cells

Since PC-3 cells expressed the highest level of PD-L1, after Raji-PDL1 cells, a test to investigate its inhibitory activity when interacting with Jurkat-Lucia cells was performed. As seen in **Figure 14**, the inhibitory effect was assessed as a measurement of the luminescent signal when NFAT activation is induced in Jurkat-Lucia with T cell TransAct or CD3/CD28. Compared to the control (Jurkat-Lucia cells alone), T cell TransAct and anti-CD3/anti-CD28 increased the activation of Jurkat-Lucia cells at 24 and 48 hours. At both time points, activation by anti-CD3/anti-CD28 was

higher. Nevertheless, the presence of PC-3 cells did not diminish the activation induced by T cell TransAct or anti-CD3/anti-CD28.

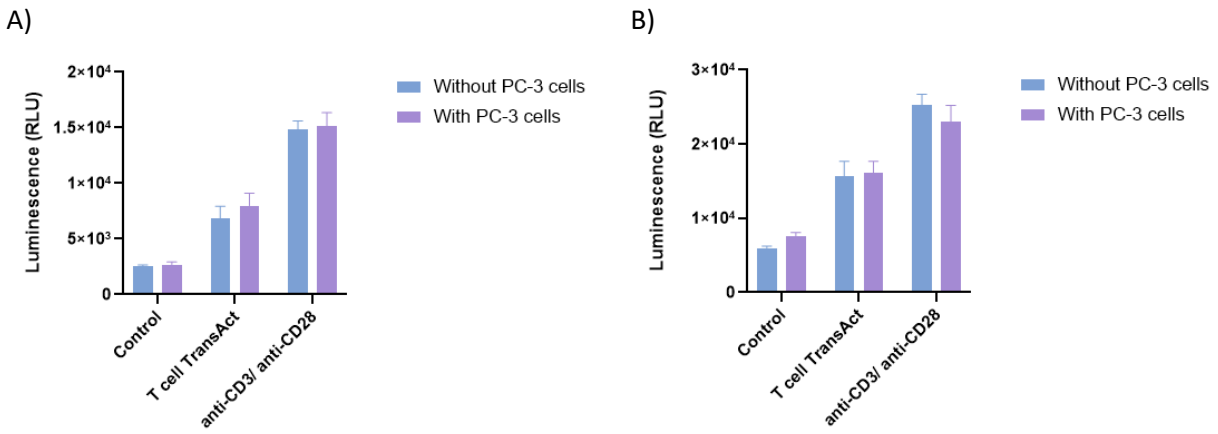

Figure 14. Comparison of the Jurkat-Lucia luciferase activity upon stimulation with T cell TransAct or anti-CD3/anti-CD28 for 24 (A) and 48 (B) hours when interacting (purple) or not (blue) with PC-3 cells. Data represented as mean ± SD of three technical replicates.

DISCUSSION

The increase in the number of clinical trials and the development of new anti-PD molecules requires approaches that allow the characterization of biological activity (Upadhaya et al., 2022; Versteven et al., 2018). Attempts to develop a cell-based assay to evaluate the activity of anti-PD inhibitors have been developed in academia as well as from the industrial sector. Although these assays generally use the NFAT controlled-luciferase reporter gene located in Jurkat T cells mechanism, the target cell line varies (Ganesan et al., 2019; Lu et al., 2022; Pandey et al., 2021; Versteven et al., 2018). Nonetheless, their value for compound screening resides in its short-term assay duration (1 day) and high-throughput potential (mostly 96-well plate automated readout) (Versteven et al., 2018). Therefore, in this work, we evaluated the capabilities of the PD-1/PD-L1 Bio-IC™ assay (InvivoGen) with FDA-approved mAb and inhibitors developed by Bristol-Myers Squibb (BMS) targeting PD-1 or PD-L1.

Particularly in the PD-blockade cell-based assays the target of soluble T cell activators employed as positive control also varies (e. g. anti-PD-1, anti-PD-L1, anti-CD3, anti-CD3/anti-TAA1, anti-

CD3/anti-TAA2, and Staphylococcal enterotoxin B) (Doronin et al., 2019; Ganesan et al., 2019). Another commercially available variant of antibodies targeting CD3 and/or CD28 is T cell TransAct. T cell TransAct is a colloidal reagent consisting of nanoscale iron oxide crystals embedded into a biocompatible polysaccharide matrix that has antibodies against CD3 and CD28 covalently attached to the matrix (Casati et al., 2013). Although it has proven to be effective in activating T cells (Casati et al., 2013), in this work its low activation yield (**Figs. 3** and **5**) might be due to intrinsic defects of the reagent associated with the expiration date.

Other common T cell activator molecules are PMA and Ionomycin. A combination of these molecules has been widely used in the study of T cell activation, and it is thoroughly reported that activates members of the NFAT family (Brignall et al., 2017; Han et al., 2013; Tomkowicz et al., 2015), which is consistent with our results in **Fig. 4**. Although PMA/Ionomycin can induce T cell proliferation and differentiation at certain concentrations, it can also promote activation-induced cell death (AICD) in lymphocytes (including the Jurkat cell line) (Chwae et al., 2002; Han et al., 2013; Lin et al., 2011). Additionally, the final assay concentration employed here is considerably higher than the one employed in the literature (Chwae et al., 2002; Han et al., 2013).

During T cell activation in cell-based assays, a strong dependency on the quality of the accessory cells has been suggested (Casati et al., 2013). Here we show that the cell lines employed in this immune checkpoint assay are sensitive enough to convey both activation and inhibition signals when interacting together. The inhibitory signal has proven to be functional since the level of activation when adding Atezolizumab is significantly higher, indicating the blockade of the PD-1/PD-L1 axis (**Fig. 6**), this in turn validates the functionality of the assay.

Several therapeutic mAb targeting PD-L1/PD-1 have been approved by the FDA for different cancers (Voli et al., 2020). Here some of those antibodies were tested, and even with some variability between biological replicates (data not shown), there was a clear dose response using the PD-1/PD-L1 Bio-IC™ assay (**Fig. 7**). As seen from the IC_{50} values, Atezolizumab and Durvalumab have dose-response curves similar to each other. A similar case happens for Pembrolizumab and Nivolumab. This clustering could be related to the antibody-differential targets (PD-L1 and PD-1 respectively). Overall, the antibody dose-response curves are consistent

with published results where it is reported that anti-PD-1 antibodies have higher IC_{50} values (and are therefore less potent) compared to anti-PD-L1 antibodies (De Sousa Linhares et al., 2019; Doronin et al., 2019). On the contrary, the antibodies' IC_{50} calculated here are around 1000 times higher than that in the literature, but comparisons should be made with caution since the assay conducted here employed different cell lines, cell densities and incubation times.

Due to limited responsiveness in some patients, the immune-related adverse events, production costs, and constraints in bioavailability, there is an increased interest in small molecule inhibitors (SMI) (Ganesan et al., 2019; T. Wang et al., 2022; Wu et al., 2021). Opposed to anti-PD antibodies that block by binding to the target receptor, the proposed mechanism for some PD-1/PD-L1 BMS SMI (such as BMS-8, BMS-202, and BMS-1001) is dimer-locking in which these molecules induce dimerization of two PD-L1 proteins via their PD-1 binding epitopes (see **Fig. 1**) (Park et al., 2021; Skalniak et al., 2017; Zak et al., 2016). This molecular packing makes it impossible for the recognition of PD-L1 by PD-1 *in vitro* (Guzik et al., 2017), which in our cell-based assay should translate into an increase in the luminescence signal.

Although we observed a dose response with the SMI employed here, the curve pattern varies between compounds (**Fig. 9**). These observations indicate that the structural differences in the compounds may play a role in interfering with the PD-1/PD-L1 interaction that the cell-based assay can detect (Ganesan et al., 2019). Although BMS-1001 was released as low toxicity and enhanced cell-based activity inhibitor, developed from previous compounds (Skalniak et al., 2017), in our work remained inactive (**Fig. 9**) but the cell viability did not drop below 70% (**Fig. 10**). A similar case happened with BMS-8, although at top concentrations it induced a 1.5-fold change in the luminescence signal.

On the other hand, it has been suggested that the NFAT-reporter assay may not be ideally suited for characterizing all classes of SMI (Sasikumar & Ramachandra, 2022). There is evidence showing that BMS-1001 and BMS-202 do not produce sigmoidal dose-response curves and that the activity of these compounds is largely constrained by the cytotoxic effect when using a similar reporter system with a different target cell line (CHO-K1) (Basu et al., 2019; Dai et al., 2021; L. Liu et al., 2021; Skalniak et al., 2017). Interestingly, Inhibitor 9, which is classified as a C_2 symmetric

compound, showed a similar IC_{50} value as another compound of the same family (known as "L7"), in a NFAT-reporter assay (L. Liu et al., 2021). Nevertheless, comparisons here are made with caution since we employed a cell assay from a different manufacturer with different target cell line and less incubation time.

Evidence shows that a high binding affinity in a biophysical assay does not equally translate into biological activity in cell-based assays (Surmiak et al., 2021). Particularly, for SMI its broad drug distribution might result in on- and off-target toxicity (Sasikumar & Ramachandra, 2022). The decrease in cell viability observed in **Fig. 10** might likely be associated with the phenomena mentioned above since it has been observed previously in other BMS inhibitors (Lu et al., 2022). Nevertheless, data from Lu and colleagues (2022) might suggest that the effects on cell viability were attenuated when studying diverse types of cancer cell lines or even human PBMCs at higher doses of the inhibitors.

Since the magnitude of the assay window was lower when testing SMI compared to mAb, different cell ratios were tested. As seen in **Table 3**, none of the target to effector cell ratios outperformed the rest of the assay windows. Therefore, the influence of the ratio between Raji-PDL1 and Jurkat-Lucia cells was discarded as a major factor in the assay window. On the other hand, the possible interference by serum proteins acting as an undesired target was tested (**Fig. 12**). However, no improvement was observed in reducing the percentage of FBS in the media of cells before treating them with either SMI or mAb.

Even though the activity of luciferase cannot give details on the antigen-specific intracellular signalling cascades or other interactions between cells (C. Liu et al., 2021); the results of this work suggest a certain degree of sensitivity to the target of the inhibitor as well as the mechanism of action that ultimately shape the dose-dependent responses. Hence, looking at the proposed biological mechanism, it is plausible that the difference by which inhibitors block the PD-1/PD-L1 interaction (physical blockade in the case of antibodies or dimerization of PD-L1 with SMI) promotes a differential reporter gene response. However, the potency and safety of these SMI have not yet reached the level of antibodies (Ganesan et al., 2019; Skalniak et al., 2017). Therefore, the overexpression of PD-L1 might play a strong suppressive role for the T cell

activation signals, and SMI are unable to disrupt T cell inhibition. Hence, a movement towards alternative cell lines with a better yield in the PD-1/PD-L1 axis was suggested.

There is evidence showing basal levels of PD-L1 in some cancer cell lines (such as HT-1080, H-460, PC-3, A549, HeLa, SH-SY5Y, etc.) based on western blotting (Voli et al., 2020; Xie et al., 2020; Zheng et al., 2019b). For the cell lines tested here, results suggest that such expression is minimal compared to that in the Raji-PDL1 cell line (**Fig. 13**). A common procedure to induce PD-L1 expression in cancer cell lines is the treatment with IFN-γ (Voli et al., 2020). Thus, for future experiments, PD-L1 expression could be induced to a moderate level in alternative cell lines using IFN- γ, which would allow for the screening of SMI in the PD-1/PD-L1 immune checkpoint cell-based assay. However, due to the dual role of IFN-γ (depending on the context can convey antitumor immunity or promote PD-L1-mediated immune escape), interpretations must be carried with compelling evidence (Mandai et al., 2016). Another alternative for this assay would be to acquire other cell lines that have proven a high baseline of PD-L1 expression.

As already mentioned, the overexpression of PD-L1 is a mechanism that allows cancer immune evasion through a reduction in cytokine production and cytotoxic activity against target cells, including tumour cells (Cha et al., 2019; Voli et al., 2020). Unfortunately, the level of expression of PD-L1 in PC-3 cells, which was lower than Raji-PDL1, was not enough to diminish the activation induced by T cell TransAct or CD3/CD28 (**Fig. 14**). Previously, a PD-1/PD-L1 blockade cell-based assay using MCF7 cells as the target cell line has been successful in downregulating T-cell activity and cytokine secretion (Zheng et al., 2019b). However, it is likely that diversification in gene expression states promoted cancer cell subpopulations that might respond differently to this type of assay (Nguyen et al., 2016). Aside from other differences regarding immune checkpoint protein in different cancer cell lines, it looks like the overexpression of PD-L1 in target cells and PD-1 in effector cells is a key factor in the system.

CONCLUSIONS

Anti-PD therapy is a promising strategy in immunotherapy, captivating the attention of public and private institutions. During this project, the proficiency of a commercially available cell-based assay suitable for screening of PD-1/PD-L1 inhibitors was tested. The aim of this project was to evaluate the activity of different compounds that block the interaction between PD-1 and PD-L1. Overall, the interaction between the cell lines from the anti-immune checkpoint cell-based assay from InvivoGen displayed a prominent level of inhibition of T cell activation. The inhibition was depleted by all the antibodies tested here at certain concentrations but that was not the case for all the SMI. Despite efforts to increase the assay window when using SMI (i.e., changing the target to effector cell ratio, modifying the concentration of serum proteins in the media, replacing the PD-L1-expressing cell line), the assay window did not increase. However, this pattern in which mAb outperform SMI during immune checkpoint luciferase reporter assays seems to be consistent with published data (Basu et al., 2019; L. Liu et al., 2021; Skalniak et al., 2017). Aside from the intrinsic molecular properties of small molecules, one of the reasons for the distinctive activity of mAb and SMI might be the mechanism of action (Figure 1). Additionally, new SMI are constantly in development to overcome issues with cell viability and dose-response behaviour, like those observed in this work.

Immune checkpoint blockade therapy has revolutionized the field of cancer immunotherapy for its powerful antitumor activity. However, the efficacy of the off-the-shelf antibodies is limited by many factors, such as cancer cell resistance in many patients and immune-related adverse events in others, low affordability, and complex production processes. Therefore, research on SMI is being pushed forward and requires suitable biological assays to assess its bioactivity before going into clinical trials. Future perspectives of this work concern the use of other cancer cell lines as target cells (expressing PD-L1) and quantification of secreted IL-2 to gain insight into the effector functions restored using this cell-based assay. Alternatively, with one of the available cancer cell lines already evaluated, a pre-stimulation with IFN-y to induce PD-L1 expression can be tested, followed by the interaction with the effector cell line. Finally, the dimerization mechanism can be evaluated by western blot analysis to investigate if the mechanism of action of the different inhibitors impacts the cell-based assay.

REFERENCES

Ai, L., Chen, J., Yan, H., He, Q., Luo, P., Xu, Z., & Yang, X. (2020). Research status and outlook of pd-1/pd-l1 inhibitors for cancer therapy. In *Drug Design, Development and Therapy* (Vol. 14, pp. 3625–3649). Dove Medical Press Ltd. https://doi.org/10.2147/DDDT.S267433

Akinleye, A., & Rasool, Z. (2019). Immune checkpoint inhibitors of PD-L1 as cancer therapeutics. *Journal of Hematology & Oncology*, *12*(1), 92. https://doi.org/10.1186/s13045-019-0779-5

Basu, S., Yang, J., Xu, B., Magiera-Mularz, K., Skalniak, L., Musielak, B., Kholodovych, V., Holak, T. A., & Hu, L. (2019). Design, Synthesis, Evaluation, and Structural Studies of C2-Symmetric Small Molecule Inhibitors of Programmed Cell Death-1/Programmed Death-Ligand 1 Protein–Protein Interaction. *Journal of Medicinal Chemistry*, *62*(15), 7250–7263. https://doi.org/10.1021/acs.jmedchem.9b00795

Brignall, R., Cauchy, P., Bevington, S. L., Gorman, B., Pisco, A. O., Bagnall, J., Boddington, C., Rowe, W., England, H., Rich, K., Schmidt, L., Dyer, N. P., Travis, M. A., Ott, S., Jackson, D. A., Cockerill, P. N., & Paszek, P. (2017). Integration of Kinase and Calcium Signaling at the Level of Chromatin Underlies Inducible Gene Activation in T Cells. *The Journal of Immunology*, *199*(8), 2652–2667. https://doi.org/10.4049/jimmunol.1602033

Casati, A., Varghaei-Nahvi, A., Feldman, S. A., Assenmacher, M., Rosenberg, S. A., Dudley, M. E., & Scheffold, A. (2013). Clinical-scale selection and viral transduction of human naïve and central memory CD8+ T cells for adoptive cell therapy of cancer patients. *Cancer Immunology, Immunotherapy*, *62*(10), 1563–1573. https://doi.org/10.1007/s00262-013-1459-x

Cha, J. H., Chan, L. C., Li, C. W., Hsu, J. L., & Hung, M. C. (2019). Mechanisms Controlling PD-L1 Expression in Cancer. In *Molecular Cell* (Vol. 76, Issue 3, pp. 359–370). Cell Press. https://doi.org/10.1016/j.molcel.2019.09.030

Chen, F.-F., Li, Z., Ma, D., & Yu, Q. (2020). Small-molecule PD-L1 inhibitor BMS1166 abrogates the function of PD-L1 by blocking its ER export. *Oncoimmunology*, *9*(1), 1831153. https://doi.org/10.1080/2162402X.2020.1831153

Chwae, Y.-J., Chang, M. J., Park, S. M., Yoon, H., Park, H.-J., Kim, S. J., & Kim, J. (2002). Molecular Mechanism of the Activation-Induced Cell Death Inhibition Mediated by a p70 Inhibitory Killer Cell Ig-Like Receptor in Jurkat T Cells1. *The Journal of Immunology*, *169*(7), 3726–3735. https://doi.org/10.4049/jimmunol.169.7.3726

Costa, B., & Vale, N. (2022). Dostarlimab: A Review. In *Biomolecules* (Vol. 12, Issue 8). MDPI. https://doi.org/10.3390/biom12081031

Dai, X., Wang, K., Chen, H., Huang, X., & Feng, Z. (2021). Design, synthesis, and biological evaluation of 1-methyl-1H-pyrazolo[4,3-b]pyridine derivatives as novel small-molecule inhibitors targeting the PD-1/PD-L1 interaction. *Bioorganic Chemistry*, *114*, 105034. https://doi.org/https://doi.org/10.1016/j.bioorg.2021.105034

De Sousa Linhares, A., Battin, C., Jutz, S., Leitner, J., Hafner, C., Tobias, J., Wiedermann, U., Kundi, M., Zlabinger, G. J., Grabmeier-Pfistershammer, K., & Steinberger, P. (2019). Therapeutic PD-L1

antibodies are more effective than PD-1 antibodies in blocking PD-1/PD-L1 signaling. *Scientific Reports*, *9*(1), 11472. https://doi.org/10.1038/s41598-019-47910-1

Dermani, F. K., Samadi, P., Rahmani, G., Kohlan, A. K., & Najafi, R. (2019). PD-1/PD-L1 immune checkpoint: Potential target for cancer therapy. *Journal of Cellular Physiology*, *234*(2), 1313–1325. https://doi.org/https://doi.org/10.1002/jcp.27172

Doronin, A. N., Gordeev, A. A., Kozlov, A. E., Smirnova, Y. A., Puchkova, M. Y., Ekimova, V. M., Basovskiy, Y. I., & Solovyev, V. V. (2019). T-Cell Engagers Based Bioassay for Evaluation of PD-1/PD-L1 Inhibitors Activity. *Biochemistry (Moscow)*, *84*(7), 711–719. https://doi.org/10.1134/S0006297919070034

Freeman, G. J. (2008). *Structures of PD-1 with its ligands: Sideways and dancing cheek to cheek*. www.pnas.orgcgidoi10.1073pnas.0805459105

Furuse, M., Kuwabara, H., Ikeda, N., Hattori, Y., Ichikawa, T., Kagawa, N., Kikuta, K., Tamai, S., Nakada, M., Wakabayashi, T., Wanibuchi, M., Kuroiwa, T., Hirose, Y., & Miyatake, S.-I. (2020). PD-L1 and PD-L2 expression in the tumor microenvironment including peritumoral tissue in primary central nervous system lymphoma. *BMC Cancer*, *20*(1), 277. https://doi.org/10.1186/s12885-020-06755-y

Ganesan, A., Ahmed, M., Okoye, I., Arutyunova, E., Babu, D., Turnbull, W. L., Kundu, J. K., Shields, J., Agopsowicz, K. C., Xu, L., Tabana, Y., Srivastava, N., Zhang, G., Moon, T. C., Belovodskiy, A., Hena, M., Kandadai, A. S., Hosseini, S. N., Hitt, M., … Barakat, K. (2019). Comprehensive in vitro characterization of PD-L1 small molecule inhibitors. *Scientific Reports*, *9*(1), 12392. https://doi.org/10.1038/s41598-019-48826-6

Guzik, K., Tomala, M., Muszak, D., Konieczny, M., Hec, A., Błaszkiewicz, U., Pustuła, M., Butera, R., Dömling, A., & Holak, T. A. (2019). Development of the inhibitors that target the PD-1/PD-L1 interaction—a brief look at progress on small molecules, peptides and macrocycles. In *Molecules* (Vol. 24, Issue 11). MDPI AG. https://doi.org/10.3390/molecules24112071

Guzik, K., Zak, K. M., Grudnik, P., Magiera, K., Musielak, B., Törner, R., Skalniak, L., Dömling, A., Dubin, G., & Holak, T. A. (2017). Small-Molecule Inhibitors of the Programmed Cell Death-1/Programmed Death-Ligand 1 (PD-1/PD-L1) Interaction via Transiently Induced Protein States and Dimerization of PD-L1. *Journal of Medicinal Chemistry*, *60*(13), 5857–5867. https://doi.org/10.1021/acs.jmedchem.7b00293

Han, S., Tie, X., Meng, L., Wang, Y., & Wu, A. (2013). PMA and Ionomycin Induce Glioblastoma Cell Death: Activation-Induced Cell-Death-Like Phenomena Occur in Glioma Cells. *PLOS ONE*, *8*(10), e76717-. https://doi.org/10.1371/journal.pone.0076717

Lee, J., Ahn, E., Kissick, H. T., & Ahmed, R. (2015). Reinvigorating exhausted T cells by blockade of the PD-1 pathway. *Forum on Immunopathological Diseases and Therapeutics*, *6*(1–2), 7–18. https://doi.org/10.1615/ForumImmunDisTher.2015014188

Lee, J. U., Kim, L. K., & Choi, J. M. (2018). Revisiting the concept of targeting NFAT to control T cell immunity and autoimmune diseases. In *Frontiers in Immunology* (Vol. 9, Issue NOV). Frontiers Media S.A. https://doi.org/10.3389/fimmu.2018.02747

Lei, Q., Wang, D., Sun, K., Wang, L., & Zhang, Y. (2020). Resistance Mechanisms of Anti-PD1/PDL1 Therapy in Solid Tumors. In *Frontiers in Cell and Developmental Biology* (Vol. 8). Frontiers Media S.A. https://doi.org/10.3389/fcell.2020.00672

Lin, H. P., Chang, J. Y., Lin, S. R., Lee, M. H., Huang, S. S., Hsu, L. J., & Chang, N. S. (2011). Identification of an in vivo MEK/WOX1 complex as a master switch for apoptosis in t cell leukemia. *Genes and Cancer*, *2*(5), 550–562. https://doi.org/10.1177/1947601911418498

Liu, C., Seeram, N. P., & Ma, H. (2021). Small molecule inhibitors against PD-1/PD-L1 immune checkpoints and current methodologies for their development: a review. *Cancer Cell International*, *21*(1), 239. https://doi.org/10.1186/s12935-021-01946-4

Liu, J., Chen, Z., Li, Y., Zhao, W., Wu, J. B., & Zhang, Z. (2021). PD-1/PD-L1 Checkpoint Inhibitors in Tumor Immunotherapy. In *Frontiers in Pharmacology* (Vol. 12). Frontiers Media S.A. https://doi.org/10.3389/fphar.2021.731798

Liu, L., Yao, Z., Wang, S., Xie, T., Wu, G., Zhang, H., Zhang, P., Wu, Y., Yuan, H., & Sun, H. (2021). Syntheses, Biological Evaluations, and Mechanistic Studies of Benzo[c][1,2,5]oxadiazole Derivatives as Potent PD-L1 Inhibitors with In Vivo Antitumor Activity. *Journal of Medicinal Chemistry*, *64*(12), 8391–8409. https://doi.org/10.1021/acs.jmedchem.1c00392

Lu, C.-H., Chung, W.-M., Tsai, C.-H., Cheng, J.-C., Hsu, K.-C., & Tzeng, H.-E. (2022). In vitro characterization of a small molecule PD-1 inhibitor that targets the PD-l/PD-L1 interaction. *Scientific Reports*, *12*(1), 303. https://doi.org/10.1038/s41598-021-03590-4

Mandai, M., Hamanishi, J., Abiko, K., Matsumura, N., Baba, T., & Konishi, I. (2016). Dual Faces of IFNγ in Cancer Progression: A Role of PD-L1 Induction in the Determination of Pro- and Antitumor Immunity. *Clinical Cancer Research*, *22*(10), 2329–2334. https://doi.org/10.1158/1078-0432.CCR-16-0224

Marin-Acevedo, J. A., Dholaria, B., Soyano, A. E., Knutson, K. L., Chumsri, S., & Lou, Y. (2018). Next generation of immune checkpoint therapy in cancer: new developments and challenges. *Journal of Hematology & Oncology*, *11*(1), 39. https://doi.org/10.1186/s13045-018-0582-8

Nguyen, A., Yoshida, M., Goodarzi, H., & Tavazoie, S. F. (2016). Highly variable cancer subpopulations that exhibit enhanced transcriptome variability and metastatic fitness. *Nature Communications*, *7*, 11246. https://doi.org/10.1038/ncomms11246

Pandey, M. S., Wang, C., Umlauf, S., & Lin, S. (2021). Simultaneous Inhibition of PD-1 and Stimulation of CD40 Signaling Pathways by Anti-PD-L1/CD40L Bispecific Fusion Protein Synergistically Activate Target and Effector Cells. *International Journal of Molecular Sciences*, *22*(21). https://doi.org/10.3390/ijms222111302

Park, J.-J., Thi, E. P., Carpio, V. H., Bi, Y., Cole, A. G., Dorsey, B. D., Fan, K., Harasym, T., Iott, C. L., Kadhim, S., Kim, J. H., Lee, A. C. H., Nguyen, D., Paratala, B. S., Qiu, R., White, A., Lakshminarasimhan, D., Leo, C., Suto, R. K., … Moore, C. B. (2021). Checkpoint inhibition through small molecule-induced internalization of programmed death-ligand 1. *Nature Communications*, *12*(1), 1222. https://doi.org/10.1038/s41467-021-21410-1

Rao, S., Anandappa, G., Capdevila, J., Dahan, L., Evesque, L., Kim, S., Saunders, M. P., Gilbert, D. C., Jensen, L. H., Samalin, E., Spindler, K.-L., Tamberi, S., Demols, A., Guren, M. G., Arnold, D., Fakih, M., Kayyal, T., Cornfeld, M., Tian, C., … Spano, J.-P. (2022). A phase II study of retifanlimab (INCMGA00012) in patients with squamous carcinoma of the anal canal who have progressed following platinum-based chemotherapy (POD1UM-202)☆. *ESMO Open*, *7*(4), 100529. https://doi.org/https://doi.org/10.1016/j.esmoop.2022.100529

Robert, C. (2020). A decade of immune-checkpoint inhibitors in cancer therapy. *Nature Communications*, *11*(1), 3801. https://doi.org/10.1038/s41467-020-17670-y

Sakuishi, K., Apetoh, L., Sullivan, J. M., Blazar, B. R., Kuchroo, V. K., & Anderson, A. C. (2010). Targeting Tim-3 and PD-1 pathways to reverse T cell exhaustion and restore anti-tumor immunity. *Journal of Experimental Medicine*, *207*(10), 2187–2194. https://doi.org/10.1084/jem.20100643

Sasikumar, P. G., & Ramachandra, M. (2022). Small Molecule Agents Targeting PD-1 Checkpoint Pathway for Cancer Immunotherapy: Mechanisms of Action and Other Considerations for Their Advanced Development. *Frontiers in Immunology*, *13*. https://www.frontiersin.org/articles/10.3389/fimmu.2022.752065

Shi, D., An, X., Bai, Q., Bing, Z., Zhou, S., Liu, H., & Yao, X. (2019). Computational Insight Into the Small Molecule Intervening PD-L1 Dimerization and the Potential Structure-Activity Relationship. *Frontiers in Chemistry*, *7*, 764. https://doi.org/10.3389/fchem.2019.00764

Shimizu, K., Sugiura, D., Okazaki, I. mi, Maruhashi, T., Takegami, Y., Cheng, C., Ozaki, S., & Okazaki, T. (2020). PD-1 Imposes Qualitative Control of Cellular Transcriptomes in Response to T Cell Activation. *Molecular Cell*, *77*(5), 937-950.e6. https://doi.org/10.1016/j.molcel.2019.12.012

Skalniak, L., Zak, K. M., Guzik, K., Magiera, K., Musielak, B., Pachota, M., Szelazek, B., Kocik, J., Grudnik, P., Tomala, M., Krzanik, S., Pyrc, K., Dömling, A., Dubin, G., & Holak, T. A. (2017). Small-molecule inhibitors of PD-1/PD-L1 immune checkpoint alleviate the PD-L1-induced exhaustion of T-cells. In *Oncotarget* (Vol. 8, Issue 42). www.impactjournals.com/oncotarget/

Surmiak, E., Magiera-Mularz, K., Musielak, B., Muszak, D., Kocik-Krol, J., Kitel, R., Plewka, J., Holak, T. A., & Skalniak, L. (2021). PD-L1 inhibitors: Different classes, activities, and mechanisms of action. *International Journal of Molecular Sciences*, *22*(21). https://doi.org/10.3390/ijms222111797

Tomkowicz, B., Walsh, E., Cotty, A., Verona, R., Sabins, N., Kaplan, F., Santulli-Marotto, S., Chin, C.-N., Mooney, J., Lingham, R. B., Naso, M., & McCabe, T. (2015). TIM-3 Suppresses Anti-CD3/CD28-Induced TCR Activation and IL-2 Expression through the NFAT Signaling Pathway. *PLOS ONE*, *10*(10), e0140694-. https://doi.org/10.1371/journal.pone.0140694

Upadhaya, S., Neftelinov, S. T., Hodge, J., & Campbell, J. (2022). Challenges and opportunities in the PD1/PDL1 inhibitor clinical trial landscape. In *Nature Reviews Drug Discovery* (Vol. 21, Issue 7, pp. 482–483). Nature Research. https://doi.org/10.1038/d41573-022-00030-4

Versteven, M., Van Den Bergh, J. M. J., Broos, K., Fujiki, F., Campillo-Davo, D., De Reu, H., Morimoto, S., Lecocq, Q., Keyaerts, M., Berneman, Z., Sugiyama, H., Van Tendeloo, V. F. I., Breckpot, K., & Lion, E. (2018). A versatile T cell-based assay to assess therapeutic antigen-specific PD-1-targeted approaches. In *Oncotarget* (Vol. 9, Issue 45). www.oncotarget.com

Voli, F., Valli, E., Lerra, L., Kimpton, K., Saletta, F., Giorgi, F. M., Mercatelli, D., Rouaen, J. R. C., Shen, S., Murray, J. E., Ahmed-Cox, A., Cirillo, G., Mayoh, C., Beavis, P. A., Haber, M., Trapani, J. A., Kavallaris, M., & Vittorio, O. (2020). Intratumoral Copper Modulates PD-L1 Expression and Influences Tumor Immune Evasion. *Cancer Research*, *80*(19), 4129–4144. https://doi.org/10.1158/0008-5472.CAN-20-0471

Wang, T., Cai, S., Cheng, Y., Zhang, W., Wang, M., Sun, H., Guo, B., Li, Z., Xiao, Y., & Jiang, S. (2022). Discovery of Small-Molecule Inhibitors of the PD-1/PD-L1 Axis That Promote PD-L1 Internalization and Degradation. *Journal of Medicinal Chemistry*, *65*(5), 3879–3893. https://doi.org/10.1021/acs.jmedchem.1c01682

Wang, X., Teng, F., Kong, L., & Yu, J. (2016). PD-L1 expression in human cancers and its association with clinical outcomes. In *OncoTargets and Therapy* (Vol. 9, pp. 5023–5039). Dove Medical Press Ltd. https://doi.org/10.2147/OTT.S105862

Wei, J., Luo, C., Wang, Y., Guo, Y., Dai, H., Tong, C., Ti, D., Wu, Z., & Han, W. (2019). PD-1 silencing impairs the anti-tumor function of chimeric antigen receptor modified T cells by inhibiting proliferation activity. *Journal for ImmunoTherapy of Cancer*, *7*(1), 209. https://doi.org/10.1186/s40425-019-0685-y

Wu, X., Meng, Y., Liu, L., Gong, G., Zhang, H., Hou, Y., Liu, C., Wu, D., & Qin, M. (2021). Insights into non-peptide small-molecule inhibitors of the PD-1/PD-L1 interaction: Development and perspective. *Bioorganic & Medicinal Chemistry*, *33*, 116038. https://doi.org/https://doi.org/10.1016/j.bmc.2021.116038

Xie, Y., Ding, J., Cui, X., Wu, M., Huang, C., Zhang, R., Wang, J., Li, X., Cen, S., & Zhou, J. (2020). Screening of kinase inhibitors downregulating PD-L1 expression via on/in cell quantitative immunoblots. *European Journal of Pharmaceutical Sciences*, *142*, 105088. https://doi.org/https://doi.org/10.1016/j.ejps.2019.105088

Xing, X., Guo, J., Ding, G., Li, B., Dong, B., Feng, Q., Li, S., Zhang, J., Ying, X., Cheng, X., Guo, T., Du, H., Hu, Y., Zhou, T., Wang, X., Li, L., Li, Q., Xie, M., Li, L., … Ji, J. (2018). Analysis of PD1, PDL1, PDL2 expression and T cells infiltration in 1014 gastric cancer patients. *OncoImmunology*, *7*(3), e1356144. https://doi.org/10.1080/2162402X.2017.1356144

Xu, Y., Wu, Y., Zhang, S., Ma, P., Jin, X., Wang, Z., Yao, M., Zhang, E., Tao, B., Qin, Y., Chen, H., Liu, A., Chen, M., Xiao, M., Lu, C., Mao, R., & Fan, Y. (2019). A Tumor-Specific Super-Enhancer Drives Immune Evasion by Guiding Synchronous Expression of PD-L1 and PD-L2. *Cell Reports*, *29*(11), 3435-3447.e4. https://doi.org/10.1016/j.celrep.2019.10.093

Xue, C., Zhu, D., Chen, L., Xu, Y., Xu, B., Zhang, D., & Jiang, J. (2019). Expression and prognostic value of PD-L1 and PD-L2 in ovarian cancer. *Translational Cancer Research*, *8*(1), 111–119. https://doi.org/10.21037/tcr.2019.01.09

Zak, K. M., Grudnik, P., Guzik, K., Zieba, B. J., Musielak, B., Dömling, A., Dubin, G., & Holak, T. A. (2016). Structural basis for small molecule targeting of the programmed death ligand 1 (PD-L1). *Oncotarget*, *7*(21), 30323–30335. https://doi.org/10.18632/oncotarget.8730

Zheng, Y., Fang, Y. C., & Li, J. (2019a). PD-L1 expression levels on tumor cells affect their immunosuppressive activity. *Oncology Letters*, *18*(5), 5399–5407. https://doi.org/10.3892/ol.2019.10903

Zheng, Y., Fang, Y. C., & Li, J. (2019b). PD-L1 expression levels on tumor cells affect their immunosuppressive activity. *Oncology Letters*, *18*(5), 5399–5407. https://doi.org/10.3892/ol.2019.10903

APPENDIX

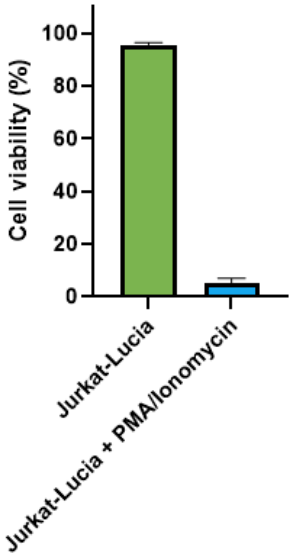

Figure S1. Impact of PMA/Ionomycin (1 µM/3 µM) on cell viability after a 24-hour incubation. Data represented as mean ± SD of four technical replicates plotted.

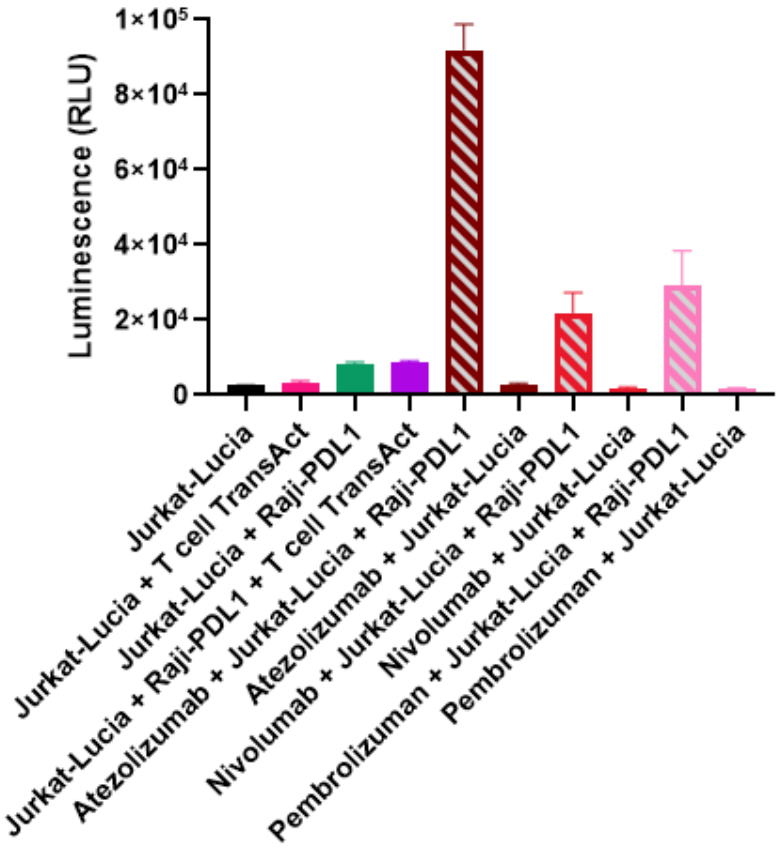

Figure S2. Comparison of the Jurkat-Lucia luciferase activity upon stimulation by T cell TransAct or an antibody (Atezolizumab at 5 µg/ml, Nivolumab at 10 µg/ml and Pembrolizumab at 10.5 µg/ml) after a 4-hour incubation. Data represented as mean ± SD of four technical replicates plotted.

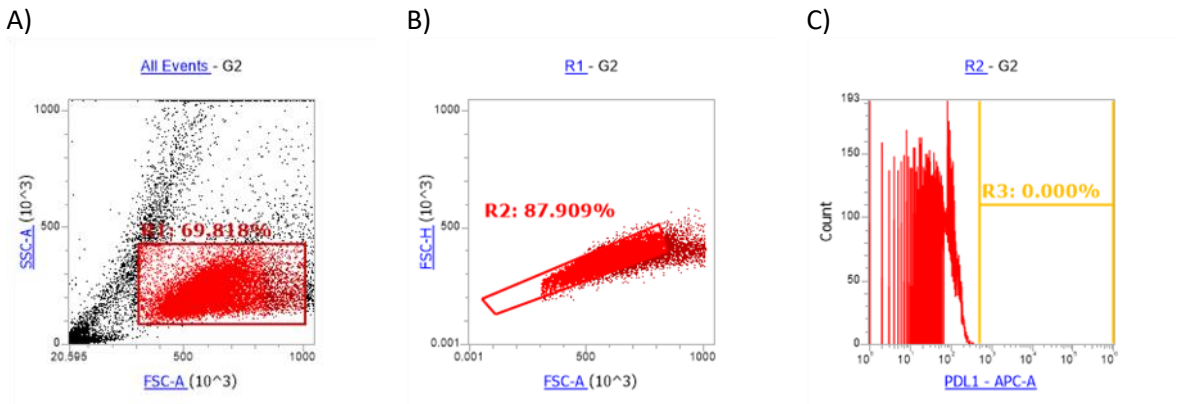

Figure S3. Representative flow cytometry plots depicting gating strategy for identification the expression of PD-L1 in unlabelled Jurkat cells (A: cell population selection, B: single cell population selection, C: PD-L1 expression in single cells).

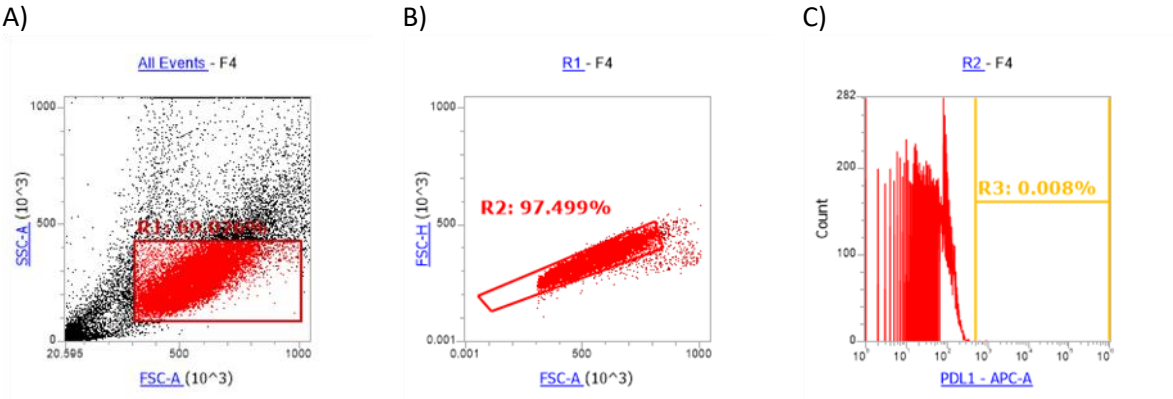

Figure S4. Representative flow cytometry plots depicting gating strategy for identification the expression of PD-L1 in unlabelled Raji cells (A: cell population selection, B: single cell population selection, C: PD-L1 expression in single cells).

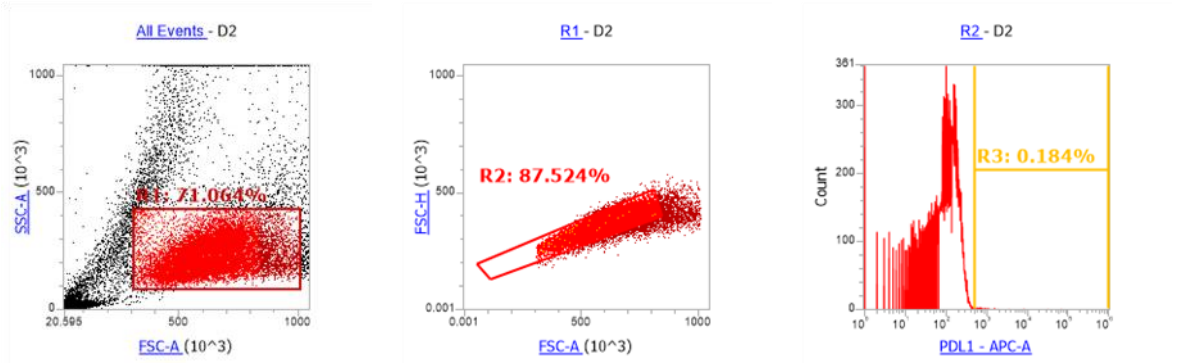

Figure S5. Representative flow cytometry plots depicting gating strategy for identification the expression of PD-L1 in Jurkat cells (A: cell population selection, B: single cell population selection, C: PD-L1 expression in single cells).

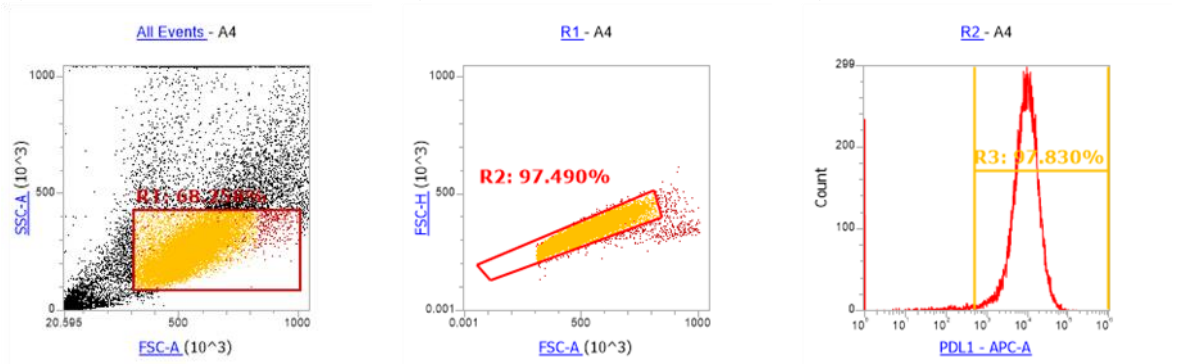

Figure S6. Representative flow cytometry plots depicting gating strategy for identification the expression of PD-L1 in Raji cells (A: cell population selection, B: single cell population selection, C: PD-L1 expression in single cells).