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REVIEW

Comprehensive assessment of TECENTRIQ® and OPDIVO®: analyzing immunotherapy indications withdrawn in triple-negative breast cancer and hepatocellular carcinoma

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Abstract

Atezolizumab (TECENTRIQ®) and nivolumab (OPDIVO®) are both immunotherapeutic indications targeting programmed cell death 1 ligand 1 (PD-L1) and programmed cell death 1 (PD-1), respectively. These inhibitors hold promise as therapies for triple-negative breast cancer (TNBC) and hepatocellular carcinoma (HCC) and have demonstrated encouraging results in reducing the progression and spread of tumors. However, due to their adverse effects and low response rates, the US Food and Drug Administration (FDA) has withdrawn the approval of atezolizumab in TNBC and nivolumab in HCC treatment. The withdrawals of atezolizumab and nivolumab have raised concerns regarding their effectiveness and the ability to predict treatment responses. Therefore, the current study aims to investigate the immunotherapy withdrawal of PD-1/PD-L1 inhibitors, specifically atezolizumab for TNBC and nivolumab for HCC. This study will examine both the structural and clinical aspects. This review provides detailed insights into the structure of the PD-1 receptor and its ligands, the interactions between PD-1 and PD-L1, and their interactions with the withdrawn antibodies (atezolizumab and nivolumab) as well as PD-1 and PD-L1 modifications. In addition, this review further assesses these antibodies in the context of TNBC and HCC. It seeks to elucidate the factors that contribute to diverse responses to PD-1/PD-L1 therapy in different types of cancer and propose approaches for predicting responses, mitigating the potential risks linked to therapy withdrawals, and optimizing patient outcomes. By better understanding the mechanisms underlying responses to PD-1/PD-L1 therapy and developing strategies to predict these responses, it is possible to create more efficient treatments for TNBC and HCC.

Keywords Atezolizumab · Nivolumab · PD-L1; PD-1 · FDA approvals · TNBC · HCC

Abbreviations

ADA	Anti-drug antibodies
ADCC	Antibody-dependent cellular cytotoxicity
AMPK	Adenosine monophosphate-activated protein kinase
c-CBL	Casitas B-lineage lymphoma
CDR	Complementarity determining regions
DOR	Duration of response
EGFR	Epidermal growth factor receptor
FDA	The United States food and drug administration
GSK	Glycogen synthase kinase
HCC	Hepatocellular carcinoma
IgV	Immunoglobulin-variable

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ICB	Immune checkpoint blockade
ITIM	Immunoreceptor tyrosine-based inhibition motif
ITSM	Immunoreceptor tyrosine-based switch motif
NEK	Never in mitosis gene A (NIMA)-related kinase
NK	Natural killer
NSCLC	Non-small cell lung cancer
OS	Overall survival
PD-L1	Programmed cell death 1 ligand 1
PD-1	Programmed cell death protein 1
PFS	Progression-free survival
RTK	Receptor tyrosine kinase
SHP	Src homology region 2 (SH2)-containing protein tyrosine phosphatase
SPOP	The substrate-binding adaptor speckle-type POZ protein
TEAE	Treatment-related adverse event
USP	Ubiquitin-specific peptidases
TAM	Tumor-associated macrophage
TNBC	Triple-negative breast cancer

1 Introduction

Immune checkpoint proteins such as programmed cell death protein 1 (PD-1), programmed cell death 1 ligand 1 (PD-L1), and cytotoxic T lymphocyte-associated protein 4 (CTLA-4) regulate the immune response, preventing autoimmune responses and allowing self-tolerance. However, these immune checkpoints can hinder the immune system's ability to recognize and attack cancer cells, enabling tumor cell evasion [1, 2]. Immune checkpoint inhibitors have emerged as promising immunotherapy options for hepatocellular carcinoma (HCC) and triple-negative breast cancer (TNBC), demonstrating the potential to reduce tumor progression, metastasis, and recurrence [3, 4].

The PD-1 receptor, expressed on various immune cells, especially cytotoxic T-cells, interacts with PD-L1 expressed on tumor cells. This interaction leads to T-cell exhaustion and inhibits proliferation and activation of $CD8^+$ T-cells. Moreover, it weakens the responses of effector T-cells and disrupts the release of cytokines [5, 6]. PD-1/PD-L1 inhibitors pharmacologically disrupt this interaction, promoting a positive immune response against tumors and reactivating anticancer immunity [7–9]. Currently, the US Food and Drug Administration (FDA) has approved six PD-1 and PD-L1 antibodies, including three PD-1 inhibitors (cemiplimab, nivolumab, and pembrolizumab) and three PD-L1 inhibitors (avelumab, atezolizumab, and durvalumab). These therapeutic agents offer new avenues for targeting the PD-1/PD-L1 pathway and enhancing the immune response against cancer [10]. Atezolizumab (MPDL3280A, TECENTRIQ®) is an engineered monoclonal antibody developed by Roche Genentech, acting as a PD-L1 antagonist [11]. It achieved FDA approval in 2016 as the first PD-L1 antibody for the treatment of metastatic non-small cell lung cancer (NSCLC), based on the positive outcomes observed in two randomized clinical trials [12]. Subsequently, in March 2019, the FDA approved the combination of atezolizumab with nab-paclitaxel for the treatment of TNBC patients, following results from the phase III IMpassion-130 trial (NCT02425891). However, the approval of this combination for TNBC was later withdrawn in October 2021 [4, 13]. The manufacturer cited the voluntary withdrawal as a result of better efficacy observed with pembrolizumab plus chemotherapy, particularly in tumors with a combined positive score (CPS) greater than ten [14].

On the other hand, nivolumab is a fully-humanized monoclonal antibody that targets PD-1 (the PD-L1 receptor), thereby enhancing the antitumor activity of suppressed effector T-cells [15, 16]. In 2017, nivolumab (BMS-936558, OPDIVO®) was conditionally approved as a second-line treatment for HCC patients whose disease had progressed or showed no response to sorafenib, a kinase inhibitor, based on encouraging results from Phases I and II of the CheckMate-040 trial (NCT01658878)[17, 18]. Although nivolumab's approvals for advanced HCC patients in combination with cabozantinib and ipilimumab remain valid, the manufacturer, Bristol Myers Squibb, voluntarily withdrew nivolumab monotherapy for HCC in July 2021. The withdrawals of atezolizumab for TNBC and nivolumab monotherapy for HCC emphasize the evolving landscape of immunotherapy in cancer treatment. As new data and evidence emerge, treatment approaches may be modified to optimize patient outcomes. Further research and clinical trials are essential to fully comprehend the effectiveness and limitations of these immune checkpoint inhibitors in different types of cancer.

Unlike atezolizumab, which has been approved for a limited number of cancer types including NSCLC, small cell lung cancer (SCLC), HCC, and melanoma, nivolumab offers a therapeutic option for a broader range of cancers. These encompass melanoma, renal cell carcinoma, NSCLC, mesothelioma, head and neck cancer, bladder cancer, and gastrointestinal cancers. As the withdrawal of an indication has significant effects on society and pharmaceutical companies, this review undertakes a comprehensive assessment of atezolizumab and nivolumab in the context of TNBC and HCC, for which these antibodies were withdrawn. The authors aim to address why PD-1/PD-L1 therapy works differently in TNBC and HCC, and how we can predict the responses to immune checkpoint inhibitors and decrease risks of withdrawals. To unravel these queries, this study adopts a thorough approach that encompasses both structural and clinical perspectives.

2 PD-1 and its ligands

2.1 Structural details

PD-1, also known as CD279, is a transmembrane receptor encoded by the *PDCD1* gene located on chromosome 2 [19]. It is a 32-kDa glycoprotein consisting of 288 amino acids. PD-1 adopts a type I transmembrane topology, with a β-sandwich immunoglobulin-variable (IgV)-type structure, wherein C54 and C123 form a disulfide bond [20, 21]. The full-length PD-1 can be divided into three domains: an ectodomain, a transmembrane domain, and an intracellular domain containing tyrosine-based signaling motifs (Fig. 1). The ectodomain encompasses the signal peptide, N-terminal loop, extracellular IgV (comprising the BC and FG loops), and the stalk region domains. The stalk region, spanning 20 amino acids, separates the IgV from the transmembrane domain [20, 22]. In the cytoplasmic tail of PD-1, there are two critical motifs: the immunoreceptor tyrosine-based inhibition motif (ITIM) and the immunoreceptor tyrosine-based switch motif (ITSM). ITIM and ITSM motifs are represented by the N-terminal sequence VDYGEL and C-terminal sequence TEYATI, respectively [23, 24]. The phosphorylation of these sites plays a significant role in PD-1's mechanism of action. Previously, it was believed that PD-1 functions as a monomer and does not form dimers like CTLA-4 [25, 26]. However, a study published in 2020 demonstrated that non-covalent homodimerization of PD-1 is possible through an interaction with the Src homology region 2 (SH2)-containing protein tyrosine phosphatase-2 (SHP-2). SHP-2, which consists of N-terminal (N-SH2), C-terminal (C-SH2), and protein tyrosine phosphatase domains, acts as a bridge, bringing two PD-1 molecules together through the phosphorylated ITSM-Y248 residue [24]. The N-SH2 and C-SH2 domains of SHP-2 are both required for PD-1 dimerization. The inhibitory function of PD-1 occurs through its interaction with two known ligands, PD-L1 (CD274 or B7-H1) and PD-L2 (CD273 or B7-DC) [27]. These ligands play a critical role in regulating the immune response and preventing excessive immune activation. The binding of PD-1 to its ligands leads to the suppression of T-cell activation, enabling immune tolerance and avoiding harmful autoimmune reactions.

PD-L1 is a 33-kDa transmembrane protein belonging to the B7 family and is composed of 290 amino acid residues [28]. It consists of two immunoglobulin-like domains, IgV (D1: F19-T127) and IgC (D2: P133-V225), a transmembrane domain (T239-F259), and a short intracellular tail (R260-T290) [29]. PD-L1 can homodimerize through hydrogen bonds between specific residues at the D1 and D2 domains. These residues include T22, E39, K41, V44, Q91 in the D1 domain, and I141, Q139, R140, V143, Q156, E228, and P230 in the D2 domain [30]. PD-L1 has a wide range of expression, being present not only on various immune cells such as antigen-presenting cells (APCs), B-cells, T-cells, and macrophages but also frequently expressed on the surfaces of both liquid and solid tumor cells [31]. In contrast, PD-L2 expression is more limited, primarily found on the surfaces of APCs, but it has a higher binding affinity for PD-1, about three to four times stronger than PD-L1. The IgV domains of PD-L2 and PD-L1 are different, with PD-L2 possessing a flexible loop between C–D β-strands around E71, providing a larger interaction area with PD-1 [32].

However, immune cell re-activation is guaranteed by an inhibitory tryptophan residue (W110) and a glycan tail on N64 in mPD-L2, which negatively regulate the PD-L2/PD-1 interaction, leading to dissociation [32].

2.2 PD-1 and PD-L1 interactions

The interaction between PD-1 and PD-L1 primarily occurs through their IgV domains, specifically involving the A´GFCC´ β-sheets, and forms a complex with 87 interatomic bonds [33]. This connection consists of 18 hydrogen bonds, involving 18 residues of mouse PD-1 (mPD-1) and 14 residues of human PD-L1 (hPD-L1). Remarkably, 15 of these residues are conserved between human and mouse PD-1. Nonetheless, there are three amino acid differences between the interactions of mPD-1/hPD-L1 and hPD-1/hPD-L1. Specifically, M64, N68, and V90 in mouse PD-1 (PDB ID: 3RNK) are substituted with V64, Y68, and G90 in humans (PDB ID: 6J14) [33]. In addition to hydrogen bonds, hydrophobic interactions also play a crucial role in facilitating the binding of PD-1 and PD-L1. Residues A121, D122, Y123, and K124 of PD-L1 (PDB ID: 7XAD) contribute to increased affinity with PD-1 (Fig. 1)[34]. These intricate interactions between PD-1 and PD-L1 are essential for regulating immune responses and maintaining immune tolerance. Understanding these interactions provides valuable insights for developing targeted therapies aimed at modulating the PD-1/PD-L1 pathway in various disease contexts [35].

On the other hand, a comparison was made between the effects of mutated residues on PD-1 binding in both PD-L1 and PD-L2. To assess the potential reduction of ligand-receptor binding by at least 50%, targeted mutations were introduced into 21 residues of mPD-L1 and 17 residues of mPD-L2, known to play a role in ligand-receptor interactions. Notably, half of these mutations affected the binding were in distinct sections known as C′-, C″-, F-, and G-sheets. Mutations directed at F67, K124, I126, and K129 of PD-L1 (PDB ID: 7XAD) and R56, S67, E71, R101, I105, D111, and K113 of PD-L2 (PDB ID: 3BOV) significantly affected the binding process [33]. Additionally, alkyl-π and π-π interactions play crucial roles in PD-1 and PD-L1 binding.

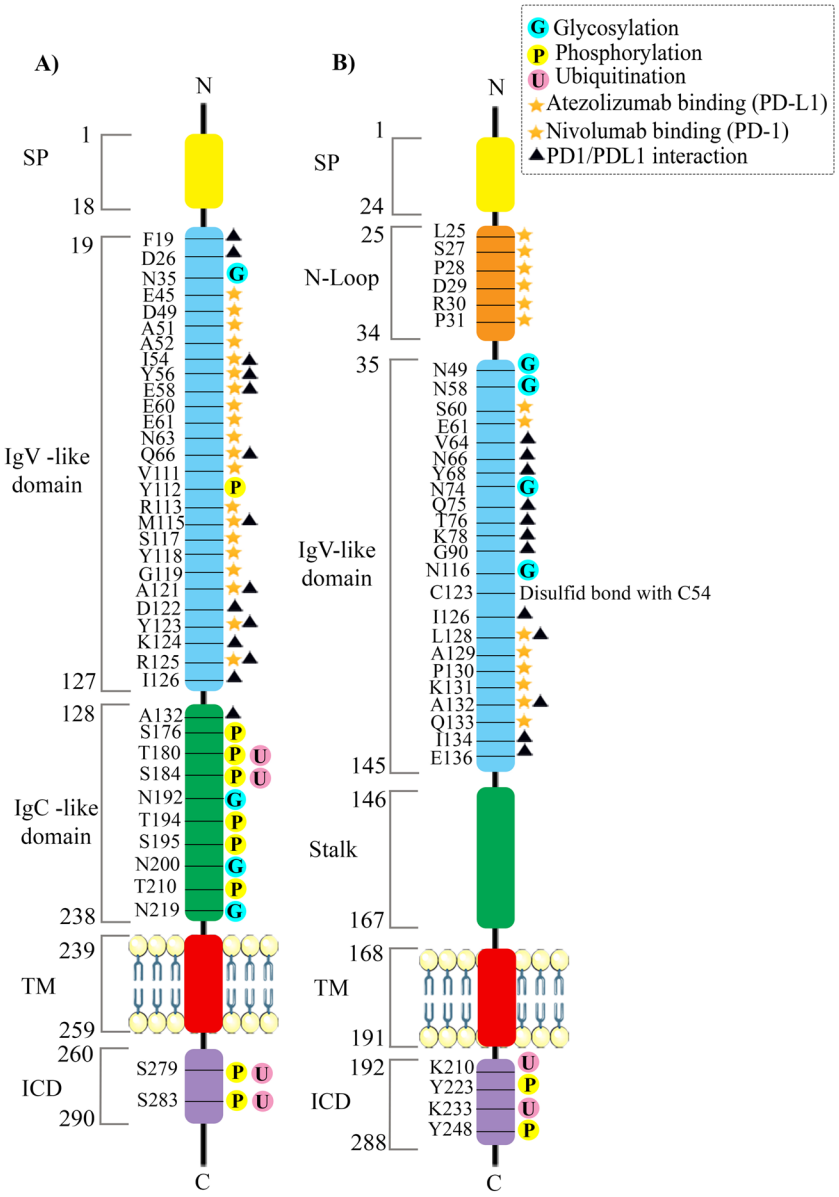


Fig. 1 Structure and key amino acid residues in PD-L1 (**A**) and PD-1 (**B**). The interaction between PD-1 and PD-L1 is primarily mediated by their IgV domains, forming 87 interatomic bonds. Essential alterations in binding were observed upon introducing mutations to specific residues, including F67, K124, I126, and K129 in PD-L1 (PDB ID: 7XAD). Alkyl-π and π—π interactions play critical roles in facilitating the binding of PD-1 and PD-L1. Both the ligand (PD-L1) and receptor (PD-1) contribute to a central hydrophobic core, with key residues such as V64, I126, L128, A132, and I134 in PD-1 (PDB ID: 6J14), and I54, Y56, M115, A121, and Y123 in PD-L1 (PDB ID: 7XAD). During the π-π stacking interaction, the phenol groups of the side chains Y68 (PD-1) and Y123 (PD-L1) align in an antiparallel manner, forming hydrogen bonds with the hydroxyl group of Y68 (PD-1) and the carboxyl group of D122 (PD-L1). Additional hydrogen bonds are formed through interactions between T76 (PD-1) and Y123 (PD-L1), Q75 (PD-1) and R125 (PD-L1), Q75 (PD-1) and D26 (PD-L1), T76 (PD-1) and K124 (PD-L1), and K78 (PD-1) and F19 (PD-L1). Furthermore, modifications to PD-1 and PD-L1 can affect their protein activity and the effectiveness of drugs targeting this pathway. These modifications include glycosylation and ubiquitination of PD-1/PD-L1, which may have implications for regulating immune responses and immunotherapy outcomes

The ligand and receptor form a central hydrophobic core involving V64, I126, L128, A132, and I134 in PD-1 (PDB ID: 6J14), and I54, Y56, M115, A121, and Y123 in PD-L1 (PDB ID: 7XAD) [21]. An in-silico study predicted that the interaction between PD-L1 and PD-1 is highly reliant on Y56, D122, and K124 residues, particularly through the π-π stacking interaction of Y56. Notably, this interaction can be disrupted by various PD-1 and PD-L1 inhibitors [36]. The hydrophobic network formed by these interactions also facilitates additional hydrogen bond interactions between

the receptor and the ligand. During the π-π stacking interaction, the phenol groups of the side chains of Y68 (PD-1) and Y123 (PD-L1) are oriented antiparallel, resulting in the formation of hydrogen bonds through interactions between the hydroxyl group of the side chain of Y68 (PD-1) and the carboxyl group of the side chain of D122 (PD-L1). Furthermore, hydrogen bonds are generated through interactions between T76 (PD-1) and Y123 (PD-L1), Q75 (PD-1) and R125 (PD-L1), Q75 (PD-1) and D26 (PD-L1), T76 (PD-1) and K124 (PD-L1), and K78 (PD-1) and F19 (PD-L1) [21]. Moreover, the stability of PD-1 and PD-L1 binding is further enhanced by interactions between N66 (PD-1) and A121 (PD-L1), A132 (PD-1) and Q66 (PD-L1), as well as water-mediated interactions of the backbone amide and carbonyl of I134 (PD-1) with two PD-L1 residues, Y56 and E58. Another important interaction involves a maintained salt bridge between E136 (PD-1) and R113 (PD-L1) [21], which is interrupted by atezolizumab, as shown in previous studies [37].

Fourteen amino acids play a critical role in mediating the binding between PD-1 and nivolumab in PD-1. Specifically, the PD-1 N-terminal loop is responsible for binding to nivolumab, which, in turn, induces the rebinding of the FG and BC loops of the IgV domain [20]. The N-terminal loop's involvement in nivolumab binding is essential; however, it is distant from the PD-1/PD-L1 interaction site, preventing nivolumab from recognizing PD-L1 [38]. The epitope recognized by nivolumab's complementarity determining regions (CDRs) on the light chain (VL) overlaps with four amino acids in the PD-L1 binding site situated at the PD-1 FG loop. This characteristic makes nivolumab a potent competitor for PD-L1 (Fig. 1) [39]. Moreover, the interaction between PD-1 N-terminal (residues 26–30) and nivolumab induces conformational rearrangements in the BC (residues 57–63) and FG (residues 127–134) loops of PD-1, leading to a more robust and stable binding state [38]. This interaction also increases the residence time of the PD-1/nivolumab complex, as suggested by Liu et al. [20]. Additionally, the N-terminal loop serves to protect the IgV-nivolumab hydrogen bonds from water, further enhancing the residence time of nivolumab on PD-1. It has been indicated that binding of the N-terminal loop with nivolumab can provide an environment with a lower dielectric constant around the BC loop, leading to lower polarity. This reduction in polarity contributes to an augmentation of the non-covalent interactions between the BC loop and nivolumab, including hydrogen bonds, van der Waals forces, and hydrophobic forces [20, 40].

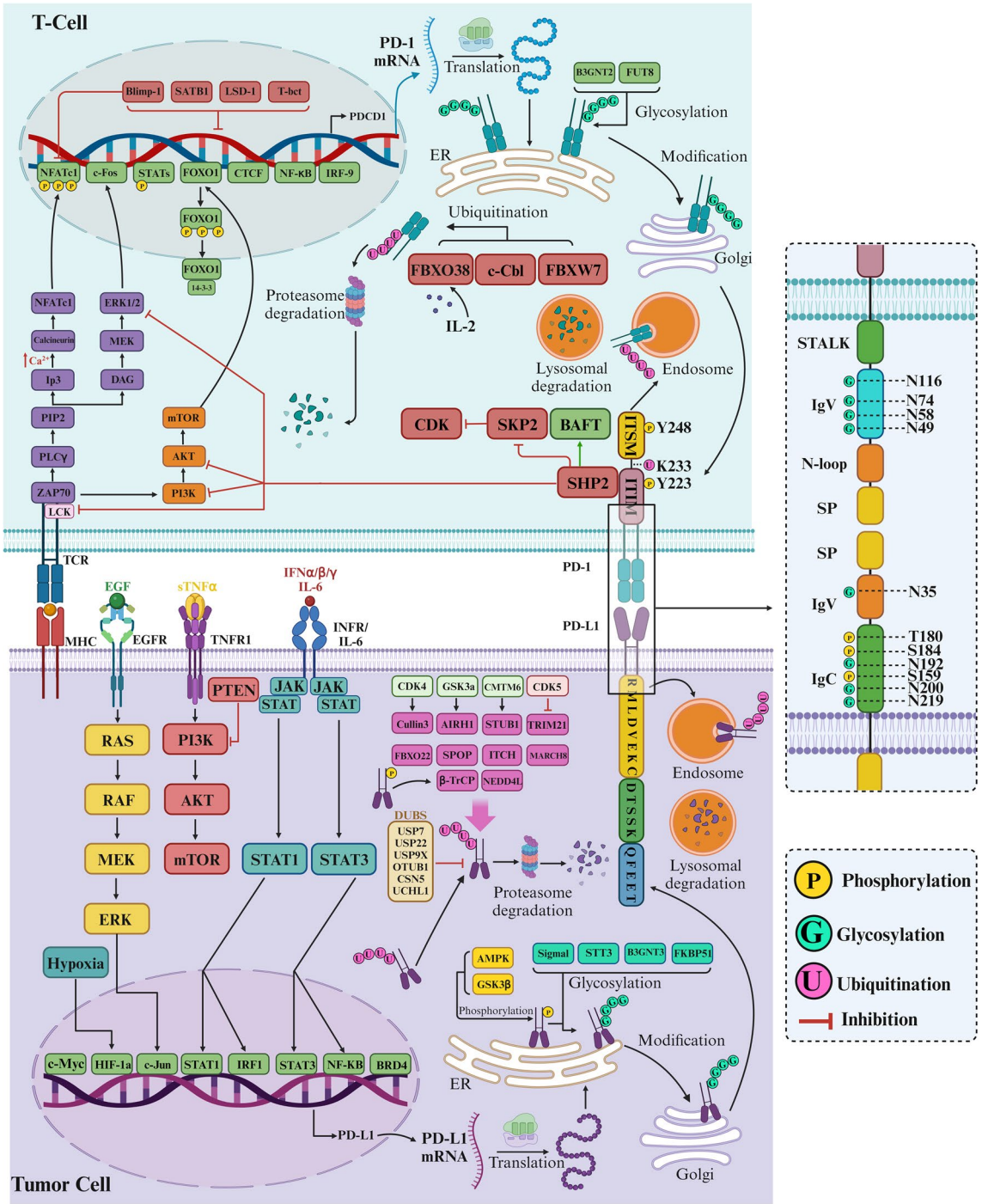
Furthermore, specific amino acids in PD-1, such as D29, R30, S60, and K131 side chain atoms, and P28, L128, A129, P130, and A132 main chain atoms, are involved in forming hydrogen bonds with nivolumab. Additionally, residues S27, P28, P31, E61, A129, P130, K131, A132, and Q133 interact with nivolumab through van der Waals interactions [41]. As mentioned earlier, the N-terminal loop of PD-1 plays a crucial role in forming many hydrogen bonds (10 out of 16) that connect nivolumab and PD-1. Specifically, residues L25, S27, P28, D29, and R30 of the N-loop interact with heavy CDR1 (S30, N31, and G33) and heavy CDR2 (W52, Y53, and K57) of nivolumab. Furthermore, the FG and BC loops also contribute to hydrogen bonding with nivolumab's heavy and light CDRs. The FG loop forms five hydrogen bonds with heavy CDR3 (D100 and D101) and light CDR2 (Y49 and T56), while the BC loop forms one hydrogen bond with heavy CDR1 (N31) (PDB ID: 5WT9) [38].

2.3 PD-1 and PD-L1 modifications

2.3.1 Glycosylation

Glycosylation is a significant post-translational modification crucial for maintaining protein stability, regulating protein interactions, and mediating signaling transduction. Alterations in glycosylation patterns, such as truncated O-glycans or modified N-glycans, have been observed in tumor cells [42]. N-glycosylation begins in the endoplasmic reticulum and continues in the Golgi apparatus, which is vital for the functionality of membranous proteins like PD-1 and PD-L1 (Fig. 2) [43, 44].

PD-L1 glycosylation Specifically, glycosylation at N192 and N219 located on the IgC domain, as well as N35 at the IgV domain, enhances the interaction between PD-1 and PD-L1 [45]. The co-expression of CD274 and regulatory proteins such as FKBP51, SIGMAR1, and MCT4 in melanoma, glioma, breast, and prostate cancers results in increased levels of glycosylated PD-L1 [46–48]. Conversely, glycogen synthase kinase (GSK)-3β induces phosphorylation-dependent proteasomal degradation of PD-L1. It has been demonstrated that glycosylation at N192, N200, and N219 stabilizes PD-L1 by inhibiting its interaction with GSK-3β [49]. However, a limitation of atezolizumab is its reduced efficacy against glycosylated PD-L1. PD-L1 glycosylation conceals interacting residues, thereby reducing patients' responsiveness to anti-PD-L1 antibodies. This limitation led to atezolizumab's withdrawal after FDA's accelerated approval for triple-negative breast cancer (TNBC). To enhance the efficacy of atezolizumab in TNBC, the level of unglycosylated PD-L1 was identified as a potential biomarker for predicting response to atezolizumab [50]. On the other hand, the development of engineered glycosylated atezolizumab, bearing core fucosylated and de-fucosylated N-glycans, demonstrated that de-fucosylated atezolizumab enhances antibody-dependent cellular cytotoxicity (ADCC) activity



against PD-L1$^+$ cancer cells through FcγRIIIa binding. Furthermore, de-fucosylated atezolizumab promotes CD8$^+$ T-cell activity [51]. Additionally, removing the fucose subunits from avelumab, another anti-PD-L1 monoclonal antibody, enhances its antitumor activity, anticancer immunity, and infiltration of T-cells in the tumor microenvironment. However, the current version of FDA-approved anti-PD-L1 monoclonal antibodies is not optimized for facilitating FcγR signaling [52].

PD-1 glycosylation The molecular weight of PD-1 purified from bacteria (Escherichia coli), where there is no glycosylation machinery for human protein, is 14 kDa. However, PD-1 purified from humans is substantially glycosylated at N49, N58, N74, and N116, resulting in an increased molecular weight of up to 35–40 kDa [38]. PD-1 glycosylation is mediated by B3GNT2 and FUT8 enzymes. Furthermore, the level of PD-1 glycosylation plays a regulatory role in PD-1-expressing cells. For instance, during activation of

◂**Fig. 2** Mechanism of PD-1 regulation in tumor immunity. This figure illustrates the diverse mechanisms governing the up-and down-regulation of PD-1 and PD-L1. PD-1, an immune cell receptor, exerts inhibitory effects on immune responses and forms homodimers through interaction with Src homology region 2 (SH2)-containing protein tyrosine phosphatase-2 (SHP-2). SHP-2 serves as a bridge between two PD-1 molecules, binding to the phosphorylated ITSM-Y248 residue, thereby regulating PD-1's inhibitory function. ZAP70, an upstream regulator of SHP-2 phosphatase activity, enhances ERK1/2 phosphorylation, a signaling pathway in immune regulation. Inhibition of SHP2 has demonstrated effectiveness in controlling tumor growth by reinforcing immune surveillance and fostering cancer cell elimination. Additionally, glycosylation, a crucial post-translational modification, significantly influences protein stability, interactions, and signaling in cancer immunity. N-glycosylation of PD-1, especially at N192, N219, and N35, enhances PD-1/PD-L1 interaction, impacting immune responses. PD-L1 glycosylation, including at N58, plays a role in immune checkpoint therapy efficacy. Furthermore, phosphorylation, ubiquitination, and deubiquitination events intricately modulate PD-1 and PD-L1, presenting potential targets for optimizing immune checkpoint therapy. Furthermore, PD-L1 experiences ubiquitin attachment, resulting in identification and degradation by the proteasome. This breakdown plays a crucial role in controlling the immune response, impacting the availability of PD-L1 on the cell surface and influencing its interaction with PD-1, thus modulating the immune checkpoint pathway. The review's comprehensive exploration of these molecular mechanisms provides valuable insights into the regulation of cancer immunity through PD-1 and PD-L1

the T-cell receptor, PD-1 glycosylation is altered, and core fucosylation is crucial for the expression of PD-1 on cell membranes, leading to the suppression of immune cells [53, 54]. Thus, targeting PD-1 glycosylation holds the potential to enhance anticancer immunity [55].

In addition to PD-1 stabilization, glycosylation affects PD-1/PD-L1 interaction, particularly at the N58 glycosylation site [54]. Among the four glycosylation sites of PD-1 (N49, N58, N74, and N116), only N58 is located close to the PD-1 and nivolumab-Fab interacting sites [38]. The N58 glycosylation site contains two N-acetylglucosamines, two mannoses, and one fucose [56]. Initially, nivolumab was reported to bind only to human PD-1 and not bacterial PD-1, likely because human PD-1 is glycosylated [57]. However, a recent study demonstrated that the binding affinity of nivolumab with either expressed PD-1 in mammalian cells or refolded PD-1 expressed in prokaryotic cells is not dependent on PD-1's N-glycosylation. The reason for nivolumab's binding preference to human PD-1 over bacterial PD-1 is due to the truncation of the PD-1 N-terminal, which is crucial for nivolumab binding, during the construction of bacterial plasmids. Thus, the effect of the N-terminal loop dominates over glycosylation in the binding of nivolumab to PD-1 [54]. Additionally, PD-1 glycosylation may play a significant role in the interaction with PD-1-targeting monoclonal antibodies [38]. Therefore, PD-1 glycosylation levels, especially at N58, impact the efficacy of immune checkpoint blockade (ICB) therapy [54]. This has led to the development of a new Fc-Engineered IgG1 PD-1-targeting antibody, penpulimab, which can also detect the glycosylated version of PD-1 [58].

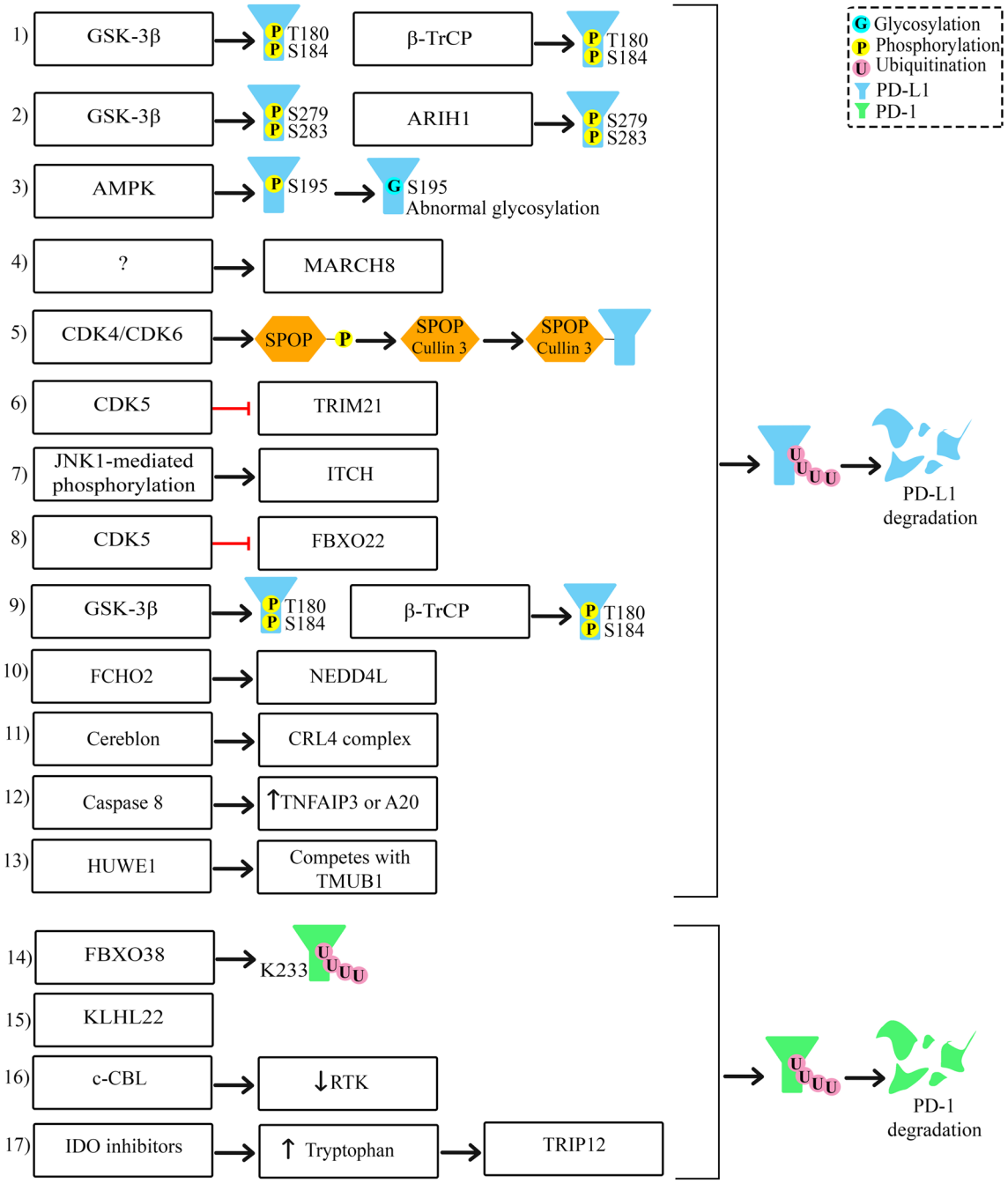
2.3.2 Phosphorylation

Serine/Threonine phosphorylation regulates PD-L1 Depending on the phosphorylation sites, PD-L1 phosphorylations have two functions and cause PD-L1 degradation and strength. First, PD-L1 degradation by phosphorylation at S195 and S283 has been discovered [59, 60]. A study performed in 2018 indicated that metformin can activate adenosine monophosphate-activated protein kinase (AMPK), and then AMPK causes S195 phosphorylation, leading to abnormal glycosylation and PD-L1 degradation. As normal glycosylation is required for normal PD-L1 membrane localization, unusual glycosylation inhibits normal localization, leading to PD-L1 accumulation in the endoplasmic reticulum and endoplasmic reticulum-associated PD-L1 degradation [59].

Moreover, the interaction of PD-L1 and Chemokine-like factor 1 (CKLF)-like MARVEL transmembrane domain 4 (CMTM4), a positive regulator for PD-L1 [61], is abolished by AMPK, which phosphorylates PD-L1 at S283, resulting in PD-L1 degradation [60]. However, as mentioned earlier, PD-L1 glycosylations at N192, N200, and N219 stabilize PD-L1. A study has shown that there is a GSK-3β phosphorylation motif, SxxxTxxxS, located at N192, N200, and N219 neighborhoods, hidden after PD-L1 glycosylation. With lower glycosylation levels, S176A, T180A, and S184A are phosphorylated by GSK-3β, leading to a phosphorylation-dependent PD-L1 degradation [49].

Furthermore, PD-L1 phosphorylation causes PD-L1 stability. JAK1 activation by IL-6 phosphorylates PD-L1 at Y112, stabilizing membrane PD-L1 and therefore promoting cancer immune escape [62]. Moreover, in contrast to GSK-3β, never in mitosis gene A (NIMA)-related kinase (NEK)-2, a subgroup of NEKs family, can interact with strongly glycosylated PD-L1 at NEK-binding F/LXXS/T motif. NEK2 suppresses ER-dependent PD-L1 degradation by PD-L1 phosphorylation at T194 and T210 [63].

Tyrosine phosphorylation regulates PD-1 The interaction of PD-L1 and PD-1 leads to the phosphorylation of two tyrosine residues in the PD-1 cytoplasmic domain. PD-1 cytoplasmic tail has two phosphorylation sites on ITIM and ITSM. Phosphorylation of PD-1 by lymphocyte-specific protein tyrosine *kinase* then calls up the cytosolic tyrosine phosphatases SHP-1 and SHP-2, and the inhibitory C-terminal Src kinase [64–66]. PD-1 is phosphorylated



at Y223 (ITIM) and Y248 (ITSM). Moreover, the lack of PD-1 phosphorylation at ITIM and ITSM diminishes the recruitment of N-SH2 and C-SH2 domains of SHP-2 [64, 67]. PD-1 and SHP-2 interaction stimulates the de-phosphorylation of the TCRs, de-activating T-cells [64]. PD-L1 can also stimulate PD-1 phosphorylation at Y248 [68]. However, CD45 tyrosine phosphatase de-phosphorylates PD-1, promoting T-cell activation [66]. Therefore, blocking PD-1 phosphorylation by developing a specific monoclonal antibody against phospho-Y248-PD-1 may enhance PD-1/PD-L1 therapy [69].

2.3.3 Ubiquitination

Poly-ubiquitination marks proteins for degradation through the ubiquitin-proteasome mechanism. The ubiquitin-proteasome system is an intracellular mechanism important in various cell activities, such as immunity, inflammation, and cancer, leading to PD-L1 degradation [70]. PD-1/PD-L1 ubiquitination can be a critical player to be targeted in cancer immunotherapy. A recent study on melanoma indicated that cancer cells might acquire resistance to MAPK inhibitors through PD-L1 accumulation and suggested a small agonist

◂**Fig. 3** Unveiling mechanisms of PD-1 and PD-L1 degradation. Targeting the ubiquitination of PD-1/PD-L1 in cancer immunotherapy carries profound implications. Poly-ubiquitination labels proteins for degradation via the ubiquitin-proteasome system, a regulated intracellular pathway governing protein breakdown and essential cellular processes. PD-L1, in this system, undergoes ubiquitin attachment, leading to recognition and breakdown by the proteasome. This degradation critically regulates the immune response by influencing PD-L1 availability on the cell surface and its interaction with PD-1, shaping the immune checkpoint pathway. Understanding these mechanisms opens new directions for modulating PD-1/PD-L1 interactions, enhancing cancer immunotherapy effectiveness. Abbreviations: AMPK: AMP-activated protein kinase; AR1H1: Ariadne RBR E3 ubiquitin protein ligase 1; β-TrCP: Beta-Transducin Repeat Containing E3 Ubiquitin Protein Ligase; c-CBL: Casitas B lymphoma is an E3 ubiquitin ligase; CDK: Cyclin-dependent kinases; CRL4: Cullin–RING ubiquitin ligase complex 4; FBXO22: F-Box Protein 22 E3 ubiquitin-protein ligase; FBXO38: F-Box Protein 38 E3 ubiquitin-protein ligase; GSK-3β: Glycogen synthase kinase-3β; HUWE1: HECT, UBA And WWE Domain Containing E3 Ubiquitin Protein Ligase 1; IDO: Indoleamine 2,3-dioxygenase; ITCH: Itchy E3 ubiquitin-protein ligase; JNK1: c-Jun N-terminal kinase 1; KLHL22: Kelch Like Family Member 22; MARCH8: Membrane-associated RING-CH 8 E3 ubiquitin-protein ligase; NEDD4L: Neural precursor cell expressed developmentally downregulated 4-like E3 ubiquitin ligase; RTK: Receptor tyrosine kinase; SPOP: The substrate-binding adaptor speckle-type POZ protein E3 ubiquitin-protein ligase; TMUB1: Transmembrane And Ubiquitin Like Domain Containing 1; TNFAIP3: Tumor necrosis factor alpha induced protein 3; TRIM21: Tripartite Motif Containing 21 E3 ubiquitin-protein ligase; TRIP12: Tryptophan-dependent E3 ubiquitin-protein ligase 12

molecule for E3 ubiquitin-protein ligase that can sensitize melanoma cells to MAPK inhibitors through activation of PD-L1 ubiquitination (Figs. 2 and 3) [71].

To activate PD-L1 and PD-1 ubiquitination, several key enzymes that promote PD-L1 degradation have been discovered as follows:

(1) *GSK-3β and β-TrCP.* As previously discussed, in TNBC patients, GSK-3β causes the phosphorylation of PD-L1 at T180 and S184. This process subsequently promotes degradation of PD-L1 through its interaction with β-Transducin Repeat Containing E3 Ubiquitin Protein Ligase (β-TrCP) and GSK-3β [49].

(2) *GSK-3α and ARIH1*. GSK-3α negatively regulates PD-L1 through its phosphorylation at S279 and S283. Subsequently, the Ariadne RBR E3 ubiquitin protein ligase 1 (ARIH1) recognizes the phosphorylated PD-L1 and initiates its degradation through proteasomal pathway [72].

(3) *AMPK*. AMPK phosphorylates PD-L1 at S195. Phospho-S195 PD-L1 is abnormally glycosylated, resulting in PD-L1 ER accumulation and ER-associated protein degradation. Metformin-treated breast cancer patients showed higher levels of activated AMPK and lower levels of PD-L1 [59].

(4) *MARCH8*. Another E3 ubiquitin-protein ligase is encoded by membrane-associated RING-CH 8 (*MARCH8*). MARCH8 interacts with the N-terminal of PD-L1 and ubiquitinates it. It has been shown that epidermal growth factor receptor (EGFR) inhibitor osimertinib downregulates PD-L1 through upregulating GSK-3β, β-TrCP, and *MARCH8* [73].

(5) *SPOP*. The substrate-binding adaptor speckle-type POZ protein (SPOP) E3 ubiquitin-protein ligase, another negative regulator of PD-L1, is mutated in endometrial cancer. Wild-type SPOP downregulates PD-L1 at the transcriptional level. SPOP promotes the ubiquitin-proteasome-dependent degradation of interferon regulatory factor 1 (IRF1), a transcriptional inducer of PD-L1 expression; however, SPOP-dependent degradation of IRF1 is impaired when SPOP is mutated in endometrial cancer [74]. On the other hand, the degradation of SPOP is triggered by its cyclin D-CDK4-dependent phosphorylation and then degraded by the anaphase-promoting complex activator Fizzy-related protein homolog 1 (FZR1). Therefore, CDK4 increases PD-L1 levels, and instead, CDK4/CDK6 inhibitors are effective in PD-L1 therapy through the upregulation of SPOP [75].

(6) *TRIM21*. Tripartite Motif Containing 21 (TRIM21) is E3 ubiquitin-protein ligase that binds PD-L1 and downregulates it through the ubiquitination-proteasome pathway. The role of CDK5 on PD-L1 level in lung adenocarcinoma cells has also been discovered. This study demonstrated that a higher level of CDK5 is correlated with a higher level of PD-L1 and a lower level of $CD3^+$, $CD4^+$, and $CD8^+$ T-cells in spleens and higher PD-1 expression in $CD4^+$ and $CD8^+$ T-cells. The role of CDK5, as a potential therapeutic target, for combined immunotherapy in promoting antitumor immunity is highlighted [76].

(7) *ITCH*. Recently, Itchy (ITCH) E3 ubiquitin-protein ligase was discovered, which can ubiquitinate and decrease cell surface PD-L1 and PD-L2 levels and, therefore, promotes T-cell activation. On the other hand, melanoma cells treated with MAPK inhibitors show higher levels of PD-L1 and PD-L2. This study has shown that reducing intrinsic ITCH expression causes elevation of cell surface PD-L1 and acquired resistance to MAPK inhibitors. Furthermore, a small-molecular ITCH activator was identified to overcome acquired resistance to MAPK inhibitors. Therefore, a PD-L1-degrading ITCH activator prolongs the antitumor response [71].

(8) *FBXO22*. F-Box Protein 22 (FBXO22) E3 ubiquitin-protein ligase sensitizes NSCLC cells to DNA damage and improves the responses of patients with NSCLC cells to radiotherapy and chemotherapy. Furthermore,

FBXO22 enhances the PD-L1 polyubiquitination [77]. On the other hand, CDK5 has a vital role in enhancing PD-L1 expression in medulloblastoma [78]. Researchers recently discovered that CDK5 inhibition elevates FBXO22, decreases PD-L1, and increases responses of NSCLC patients to ICB alone [77].

(9) *FBXO38*. The cytoplasmic tail of PD-1 has two lysine residues, K210 and K233, that can be targeted as ubiquitination sites. It has been shown that the FBXO38 E3 ubiquitin-protein ligase induces PD-1 K48-linked poly-ubiquitination at the K233 site, resulting in subsequent proteasomal degradation of PD-1. Conditional knockout of FBXO38 in T-cells caused faster tumor progression by increasing PD-1 levels in tumor-infiltrating T-cells, while IL-2 therapy could repress tumor progression by rescuing FBXO38 transcription and then downregulating PD-1 levels [79].

(10) *NEDD4L*. The effects of NEDD4 Like (NEDD4L) E3 ubiquitin-protein ligase have been studied in NSCLC. Downregulation of NEDD4L negatively correlated with PD-L1 upregulation in NSCLC tissues. NEDD4L overexpression increases PD-L1 ubiquitination, which results in a reduced level of PD-L1 protein, decreased tumor volume and weight, and elevated proportion of $CD8^+$ T-cells and contents of IL-2 and INF-γ [80].

(11) *KLHL22*. KLHL22 is an adaptor of the Cul3-based E3 ligase. PD-1 is degraded by KLHL22 before being transported to the cell membrane. Downregulation of KLHL22 with 5-fluorouracil or with introducing loss of function mutations causes accumulation of PD-L1. KLHL22 level was also decreased in tumor-infiltrating T-cells obtained from colorectal cancer patients. This study suggested that KLHL22 can play as a complementary therapeutic target to optimize PD-1/PD-L1 therapy [81].

(12) *c-CBL*. Casitas B-lineage lymphoma (c-Cbl) is an E3 ubiquitin protein ligase, which negatively regulates downstream signals of the T-cell receptor and B cell receptor [82]. Moreover, c-Cbl downregulates receptor tyrosine kinases (RTK) such as EGFR, c-mesenchymal-epithelial transition factor (c-MET), platelet-derived growth factor receptor (PDGFR), and fibroblast growth factor receptor (FGFR) [83]. Introducing RING finger mutations on c-Cbl, which diminishes polyubiquitination, showed that PD-1 is another target for c-Cbl. However, the use of specific lysosomal inhibitors revealed that PD-1 undergoes proteasomal degradation but not lysosomal. Furthermore, decreasing c-Cbl activity improves the efficacy of PD-1/PD-L1 therapy [84].

(13) *TRIP12*. Tryptophan-dependent E3 ubiquitin-protein ligase (TRIP12) degrades a PD-1 transcription activator, NFATc1, and as a result, PD-1 is also downregulated. Indoleamine 2,3-dioxygenase (IDO) inhibitors increase intracellular levels of tryptophan. Then, tryptophan accumulation activates TRIP12 and lowers the PD-1 level at the T-cell membrane. On the other hand, Sirt1—a nicotinamide adenosine dinucleotide (NAD)-dependent deacetylase that removes acetyl groups from a wide range of proteins – can de-tryptophanylate TRIP12 and rescues surface PD-1 level [85].

(14) *Cereblon*. The E3 ubiquitin-protein ligase substrate adapter cereblon targets some therapeutic agents, such as anandamide and lenalidomide, used to treat hematopoietic malignancies. The role of cereblon is to target degradation motifs (degrons) on the target proteins and remove them from the protein, which leads to protein degradation [86]. Chronic lymphocytic leukemia (CLL) partially responded to anti-PD-L1 and anti-PD-1 therapy. A recent study showed that avadomide, a cereblon E3 ubiquitin-protein ligase modulator (CELMoD), has an enhanced immunostimulatory effect that promotes the number of $CD4^+$ or $CD8^+$ T-cells targeting CLL cells and enhances the formation of T-cell immune synapses. Furthermore, the dominant immunomodulatory mechanism of anandamide is enhancing the degradation of Aiolos and Ikaros in T-cells. However, c increases PD-L1 expression on both T-cell subsets and CLL cells and sensitizes CLL to anti-PD-L1 and anti-PD1 therapies. Moreover, the combination of avadomide with nivolumab (anti-PD-1) and durvalumab (anti-PD-L1) increased the efficacy of anti-PD-L1 and anti-PD1 therapies [87].

(15) *Caspase 8 and TNFAIP3*. Caspase 8 is another regulator of PD-L1 ubiquitination, which overexpresses ubiquitin-editing enzyme tumor necrosis factor, alpha-induced protein 3 (TNFAIP3 or A20). Inhibition of caspase 8 enhances tumor progression in mouse melanoma cells. Therefore, caspase 8 inhibition or caspase 8 low-level sensitizes cancer cells to PD-1 therapy [88].

(16) *HUWE1*. HECT, UBA, And WWE domain containing E3 ubiquitin protein ligase 1 (HUWE1) is involved in PD-L1 ubiquitination. Furthermore, transmembrane and ubiquitin-like domain-containing protein 1 (TMUB1) has been recently discovered [89], which plays a role in regulating the modifications that occur after the production of PD-L1 in tumor cells. TMUB1 works by competing with HUWE1 to interact with PD-L1. This interaction prevents PD-L1 from being modified by the addition of multiple ubiquitin molecules at a specific location (K281) in the ER. Additionally, TMUB1 promotes the attachment of sugar molecules (N-glycosylation) to PD-L1 and stabilizes it by recruiting another protein called STT3A oligo-

saccharyltransferase. These processes contribute to the maturation of PD-L1 and facilitate the evasion of the immune system by the tumor. The levels of TMUB1 protein in human tumor tissue are found to be correlated with the expression of PD-L1, and high levels of TMUB1 are associated with lower survival rates in patients [89].

Unlike ubiquitination, some enzymes deubiquitinate PD-L1 and remove ubiquitin chains, leading to PD-L1 stabilization. Furthermore, as mentioned earlier, specific glycosylations and phosphorylations cause PD-L1 stabilization and increase PD-L1 half-life. For instance, CMTM6 binds PD-L1 and stabilizes its cell membrane expression. However, CMTM4 and CMTM6, not required for PD-L1 maturation, accompanies PD-L1 at the plasma membrane and in recycling endosomes to save PD-L1 from lysosome-mediated degradation [61, 90]. In addition, CMTM6 plays a crucial role in maintaining the stability of CD58 and increasing the levels of PD-L1 when CD58 is lost. The rate at which CD58 and PD-L1 are recycled in endosomes versus degraded in lysosomes depends on their competition for binding to CMTM6 [91].

Ubiquitin-specific peptidases (USPs) USPs are the main members of the deubiquitinases, deubiquitinating the target protein, promoting the target half-life, and may be a potential; therapeutic target to optimize immune checkpoint therapy [92]. USP22 has been recognized as a positive regulator for PD-L1, which directly deubiquitinates PD-L1. USP22 removed K6, K11, K27, K29, K33 and K63-linked ubiquitination of PD-L1 [93]. On the other hand, COP9 signalosome 5 (CSN5), necessary for TNF-α-mediated PD-L1 stabilization, is activated by NF-κB p65. CSN5 inhibits the ubiquitination and degradation of PD-L1 [94]. Furthermore, USP22 also deubiquitinates *CSN5*. Therefore, USP22 and CSN5 collaboratively stabilize PD-L1 [93]. Moreover, USP9X is suggested to be another PD-L1 stabilizer that prevents PD-L1 ubiquitination in oral squamous cell carcinoma. By inhibition of USP9X expression, tumor cell growth was blocked [95]. In NSCLC, USP5 was reported as PD-L1 deubiquitinase. USP5 interacts with PD-L1 and deubiquitinates PD-L1, enhancing PD-L1 stability. Furthermore, USP5 protein levels increased in NSCLC tissues and positively correlated with PD-L1 protein levels [96]. In addition, USP21 frequently upregulated deubiquitinase in lung cancer patients, especially in lung squamous cell carcinoma, and deubiquitinates PD-L1. USP21 amplification is positively correlated with a higher level of PD-L1. The potential ubiquitination sites are mainly located in the intracellular domain of PD-L1, including K280, K281, and K270 [97]. In glioma and gastric cancer cells, higher USP7 expression decreases PD-L1 protein levels without affecting PD-L1 mRNA levels, suggesting that USP7 potentiates PD-L1 deubiquitination [98, 99]. Furthermore, OTU domain-containing ubiquitin aldehyde-binding protein 1 (OTUB1) is an enzyme that removes K48-linked ubiquitin chains of the PD-L1 and stabilizes PD-L1 to prevent ubiquitination. OTUB1 is the only member of the OTU subfamily that regulates PD-L1 and prevents PD-L1 accumulation in ER for ER-associated degradation of PD-L1 [100].

These findings shed light on an essential but overlooked aspects of cancer immunity. Focusing on regulation of PD-L1 and PD-1 to manage both immune inhibitory and stimulatory signals on a molecular level illustrates other therapeutic options to overcome the lack of response to anti-PD-L1 and PD-1 therapy.

3 Immune checkpoint blockade therapy and immune cell dynamics

The burgeoning field of ICB therapy has revolutionized cancer treatment by unleashing the body's immune system against tumors. While previous sections explored the structural aspects and interaction mechanisms of key immune checkpoints like PD1/PDL1, understanding their functional implications on immune cell dynamics remains pivotal for comprehending therapeutic efficacy.

3.1 Immune cell dynamics after chemotherapy

Approving immunotherapies like atezolizumab and nivolumab in combination with or after chemotherapy as adjuvant therapy highlights the significance of understanding how immune cells respond to chemotherapy [101, 102]. Studies have elucidated that the body's reaction to ICB hinges on these responses. Hence, researchers explored chemotherapy's influence on the tumor-immune microenvironment, revealing associations between immune cell profiles and treatment response.

In residual disease cases, tumor-infiltrating lymphocytes (TILs) counts and PD-L1 expression remained unchanged, while genes related to cellular stress, hypoxia (DUSP1, EGR1), IL6, CD36, CXCL2, CD69, and the IL8/VEGF metagene increased. Activated T-cells in the tumor environment were linked to achieving a complete treatment response, while stromal functions were associated with residual disease. Most immune-related functions declined during neoadjuvant chemotherapy, although specific targets for immunotherapy (such as PD-L1, IL6, IL8) persisted in residual disease cases, indicating potential avenues for therapeutic strategies [103]. Another research delved into immune cell variations in breast cancer patients' blood before and after chemotherapy. It revealed

heightened immunosuppressive cell levels, especially in advanced stages, irrespective of specific tumor characteristics. Chemotherapy led to decreased B cell counts, increased monocyte numbers, and changes in natural killer (NK) cell receptors. Notably, patients with breast cancer, especially those in advanced stages, exhibited increased percentages of immunosuppressive cells like granulocytic Myeloid-derived suppressor cells (MDSCs), intermediate $CD14^{++}CD16^{+}$ monocytes, and $CD127^{-}CD25^{high}FoxP3^{+}$ Treg cells. Transcriptomic analysis identified a subset of TNBC patients with elevated inflammation-related genes. In advanced breast cancer, there was an increase in certain immunosuppressive cells. Though chemotherapy did not alter specific immune cell percentages, it affected NK cell receptor expression. Moreover, specific genes (CD163, CXCR4, THBS1) in TNBC tumors were linked to predicting relapse-free survival, suggesting potential utility in assessing peripheral blood for identifying relapse risk after chemotherapy [104].

On the other hand, a study focused on chemotherapy's impact on fighting breast cancer, particularly in incomplete treatment cases, particularly in TNBC. Scientists examined changes in immune-related genes and specific immune cells in tumors and blood post-chemotherapy—an elevated expression of immune-related genes linked to cancer-killing cells correlated with better outcomes after TNBC surgery. Signature genes (PDCD1, NKG7, LAG3, GZMH, GZMB, GNLY, FGFBP2, HLA-DRB5, and HLA-G) reflected disease progression, varying across untreated, residual, and wholly treated patients. Researchers expected distinct patterns, particularly with genes like HLA-G affecting immune response regulation. While these genes could collectively indicate persistent disease, variations among patients suggested complexities in their interpretation of breast cancer prognosis [105].

Overall, these studies underscore the critical role of immune cell responses in shaping the effectiveness of immunotherapies combined with chemotherapy. Understanding these responses becomes pivotal in optimizing treatment strategies and predicting patient outcomes.

3.2 Immune cell dynamics and ICB

The tumor microenvironment (TME) is made up of diverse elements such as immune cells, blood vessels, the extracellular matrix, fibroblasts, and lymphocytes encompassing cancer stem cells, exerting a significant influence on cancer progression, treatment, and prognosis. While the involvement of T lymphocytes in adaptive immune responses against tumors is extensively known, the significance of B lymphocytes has more recently gained attention [106]. The research underscores the association of tumor-infiltrating B cells (TIL-B) with patient prognosis. However, the precise function of different B-cell subsets in breast cancer and their underlying mechanisms remain subject to debate [107].

Besides, tertiary lymphoid structures (TLS) represent ectopic lymphoid formations found in nonlymphoid tissues, including cancerous tissues, characterized by regions rich in B cells. Emerging evidence links the existence of TLS to reduced chances of tumor recurrence and heightened effectiveness of ICB in various solid tumor categories [108]. Despite the success observed with ICB in specific cancers, its efficacy remains restricted in the majority of breast cancer subtypes [109]. A recent study examining the presence of TLS in breast cancer using a 9-gene TLS signature (CCR6, CD1D, CD79B, CETP, EIF1AY, LAT, PTGDS, RBP5, and SKAP1) uncovered a positive relationship with an early tumor stage and improved prognosis among breast cancer patients with high TLS [110, 111]. In addition, TME influences the characteristics of tumor-associated neutrophils (TAN). Pathway analysis comparing TLS^{+} and TLS^{-} groups revealed distinct signaling pathways. TLS-negative groups showed elevated inflammatory pathways like PI3K, MAPK, NOD-like receptor, and NF-kB signaling pathways, linked to local inflammation and potential immunosuppression, possibly impacting ICB resistance [112, 113].

Further, the TLS-negative group displayed increased PD-L1 expression and pathways associated with cancer invasiveness and resistance to treatment, such as EGFR tyrosine kinase inhibitor resistance, TGF-β, and HIF-1 signaling pathways [114, 115]. Mature TLSs demonstrate a more vital prognostic value compared to TIL-B cells. The clinical evidence indicated that this distinction, showing that patients with Luminal B breast cancer, typically less responsive to chemotherapy, exhibited improved responses in the presence of mature TLSs. Similarly, patients with TNBC, often less responsive to ICB, showed enhanced immunotherapeutic outcomes when mature TLSs were present [114]. Interestingly, reactive oxygen species (ROS) accumulation activates NF-κB signaling, promoting PD-L1 production and the release of immune-suppressing chemokines. *In vivo* experiments in a TNBC mouse model confirmed increased PD-L1 on tumor-associated macrophages (TAMs) upon paclitaxel treatment, aligning with *in vitro* findings. Combining paclitaxel with an anti-mouse PD-L1 antibody significantly enhanced paclitaxel's effectiveness, reducing tumors and elevating tumor-associated cytotoxic T-cells [116]. On the other hand, another research uncovered a resistance mechanism to PD-1 ICB and highlighted the potential of using an anti-Δ42PD-1 antibody as a promising immunotherapeutic strategy for treating HCC. The study revealed specific T-cell subsets lacking PD-1 expression but harboring its isoform Δ42PD-1, accounting for a significant proportion, up to 71%, of cytotoxic T-cells in untreated HCC patients. These $\Delta 42PD\text{-}1^{+}$ T-cells were found in

tumors and correlated positively with HCC severity, demonstrating higher exhaustion levels than PD-1$^+$ T-cells. Significantly, in murine HCC models, the anti-Δ42PD-1 antibody, rather than nivolumab, exhibited efficacy in suppressing tumor growth [117].

In addition, typically displaying M2-like traits, TAMs contribute to disease progression, drug resistance, and unfavorable prognoses. Modern cancer therapies extend beyond traditional radiotherapy or chemotherapy, embracing targeted therapy and immunotherapy. Using single-cell transcriptome profiling in mice with liver metastatic tumors, they observed that TAMs with a pro-tumor phenotype and terminal differentiation exhibit increased purine metabolism. This feature was also noted in heterogeneous human TAMs, where those with elevated purine metabolism displayed a pro-tumor phenotype and were associated with reduced responsiveness to ICB therapies [118].

Hence, comprehending signaling pathways linked to TAM polarization and methods to regulate TAM repolarization offers a novel perspective for cancer treatment [119]. Additionally, the combination of chimeric antigen receptor (CAR) T-cells and ICB alters the immune environment in solid tumors, enhancing the effectiveness of CAR T-cells. Researchers found M2 macrophages hinder CAR T-cell activity and display increased PD-L1 expression, also observed in TAMs after CAR T-cell therapy in humanized mice. Blocking PD-L1, in combination with CAR T-cell therapy, transformed these cells into more M1-like subsets and reduced CD163$^+$ M2 macrophages through interferon-γ (IFN-γ) signaling, ultimately improving the CAR T-cells' ability to fight tumors (Fig. 4) [120].

Nonetheless, disrupting the tumor immune barrier (TIB) structure, formed by secreted phosphoprotein 1 (SPP1)$^+$ macrophages and cancer-associated fibroblasts (CAFs) interaction, could be a promising therapeutic strategy to boost the efficiency of ICB in treating HCC. The research identified a distinct TIB structure composed of SPP1$^+$ macrophages and CAFs near the tumor edge, impacting the effectiveness of ICB. In macrophages, SPP1 was associated with the M2 phenotype. The study unveiled the ligand-receptor networks among malignant cells, SPP1$^+$ macrophages, and CAFs, indicating that the hypoxic environment triggers SPP1 expression. SPP1$^+$ macrophages interact with CAFs to induce extracellular matrix remodeling, forming the TIB and limiting immune cell infiltration into the tumor core. In mouse liver cancer models, inhibiting SPP1 or deleting *Spp1* in macrophages enhanced anti-PD-1 treatment efficacy, reducing CAF infiltration and increasing cytotoxic T-cell presence [121].

On the other hand, researchers found hypersialylation, a common feature in cancer-related glycosylation, impacts disease progression and immune evasion by engaging Siglec receptors on tumor immune cells. Tumor sialylation levels were linked to distinct immune profiles and reduced survival in human cancers. They demonstrated that targeting Siglec ligands using an antibody-sialidase combination in the TME improved antitumor immunity and halted tumor growth in various mouse models. Single-cell RNA sequencing showed that desialylation changed the characteristics of TAMs, with Siglec-E identified as a primary receptor for hypersialylation on TAMs. Furthermore, genetic or therapeutic desialylation and Siglec-E loss boosted the effectiveness of ICB. This study suggested therapeutic desialylation could reshape macrophage behavior and enhance the adaptive antitumor immune response [122].

In summary, these extensive studies examining the complex interactions among the tumor microenvironment, various immune cell types, and treatment responses indicate an optimistic prospect for advancing precision medicine and tailored therapies, potentially transforming cancer treatment approaches in the times ahead.

3.3 Immunotherapeutic effects of atezolizumab and nivolumab

Analyzing gene expression, T-cell infiltration, and T-cell receptor signatures in tumors responding to the anti-mouse PD-L1 antibody, akin to human PD-L1 antibodies durvalumab and atezolizumab, showed a correlation between CD8$^+$ T-cell infiltration and treatment response. Furthermore, the response signature indicated augmented antigen processing, interactions in cytokine-cytokine receptors, and increased NK cell-mediated cytotoxicity. These findings imply that successful regression due to anti-PD-L1 treatment involves two key factors: the expansion of a distinct T-cell receptor repertoire and the tumor's accessibility to specific T-cell receptors. These observations parallel the variable ICB responses observed in patients (Fig. 5) [123].

Besides, an increasing body of research indicates that T-cell reactions directed at neoantigens play a vital role in governing the effectiveness of ICB. In patients with lung cancer who responded to atezolizumab, the circulating CD8$^+$ T-cells specific to neoantigens displayed a distinct profile marked by elevated CD57, CD244, and KLRG1 [124]. Additionally, patients with urothelial cancer who responded positively to atezolizumab displayed a notable prevalence of CD57 in their neoantigen-specific CD8$^+$ T-cells. This trend extended to bulk CD8$^+$ T-cells, where higher CD57 expression was observed before treatment among responders to atezolizumab, not in response to chemotherapy. Single-cell RNA-seq analysis also identified an enriched clonal cluster in CD57$^+$ CD8$^+$ T-cells among responders, expressing genes linked to activation, cytotoxicity, and tissue-resident memory. This underscores the potential of using CD57-expressing circulating CD8$^+$ T-cells as a convenient blood-based biomarker to select patient with urothelial cancer

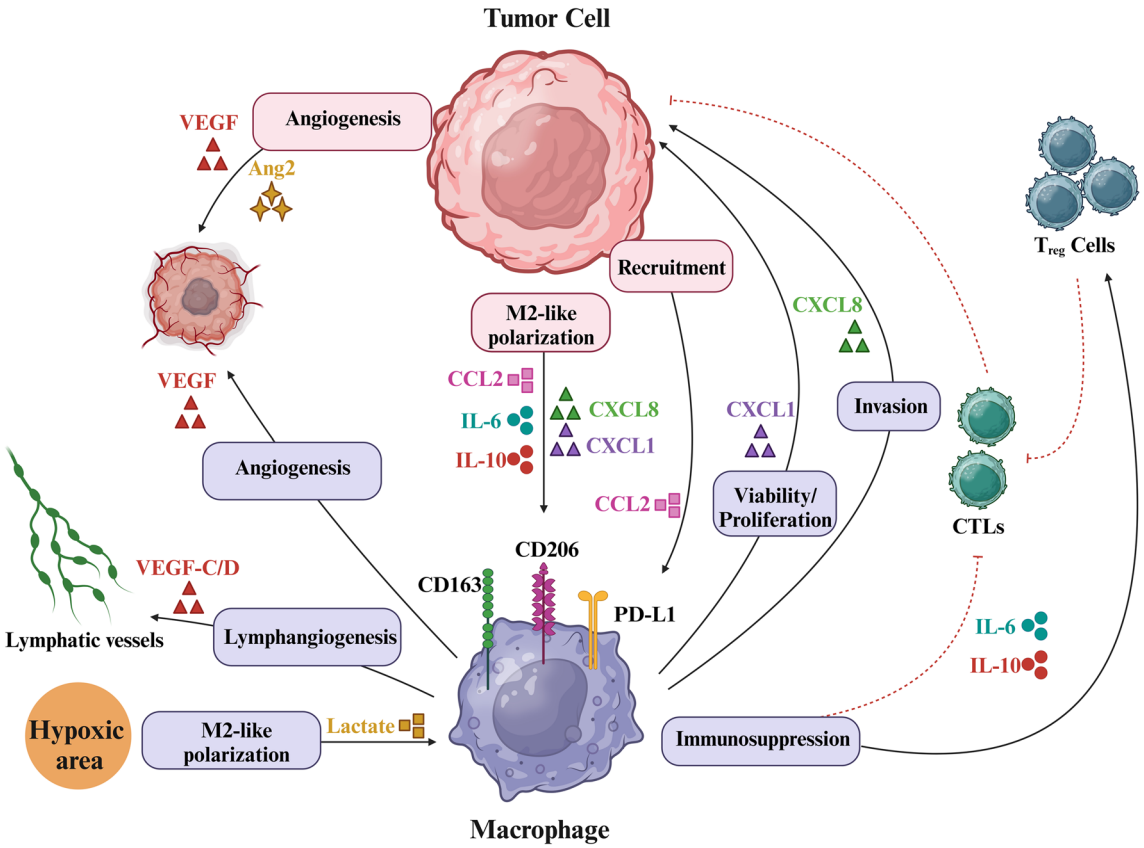


Fig. 4 Unraveling the intricate dynamics of Tumor Cell Progression and TAM Polarization. This illustration delves into the complex interplay of M2-polarization by tumor cells, spotlighting the regulatory roles of IL-10, IL-6, and CXCLs in angiogenesis, invasion, lymphangiogenesis, and hypoxia in the tumor microenvironment. These cytokines also play pivotal roles in shaping Tumor-Associated Macrophage (TAM) polarization by significantly contributing to developing Treg and M2 macrophages. IL-10, an anti-inflammatory cytokine, guides M2 macrophage polarization linked with tumor-promoting activities, while IL-6 fosters M2 polarization, supporting tumor growth. CXCLs, acting as chemokines, regulate immune cell migration, influencing macrophage polarization. Additionally, PD-L1, an upregulated immune checkpoint in cancer and M2 macrophages suppress immune responses, notably contributing to the immunosuppressive microenvironment in M2 macrophages. Understanding these intricate interactions is essential for developing targeted cancer therapies to modulate immune responses

for atezolizumab therapy [125]. The study used single-cell flow cytometry to assess the impact of PD-1/PD-L1 immune checkpoint inhibitors (atezolizumab, durvalumab, or avelumab) on immune cell subsets in metastatic NSCLC patients. This retrospective bioinformatics analysis revealed treatment-related changes in specific peripheral immune cell populations. Patients showed decreased $CD4^+$ T-cells, B cells, and neutrophil-to-lymphocyte ratio and increased levels of NK T-cells, $CD8^+PD1^+$ T-cells, and eosinophils post-treatment. This highlights the need for further comprehensive studies to understand the effects of PD-1/PD-L1 blockade on immune subsets and associated adverse events [126].

On the other hand, when NK cells are paired with anti-PD-L1 monoclonal antibodies (mAbs) that maintain Fc receptor (FcR) binding, they exhibit cytotoxicity against $PD\text{-}L1^+$ cancer cells through a process called ADCC. Researchers explored how NK cells, in combination with atezolizumab and IMC-001 anti-PD-L1 mAbs, can potentially improve the effectiveness of immunotherapy through ADCC *in vitro*. This research confirmed that NK cells are involved in ADCC against $PD\text{-}L1^+$ tumors, reinforcing the role of ADCC-driven NK cell cytotoxicity. The study examines various anti-PD-L1 mAbs across different cancer cell lines expressing PD-L1. Notably, atezolizumab demonstrated higher efficacy in ADCC than IMC-001, suggesting a varied binding affinity with NK cells. Nevertheless, this finding underscores the potential of NK cells in driving effective ADCC when paired with ADCC-capable anti-PD-L1 mAbs, suggesting PD-L1 as a target for ADCC-based therapy. However, the study acknowledges limitations in its *in vitro* setup and highlights the need for further *in vivo* or *ex vivo* studies to confirm these findings [127]. On the other hand, a novel 3D co-culture platform was designed to simulate the

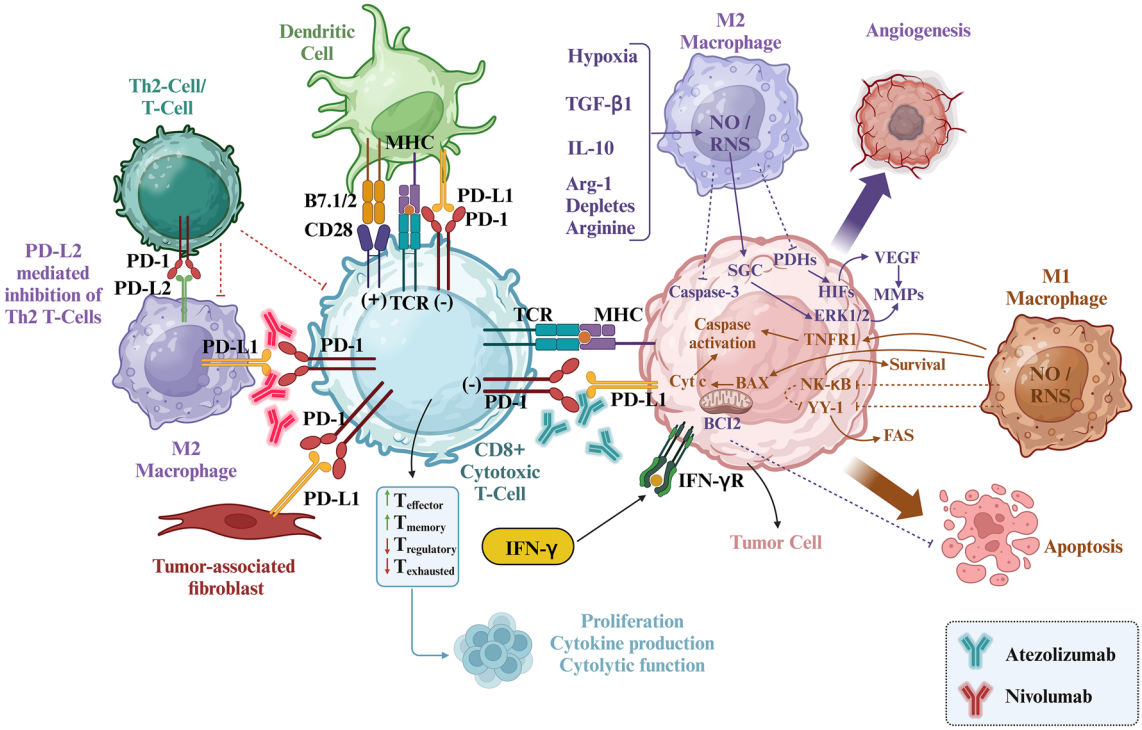


Fig. 5 Impact of immune and microenvironmental factors on PD-1/PD-L1 therapy. M2-macrophages, NK cells, Th2-cells, hypoxia, and regulatory cytokines (TGF-β, IL-10, IFN-γ) intricately shape the tumor microenvironment, impacting nivolumab (PD-1 therapy) and atezolizumab (PD-L1 therapy) immunotherapeutic effects. M2 macrophages induce immunosuppression, NK cells enhance cytotoxicity, and Th2 cells skew responses. Hypoxia and regulatory cytokines establish an immunosuppressive milieu, compromising immune checkpoint inhibition outcomes. Notably, TGF-β inhibits T cells and NK cells, while IL-10 suppresses antigen-presenting cells and cytotoxic T cells, hindering immune checkpoint inhibitors. Understanding these complexities is crucial for optimizing cancer immunotherapies. Additionally, IFN-γ is pivotal in modulating responses to nivolumab and atezolizumab, particularly in M2 macrophages. IFN-γ activates anti-tumor immune responses and can drive macrophages towards the M1 phenotype, counteracting M2 macrophage-mediated immunosuppression, offering potential therapeutic benefits for PD-1/PD-L1 blockade

TME and investigate the efficacy of combining NK cells with atezolizumab and trastuzumab antibodies against solid tumors. This platform facilitated interaction between NK92-CD16 cells and pancreatic and breast cancer cell lines (MiaPaCa-2, MCF-7, and MDA-MB-231). The findings revealed potential synergies among NK cells, antibodies, and chemotherapeutic drugs against cancer cells. This innovative tool also demonstrated promise for personalized immunotherapy by evaluating individualized cancer responses to drug and immune cell combinations in a 3D TME-like setting [128].

Additionally, co-culturing of newly created PD-1 and IL-2 gene-transfected NK cell line and a PD-L1 gene-transfected target cell line indicated that nivolumab and atezolizumab with dose-dependent modulation of cytotoxic activity, unlike the control antibody. The newly established assay system offers a quantitative method to evaluate PD-1/PD-L1 inhibitors' impact on cytotoxic activity, which is pivotal in assessing innate immunity's role in antitumor effects [129]. Furthermore, co-culturing the newly developed PD-1 and IL-2 gene-transfected NK cell line with a PD-L1 gene-transfected target cell line revealed a dose-dependent modulation of cytotoxic activity by nivolumab and atezolizumab, in contrast to the control antibody. This newly established assay system provides a quantitative approach for assessing the impact of PD-1/PD-L1 inhibitors on cytotoxic activity, which is crucial in evaluating the role of innate immunity in antitumor effects [129]. Alternatively, the efficacy of atezolizumab was notably augmented through the development of an oncolytic adenovirus that secretes a unique cross-hybrid Fc-fusion peptide, specifically targeting PD-L1. This fusion peptide harnessed the Fc-effector mechanisms of both IgA1 and IgG1, effectively activating multiple immune components. The adenovirus expressing this peptide successfully initiated IgA1-induced neutrophil activation and IgG1-mediated NK cell and complement activation. This concurrent activation of diverse effector mechanisms significantly heightened tumor eradication in various *in vitro* and *in vivo* models, including patient-derived organoids, surpassing the efficacy of FDA-approved IgG, atezolizumab, which contains an N298A mutation abolishing Fc-γ binding. Crucially, this strategy did not rely on $CD8^+$ T-cells, ensuring broader efficacy and safety, highlighting a promising avenue

for augmenting the therapeutic efficacy of atezolizumab and similar PD-L1 inhibitors [130].

In a study focused on NSCLC, researchers explored PD-L1 expression across immune cell types to identify its predictive value in anti-PD-1 axis immunotherapy. Analyzing multiple NSCLC cohorts, they found that PD-L1 expression was notably higher in $CD68^+$ macrophages than other immune cells, correlating with elevated tumor levels and association with $CD8^+$ T-cell infiltration. High PD-L1 expression in macrophages was linked to better overall survival in treated patients, suggesting their significance as predictive markers for anti-PD-1 therapy efficacy in NSCLC cases. Contrary to previous associations, early-stage TAM displayed M1 and M2 markers without clear links to tumor aggressiveness or T-cell impact. While TAMs in early-stage lung cancer seemed not to hinder T-cell function through PD-L1 expression, recent findings suggest intratumoral dendritic cells may contribute to atezolizumab therapy efficacy via PD-L1 interactions [131].

Nonetheless, the recent analysis was performed using data from the nivolumab and everolimus groups of the CheckMate-025 study, the atezolizumab arm of IMmotion-150, and the atezolizumab combined with bevacizumab group from the IMmotion-151 cohorts. This study established mRNA signatures related to fatty acid metabolism, demonstrating robust predictive capabilities through time-dependent survival analyses. Notably, the high-risk group, identified via these signatures, displayed poorer responses to anti-PD-1/PD-L1 therapy than the low-risk group, accompanied by higher levels of M2-like macrophages in the TME. These M2-like macrophages had an elevated immune score in the high-risk group. The model effectively predicted chemotherapy efficacy and sensitivity while highlighting the significance of the IL6-JAK-STAT3 pathway [132].

Besides, the Phase III IMbassador-250 clinical trial demonstrated that the combination therapy involving atezolizumab and enzalutamide did not result in increased OS among patients diagnosed with castration-resistant prostate cancer. On the other hand, PD-L1 expression increased and was under negative regulation by androgen receptor signaling in prostate cancer cells, potentially impacting the efficacy of immune checkpoint inhibitors. Interestingly, a recent study indicated that enzalutamide antiandrogen treatment was linked to fewer $CD8^+$ T-cells but increased populations of M-MDSCs and PD-L1 expression in murine tumors. Notably, the study observed an elevation in M2-like macrophage populations in the enzalutamide-resistant cells, suggesting a potential immunosuppressive environment that might limit the effectiveness of immune checkpoint inhibitors [133]. Furthermore, researchers analyzed 755 patients treated with pembrolizumab and 144 with atezolizumab, along with 59 patients having available metastatic urothelial carcinoma. Liver metastasis correlated with increased peripheral monocytes, reduced lymphocytes, and a poorer response to ICB therapy than other metastatic sites. Notably, the ratio of $CD163^+$ M2-like TAMs to $CD8^+$ TILs was significantly associated with the peripheral monocyte-to-lymphocyte ratio in both primary and metastatic urothelial carcinoma lesions. Molecular assessments revealed that resistance to ICB treatment, indicated by lower tumor mutation burden, decreased $CD8^+$ TILs, heightened M2-like TAM markers, and altered immune checkpoint signatures, was observed in primary tumors with liver metastasis. In metastatic lesions, liver metastases showed a higher $CD163^+$ M2-like TAM/$CD8^+$TIL ratio and greater expression of cancer-associated fibroblasts triggered by the TGFβ signaling pathway compared to lung metastases [134].

3.4 Significance

These studies have uncovered pivotal insights by exploring PD-1/PD-L1 immune checkpoint inhibitors on immune cell subsets. They elucidate the intricate dynamics between immunotherapy and TME, unveiling nuanced connections across various cancer types. The thorough evaluation of immune cell populations post-treatment, particularly in response to atezolizumab and other PD-1/PD-L1 inhibitors, illuminates shifts in peripheral monocytes, lymphocytes, and immune cell ratios. Scientists delved deeply into the intricate interactions among immune cells and therapeutic efficacy, shedding light on potential biomarkers and predictive models for treatment responses. The investigations into NK cells' role in ADCC against $PD\text{-}L1^+$ tumors highlight the potential synergy when combining immunotherapies with NK cell-mediated mechanisms for enhanced effectiveness. Innovative platforms simulating the tumor microenvironment and assessments of novel gene-transfected NK cell lines offer valuable insights into the role of innate immunity in antitumor responses and the influence of PD-1/PD-L1 inhibitors on cytotoxic activity.

Moreover, these findings underscore the significance of M2-like macrophages in mediating treatment resistance and the link between liver metastases and altered immune responses, which could potentially limit the effectiveness of immune checkpoint blockade. The discoveries underscore the multifaceted interplay between immune cells, tumor characteristics, and therapeutic responses. They delineate avenues for personalized immunotherapy while emphasizing the necessity for further investigations to validate these observations.

4 Clinical studies

4.1 Atezolizumab (TECENTRIQ.®)

4.1.1 IMpassion-130 vs. IMpassion-131 clinical trials

The IMpassion-130 (NCT02425891) and IMpassion-131 (NCT03125902) are both clinical trials that evaluated the efficacy and safety of atezolizumab in combination with chemotherapy for the treatment of different subtypes of breast cancer. IMpassion-130 (NCT02425891) was a Phase III trial that studied the use of atezolizumab in combination with nab-paclitaxel chemotherapy for treating patients with locally advanced or metastatic TNBC (refer to Table S1). The trial enrolled 902 patients without prior systemic therapy and randomized them to receive either atezolizumab plus nab-paclitaxel or placebo plus nab-paclitaxel. The trial's primary endpoints were progression-free survival (PFS) and OS, and the secondary endpoints included the objective response rate, duration of response (DOR), and safety. The trial demonstrated a significant improvement in PFS in patients who received atezolizumab plus nab-paclitaxel compared to those who received placebo plus nab-paclitaxel, with a median PFS of 7.2 months in the atezolizumab group and 5.5 months in the placebo group [135]. The objective response rate for the intention-to-treat population was also higher in the atezolizumab group (56%) compared to the placebo group (46%). Interestingly, among the patients in the atezolizumab and the placebo groups, 10.3% and 1.1% of patients showed a complete response, respectively. Additionally, a DOR of 7.4 months was observed in the atezolizumab group and 5.6 months in the placebo group.

Furthermore, in the PD-L1$^+$ subgroup, the objective response rate was 58.9% and 42.6% for atezolizumab and placebo groups, respectively [136]. Moreover, the median overall survival (OS) for the intention-to-treat population was longer in the atezolizumab group (21.3 months) compared to the placebo group (17.6 months); however, OS benefit for the intention-to-treat population was not statistically significant [137, 138]. On the other hand, the median OSs for patients with PD-L1$^+$ tumors treated with atezolizumab and placebo were 25.0 months and 15.5 months, respectively [136]. Based on the positive results of the IMpassion-130 trial, atezolizumab, in combination with nab-paclitaxel, was granted FDA's approval for metastatic TNBC in March 2019 [139].

IMpassion-131 (NCT03125902), on the other hand, was a Phase III trial that evaluated the treatment of patients with inoperable locally advanced or metastatic TNBC with atezolizumab in combination with paclitaxel chemotherapy. For this study, 651 previously untreated patients participated. Patients were randomized to receive either atezolizumab plus paclitaxel or placebo plus paclitaxel. The trial's primary endpoint was PFS, and the secondary endpoints were OS, objective response rate, DOR, and safety. However, the trial failed to demonstrate a significant improvement in the primary endpoint of PFS in patients receiving drug combinations compared to those receiving paclitaxel alone (NCT03125902).

Among the patients with PD-L1-expressing tumors, the median PFS was six months in the atezolizumab group and 5.7 months in the placebo group, and the hazard ratio for PFS was 0.82 (95% CI, 0.60–1.12). However, a more favorable overall response rate was observed. The overall response rate was 63% and 55% for atezolizumab and placebo groups, respectively [140]. The safety profile of the combination treatment was consistent with the known safety profiles of both drugs, and no new safety signals were observed. Based on the negative results of the IMpassion-131 trial in October 2021, the application for approval of atezolizumab in combination with paclitaxel for treating TNBC was withdrawn.

Despite their similarities, these two trials exhibit distinct differences. One potential reason for the varying outcomes could be the use of different chemotherapy regimens. The IMpassion-130 trial used nab-paclitaxel (albumin-bound paclitaxel), while IMpassion-131 trial used paclitaxel, both of which are types of taxane chemotherapies.

4.1.2 IMpassion-132 clinical trial

As discussed above, atezolizumab received FDA's accelerated approval as a treatment for PD-L1-positive metastatic TNBC. In IMpassion-130, combined atezolizumab with first-line nab-paclitaxel for metastatic TNBC showed improved median PFS and a clinically meaningful effect on OS in patients with PD-L1$^+$ TNBC tumors. In contrast, in IMpassion-131, atezolizumab combined with paclitaxel did not show improvement between atezolizumab and control groups, leading to approval withdrawal. Therefore, the ongoing IMpassion-132 (NCT03371017) recruits patients to evaluate the efficacy and safety of atezolizumab in combination with chemotherapy (gemcitabine, capecitabine, and carboplatin). Notably, this trial excluded taxol-associated drugs such as paclitaxel or nab-paclitaxel, as a first-line treatment for patients with early relapsing recurrent TNBC. The outcome of IMpassion-132 trial, which has not been published yet, might provide insights into additional combinations involving atezolizumab for TNBC patients [139].

4.1.3 OAK vs. IMpower-150 clinical trials

Atezolizumab has been approved for the treatment of NSCLC by several regulatory agencies, including the FDA and the European Medicines Agency (EMA). The

approval of atezolizumab for NSCLC was based on the results of various clinical studies, notably the critical Phase III trials (refer to Table S2): OAK (NCT02008227), and IMpower-150 (NCT02366143). As atezolizumab combined with nab-paclitaxel is no longer used for TNBC, though it remains approved for NSCLC, the authors' aim is to compare the outcomes of vital clinical trials in TNBC (IMpassion-130–132, previously discussed) and NSCLC (OAK and IMpower-150). Besides, comparing the OAK and IMpower-150 trials, performed on the combinatorial treatment of atezolizumab in NSCLC, may provide useful insight for improving future/ongoing clinical trials investigating the role of atezolizumab in TNBC.

The OAK trial (NCT02008227) was a Phase III, randomized, open-label study to compare the efficacy and safety of atezolizumab and docetaxel in patients with locally advanced or metastatic NSCLC who had previously received platinum-containing chemotherapy. The study recruited 1,225 patients who were randomized in a 1:1 ratio to receive either atezolizumab (425 patients) or docetaxel (425 patients). The study's primary endpoint was OS, and the secondary endpoints were the objective response rate, PFS, and safety. In patients with NSCLC, the study showed that atezolizumab significantly improved OS compared to docetaxel. The median OS was 13.8 months in the atezolizumab group and 9.6 months in the docetaxel group. The improvement in OS was observed across all prespecified subgroups, including patients with high and low PD-L1 expression levels, squamous and non-squamous histology, and the presence or absence of brain metastases [141]. The objective response rate in the intention-to-treat population was almost similar between the atezolizumab and docetaxel groups (14% vs. 13%). Median PFS was 2.8 months for atezolizumab and 4.0 months for docetaxel treatment groups. However, the median DOR in the intention-to-treat population was dramatically more prolonged in the atezolizumab group (16.3 months) compared with 6.2 months in the docetaxel group. The most common adverse events (AEs) were fatigue, decreased appetite, and nausea. The incidence of grade 3–4 treatment-related AEs (TRAEs) was lower in the atezolizumab group compared to the docetaxel group (15% vs. 43%) [141].

On the other hand, the patients were followed up for more than two years, and those who survived for more than two years were labeled as long-term survivors. Atezolizumab showed durable survival benefits compared with docetaxel, with tolerable safety. The atezolizumab group exhibited higher long-term survival rates (28% vs. 18%). Moreover, long-term survival was not limited to PD-L1 expression in survivors [142]. Furthermore, the IMpower-150 (NCT02366143) trial, another Phase III (randomized, open-label) study, demonstrated that the addition of atezolizumab to chemotherapy significantly improved PFS and OS in patients with advanced non-squamous NSCLC. In addition, the atezolizumab-containing regimens were well-tolerated, with an acceptable safety profile. Therefore, an IMpower-150 trial was started to evaluate atezolizumab combined with chemotherapy in patients with advanced non-squamous NSCLC. In this trial, 1,202 patients were randomized and divided into three arms to receive either atezolizumab plus carboplatin and paclitaxel (ACP group), atezolizumab plus carboplatin, paclitaxel, and bevacizumab (ABCP group), or carboplatin, paclitaxel, and bevacizumab (BCP group). This study's primary endpoint was PFS; the secondary endpoints were OS, objective response rates, DOR, and safety. In the wild-type population, the study showed that median PFS significantly improved in ACP and ABCP compared to BCP in patients with advanced non-squamous NSCLC. The median PFS was 8.3 and 6.8 months for ACP and ABCP groups, respectively [143]. Furthermore, the median OS was numerically, but not significantly, improved for wildtype ACP (19.2 months) and ABCP (19.8 months) compared with wildtype BCP (14.7 months).

In addition, patients were prespecified in different subgroups, including patients with high and low PD-L1 expression levels and those with EGFR and KRAS mutations. As a result, the median OS was enormously extended and determined as 26.3 and 30 months for ACP and ABCP groups, respectively, compared with BCP with a median OS of 15 months [144]. Moreover, the median objective response rates for wildtype ABCP and BCP groups were determined as 63% and 48%, respectively; 3.7% of the patients in the ABCP group completely responded [143].

4.2 Nivolumab (OPDIVO.®)

4.2.1 CheckMate-040 clinical trials

Table S3 presents an overview of the historical progression of clinical trials involving nivolumab, specifically those conducted or currently in progress for HCC patients. The CheckMate-040 study, characterized by its multicohort design and Phase I/II approach conducted in an open-label manner, stands as a significant milestone in the history of HCC immunotherapy. There were no randomized control arms in this study. Patients with HCC who had disease progressing on the first line of systemic therapy or were resistant to sorafenib, regardless of their hepatitis viral infections status were included in this trial [18]. The CheckMate-040 trial (NCT01658878) was designed to assess the efficacy and safety of nivolumab.

According to CheckMate-040, a total of 262 patients were recruited, encompassing both the dose escalation phase (48 patients) and the subsequent dose expansion phase (214 patients) [18]. This trial investigated various cohorts, including monotherapy and combination therapy, intending

to assess nivolumab's potential in HCC treatment. Three cohorts were assigned to the dose escalation phase, and the four cohorts were investigated in the dose expansion phase.

(1) *Monotherapy (Sorafenib-Naive):* This cohort enrolled sorafenib-naive patients with advanced HCC. Nivolumab was administered as monotherapy, and the primary endpoint was to assess its efficacy and safety in this specific patient population [145].
(2) *Monotherapy (Sorafenib-Experienced):* This cohort included patients with advanced HCC who had received therapy with sorafenib. The objective was to assess nivolumab's efficacy and safety in sorafenib-experienced patients [146].
(3) *Combination Therapy (Nivolumab + Ipilimumab):* In this cohort, nivolumab combined with ipilimumab was studied in patients with advanced HCC with a history of receiving sorafenib. The safety and efficacy of the nivolumab combination therapy were evaluated in this cohort [147].
(4) *Combination Therapy (Nivolumab + Cabozantinib):* This cohort investigated the combination of nivolumab and cabozantinib (Cabometyx) in treatment-naive patients with advanced HCC [148].

The results of these cohorts provided valuable insights into the potential benefits of nivolumab, both as monotherapy and in combination with targeted therapy (cabozantinib) and immunotherapy (ipilimumab), for the treatment of advanced HCC. The trial outcomes contributed to the accelerated approval of nivolumab by the FDA for specific HCC patient populations. As a result, nivolumab was granted accelerated approval as monotherapy in 2017 and in combination with ipilimumab (anti-CTLA-4 antibody) and cabozantinib (anti-RTK) in 2020 and 2021, respectively, for the treatment of patients with advanced HCC with a background of receiving sorafenib, a standard systemic therapy for advanced HCC [101, 149]. However, the accelerated approval of nivolumab monotherapy for patients with advanced HCC who received prior treatment with sorafenib was withdrawn in July 2021 [150].

Nivolumab monotherapy The dose expansion phase of CheckMate-040 (NCT01658878) recruited 214 patients to evaluate the response rates, DOR, PFS, OS, and safety outcomes of nivolumab monotherapy in patients with advanced HCC. Patients were assigned to four cohorts, including patients without HCV or HBV that had not received sorafenib previously or were intolerant (56 patients), sorafenib cohort with disease progression after prior treatment of sorafenib (57 patients), HCV cohort (50 patients), and HBV cohort (51 patients). In this phase, 145 patients (68%) had previously been treated with sorafenib. Grade 3/4 TRAEs were observed in 19% of patients, and grade 3/4 serious TRAEs in 4% [18].

On the other hand, there were no treatment-related deaths. Based on the investigated cohort, a response rate of 23% and a median OS of 9 months were observed in patients without viral infections (without previously being treated with sorafenib or were intolerant) and a response rate of 21% and a median OS of 13.2 months was observed in sorafenib group (without viral hepatitis). The PFS rates of six and nine months were observed in 37% and 28% of patients, respectively [18] (NCT01658878). Furthermore, the median OS for all Child–Pugh B patients decreased to 7.6 months. The median OS for sorafenib-naive and -treated patients was 9.8 months and 7.4 months, respectively. The median PFS for all Child–Pugh B patients was 2.7 months; the median PFS for sorafenib-naive and -treated patients was 3.4 months and 2.2 months, respectively [145].

Moreover, PD-L1 expression levels were considered a secondary endpoint and a potential biomarker for nivolumab therapy in the 174 patients in the dose-expansion phase. Eighty percent of patients had less than 1% PD-L1 expression, and 20% had at least 1% PD-L1 expression on tumor cells. Based on the PD-L1 expression, the objective response rate was 19% and 26% in patients with less than 1% and more than 1% PD-L1 expression, respectively. In a larger patient population, PD-L1 can be considered a stable and reliable biomarker among tumor types; however, HCC patients with low PD-L1 expression also responded to nivolumab. Furthermore, the median OS of 28.1 and 16.6 months were observed for patients with tumor PD-L1 $\geq 1\%$ vs. $< 1\%$, suggesting tumor PD-1 and PD-L1 expression were associated with improved OS. Therefore, nivolumab, through inhibition of PD-L1 signaling in non-tumor cells, may contribute to the efficacy of nivolumab in patients with low ($< 1\%$) levels of PD-L1 expression in tumor cells [18, 151].

Nivolumab combinatorial therapy In addition to the proven effectiveness of the nivolumab monotherapy, the CheckMate-040 trial (NCT01658878) evaluated the combination of nivolumab and ipilimumab (a CTLA-4 blocker) as well as the combination of nivolumab and cabozantinib (a non-specific c-MET RTK inhibitor). In the ipilimumab cohort, 148 patients with advanced HCC were enrolled. These patients had previously been treated with sorafenib. They were randomized into three arms, and received two different doses of nivolumab (arm A: 1 mg/kg or arm B: 3 mg/kg) and ipilimumab (arm B: 1 mg/kg or arm A: 3 mg/kg) every three weeks, followed by nivolumab 240 mg every two weeks or one dose (arm C) of nivolumab (3 mg/kg) every two weeks plus ipilimumab (1 mg/kg) every six weeks followed by nivolumab 240 mg every two weeks [152].

The primary endpoints for this cohort included safety, tolerability, and objective response rate. The objective response rate of 32% was observed in the patients assigned to arm A, with an 8% complete response. However, the 27% and 29% lower objective response rates were measured in arms B and C, respectively. Compared with the results of nivolumab monotherapy (the median OS of 13.2 months and objective response rates of 20%), the combination therapy of nivolumab and ipilimumab in arm A display notably more promising outcomes. In arm A, the median OS of 22.8 months and objective response rates of 32% were observed. However, arms B and C exhibited median OS values of 12.5 months and 12.7 months, respectively, which were nearly comparable to the median OS observed with nivolumab monotherapy (13.2 months). On the other hand, five patients in arm A, two in arm B, and one in arm C showed grade 3/4 TRAEs. Based on the encouraging results from this study, nivolumab 1 mg/kg plus ipilimumab 3 mg/kg every three weeks and then followed by nivolumab 240 mg every two weeks, granted accelerated approval as a second-line option for treatment of advanced HCC [152].

4.2.2 CheckMate-459 clinical trial

The CheckMate-459 trial registered as NCT02576509, conducted a randomized Phase III study comparing nivolumab and sorafenib as initial treatments for advanced HCC. A total of 743 patients who had previously received systemic treatment for advanced HCC took part in the study. Among them, 371 patients were randomly assigned to receive nivolumab, while 372 received sorafenib. The study evaluated the objective response rate and median OS for both treatments. The results showed that nivolumab had an objective response rate of 15%, a complete response rate of 4%, and a median OS of 16.4 months [153]. In contrast, sorafenib had an objective response rate of 7%, a complete response rate of 1%, and a median OS of 14.7 months. When analyzing patients according to the PD-L1 expression, those with a PD-L1 expression of 1% or higher showed an objective response rate of 28%, while those with PD-L1 expression below 1% demonstrated an objective response rate of 12% [153]. The 12-month and 24-month OS rates were 59.7% and 36.8% in the nivolumab group, respectively, and 55.1% and 33.1% in the sorafenib group. The median PFS of 3.8 and 3.9 months were observed for nivolumab and sorafenib, respectively. Although the OS results did not meet the predefined statistical significance threshold, nivolumab demonstrated a higher objective response rate than sorafenib (NCT02576509).

Additionally, nivolumab exhibited a less toxic profile and a lower incidence of grade 3/4 AEs (22%) compared to sorafenib (49%), which included diarrhea, rash, pruritus, and other side effects. The most common grade 3 TRAEs were palmar-plantar erythrodysesthesia (< 1% in the nivolumab group vs. 14% in the sorafenib group), hypertension (0% vs. 7%), and aspartate aminotransferase increase (6% vs. 4%). Serious TRAEs occurred in 12% of patients receiving nivolumab and 11% receiving sorafenib. In addition, the study reported four treatment-related deaths in the nivolumab group, whereas there was one in the sorafenib group. Based on the negative findings of the CheckMate-459 trial and the low objective response rate, the drug advisory committee voted against maintaining the accelerated approval of nivolumab for second-line treatment following progression on sorafenib, led to the withdrawal of nivolumab monotherapy [153].

However, regarding the combination of nivolumab with ipilimumab, which was previously assessed in Phase I/II CheckMate-040 (NCT01658878) and granted accelerated approval, the Phase III CheckMate-9DW (NCT04039607) and CheckMate-74W (NCT04340193) trials are currently active. These trials aim to compare the OS of a combination treatment consisting of ipilimumab plus nivolumab with sorafenib or lenvatinib kinase inhibitors as the first-line treatment for patients with advanced HCC. The OS is the study's primary endpoint, while secondary endpoints include objective response rate, DOR, and time to symptom deterioration. Furthermore, Trans-Arterial ChemoEmbolization (TACE) will also be evaluated in CheckMate-74W trial.

5 Limitations and future prospective

The withdrawal of drug indications by the FDA can have significant implications for patients, healthcare providers, and the pharmaceutical industry (Table 1)[154]. In this comprehensive review, we have focused on atezolizumab and nivolumab to provide fresh insights into the withdrawals of immunotherapeutic indications, taking both structural and clinical approaches into account. Specifically, the withdrawal of atezolizumab in combination with nab-paclitaxel for TNBC and nivolumab monotherapy for HCC could have significant consequences for patients with TNBC and HCC, respectively. Losing access to these treatment options may potentially diminish their chances of successful treatment. It is essential to understand the reasons behind these withdrawals and assess the structural and clinical factors that contributed to the decisions.

In the field of cancer pharmacology, it is important to consider baseline patient characteristics and prognostic factors when assessing the influence of immunogenicity on pharmacokinetics (PK) and efficacy [155]. Previous research has explored the impact of anti-drug antibodies (ADA) on the PK of nivolumab and atezolizumab. Along with its effects on drug processing, safety, and effectiveness in patients with solid tumors, the immunogenicity of nivolumab has been studied. Out of the 1,086 patients who received nivolumab,

Table 1 Immunotherapeutic indications whose accelerated approvals (AA) were withdrawn

Drug names	Target	Accelerated approval indication	AA Date	Withdrawal date	Reason for withdrawal
Keytruda (pembrolizumab)	PD-1	Metastatic SCLC with disease progression on or after platinum-based chemotherapy and at least one other prior line of therapy	6/17/2019	3/30/2021	Failure to demonstrate clinical benefit in a post-marketing study
Tecentriq (atezolizumab)	PD-L1	In combination with paclitaxel protein-bound for unresectable locally advanced or metastatic TNBC whose tumors express PD-L1 (PD-L1 stained tumor-infiltrating immune cells of any intensity covering = 1% of the tumor area), as determined by an FDA-approved test	3/8/2019	10/6/2021	Data did not confirm clinical benefit, failure to meet requirement
Opdivo (nivolumab)	PD-1	Metastatic SCLC with progression after platinum-based chemotherapy and at least one other line of therapy	8/16/2018	12/29/2020	Confirmatory trial results did not show clinical benefit
Keytruda (pembrolizumab)	PD-1	For patients with recurrent or locally advanced or metastatic gastric or GEJ adenocarcinoma whose tumors express PD-L1 [CPS ≥ 1] as determined by an FDA-approved test, with disease progression on/after two or more prior lines of therapy including fluoropyrimidine and platinum-containing chemotherapy and if appropriate, HER2/NEU targeted therapy	9/22/2017	2/4/2022	Data did not confirm clinical benefit
Opdivo (nivolumab)	PD-1	HCC previously treated with sorafenib	9/22/2017	7/23/2021	Post-marketing trial did not verify clinical benefit
Imfinzi (durvalumab)	PD-L1	Locally advanced or metastatic urothelial carcinoma that progressed during or following platinum-containing chemotherapy or within 12 months of neoadjuvant or adjuvant treatment with platinum-containing chemotherapy	5/1/2017	2/19/2021	Failure to confirm clinical benefit, no improvement in survival
Tecentriq (atezolizumab)	PD-L1	Patients with locally advanced or metastatic urothelial carcinoma who are not eligible for cisplatin-containing chemotherapy and whose tumors express PD-L1 as determined by an FDA-approved test or who are not eligible for any platinum-containing chemotherapy regardless of PD-L1 status	4/17/2017	12/2/2022	Failure to meet post-marketing requirement, insufficient evidence
Tecentriq (atezolizumab)	PD-L1	Locally advanced or metastatic urothelial carcinoma that progressed during or following platinum-containing chemotherapy or within 12 months of neoadjuvant or adjuvant treatment with platinum-containing chemotherapy	5/18/2016	4/13/2021	Withdrawal of indication due to failure to confirm clinical benefit

The order of drugs is based on the AA date (Source: https://www.fda.gov)

138 patients (12.7%) exhibited ADA positivity, of which only three patients (0.3%) consistently tested positive for ADAs, and nine patients (0.8%) tested positive for neutralizing antibodies (Nabs) at a single time point. The presence of ADAs did not demonstrate any association with hypersensitivity, infusion reactions, or a decline in the drug's effectiveness. Additionally, it had minimal impact on the elimination of nivolumab from the body [156]. n contrast, data from around 4,500 patients participating in 12 clinical trials involving various tumor types, treatment approaches, and dosing plans were analyzed. The study revealed that nearly 30% of patients (ranging from 13 to 54%) developed ADA during treatment. ADA^+ patients showed lower levels of atezolizumab exposure. The presence of ADA resulted in a minimal increase in atezolizumab clearance (9%), but it did not significantly impact drug exposure or OS [157]. Thus, immunogenetic factors alone are not significant predictors with clinical relevance [158].

On the other hand, post-translation modifications play a significant role in the activity and efficacy of immunotherapeutic antibodies like atezolizumab and nivolumab, particularly in their interaction with PD-1 and PD-L1. Certain glycosylation sites on PD-L1, namely N192, N219, and N35, enhance the interaction between PD-1 and PD-L1 [49]. However, glycosylated PD-L1 reduces the responsiveness of patients to anti-PD-L1 antibodies, which played a role in the withdrawal of atezolizumab as a treatment option for TNBC. The level of unglycosylated PD-L1 has been suggested as a potential biomarker for anticipating the response of TNBC patients to atezolizumab [50]. Likewise, the efficacy of nivolumab is influenced by the glycosylation of PD-1 at N49, N58, N74, and N116. Human-derived PD-1 undergoes extensive glycosylation at specific locations, leading to an increase in molecular weight [38]. Similarly, although the binding affinity of nivolumab to PD-1 is not exclusively dependent on N-glycosylation, the N-terminal of PD-1 plays a dominant role in the binding process. PD-1 glycosylation might also play a significant role in the interaction with monoclonal antibodies targeting PD-1 [38]. The levels of PD-1 glycosylation, particularly at N58, affect the effectiveness of ICB therapy. These factors have spurred the development of a novel antibody called penpulimab, which specifically targets PD-1 and can identify the glycosylated form of PD-1 [54].

Besides, atezolizumab was obtained through a screening process using a human phage display library (Genentech) and a recombinant extracellular domain (ECD)–Fc fusion of human PD-L1. A highly potent antibody with strong binding affinity was selected from a single phage clone on a human IgG1 backbone. Furthermore, the FDA-approved drug atezolizumab lacks glycosylation. To eliminate effector functions, such as ADCC, the Fc domain of atezolizumab was modified by introducing a mutation (Asp to Ala) at position 297/298 in each heavy chain. This mutation affects the glycosylation of the antibody at position 297, resulting in glycosylation of the antibody [11]. The glycosylated form of atezolizumab is, however, highly unstable and prone to aggregation, resulting in the rapid development of ADA in 41% of cancer patients treated with atezolizumab, ultimately leading to a loss of effectiveness. Therefore, the development of a glycosylated version of atezolizumab, named Maxatezo, which retains no ADCC activity, exhibits improved thermal stability, and demonstrates significantly enhanced antitumor activity *in vivo*. Glycosylation was reintroduced by reversing the A297N mutation. Additionally, the levels of ADA in mice treated with Maxatezo were significantly lower compared to those treated with atezolizumab. Most notably, at the same dose of 10 mg/kg, Maxatezo achieved a tumor growth inhibition rate of 98% compared to 68% for atezolizumab [159]. Additionally, the development of engineered glycosylated atezolizumab with specific modifications has shown promising results. De-fucosylated atezolizumab, compared to the commercial version, has demonstrated enhanced ADCC activity against $PD\text{-}L1^+$ cancer cells by binding to FcγRIIIa [57].

To further overcome these limitations, researchers have also explored the use of the human PD-1 ectodomain, a small protein component weighing 14–17 kDa, as a potential therapeutic target. Anti-PD-1 antibody was developed using a bacterial display-based high-throughput directed evolution approach to successfully identify human PD-1 variants that are glycan-controlled, either glycosylated or carrying a single N-linked glycan. The resultant variants namely glycosylated JYQ12 and JYQ12-2 with a single N-linked glycan chain, demonstrated exceptionally high affinity for hPD-L1 and strong affinity for both hPD-L2 and mPD-L1. Furthermore, JYQ12-2 effectively enhanced the proliferation of human T-cells. These hPD-1 variants with significantly improved binding affinities for hPD-1 ligands offer the potential for developing highly effective therapeutics or diagnostics that differ from large-sized IgG antibodies, showing the effective role of glycosylation in developing anti-PD-1/PD-L1 drugs [160].

Limited tumor penetration is a significant drawback of macromolecular therapeutics like antibodies. Increasing the affinity of a macromolecule is commonly thought to improve its tumor retention. Although antibodies strongly bind to the surface of tumor cells, they struggle to diffuse into the tumor microenvironment [161]. On the other hand, peptides are characterized by their smaller size and relatively lower binding affinity compared to antibodies, enabling them to penetrate tumor spheroids more effectively. As a result, the use of phage display to identify small peptide-based checkpoint inhibitors has been demonstrated, leading to the discovery of several anti-PD-L1 peptide inhibitors that disrupt the PD-1/

PD-L1 interaction. These peptides exhibit a high affinity and specificity for the human PD-L1 protein, showcasing their potential as low-molecular-weight checkpoint inhibitors in cancer immunotherapy [162].

Recently, there has been significant progress in the development of various protein degradation strategies for treating different types of cancer. These strategies include the use of proteolysis-targeting chimeras (PROTACs), CELMoDs, and immunomodulatory drugs (IMiDs) to induce targeted protein degradation [163, 164]. Studies have investigated the effectiveness of novel compounds like iberdomide and CC-92480 in multiple myeloma, both in cell lines and patient samples. Additionally, researchers have explored the synergistic effects of combining these compounds with other drugs, such as bortezomib, dexamethasone, and daratumumab [165]. In the context of gastrointestinal cancers, the development of MDEG-541, an MYC-specific PROTAC, has shown potential as a therapeutic approach for targeting MYC. MDEG-541 selectively degrades GSPT1/2 and PLK1 and regulates MYC expression in a manner dependent on cereblon, proteasome, and ubiquitin [164]. While targeted protein degradation is still an emerging field, the development of antibody-based PROTACs (AbTACs) that can degrade cell surface proteins has been instrumental. Researchers have used rational protein engineering strategies to optimize these AbTACs for efficient degradation of membrane proteins like PD-L1 and EGFR. Importantly, these AbTACs have demonstrated no undesired signaling effects and can be further enhanced through variations in the configuration of their binding arms [166].

6 Conclusion

The withdrawal of immunotherapeutic indications underscores the importance of continuously evaluating drug safety and efficacy to ensure optimal patient outcomes. While immunogenicity and glycosylation can impact the pharmacokinetics and effectiveness of immunotherapies, further research is needed to fully understand their clinical relevance. Continued advancements in protein engineering and targeted protein degradation strategies offer promising avenues for enhancing cancer treatments. Overall, the ongoing efforts to optimize immunotherapeutic interventions provide hope for improving cancer therapy and patient care in the future.

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Declarations

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Competing interests The authors declare no competing interests.

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