



Enhancing personalized immune checkpoint therapy by immune archetyping and pharmacological targeting

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ABSTRACT

Immune checkpoint inhibitors (ICIs) are an expanding class of immunotherapeutic agents with the potential to cure cancer. Despite the outstanding clinical response in patient subsets, most individuals become refractory or develop resistance. Patient stratification and personalized immunotherapies are limited by the absence of predictive response markers. Recent findings show that dominant patterns of immune cell composition, T-cell status and heterogeneity, and spatiotemporal distribution of immune cells within the tumor microenvironment (TME) are becoming essential determinants of prognosis and therapeutic response. In this context, ICIs also function as investigational tools and proof of concept, allowing the validation of the identified mechanisms. After reviewing the current state of ICIs, this article will explore new comprehensive predictive markers for ICIs based on recent discoveries. We will discuss the recent establishment of a classification of TMEs into immune archetypes as a tool for personalized immune profiling, allowing patient stratification before ICI treatment. We will discuss the

Abbreviations: ADC, antibody-drug conjugates; ADME, absorption, distribution, metabolism, and excretion; AML, acute myeloid leukemia; APC, antigen-presenting cell; BCC, metastatic basal cell carcinoma; BRAF, B-Raf proto-oncogene; BMS, Bristol-Myers Squibb; BTC, biliary tract cancer; CAF, cancer-associated fibroblast; CAR-T, chimeric antigen receptor T-cells; cDC, conventional DC; CDKN2A, cyclin-dependent kinase inhibitor 2 A; COX-2, cyclooxygenase-2; CRC, colorectal cancer; CSCC, cutaneous squamous cell carcinoma; CCL2/5, chemokine (C-C motif) ligand 2/5; CSF-1R, colony-stimulating factor 1 receptor; CTLA-4, cytotoxic T-lymphocyte-associated protein 4; CXCL10/12, chemokine (C-X-C motif) ligand 10/12; CXCR4, chemokine (C-X-C motif) receptor 4; DC, dendritic cell; dMMR, mismatch-repair deficient; ECM, extracellular matrix; EGFR, epithelial growth factor receptor; ESCC, esophageal squamous cell carcinoma; EMT, epithelial-mesenchymal transition; FDA, Food and Drug Administration; FGf1, fibrinogen-like protein 1; FLT3L, Fms-like tyrosine kinase ligand 3; GAL-3, galectin-3; GEJ, gastroesophageal junction; GITR, glucocorticoid-induced TNFR-related; HCC, hepatocellular carcinoma; HIP1R, huntingtin interacting protein 1 related; HLA, human leukocyte antigen; HMGB1, high-mobility group box 1; HNC, head and neck cancer; HNSCC, head and neck squamous cell carcinoma; HTRF, homogeneous time-resolved fluorescence; ICB, immune checkpoint blockade; ICI, immune checkpoint inhibitor; IFN γ , interferon γ ; IgG, immunoglobulin; IL-1 α /IL-1 β /6/10, interleukin 1 α /1 β /6/10; IHC, immunohistochemistry; ITAM, tyrosine-based activation motif; ITIM, tyrosine-based inhibitory motif; KIRs, killer cell immunoglobulin-like receptors; LAG-3, lymphocyte activation gene 3; LCMV, lymphocytic choriomeningitis virus; LLC, lung Lewis carcinoma; LRRC15, leucine-rich repeat containing 15; LSECtin, liver sinusoidal endothelial cell lectin; LYTAc, lysosome-targeting chimera; M6PR, mannose 6-phosphate receptor; MB, megabase; MCC, Merkel cell carcinoma; MDS, myelodysplastic syndrome; MDSC, myeloid-derived suppressor cell; MEK, mitogen-activated protein kinase kinase; MHC-II, molecular histocompatibility complex class II; MMP-2/9, metalloproteinase 2/9; MSI-H, microsatellite instability-high; NK, natural killer; NKG2A, NK group 2 member A; NMIBC, non-muscle invasive bladder cancer; NSCLC, non-small cell lung cancer; OCR, open chromatin region; ORR, overall response rate; OS, overall survival; PC, prostate cancer; PD-1, programmed death 1; PDAC, pancreatic ductal adenocarcinoma; PD-L1/2, programmed death-ligand 1/2; PFS, progression-free survival; PMBCL, primary mediastinal large B-cell lymphoma; PS, phosphatidylserine; PROTAC, proteolysis-targeting chimera; PTEN, phosphatase and tensin homolog; RAR, retinoic acid receptor; RCC, renal cell carcinoma; RFS, recurrence-free survival; SCLC, small cell lung cancer; Siglec, sialic acid-binding immunoglobulin-like lectin; SMARC4, SWI/SNF Related, Matrix Associated, Actin Dependent Regulator of Chromatin, Subfamily A, Member 4; SPR, surface plasmon receptor; STK11/LKB1, serine/threonine kinase 11/liver kinase B1; TAM, tumor-associated macrophage; TCR, T-cell receptor; TCR-seq, T-cell receptor sequencing; TEAD, TEA domain; Teff, effector T-cell; Tex, exhausted T-cell; TGF β , tumor growth factor β ; Tfh, T follicular helper; Th, T helper; TIGIT, T-cell immunoreceptor with Ig and ITIM domains; TIL, tumor-infiltrating lymphocyte; TIM-3, T-cell immunoglobulin and mucin domain 3; TMB, tumor mutational burden; TME, tumor microenvironment; Tmem, memory T-cell; Tn, naïve T-cell; TNBC, triple-negative breast cancer; TNF α , tumor necrosis alpha; TOX, thymocyte selection associated high mobility group box; Treg, regulatory T-cell; T_{ST}, T-cell response stress state; UC, urothelial cancer; VEGF, vascular endothelial growth factor; VISTA, V-domain Ig suppressor of T-cell activation; VSIG, V-set and immunoglobulin domain-containing protein; VSIR, V-set immunoregulatory receptor; VSTM, V-set and transmembrane domain-containing protein; WUCAM, Washington University cell adhesion molecule; XCL1, X-C motif chemokine ligand 1.

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developing comprehension of T-cell diversity and its role in shaping the immune profile of patients. We describe the potential of strategies that score the mutual spatiotemporal modulation between T-cells and other cellular components of the TME. Additionally, we will provide an overview of a range of synthetic and naturally occurring or derived small molecules. We will compare compounds that were recently identified by *in silico* prediction to wet lab-validated drug candidates with the potential to function as ICIs and/or modulators of the cellular components of the TME.

1. Introduction

Immunotherapy is an innovative and fast-expanding anticancer treatment approach. Recent research has aimed to initiate or reinvigorate the immune system's reaction against cancer cells. The recent clinical approval of immunomodulators belonging to the immune checkpoint inhibitor (ICI) class has profoundly affected cancer patients' therapeutic outcomes. Ipilimumab, the first-in-class ICI targeting cytotoxic T lymphocyte antigen 4 (CTLA-4), induced complete and long-lasting tumor regression in a subset of patients affected by metastatic melanoma [1,2]. Similarly, complete tumor resorption was achieved in patients treated with pembrolizumab or nivolumab. These two first-generation antibodies block programmed death 1 (PD-1) and its ligands 1 and 2 (PD-L1 and PD-L2) [1,3]. These remarkable therapeutic achievements have led to the development of additional ICIs that improved the treatment of multiple types of cancer. Immunotherapeutic approaches have recently included neoantigen vaccines and adoptive cell therapies based on chimeric antigen receptor T-cells (CAR-T). All these therapeutic approaches have generated durable (> 10 years) and curative effects in sensitive patients. However, these exciting outcomes are counterbalanced by low response rates and ineffective predictive biomarkers. A significant number of patients are indeed refractory to immunotherapies. In addition, initial responders may acquire resistance and eventually progress, as observed in chemotherapy or targeted therapies. Much attention is focused on understanding why certain patients respond well to immunotherapy while others do not. The goal is to identify the reasons behind this distinction and find ways to convert non-responders into responders.

Cancer immunity involves multiple cellular components interacting and mutually modulating each other. Changes in any of the steps can impede the anticancer immune response. Modulatory signals occur within the tumor tissue and other areas of the body. Recent findings have identified critical onco-immunological regulatory mechanisms, providing the rationale for cutting-edge personalized approaches. Considering the current advancements, we provide an overview of the anticancer immune response and discuss therapeutic strategies to overcome resistance using synthetic and natural immune checkpoint modulators.

2. Development and rationale behind utilizing ICIs as a curative treatment for cancer

The anticancer activity of ICIs was initially predicted on a theoretical basis. Later, experimental approaches that involved the development of neutralizing antibodies validated their anticancer potential. In the early 1990s, researchers discovered that in addition to the T-cell receptor (TCR), T-cells needed costimulatory signals for full activation after interacting with antigen-presenting dendritic cells (DCs). It has been discovered that the CD28 receptor on T-cells binds with the B7 family proteins situated on the surface of DCs. [4]. Following engagement, the inhibitory receptor CTLA-4 moves from the endoplasmic reticulum to the plasma membrane and takes the place of CD28 in its interaction with B7 proteins. The higher binding affinity of CTLA-4 eventually turns off T-cell stimulation. Such an evolutionary process is a safety mechanism to avoid uncontrolled and deleterious immune responses. The hypothesis suggested that by neutralizing CTLA-4, T-cells can be (re)activated, resulting in enhanced anticancer immunity [5]. This approach

represented a shift in the philosophy of cancer treatment: the primary therapeutic targets were no longer cancer cells but the patient's immune system. The anti-CLTA-4 ICI ipilimumab was the first-in-class immunotherapy to be used in clinical practice for treating metastatic melanoma. After conducting clinical trials and 5-year survival studies, certain patients experienced complete tumor resorption, as seen in their follow-up results spanning over a decade. [1,2]. Ipilimumab is now being used clinically for advanced stages of other solid tumors. (i.e., non-small cell lung cancer (NSCLC)). The adverse side effects of prolonged T-cell activation appeared manageable with steroids. Although several follow-up studies were conducted, they all indicated that the treatment provided a cure for only about 20% of patients. Most of the enrolled patients did not show any initial objective response. This challenging outcome led to the theory that there may be more checkpoints. The identification of the PD-1 receptor on T-cells [6] and its corresponding ligands, PD-L1 and PD-L2 [7], on cancer cells marked a significant milestone in comprehending the role of T-cell-mediated anticancer immunity and has had a profound impact on therapy. PD-1/PD-L1 is a late-occurring checkpoint blockade that prevents activated T-cells from killing cancer cells. In cancer cells, PD-L1 upregulation can be caused by oncogenic signaling pathways. PD-L1 is also under the control of interferon-gamma (IFN γ) or tumor necrosis alpha (TNF α) released in the tumor microenvironment (TME) [8]. In addition, tumor-associated macrophages (TAMs) and T lymphocytes also upregulate PD-L1/PD-L2. The mechanism of action for this checkpoint blockade differs from that of CTLA-4 blockade, as it corresponds to a resistance mechanism developed by cancer cells. Cancer co-opts a physiological, protective mechanism used by healthy tissues or embryonal/fetal cells to prevent an attack from the maternal immune system. This discovery led to the development of pharmacological agents neutralizing PD-1 (nivolumab, pembrolizumab, cemiplimab) or its cognate receptors PD-L1 and PD-L2 (atezolizumab, durvalumab, avolumab). These antibody drugs are now approved for treating solid tumors with a well-tolerated profile (Table 1, and Fig. 1). Other PD-1/PD-L1 inhibitors are under advanced investigation. However, cancer patients can also become refractory/resistant to PD-1/PD-L1 inhibitors (approximately 50% of responders). Combinatorial strategies were assessed because the CTLA-4 and PD-1/PD-L1 immune checkpoints have distinct, non-redundant mechanisms and timing of action. Combinations improved tumor rejection rates in murine melanoma models. In addition, specific therapeutic schemes appeared to be more effective. The administration of nivolumab followed by ipilimumab showed an overall response rate (ORR) of approximately 56%, despite increased adverse effects. On the other hand, the opposite approach resulted in a 31% ORR. [9]. A 5-year survival study on melanoma patients treated with ipilimumab or nivolumab administered alone or in combination showed superior overall survival (OS) and progression-free survival (PFS) in patients receiving nivolumab alone (44% OS; 36% PFS) or nivolumab plus ipilimumab (52% OS; 29% PFS) compared to ipilimumab alone (26% OS; 8% PFS), with a similar safety profile [1]. Additional combinatorial strategies involving agents besides immune modulators are used in advanced clinical trials or clinics. These trials involve triplet-combined therapies with anti-PD-1 pembrolizumab, inhibitors of B-Raf proto-oncogene (BRAF) and mitogen-activated protein kinase kinase (MEK) [10,11]. The anti-PD-1 spartalizumab was combined with dabrafenib and trametinib in BRAF $V600$ -mutated metastatic melanoma patients [12]. Another trial tested the combination of

Table 1
FDA-approved ICIs.

Target	Name	Mechanism of action; effect	Indication
CTLA-4 PD-1	Ipilimumab	humanized IgG1 anti-CTLA-4 monoclonal antibody; disruption of CTLA-4/B7.1 or B7.2 interaction	monotherapy (melanoma, MSI-H, and dMMR CRC); with anti-PD-1 nivolumab (melanoma, HCC, NSCLC, mesothelioma, RCC, ESCC)
	Nivolumab	humanized anti-PD-1 IgG4 monoclonal antibody; disruption of PD-1/PD-L1 interaction	monotherapy (NSCLC, ESCC); with the anti-CTLA4 ipilimumab (melanoma, HCC, NSCLC, mesothelioma, RCC, ESCC), with the anti-LAG-3 relatlimab (melanoma), with chemotherapy-based regimens (NSCLC, RCC, esophageal, GEJ, and gastric cancer); adjuvant (UC)
PD-1	Pembrolizumab	humanized anti-PD-1 IgG4k monoclonal antibody; disruption of PD-1/PD-L1 interaction	monotherapy (advanced cervical cancer, CSCC, MCC, MSI-H, and dMMR CRC and advanced endometrial carcinoma, NMIBC, HNSCC, PMBCL, SCLC, TMB-H solid tumors); with anti-angiogenic bevacizumab (cervical cancer); with tyrosine kinase inhibitors (axitinib and lenvatinib for RCC); with chemotherapy-based regimens (esophageal and GEJ carcinoma, NSCLC, TNBC); adjuvant (melanoma, RCC, TNBC)
PD-1	Cemiplimab	humanized hinge-stabilized anti-PD-1 IgG4 monoclonal antibody; disruption of PD-1/PD-L1 interaction	monotherapy (locally advanced or metastatic BCC, untreatable CSCC, NSCLC); with chemotherapy-based regimens (NSCLC)
PD-L1	Atezolizumab	humanized anti-PD-L1 IgG1 monoclonal antibody; disruption of PD-1/PD-L1 interaction	monotherapy (NSCLC, UC); with anti-angiogenic bevacizumab (HCC, NSCLC); with tyrosine kinase inhibitor (cobimetinib for metastatic melanoma); BRAF inhibitor (vemurafenib for metastatic melanoma); with chemotherapy-based regimens (NSCLC, SCLC, TNBC); adjuvant (NSCLC)
PD-L1	Durvalumab	human anti-PD-L1 IgG1k monoclonal antibody; disruption of PD-1/PD-L1 interaction	with anti-CTLA4 tremelimumab (HCC, NSCLC); with chemotherapy-based regimens (BTC, NSCLC, SCLC)
PD-L1	Avelumab	human anti-PD-L1 IgG1 monoclonal antibody; disruption of PD-L1/PD-1 interaction and antibody-dependent mediated cytotoxicity	monotherapy (MCC); with tyrosine kinase inhibitors (axitinib for RCC); maintenance therapy (UC)
LAG-3	Relatlimab	human anti-LAG-3 IgG4 monoclonal antibody; disruption of LAG-3/MHC-II or FGL1 interaction	with anti-PD-1 nivolumab (melanoma)

Abbreviations: BCC: basal cell carcinoma; BTC: biliary tract cancer; CRC: colorectal cancer; CSCC: cutaneous squamous cell carcinoma; dMMR: mismatch repair deficient; ESCC: esophageal squamous cell carcinoma; GEJ: gastroesophageal junction GEJ; HCC: hepatocellular carcinoma; HNSCC: head and neck squamous carcinoma MCC: Merkel cell carcinoma; MSI-H: microsatellite instability-high; NMIBC: nonmuscle invasive bladder cancer; NSCLC: non-small cell lung cancer; PMBCL: primary mediastinal large B-cell lymphoma; RCC: renal cell carcinoma; SCLC: small cell lung cancer; TMB: tumor mutational burden; TNBC: triple-negative breast cancer; UC: urothelial cancer.

PD-1/PD-L1 inhibitors with vascular endothelial growth factor (VEGF) or tyrosine kinase inhibitors in endometrial and/or renal cell carcinoma (RCC). ICIs combined with chemotherapy have been clinically approved for treating certain types of cancer (e.g., NSCLC). Additional clinical trials investigate the efficacy of the combination of PD-1/PD-L1 with chemotherapeutic agents and/or additional investigational drugs in NSCLC (NCT05555732; NCT5687266; NCT03322566), triple-negative breast cancer (TNBC; NCT02685059 [13]; NCT02622074; [14]; NCT05402722; NCT05556200; NCT05088057), and head and neck cancers (HNC; NCT05758389, NCT04429542; NCT04954599; NCT05777824). These studies are aimed at investigating survival advantages and improving patient stratification. Furthermore, there is evidence suggesting that the combination of ICIs and radiation therapy may have a synergistic effect. Nevertheless, the extent of their therapeutic benefits in certain cases remains a topic of debate.

Studies on primary or acquired resistance against the two central immune checkpoint blockade (ICB) pathways contributed to identifying compensatory mechanisms, in most cases arising from alternative IC modulators (Fig. 2). We will provide a brief overview of inhibitory and stimulatory immune checkpoints (ICs), with a primary emphasis on ICIs that have been approved for therapeutic use or are nearing approval. We suggest some reviews for a detailed description of inhibitory and stimulatory checkpoints [15,16].

2.1. Inhibitory checkpoints

VISTA (V-domain Ig suppressor of T-cell activation, also known as V-set immunoreceptor, VISR) is an instructive example of experimental validation of acquired resistance to first-generation ICIs. Prostate cancer (PC) is refractory to anti-PD-1/PD-L1 inhibitors and is characterized by low T-cell infiltration (see Section 3). The administration of the anti-CTLA-4 ipilimumab to PC patients promotes T-cell infiltration. The PC patients who participated in the clinical trials did not experience any advantages from taking ipilimumab as a standalone treatment or in combination with hormonal therapy. VISTA (in addition

to PD-L1) was upregulated on CD8 T-cells, CD4 T-cells, and CD68⁺ TAMs in tumor tissue [17].

LAG-3 (lymphocyte activation gene 3, also known as CD223) is a receptor expressed on the surface of effector T-cells after progressive exposure to (neo)antigens. Natural killer (NK)-activated B cells, regulatory T-cells (Tregs), and plasmacytoid DCs also express LAG-3. LAG-3 is transcriptionally upregulated downstream of TCR activation and cytokine production [18]. The primary ligand on APCs is the molecular histocompatibility complex class II (MHC-II) [19]. Additional ligands include galectin-3 (GAL-3), liver sinusoidal endothelial cell lectin (LSECtin), fibrinogen-like protein 1 (FGL1), and α -synuclein. When oligomeric LAG-3 binds to the MHC-II, its cytosolic domain can inhibit the TCR intracellular signaling pathway, thereby blocking the activation of transcription factors and cytokine production [18]. To prevent autoimmunity, the self-induced expression of LAG-3 is associated with a dysfunctional exhausted phenotype of T-cells [20]. However, LAG-3 upregulation can occur in parallel or following PD-1 upregulation in tumor-infiltrating lymphocytes, neutralizing the activity of anti-PD1/PD-L1 agents [21]. Relatlimab (BMS-986016) is a humanized immunoglobulin G4 (IgG4) anti-LAG3 monoclonal blocking antibody. In stage III/IV metastatic melanoma patients, a relatlimab-nivolumab combination improved OS and PFS. No additional adverse effects were observed compared to nivolumab alone [22]. Based on these results, the FDA (Food and Drug Administration) recently approved relatlimab in combination with nivolumab against pediatric and adult forms of advanced unresectable or metastatic melanoma. The combination of experimental LAG-3 inhibitors and anti-PD-1/PD-L1 agents is under investigation [23]. The monoclonal humanized IgG4 antibody ieramilimab (LAG-525) blocks the interactions between LAG-3 and the ligands MHC-II and FGL1. In a phase I/II study, an unselected patient population with various forms of cancer experienced only a modest improvement when ieramilimab was combined with spartalizumab (PDR001), a humanized IgG4 anti-PD-1 monoclonal antibody [24]. Favezelimab (MK-4280) is a LAG-3 antibody investigated in combination with the PD-1 neutralizing agent pembrolizumab in colorectal

cancer (CRC), showing promising increased antitumor activity in some patient subsets [25].

Similar to CD28, CD226 is a costimulatory receptor that is necessary for complete T-cell activation. Like CD28, CD226 activity is time-limited by TIGIT (T-cell immunoreceptor with Ig and ITIM domains, also known as Washington University cell adhesion molecule, WUCAM; V-set and immunoglobulin domain-containing protein 9, VSIG9; and V-set and transmembrane domain-containing protein 3, VSTM3). TIGIT competes with the costimulatory receptor CD226 to bind nectin/nectin-like (PVR) family receptors. These receptors include CD155 (PVR) or CD112 (PVRL2 or nectin-2), which can be expressed on both tumor cells and APCs, thereby inhibiting T-cell priming. TIGIT is broadly expressed in different immune cell types and plays differential immunosuppressive roles. Not only does it affect the priming and differentiation of T-cells, but it also obstructs the ability of T-cells and NK cells to destroy cancer cells. TIGIT also induces immunosuppressive DCs [26]. Currently, combination strategies of inhibitors of TIGIT and the PD-1/PD-L1 pathway are expected to provide a clinical benefit. The TIGIT inhibitor tiragolumab has been investigated in combination with the anti-PD-1 agent atezolizumab as a first-line treatment for unresectable, chemotherapy-naïve, PD-L1-positive NSCLC (phase II Cityscape study). The results indicate that patients who received the combination treatment had a longer survival period compared to those who only received atezolizumab [27].

Nectin/nectin-like family Ca^{2+} -independent immunoglobulins are ubiquitously expressed on the plasma membrane of immune cells. They are involved in multiple cellular processes, including cell-cell adhesion, cell polarization, and tissue reorganization. Several family members, such as TIGIT and CD226, possess immunomodulatory functions by acting as ligands for specific immune receptors (recently reviewed by Johnston and colleagues [28]). Other family members are aberrantly expressed in cancer and promote oncogenic signals. Elevated levels of CD155 were reported in many solid tumors, where its expression correlates with poor prognosis and cancer progression [29]. Furthermore,

CD155 overexpression increased cancer cell proliferation and metastasis spreading. Loss of CD112 and CD115 correlated with resistance to NK cell-mediated cytotoxicity in acute myeloid leukemia (AML) [30]. NK cell-mediated cytotoxicity was inhibited following the binding of CD112 to TIGIT. The oncogenic role of other nectin/nectin-like molecules has emerged in several tumors. Nectin-4 (PVRL4) is overexpressed in urothelial carcinoma [31]. The antibody-drug conjugate (ADC) enfortumab vedotin is a human anti-nectin-4 antibody linked to the cytotoxic microtubule-disrupting agent monomethyl auristatin E. It received accelerated approval from the FDA in 2021 to treat advanced/metastatic urothelial carcinoma [32]. Similarly, the combination of enfortumab vedotin with pembrolizumab was approved for advanced/metastatic urothelial carcinoma patients ineligible for cisplatin treatment [33].

The TIM-3 (T-cell immunoglobulin and mucin domain 3) receptor is expressed by different immune cells including T-cells, NK cells, and myeloid cells. It inhibits T-cell-mediated antitumor immunity through mechanisms that remain to be understood [34]. Interestingly, both the ligand and the cell context may be crucial factors in determining the outcome. In chronic infections and cancer, exhausted T-cells co-express TIM-3 and PD-1. The ligand galectin-9 (L GAL9) can bind to TIM-3 and induces programmed cell death of exhausted T-cells. In tumors, these exhausted T-cells persist. PD-1 can also bind galectin-9, forming PD-1/galectin-9/TIM-3 complexes. As a result, cell death is inhibited, and the exhausted PD-1^+ TIM-3^+ T-cell population survives [35]. Accordingly, galectin-9 may become a promising target to enhance anticancer immunity. TIM-3 senses phosphatidylserine (PS) and the danger signal high-mobility group Box 1 (HMGB1) released by dying cells [36]. Tumor-infiltrating activated PD-1^+ TIM-3^+ CD8 T-cells upregulate PS and interact with TIM-3 expressed on the surface of tumor-infiltrating myeloid APCs. As a result of this interaction, membrane fragments from the APCs are acquired by PD-1^+ TIM-3^+ CD8 T-cells, a phenomenon called trogocytosis [37]. These myeloid membrane fragments also contain MHC-II complexes. As a result, PD-1^+ TIM-3^+ CD8 T-cells are targeted by PD-1^+ TIM-3^- CD8 T-cells, causing

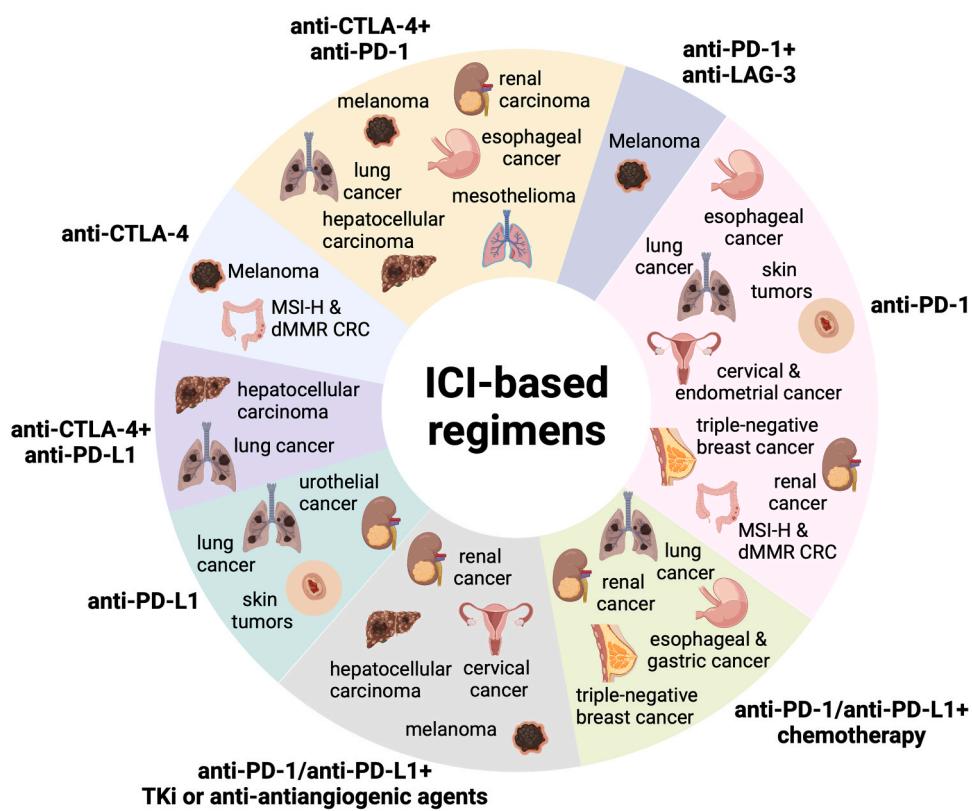


Fig. 1. ICI-based regimens by cancer type. Abbreviations: dMMR: mismatch repair deficient; MSI-H: microsatellite instability-high.

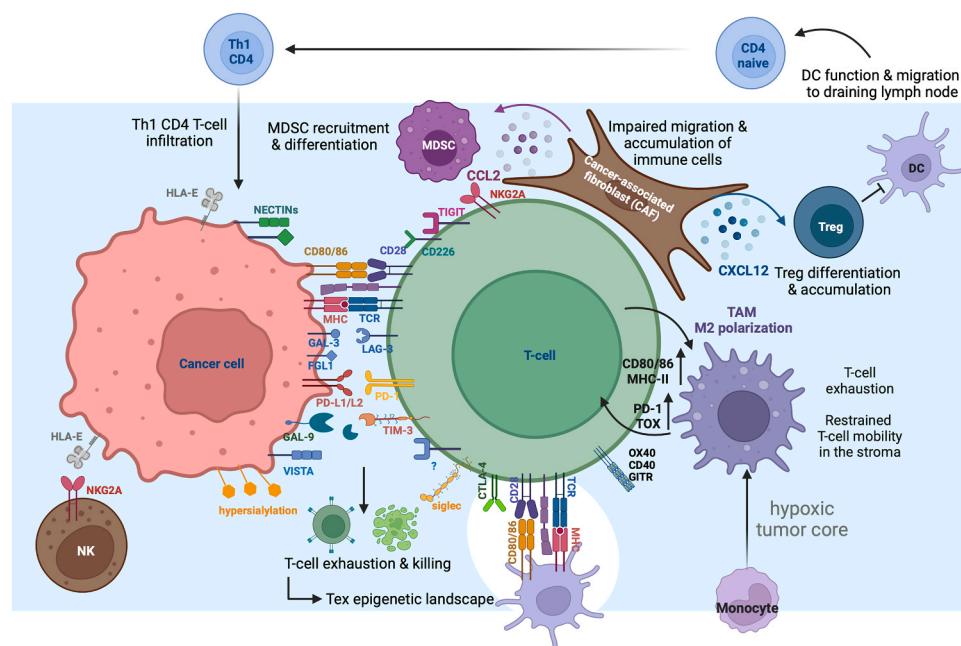


Fig. 2. Molecular and cellular mechanisms of ICI resistance. Mechanisms avoiding the prolonged activation of T-cells are exploited by cancer cells to elude the immune system and inhibit anti-cancer immunity. Molecular and cellular determinants in the TME promote immunosuppression. CTLA-4 neutralization promotes T-cell tumor infiltration; however, the interaction between L1 and PD-1 in the tumor microenvironment (TME) hinders the cytotoxic activity of infiltrated T-cells. The targeting of PD-1/PD-L1 interaction is frequently insufficient to restore anti-cancer immunity due to compensatory mechanisms activated downstream of TCR/CD28 activation (i.e., LAG-3 and TIM-3) and interacting with upregulated conjugate ligands overexpressed by cancer cells (i.e., GAL-3 and FGL1 for LAG-3; GAL-9 for TIM-3). This interaction promotes the accumulation of exhausted T-cells in the TME, associated with specific epigenetic profiles, which determine ICI resistance. Cancer cells may over-express additional surface molecules promoting ICI resistance, including nectins, protein hypersialylation, HLA-E, which may modulate other immune cell types (i.e., NK cells). Various cellular components contribute to ICI refractoriness in a direct and indirect manner. The figure depicts several mechanisms that have been discussed in the text. Tumor-recruited

monocytes are converted in TAMs by activated T-cells (upregulation of MHC-II, CD80/86), which in turn promote T-cell exhaustion by upregulating TOX, PD-1 and CD38 in the hypoxic tumor core. TAMs and stromal components (i.e., CAFs) prevent T-cell migration to the tumor center and restrain them into the stroma. CAFs alter the migration and the accumulation of various immunosuppressive immune cell types (i.e., MDSCs by e.g., CCL2 secretion; and Tregs by e.g., CXCL12 secretion). Tregs control a subtype of conventional DCs inducing the migration of Th1 CD4⁺ T-cells promoting anti-tumor immunity. These abnormal cellular characteristics can predict how a patient will respond to immunotherapy-based treatments. Their targeting restores the therapeutic efficacy of the investigated ICIs. Abbreviations: CAF: cancer-associated fibroblast; CDKN2A: cyclin-dependent kinase inhibitor 2A; CCL2: chemokine (C-C motif) ligand 2; DC: dendritic cells; FGL1: fibrinogen-like protein 1; GAL-3/9: galectin-3/9; HLA: human leukocyte antigen; GITR: glucocorticoid-induced TNFR-related; LAG-3: lymphocyte activation gene 3; MHC: molecular histocompatibility complex; MDSC: myeloid-derived suppressor cell; NK: natural killer; PD-1: programmed death 1; PD-L1/2: programmed death-ligand 1/2; TAM: tumor-associated macrophage; TCR: T-cell receptor; Tex: exhausted T-cell; Th: T helper; TIGIT: T-cell immunoreceptor with Ig and ITIM domains; TIM-3: T-cell immunoglobulin and mucin domain 3; TOX: thymocyte selection associated high mobility group box; VISTA: V-domain Ig suppressor of T-cell activation.

their death. Blocking antibodies against TIM-3 prevents trogocytosis and the subsequent elimination of PD-1⁺ TIM-3⁺ CD8⁺ T-cells. Despite their exhausted phenotype, these T-cells maintain higher cytolytic functions than PD-1⁺ TIM-3⁺ CD8⁺ T-cells [37].

Of note, TIM-3 suppresses antitumor immunity by directly modulating DCs. The loss of TIM-3 on DCs (but not on CD4⁺ and CD8⁺ T-cells) promoted the anticancer effect by enhancing antigen-specific immunity and inflammasome activation [38]. Furthermore, TIM-3 restricts HMGB1-dependent endocytosis of extracellular DNA and limits the activation of the cGAS-STING pathway in intratumoral conventional DCs (cDCs) [39]. This modulation may contribute to chemotherapy resistance (i.e., in mammary carcinoma). In summary, these discoveries suggest implementing strategies to block TIM-3 during treatment. Clinical trials are currently investigating the effectiveness of various anti-TIM-3 antibodies alone or in combination with PD-1 neutralizing agents for the treatment of solid tumors. They include cobolimab (TSR-022) tested in combination with anti-PD-1 inhibitors (e.g., dostarlimab, TSR-042) on PD-L1-positive PD-1 NSCLC and liver cancer. Sabatolimab (MBG453) is combined with the anti-PD-1 spartalizumab. LY3321367 is combined with the anti-PD-L1 LY3300054, and BMS986258 is co-administered with nivolumab.

Sialic acid-binding immunoglobulin-like lectin (Siglec) receptors are expressed by various immune cells. They interact and bind to sialic acid-containing glycans (sialoglycans). The interaction may occur between ligands and receptors on different (in *trans*) or the same T-cells (in *cis*). Numerous receptors belong to this family (recently reviewed by Murugesan and colleagues [40] and Stanczak and Laubli [41]). Most Siglec

receptors display inhibitory functions, avoiding forms of immune autoreactivity against the self. The intracellular domain of inhibitory Sigecls contains a tyrosine-based inhibitory motif (ITIM) and ITIM-like motifs interacting with SHP1/SHP2 phosphatases. A few members, such as Siglec-1 and Siglec-4, exert stimulatory activity. In this instance, the interaction and binding of sialoglycans trigger the recruitment of DAP12 (alias TYROBP), which contains a tyrosine-based activation motif (ITAM).

The TME shows elevated glycosylation; cancer cells exhibit hypersialylation [42]. Increased sialylation characterizes many solid tumors (i.e., pancreatic ductal adenocarcinoma (PDAC), melanoma, CRC, HCC, NSCLC, breast, and cervical cancer [42]) and hematological malignancies (i.e., multiple myeloma [43]). Increased sialylation of specific receptors (i.e., CD43 in CML K562 cells [44]; CD24 in ovarian and breast cancer [45]) correlates with phenomena of immune evasion by inhibiting the functions of various immune cell types expressing Siglec receptors, including NK cells [46] and T-cells [47]. Furthermore, sialoglycans induce the tumor-associated macrophage phenotype [45, 48]. In addition, hyperglycosylation was shown to impair the efficacy of CAR T-cells targeting CD19 [49]. ADCs against Siglec receptors are being developed to disrupt the sialoglycan-Siglec interaction in the TME. Inotuzumab ozogamicin targeting Siglec-2 (CD22) showed efficacy in relapsed acute lymphoblastic leukemia [50]. The anti-CD33 (Siglec-3) gemtuzumab ozogamicin is currently approved for AML therapy. Genetic ablation or antibody blockade of Siglec-15 amplified the antigen-specific T-cell response in the TME of mouse cancer models [47]. A phase 1/2 trial initiated in 2021 is investigating the first-in-class

humanized IgG₄ monoclonal antibody NC318 against Siglec-15. NC318 is assessed alone or in combination with the anti-PD-1 pembrolizumab in Siglec-15-positive solid tumors (NCT04699123). Preclinical studies used Fc-engineered blocking antibodies against Siglec-7 and Siglec-9 that reduced the tumor burden in mouse models [51]. Blocking sialo-glycans is an alternative strategy currently being investigated. Genetic and therapeutic desialylation with an antibody-sialidase conjugate enhanced antitumor immunity by repolarizing TAMs. The loss of Siglec-E, the main receptor for sialylation, enhanced ICB efficacy [52]. Antibodies blocking the interaction between the disialoganglioside GD2 and Siglec-7 on macrophages synergized with anti-CD47 therapy in syngeneic and xenograft mouse neuroblastoma models [53]. Alternatively, the inhibition of glycosylation by deoxy-2-glucose or bacterial sialidases aims to enhance the therapeutic response to CAR T-cells [54] or approved therapeutic agents [55].

NK cells are involved in the first-line innate immune defense against infections and cancer cells. Their activation and cytotoxic activity are controlled by the sophisticated and balanced activity of inhibitory and activating surface receptors to avoid immunoreactivity [56,57]. NK cells express inhibitory receptors that recognize human leukocyte antigen (HLA)-class I (killer cell immunoglobulin-like receptors, KIRs) and HLA-E class Ib (the heterodimer CD94/NKG2A (NK Group 2 member A)) in addition to the inhibitory checkpoints PD-1, TIM-3, and CD96. Cognate ligands of these receptors are overexpressed in cancer cells, preventing NK activation. Cancer cells may circumvent NK activation by inducing the downregulation of activating NK receptors or modulating the expression of their cognate ligands in the TME.

Recently, the C-type lectin-like NKG2A receptor has emerged as a critical checkpoint for NK and T-cell activation, and its blockade could potentially be utilized for cancer therapy [58]. HLA-E, a nonclassical MHC class I molecule, inhibits NK cell functions upon binding to NKG2A. Many tumors overexpress HLA-E, inducing NK cell dysfunction. HLA-E was overexpressed in circulating tumor cells from PDAC patients who are generally refractory to immunotherapy [59]. Genetic ablation of HLA-E or NKG2A blockade with the humanized monoclonal antibody monalizumab limited liver metastasis formation, suggesting the potential of NKG2A blockade as neoadjuvant therapy in combination with standard treatments. Monalizumab was tested alone or combined with other antibodies (i.e., cetuximab [60], anti-PD1/PD-L1 [61]) or cancer vaccines [62] in various tumor models. Its administration restored NK and CD8⁺ T-cell functions [59]. In advanced bladder cancer, NKG2A expression was associated with improved survival upon anti-PD-L1 treatment. HLA-E-expressing bladder tumors, however, restrict NKG2A⁺ CD8⁺ T-cell function, which was restored upon NKG2A blockade with monalizumab [63]. The adoptive transfer of NGK2A-lacking NK cells refractory to the inhibitory effects of HLA-E overcame HLA-E tumor resistance in mouse models, thus representing a potential alternative approach to NGK2A blockade to circumvent the inhibitory role of the NGK2A/HLA-E axis [64].

2.2. Stimulatory checkpoints

OX40 (also known as TNFRSF4 or CD134) is a receptor belonging to the TNF family. It is expressed on T-cell subsets, including Tregs and NK cells. It binds to OX40L which is expressed on the surface of APCs, such as DCs. This interaction improves their maturation and overall function. OX40 expression promotes T-cell survival and proliferation. It elicits cytokine secretion and contributes to Treg depletion from the TME. OX40 emerged as a critical factor regulating the expansion of CD4⁺ T-cells and the subsequent establishment of memory T-cells upon activation. These properties of OX40 make it a promising candidate for the development of agonistic antibodies that can elicit antitumor immunity. Humanized antibodies are under investigation in phase I dose-escalation studies on adults with advanced solid tumors. Patients with advanced or metastatic refractory solid tumors were involved in a monotherapy trial, which utilized the OX40 agonist MOXR0916 (NCT02219724) [65].

Another recent phase I dose-escalation study of the OX40 agonist humanized monoclonal antibody MEDI0562 was conducted on adults with advanced solid tumors (NCT02318394) [66]. The favorable safety profile and the observed immune activation suggest future combinations with PD-1/PD-L1 antagonists, despite the low general objective response to agonist monotherapy.

Glucocorticoid-induced TNFR-related (GITR, also known as TNFS18 or CD257) is an OX-40-like protein. Its expression promotes Teff cell proliferation and cytokine production. Additionally, it impairs Treg tumor infiltration. This dual activity makes GITR a potential candidate for agonist antibodies. GITR agonists have shown efficacy as monotherapies and in combination with other ICIs [67–70]. In a dose-escalation study, the anti-GITR antibody TRX-518 was found to be safe for patients with advanced malignancies (NCT01239134) [71].

4-1BB (CD137 or TNFRS9) is another TNFR family member expressed on various immune cell types, including several T cell subsets, NK cells, Tregs, and monocytes. Its ligand, 4-1BBL, is expressed on the surface of DCs. 4-1BB promotes T-cell survival and anti-tumor immunity by triggering NF-κB-dependent mechanisms [72,73]. Combining anti-4-1BB agonists (urelumab and PF-582566) with anti-PD-1 antibodies induced tumor regression in different cancer models. Studies are currently in progress to confirm the safety profile of these agents.

CD40 is a TNFR family member expressed by B cells, DCs, and macrophages. Its ligand (CD40L) is expressed on the surface of CD4⁺ T-cells. CD40 promotes MHCII upregulation on APCs and cytokine secretion, leading to CD8⁺ T-cell priming. CD40 agonism promotes immune cell infiltration in different tumor models. CD40 agonism alone or combined with other agents promotes priming or reprogramming and infiltration of immune cells in the TME of solid tumors [74–77]. This finding shows that activating CD40 can change a non-responsive cold tumor into a responsive hot one, and it could be effective when used together with anti-PD-1/PD-L1 antibodies.

3. TME determinants of ICI resistance

3.1. PD-L1 as an ICI biomarker

The therapeutic use of anti-PD-1/PD-L1 antibodies is subordinated to testing PD-L1 positivity in cancer patients with an approved companion or complementary diagnostics. At present, the FDA has approved four distinct assays for using immunohistochemistry (IHC) to monitor PD-L1 levels in order to make therapy decisions: Dako 22C3 for pembrolizumab administration in several solid tumors; Ventana SP142 for administrating atezolizumab in patients with urothelial carcinoma, TNBC, and NSCLC; Dako 28-8 for ipilimumab and nivolumab combination in patients with NSCLC; and Ventana SP263 as a complementary diagnostic for nivolumab in patients with advanced NSCLC and durvalumab in patients with advanced urothelial carcinoma [78].

Through clinical trials involving patients receiving ICI treatment, researchers were able to determine the level of PD-L1 expression that could accurately predict the clinical benefits of anti-PD-1/PD-L1 treatments. The authors of the phase 1b KEYNOTE-028 multicohort study (patients with advanced solid tumors receiving pembrolizumab) used the 22C3 assay to define PD-L1 positivity when ≥ 1% of tumor, inflammatory, or stromal cells were stained. With this cutoff, 48% of advanced or metastatic endometrial cancer patients were classified as PD-L1 positive; among the positive patients further enrolled in the efficacy analysis, the ORR was 13% [79]. The ORR was 17% in the cohort of 24 PD-L1 patients with advanced cervical cancer [80], 33% in the cohort of advanced small cell lung cancer (SCLC) patients [81], and 17.4% in the cohort of advanced prostate carcinoma patients [82] included in this multicohort study. Based on the results from all the cohorts included in the KEYNOTE-028 open-label study, pembrolizumab was considered promising in treating PD-L1-expressing solid tumors. The KEYNOTE-001 study assessed the efficacy of the anti-PD-1 pembrolizumab and validated the PD-L1 expression level to indicate clinical

benefit in advanced NSCLC patients. Patients with a proportion score $\geq 50\%$ (percentage of neoplastic cells with membranous staining for PD-L1 using the 22C3 assay), which represents a quarter of patients with advanced or metastatic disease, were associated with a higher response rate and longer PFS and OS [83]. This study accelerated FDA approval of pembrolizumab for advanced NSCLC and the companion diagnostic 22C3 assay [84]. More recently, the phase II KEYNOTE-158 study confirmed the durable response of pembrolizumab in advanced cervical cancer. This has led to an accelerated FDA approval of this treatment in PD-L1-positive patients experiencing progression during or after chemotherapy. PD-L1 positivity was assessed by determining the percentage of PD-L1-positive cells (including tumor cells, lymphocytes, and macrophages) in relation to the total number of tumor cells. This was done through the 22C3 assay and PD-L1 positivity was defined as $\geq 1\%$ of this combined positive score [85]. Monitoring of PD-L1 positivity with SP142 and 22C3 IHC assays confirmed that PD-L1-positive tumors showed the most significant response to atezolizumab treatment, despite variations in the sensitivity levels of the assay [86]. However, other studies showed durable responses to pembrolizumab in PD-L1-negative melanoma [87]. NSCLC cancer patients were treated with atezolizumab regardless of their PD-L1 expression level [88]. Furthermore, the effectiveness of clinically-used IHC assays is restricted by tumor heterogeneity and assay sensitivity, as discussed in the review by Doroshow et al. [78]. As a result, these assays are only suitable for certain therapeutic situations. In one instance, research conducted to compare the PD-L1 positivity in TNBC for determining the eligibility of atezolizumab revealed a disparity in the scores obtained from the Ventana SP142 and Ventana SP263 assays [89]. Altogether, there is a considerable risk of selecting the wrong patients for treatment due to these discrepancies.

3.2. Tumor mutational burden, mismatch repair deficiency, and microsatellite instability

Tumors with a high mutational burden (TMB) are generally the most responsive to immunotherapy. Similarly, melanoma, lung, colorectal, bladder, and kidney cancer exhibit a high frequency of somatic mutations [90]. Responsiveness is directly linked to the intrinsic ability of these tumors to generate multiple neoantigens, essential for T-cell priming and activation. A notable correlation exists between the response rate to PD-1/PD-L1 inhibition and TMB [91]. It is interesting to note that certain previously identified outliers have since been explained due to advancements in technology. These have allowed the detection of new types of mutations, such as insertion-and-deletion and fusion proteins, which serve as additional sources of neoantigens [92]. In addition, cancer patients with mismatch repair-deficient (dMMR) solid tumors are also favorable candidates for immunotherapy. A recent study explored the effectiveness of pembrolizumab, an anti-PD-1 medication, on patients with 12 different types of solid tumors. The study found that 53% of patients experienced objective radiographic responses, with 21% achieving a complete response. Deep sequencing of T-cell receptor CDR3 regions (TCR-seq) was used to evaluate T-cell clonal representation in tumors and blood from responders. After administering pembrolizumab, tumor neoantigen-specific T-cell clones were rapidly expanded in the patient's peripheral blood [93]. Similarly, cancer with microsatellite instability (MSI) can create more neoantigens. Anti-PD-1 strategies are approved against adult, and pediatric MSI-high (MSI-H) advanced solid tumors. Phase III studies have shown that MSI-H patients respond much better to ICIs than chemotherapy, whether they receive them as first-line treatments or as previously treated patients [94,95].

Despite the association with improved response rates, not all tumors presenting these alterations respond to current immunotherapies. Consequently, TMB, dMMR, and MSI-H do not represent specific predictive markers of response to ICIs. The discovery of multiple JCB pathways is just the tip of the iceberg when it comes to understanding the complex immune cancer landscape. The cellular heterogeneity of the TME plays a crucial role in determining the efficacy of these pathways.

3.3. Diversified TME immune profiles as determinants of ICI response

The histological observation of tumor tissues allows to define different TME immune profiles. The cellular composition of the TME indicates the progression of the cancer-immunity cycle and the diagnosis of the response to a specific class of ICIs. The complete absence of cytotoxic CD8-positive T-cell infiltration defines an immune desert TME. This histological pattern reflects early alterations in the anticancer immune response. Specifically, it suggests that T-cells are not being properly primed to recognize and attack tumor neoantigens. Tumors exhibiting this phenotype are generally refractory to PD-1/PD-L1 inhibitors, with the PD-1/PD-L1 axis being a late-occurring checkpoint in the cancer-immunity cycle.

The spatial distribution of infiltrated T-cells and the abundance of stromal cells in the TME further distinguish an immune-excluded TME from an inflamed TME. The excluded TME is enriched in stromal cells. PD-1/PD-L1-resistant forms of pancreatic and bladder cancer are paradigmatic examples. Stromal cells caused resistance to the PD-L1 inhibitors nivolumab and atezolizumab in large cohorts of urothelial cancer (UC) patients. Earlier research using real-time fibronectin and CD3 staining on viable slices of human lung tumors allowed to track the movement of individual T-cells within the tumor microenvironment (TME). This study established a relationship between the density and orientation of the stromal extracellular matrix (ECM) and T-cell migration [96]. A dense ECM may therefore contribute to the lack of response to ICIs, as it can limit T-cells from interacting with cancer cells by trapping them in stromal regions. Transforming growth factor β (TGF β) can be released from cancer cells or other cellular components to activate fibroblasts to produce ECM. Increasing layers of ECM drive T-cell entrapment in the stromal region. The TGF β -related gene signature was associated with a lower response and inferior OS to the PD-L1 inhibitor atezolizumab [97]. In line with these findings, an ECM transcriptional signature was upregulated and associated with TGF β signaling and resistance to ICIs across various tumor types [98]. Other cellular components of the TME may also promote T-cell entrapment. They include cancer-associated fibroblasts (CAFs) and subsets of myeloid cells (see Section 4; Fig. 2).

An inflamed TME is characterized by low stromal cell abundance and infiltrating T-cells. This tumor phenotype is well adapted for the therapeutic use of ICIs. The TMB further subclassifies this TME. A study analyzing the association between the PD-L1 expression level and the TMB was analyzed across a large panel of ICI-responsive cancer patients. Four recurrent immunologic TME patterns could be established based on the TMB and PD-L1 status, which improves the prediction of the patient response to anti-PD-1/PD-L1 inhibitors [99]: 1) hypermutated and inflamed, displaying high TMB (≥ 10 mutations/megabase (MB)) and high PD-L1 expression (such as melanoma and smoking-induced lung cancer); 2) inflamed with a consistent presence of tumor infiltration lymphocytes (TILs), with low TMB (< 10 mutations/MB, likely due to undetected insertion-and-deletion alterations or fusion proteins) and high PD-L1 levels (i.e., renal cancer), typically responsive to anti-PD-1/PD-L1 agents; 3) hypermutated, with high TMB but no PD-L1 expression, which mirrors the absence of T-cells in the TME and may benefit of ICIs to attract TILs; and 4) not hypermutated and not inflamed, presenting low TMB and no PD-L1 expression, which include immune-resistant malignancies such as pancreatic and prostate cancer.

These studies emphasize that the cellular heterogeneity of the TME plays a crucial role in determining a patient's response to immunotherapy. This underscores the importance of considering the TME itself as a biomarker. Based on published literature, an integrated transcriptomic and genomic analysis of the TME led to the elaboration of a gene expression signature with 29 genes discriminating four types of TMEs. The authors subdivided malignant and nonmalignant cellular components (immune-enriched, fibrotic; immune-enriched, nonfibrotic; fibrotic; and depleted), which are conserved across different tumor types [100]. Notably, the four identified TME subtypes predict ICI response.

For example, the percentage of responders to anti-CTLA-4 ipilimumab was higher in melanoma patients exhibiting an immune-enriched, nonfibrotic TME phenotype than those with the fibrotic phenotype (82% vs. 10%). Similar results were observed in patient cohorts affected by bladder, lung, and gastric cancer treated with anti-PD-1/PD-L1 regimens, confirming the fibrotic phenotype associated with inferior response and survival.

3.4. Novel TME categorization by immune archetypes

Recent findings refined the classification of TMEs. Cancers do not engage in infinite immunological patterns. Instead, they fall into a limited number of tumor immune archetypes, corresponding to conserved collections of immune cells involving both myeloid and T-cell subsets. These cells exhibit specific gene expression patterns, work together in a coordinated manner, and interact with other TME cell components, including cancer and stromal cells [101]. Twelve archetypes have been recently defined based on three hierarchical analyses of the frequency of 10 different cell types: first, T-cells, myeloid cells, and stromal cells; then, regulatory T-cells (Tregs), CD8 T-cells, and CD4 T-cells; and eventually, type 1 and 2 cDCs (cDC1 and cDC2), monocytes, and macrophages [102]. Based on the analysis of fresh biopsies taken from 304 patients with 12 different types of solid tumors, it has been found that these archetypes are independent of the tissue they originate from. Instead, they are associated with tumor proliferation, OS, and enriched gene signatures for inflammatory (i.e., chemokines), functional (i.e., senescence, damage, fibrosis), and additional cell type abundance (i.e., NK cells). Identifying the prevailing immune archetypes in cancer can define novel cancer targets and the development of personalized treatments for patients. For example, the profiling of biopsies from melanoma patients before anti-PD-1 treatment showed that responders tended to fall into two predominant phenotypes. In one instance, tumor tissues exhibit high cDC1 and NK cells and CD8 TILs (Antiviral Class I). Otherwise, an increased presence of CD4 T-cells was observed together with high cDC2 and few Tregs (Antiviral Class II) [103]. Such phenotypes provide mechanistic insights into ICB responsiveness and identify vulnerabilities that may generate resistance. In Class I, NK cells stimulate CD103⁺ cDC1 cells in the TME (via the release of Fms-like tyrosine kinase ligand 3 (FLT3L), chemokine (C-C motif) ligand 5 (CCL5), and X-C motif chemokine ligand 1 (XCL1)). cDC1s migrate to the tumor-draining lymph node, where they prime CD8 T-cells. When activated T-cells migrate toward the tumor, they develop the ability to trigger tumor rejection upon interaction with cDC1 cells. This anticancer immune response is CD8-associated and relies on the functional ability of NK cells to harness CD8 T-cells. In Class II, CD11b⁺ cDC2s can migrate into the lymph node, activating CD4 T-cells (T helper1-like CD4⁺ T-cells) and promoting tumor rejection once infiltrating the TME. CD11b⁺ cDC2 cells thus play the same role as CD103⁺ cDC1 cells; however, they can be blocked by Tregs. Interestingly, cDC2⁺/CD4⁺ is a common profile in head and neck squamous cell carcinoma (HNSCC). Nevertheless, in this instance, an enrichment of Tregs in the TME is frequently observed [104], which may predict the requirement of anti-Treg strategies to overcome intrinsic ICB resistance.

4. Mechanisms contributing to T-cell dysfunction and ICI resistance

4.1. The stromal compartment

The stromal compartment contributes to tumor tissue heterogeneity and determines clinical outcomes. Immune-resistant PDAC contains 10–40% of cancer cells dispersed as islets in a stromal scaffold. At least four different cancer subtypes can be classified by single-cell analyses (proliferating Ki-67-positive, undergoing epithelial-mesenchymal transition (EMT), dual positive or negative to these two parameters), which gave rise to 8 different types of tumor glands [105]. The common

phenotype found in distant liver metastasis is the dual-positive phenotype that is linked with high stromal CAF content. Additionally, patients presenting double-positive phenotypes with high or medium stroma content surrounding the cells before treatment more frequently developed resistance against the standard of care FOLFIRINOX [105]. In line with these results, the enrichment of a TGFβ-driven high leucine-rich repeat containing 15 (LRRC15) CAF lineage predicted resistance to anti-PD-1 atezolizumab [106]. Several mechanisms contribute to the role of cancer-associated fibroblasts (CAFs) in promoting tumor growth (Fig. 2). CAFs release C-C motif chemokine ligand 2 (CCL2) to recruit and differentiate myeloid-derived suppressor cells (MDSCs) [35]. By activating cyclooxygenase-2 (COX-2)-dependent signaling, CAFs hinder the efficiency of antigen presentation by DCs [107]. Via the production of chemokine (C-X-C motif) ligand 12 (CXCL12), a specific CAF subset (CAF-S1) promotes Treg differentiation and accumulation [108]. All these mechanisms were linked to forms of resistance toward ICIs or antigen vaccination therapies. These findings indicate that CAFs stage cancer aggressiveness and differential sensitivity to chemotherapy and immunotherapy. However, identifying and targeting relevant CAF subsets is challenging. It was found that the removal of CAFs in mouse models of PDAC did not result in beneficial outcomes; instead, their depletion accelerated tumor progression and was associated with a lower survival [109,110]. An alternative to CAF depletion is to target CAF-mediated signals. The combination of TGFβ-targeting approaches with anti-PD-L1 antibodies in an immune-excluded mouse model of breast cancer triggered tumor regression. The response was associated with a higher penetration of T-cells into the center of the tumors [97]. The inhibition of chemokine (C-X-C motif) receptor 4 (CXCR4), the CXCL12 receptor, potentiated the effects of anti-PD-1 strategies in murine models and clinical trials [111–114]. The CXCR4/CXCL12 axis correlates with poor intratumoral T-cell infiltration. In PDAC and CRC, it has been shown that CAF-derived CXCL12 coats cancer cells. Its interaction with CXCR4 expressed by most immune cells impairs the migration and accumulation of immune cells within tumors [115]. CXCR4 expression could potentially serve as a predictive marker of therapy response in patients undergoing anti-PD-1/PD-L1 therapy. There are ongoing efforts to develop inhibitors of CXCR4.

Potential curative approaches based on CAF targeting will require a deeper classification of CAF heterogeneity as a first step to clarify their functions. CAFs have been divided into TGFβ-driven myofibroblastic myCAF, IL-1β-dependent inflammatory iCAF, and antigen-presenting apCAF; their relative abundance varies depending on the type of cancer [116]. Very recently, an additional single-cell RNA sequencing-based study suggested the existence of at least four main types of CAFs, two of which promote T-cell exclusion from the TME by the deposition of distinct ECM molecules [117]. Of note, these two subpopulations characterize different phases of tumor progression and give rise to differential ECM scaffolds. Whereas MYH11⁺ αSMA⁺ CAFs are predominant in early carcinogenesis, FAP⁺ αSMA⁺ CAFs are abundant in more advanced tumor stages. The observed spatiotemporal modulation could inspire novel methods to target specific subsets of CAFs in different ways.

4.2. The role of tumor-associated macrophages (TAMs)

TAMs promote T-cell dysfunction through different spatiotemporal mechanisms (Fig. 2). Similar to ECM fibers and fibroblasts, TAMs can also confine T-cell mobility into the stromal regions. The analysis of cell type distributions in lung squamous cell carcinoma tissue revealed that CD206⁺/CD163⁺ macrophages are enriched in the stroma compartment. The real-time 3D tracking of T-cell trajectories in tissue sections documented that T-cells immobilize in macrophage-enriched stromal areas [118]. Inhibition of the colony-stimulating factor 1 receptor (CSF-1R) leads to TAM depletion, promotes T-cell accumulation, and reinvigorates intratumoral T-cell motility. CSF-1R inhibition per se has no relevant effects but strongly potentiates tumor regression when

combined with anti-PD-1 antibodies. This sensitization was associated with T-cell tumor-stroma redistribution, accompanied by prolonged contact of T-cells with tumor cells [118].

Tumor-infiltrated T-cells become dysfunctional due to chronic exposure to neoantigens and inflammatory conditions in the TME. This status is frequently associated with the upregulation of T-cell exhaustion markers PD-1, CD38, and TOX, thymocyte selection-associated high mobility group box. Mechanistically, there is a mutual modulation between T-cells and TAMs. On the one hand, exhausted T-cells show a superior ability to attract monocytes compared to naïve T-cells or intermediate progenitors; once recruited, exhausted T-cells facilitate the differentiation of these monocytes toward antigen-presenting macrophages *in vitro/ex vivo* conditions. On the other hand, differentiated TAMs form synapses with T-cells; instead of activating and proliferating T-cells, this interaction acts as a barrier as the TAM subsets are ineffective in priming them. Instead, their prolonged interaction exhausts T-cells characterized by PD-1 upregulation [119]. In line with this mechanistic model, TAM depletion with anti-CSFR1 attenuates the expression of T-cell exhaustion markers PD-1, CD38, and TOX. There is an increase in the production of inflammatory cytokines TNF α or IFN γ , which are usually secreted by effector T-cells (Teff). Of note, this dual modulation is spatiotemporally regulated. We can distinguish an outer/intermediate and an inner tumor area. As the T-cells move into the inner part of the tumor, they progressively acquire an exhausted phenotype, exhibiting an increased glycolytic metabolism due to hypoxic conditions. As monocytes move inward, they undergo terminal differentiation and become macrophages. Their antigen presentation activity is downregulated during this process while T-cell exhaustion increases in parallel [119,120].

4.3. Atlas of T-cell heterogeneity and epigenetic features of exhausted T-cells

T-cells play a relevant role when deciphering the complexity of the TME and the determinants of the ICI response. Here, we discuss emerging aspects of CD8 T-cell biology mainly associated with the exhausted phenotype. Although the definition of exhausted CD8 T-cells (Tex) is far from being definitive, there is consensus that reduced functions characterize Tex compared to effector T-cells (Teff). Major changes include cytokine/chemokine production, reduced proliferation, and increased expression of PD-1, TOX, LAG-3, TIGIT, or TIM-3 [121]. The inhibition of inhibitory receptors reinvigorates T-cell functions and restores an effector-like phenotype [20,122,123].

Similar to other TME cellular components, Tex are organized into different subsets with distinct transcriptional programs and a unique epigenetic landscape. The use of mice infected with chronic lymphocytic choriomeningitis virus (LCMV) allowed investigators to define a four-cell-stage hierarchical development framework for Tex based on transcriptional and epigenetic analyses. These results were confirmed in murine and human tumors. These analyses identified quiescent and blood inaccessible (Tex^{prog1}) and cycling and blood circulating (and Tex^{prog2}) early progenitor Tex subpopulations. Tex^{prog2} gives rise to the third intermediate Tex subset with high T-bet expression (Tex^{int}) that has yet to reacquire Teff features. Eventually, Tex^{int} may terminally differentiate into a definitive dysfunctional state, driven by TOX expression and characterized by a permanent exit from cell cycling, loss of Bet1, and gain of EOMES expression (Tex^{term}) [124]. Of note, PD-L1 blockade amplified Tex^{prog2} and Tex^{int}, thus suggesting a cell redistribution favoring Tex^{prog2}/Tex^{int} vs. Tex^{term} subsets. This phenomenon supports the clonal replacement of tumor-specific T-cells detected with paired single-cell RNA and T-cell sequencing analyses applied to site-matched tumors from patients with basal or squamous cell carcinoma before and after anti-PD-1 therapy [125].

Tex subsets show a unique epigenetic landscape. The comparative analysis of the open chromatin landscape by assay for transposase-accessible chromatin sequencing (ATAC-seq) between naïve T-cells

(Tn), Teff, memory T-cells (Tmem), and Tex from murine models documented a distinct and divergent state for Tex, with approximately 6000 open chromatin region (OCR) changes unique to Tex. Treatment with anti-PD-L1 strategies can reinvigorate Tex cells but does not reprogram their epigenetic landscape [126]. CRISPR engineering of Tex to delete enhancers did not abolish the expression of PD-1. Instead, it restored the PD-1 expression levels to those observed in Teff, suggesting the presence of Tex-specific regulatory elements supporting PD-1 expression. These critical regions are indeed associated with distinct transcription factor patterns, e.g., Sox-3, T-bet (Tbx21), and retinoic acid receptor (RAR) [127]. These results suggest the editing of exhaustion-specific enhancers as a potential therapeutic approach. Such a strategy would require a functional map of Tex-specific enhancers. Accordingly, a transcriptomic and open chromatin landscape atlas of T-cells has been established. Using a cohort of 24 healthy donors, 14 T-cell subsets were identified and clustered by the global relationship between the chromatin landscape and gene expression. RNA-seq and ATAC-seq data comparisons discriminated Tex from the other T-cell subsets. The application of this framework to anti-PD-1 melanoma and basal cell carcinoma (BCC) datasets identified the predominant infiltrating T-cell subsets [128]. Furthermore, this approach is helping to uncover additional novel Tex subsets, anticipating a higher level of complexity and heterogeneity of the Tex clones [129,130].

By combining single-cell RNA-seq, TCR-seq analyses, and computational methods, a high-resolution pan-cancer tumor-infiltrating T-cell atlas was built for 316 cancer patients across 21 different cancer types. A systematic T-cell immune-typing approach has been established for both prognostic and diagnostic purposes [131]. The study identified multiple potentially tumor-reactive T-cell clones, highlighting similarities and divergences in the TME of different cancers. A more recent single-cell transcriptomic atlas of tumor-infiltrating cells across 16 cancer types defined up to 32 T-cell states in the TME by integrating in-house and publicly available datasets [132]. Besides further dissecting known T-cell subsets (e.g., Treg and T follicular helper, Tfh), the authors discovered a novel T-cell response stress state (T_{STR}), characterized by upregulated heat shock gene expression. Different spatial profiling approaches can be used to track this T_{STR} *in situ* which emerged as a distinct feature associated with immunotherapy resistance. Mechanisms triggering T_{STR} are still under investigation.

4.4. Genomic alterations contributing to immunosuppressive signals

Several genomic alterations sustain immunosuppressive TME signals. The absence of T-cell recognition by cancer cells is caused by the mechanisms that hinder or prevent the exposure of tumor antigens. Mutation or reduced expression of proteins of the antigen-presenting machinery or the MHC complex promote cancer cell immune evasion [133,134]. Specifically, the MHC machinery component beta-2-microglobulin (B2M) is essential for HLA class I folding and transport to the cell surface. Alterations leading to B2M loss-of-function, e.g., truncating mutations or homozygous genetic loss, impair (neo)antigen processing and presentation [134] and contribute to primary or acquired resistance to ICIs [135,136]. Notably, MHC class I and II expression predicts ICI susceptibility. The loss of the MHC I complex in melanoma was associated with the transcriptional repression of HLA-A, HLA-B, HLA-C, and B2M and predicted resistance to anti-CTLA-4 but not to anti-PD-1. MHC II complex expression correlates with interferon-gamma (IFN γ)-mediated gene signatures and the anti-PD-1 response in melanoma [137]. Truncating mutations in Janus kinase 1/2 (JAK1/2) determine a lack of response to IFN γ [136]. Consecutive B2M loss and impaired JAK1 signaling can lead to pan-T immune cell escape [138]. These findings further explain the improved clinical response rates observed with the anti-CTLA-4/anti-PD-1 combination and identify potential resistance mechanisms to this regimen.

Loss-of-function (LOF) mutations in the STK11/LKB1 (serine/threonine kinase 11/liver kinase B1), KEAP1 (kelch-like ECH-associated

protein 1), CDKN2A (cyclin-dependent kinase inhibitor 2A), SMARC4 (SWI/SNF Related, Matrix Associated, Actin Dependent Regulator of Chromatin, Subfamily A, Member 4), and PTEN (phosphatase and tensin homolog) genes are associated with cancer immune evasion and ICI resistance. Longitudinal and retrospective studies of cancer patients profiled by next-generation sequencing (NGS) document that these alterations correlate with reduced T and NK cell infiltration in the TME (cold tumors). In addition, modulation of PD-L1 expression levels is frequently observed. Mechanisms involving mitogen-activated protein kinase (MAPK) and phosphoinositide-3 kinase (PI3K) pathways contribute to the immunosuppressive effects.

STK11/LKB1 is a tumor suppressor gene encoding a serine-threonine kinase involved in cell growth, polarity, and metabolism by interacting with multiple downstream effectors including the adenosine monophosphate-activated protein kinase, AMPK. KEAP1 negatively regulates nuclear factor erythroid-2-related factor 2 (Nrf2), a redox-sensitive transcription factor activated in response to oxidative stress. STK11/LKB1 and KEAP1 LOF worsen prognosis and predict ICI resistance when cooccurring with KRAS mutations in lung adenocarcinoma (LUAD) patients [139]. The immunosuppressive effects of STK11/LKB1 deficiency include the downregulation of MHC genes [139], activation of AMPK-downstream mediators, such as mTOR [140], altered cytokine/chemokine balance [141], metabolic restriction of T-cells [142], and decreased PD-L1 expression and anti-PD-L1 response [139]. The negative impact of STK11/LKB1 LOF on the PD-1/PD-L1 blockade axis also occurred in PD-L1-positive lung cancer subtypes. These observations suggest that the immunosuppressive impact of STK11/LKB1 LOF is not solely dependent on low levels of PD-L1 expression to mediate ICI resistance [65]. KEAP1-mutated tumors show increased glutamine addiction and rely on glutaminolysis [143]. The cooccurrence of STK11B/LKB1 and KEAP1 mutations in LUAD patients promotes metabolic reprogramming toward glutamine dependency [144]. In addition, KEAP1/NRF2 upregulates PD-L1 after IFN γ stimulation [145]. NRF2 depletion prevented PD-L1 upregulation and promoted CD4/CD8 T and NK cell infiltration in melanoma; additionally, NRF2 inhibition potentiated the effects of anti-PD-1 treatment [146]. Of note, NRF2 is an important modulator of the innate immune response by directly or indirectly modulating innate immune mediators, the IFN γ , and NF- κ B pathways (recently reviewed by van der Host and colleagues [147]). The identified mechanisms pave the way for the clinical trial validation of specific therapeutic interventions. Currently, mTOR and glutaminase inhibitors are under therapeutic investigation to resume immunotherapy sensitivity. Interesting therapeutic results have also been observed after the systemic inhibition of AXL, a receptor tyrosine kinase regulating APC activation. The authors used a murine LUAD model that had a Stk11/Lkb1 mutation, Kras, and Tp53 loss. These alterations recapitulate the lack of TCF1 $^+$ PD1 $^+$ CD8 T-cells observed in human STK11/LKB1 mutant NSCLCs [148]. AXL inhibition increased type I IFN secretion from DCs with subsequent expansion of tumor-associated TCF1 $^+$ PD1 $^+$ CD8 T-cells. These modulations restored the therapeutic response to anti-PD-1 treatment in mutated mice. Furthermore, clinical trials involving patients with LUAD who received a combination of the AXL inhibitor bemcentinib and the anti-PD-1 pembrolizumab have shown an objective clinical response [148]. STK11/KEAP1-mutated tumors have a poor prognosis regardless of KRAS status. It is interesting to note that genes protecting against ferroptosis may be a potential weakness that could be used in therapy [149]. Despite these promising results, the pharmacological targeting of specific alterations still requires further investigation and validation. Recent research has shown that inhibitors of glutaminase can affect the dependency on glutamine of anti-PD-1-activated cytotoxic CD8 T-cells. These inhibitors impair clonal expansion and the immunotherapy response in STK11/LKB1- and KEAP1-mutated lung cancer [150].

Several studies have correlated CDKN2A LOF with ICI resistance in different solid tumors [151]. Recent NGS profiling of LUAD patients confirmed a significant correlation between CDKN2A LOF and poor

response to ICB in lung cancer. In addition, genetic deficiency of CDKN2A is associated with a twofold increased rate of disease progression and lower OS [152]. Retrospective studies of NSCLC patient cohorts have shown that CDKN2A gene alterations can negatively affect long-term outcomes when treated with a combination of ICIs and chemotherapy [153]. Mechanistically, it has been observed that active CDK4/CDK6, which are inhibited by functional CDKN2A, can cause resistance to anti-PD-1 treatment. In melanoma, this resistance is concomitant with T-cell exclusion and immune evasion. In line with these observations, CDK4/CDK6 inhibitors sensitize melanoma xenografts to immunotherapy [154]. Furthermore, CDKN2A-mutated tumors show increased CTLA-4 and PD-1 expression [155]. When both CDKN2A and JAK2 are lost, melanoma cells become resistant to IFN γ , which then impairs their response to ICB treatment using anti-CTLA-4 and anti-PD-1 antibodies [156].

PTEN plays a crucial role in regulating the innate immune response through various mechanisms. It dephosphorylates the interferon regulatory transcription factor 3 (IRF3) at Ser97 and subsequently promotes its activation and nuclear translocation [157]; furthermore, a variant of PTEN (PTEN-L) promotes the nuclear import of subunit p65 of NF- κ B [158]. Both of these events are involved in the transcription of type I IFN-response genes. Consequently, the loss of PTEN impairs the activation of type I IFN and NF- κ B pathways. In addition, tumors with PTEN loss increase PI3K signaling [159]. They are associated with increased indoleamine-2,3-dioxygenase 1 (IDO-1) [160] and PD-L1 expression and resistance to anti-PD-1/PD-L1 therapies [159]. The loss of PTEN is linked to changes in the cellular composition of the TME. Increased densities of TAMs [161], Tregs [160], and MDSCs [162] have been documented. Additionally, PTEN loss correlated with reduced CD8 $^+$ T-cell density and activation in the TME of melanoma, sarcoma, lung cancer, and breast cancer [163–165].

Mutated KRAS $G12D$ promotes mechanisms that contribute to tumor growth, including metabolic changes, excessive cell division, and tumor stroma formation. Immunosuppressive mechanisms involve MDSC differentiation by GM-CSF and TAM attraction to the TME by ICAMs. The stromal compartment is coopted to secrete additional immunosuppressive cytokines via an IL-6/Sonic Hedgehog-mediated pathway. Beyond RAS, TP53 and BRAF mutations also shape the tumor immune landscape. Loss of TP53 in the tumor stroma induces the conversion of M1 macrophages to the T-cell-immunosuppressive M2 status. BRAF $V600E$ -mutated cells express interleukin 1 α (IL-1 α) and interleukin 1 β (IL-1 β) that promote CAF differentiation. Mutated cells also secrete interleukin 10 (IL-10), VEGF, and interleukin 6 (IL-6), which promote tolerogenic dendritic cells [166].

4.5. Spatiotemporal T-cell distribution as a prognostic index

TILs are an essential and favorable predictor of the anti-PD-1/PD-L1 therapy response. T-cell entrapment and inhibition by TME components within specific areas of the tumor tissue indicate that intratumoral patterns of TIL distribution should also be considered. Recently, spatial computational analyses have been developed to meet this need and allow to establish a score. Several patient cohorts show that a better prognosis and longer overall survival are associated with higher densities of CD3 and CD8 T-cells in the center of CRC and PDAC tumors compared to the margins [167,168]. T-cell density-based analyses are currently integrated into patient stratification and personalized therapy decisions, for example, in CRC stage I-III [167,169]. Furthermore, there have been advancements in machine-learning TIL algorithms that are automated or optimized to correlate TIL with OS and recurrence-free survival (RFS), which aim to enhance the prognostication of melanoma patients [65,170].

Besides quantifying TILs, novel methods are emerging to evaluate the colocalization of T-cells and cancer cells, specifically through T-cell proximity scoring. To obtain a T-cell proximity score, authors combined CD3/CD8 immunohistochemistry positivity with a 20 μ m radius

distance cutoff between each cancer cell and immune cells in the surrounding area. This threshold determined the likelihood of effective interaction. A strong correlation between an elevated T-cell proximity score and favorable clinical outcomes could be documented in CRC patients. Importantly, this score was a robust and independent tumor-immune marker [171]. Similarly, the spatial distribution of cytotoxic T-cells near cancer cells is significantly associated with longer OS in PDAC [172].

The spatial sublocalization of TILs also provides important information about specific interactions within the TME. The combination of transparent tissue tomography (T3), 3D imaging cytometry, and machine learning allows optimization of a 3D view of the TME architecture. This approach allowed to colocalize CD3/CD8 T-cells and tumor microvasculature in epithelial growth factor receptor (EGFR)-positive parenchyma in HNC patients [173]. Imaging mass cytometry allowed the monitoring of stromal, immune, and cancer cells in lung tumor samples. The authors used 26 cell-type specific and immune markers including CD3, CD8, or CD8, FoxP3 for T-cell subsets; CD45RO for the myeloid compartment; CD68 for macrophages, CD206 and CD163 to monitor M2-macrophages; CD20 for B cells; TCF1 and CD103 for additional T-cell clustering; PD-1, PD-L1 granzyme B, and CD95. The resulting spatial variant immune infiltration score (SpatialVizScore) allows patient stratification based on multicellular immune profiling with improved information about the presence of immunoinhibitory vs. immunostimulatory proteins [174]. Similarly, multiplex high-dimensional tissue imaging that quantifies cell-to-cell interactions and spatial cellular organization has a prognostic potential in TNBC [175]. In addition, the spatial immune profiling of intratumoral and peritumoral tissues of renal cell carcinoma (RCC) patients showed that T-cell exhaustion and poor OS were associated with elevated levels of peritumoral CD45RO⁺ T-cells [176].

Overall, TIL quantity and quality scoring have the potential to become robust integrated predictive markers of response to immunotherapy [177,178]. Furthermore, the sophisticated imaging analysis required for this approach may help dissect mechanisms of cell-to-cell interaction within the TME, which is essential for a better understanding of anticancer immunity.

5. Natural or hemisynthetic modulators of ICIs and TME cellular components

Natural compounds possess anticancer potential and have been investigated in multiple preclinical settings alone or in combination with anticancer therapies. Many classes of natural compounds exhibit anti-inflammatory and antioxidant properties, thus predicting the modulation of immunomodulatory mediators as targets. In addition, a diet enriched in fibers and polyphenols exerts beneficial effects on the microbiota, emerging as an essential actor of anticancer immunity [179]. Recent comprehensive reviews described the direct and indirect immunomodulatory activities of natural compounds, subclassified into different chemical classes [180–182]. This review focuses on recent experimental evidence supporting the targeting of ICB and TME cellular heterogeneity by naturally occurring or hemi-synthetic modulators (Table 2 and Fig. 3). Our overview includes molecules recently identified as potential ICI candidates by *in silico* and *in vitro* approaches, which still require wet-lab validation. We also added molecules that were validated by using *in cellulo* and *in vivo* experimental models. This information is provided in the text and Table 2.

5.1. Predicting new ICIs by *in silico* and binding affinity approaches

In silico methodologies offer the advantage of screening thousands of compounds against one target of interest. After screening a library of over 50,000 marine natural compounds and creating a structure-based pharmacophore model using chemical properties and docking studies, a limited selection of 12 compounds was identified. One of these

compounds showed potential as a small molecular inhibitor of PD-L1 with favorable ADME (absorption, distribution, metabolism, and excretion) properties [183]. Future functional assays and dose-dependent studies will be required to validate these screening methods and progress toward the pre-clinical evaluation of the identified naturally occurring molecules as direct PD-L1/PD-1 inhibitors.

VISTA represents a promising therapeutic target to prevent cancer-mediated immunosuppression; however, its ligands remain unknown, challenging the development of effective targeting strategies. A collection of phytochemicals was screened for their ability to inhibit VISTA in silico. Paratocarpin K (1), 3-(1 H-indol-3-yl)- 2-(trimethylazaniumyl) propanoate (2), and 2-[(5-benzyl-4-ethyl-1,2,4-triazol-3-yl) sulfanylmethyl]- 5-methyl-1,3,4-oxadiazole (3) were found to establish two interactions with residues of the active site of VISTA, with (1) and (3) showing higher binding stability and encouraging further wet-lab validation [184].

Several studies are currently attempting to identify new classes of ICIs in addition to antibodies by predicting the binding affinities of specific classes of natural compounds based on their chemical scaffolds. These studies aim to offer improved and complementary properties to antibodies regarding half-life, cell penetration, and low production costs. The hydrophobic and flat nature of the binding interface between PD-L1 and PD-1 might generate false positive hits during the screening processes. Some compounds may have the ability to bind to PD-L1/PD-1 but may not be effective at inhibiting their interaction. The combination of a PD-1/PD-L1 blockade assay (ELISA) with a PD-1/PD-L1 binding assay (surface plasmon resonance, SPR) was used to confirm the PD-1/PD-L1 binding activity of a panel of natural compounds, including kaempferol [185], cosmoisin [186], tannic acid, pentagalloyl glucose, ellagic acid, resveratrol [187], urolithin A, and rifabutin [188]. This approach allowed for distinguishing genuine PD-1 and/or PD-L1 binders. (e.g., kaempferol, tannic acid, resveratrol, and cosmoisin). Furthermore, the authors identified binders without blockade capacity (pentagalloyl glucose and ellagic acid) [189]. Further validation by functional and cellulo/*in vivo* assays of the ICI candidates identified by these studies is warranted.

5.2. Potential ICIs with *in cellulo/in vivo* validated activities

A widely researched strategy involves disrupting the interaction between PD-L1 and PD-1 to restore the functions of T-cells. The hydrophobic and tunnel-shaped binding pocket of PD-L1 inspired several hydrophobic molecules capable of docking in the tunnel and interacting with critical residues (i.e., Tyr56, Ala121, Asp122, Ile54, Tyr123). The biphenyl-based molecules, originally designed from Bristol-Myers Squibb (BMS compounds), represent a common backbone to discover and develop more effective biphenyl-based PD-L1 inhibitors with different chemical scaffolds (recently reviewed by Sasmal and colleagues [190]). Homogeneous time-resolved fluorescence (HTRF) binding assays have shown that biphenyl-based compounds can suppress PD-L1 activity. These molecules can act through different mechanisms. They may promote PD-L1 dimerization, internalization, and degradation [191]. Additionally, the BMS-1166 small molecule abrogates PD-L1 glycosylation and maturation, thus inhibiting its export from the ER to the Golgi apparatus and subsequently to the plasma membrane [192].

The design, synthesis, and evaluation of o-(biphenyl-3-ylmethoxy) nitrophenyl derivatives showed significant inhibitory activity against PD-1/PD-L1 *in vitro* (IC_{50} values between 2.7 and 87.4 nM), accompanied by *in cellulo* and *in vivo* anticancer validation using immunohistochemistry and flow cytometry assays [193,194]. Through molecular docking studies, it was discovered that the B2 compounds have the greatest effectiveness in localizing into the hydrophobic cavity formed by the PD-L1 dimer. Furthermore, these compounds form specific hydrogen bonding networks. These results may explain the higher inhibitory activity compared with the reference compound BMS-202. B2

Table 2
Natural and hemisynthetic compounds with immunomodulatory activities.

Compound	PubChem ID	Chemical structure	Target	Methodology	Experimental model	Mechanism of action	Doses	Refs
51320	NA		PD-L1	Structure-based pharmacophore model, virtual screening, molecular docking, ADME, molecular dynamics simulation	In silico	Predicted interaction with Ala121, Asp122, Ile54, Tyr123 of PD-L1	NA	[183]
2-[(5-Benzyl-4-ethyl-1,2,4-triazol-3-yl)sulfanyl]methyl]-5-methyl-1,3,4-oxadiazole	6494266		VISTA	Virtual screening, molecular docking, molecular dynamics simulation, free energy estimation, ADME	In silico	Interaction with Arg54 and Arg127 in the active site of VISTA	NA	[184]
3-(1 H-indol-3-yl)-2-(trimethylazaniumyl)propanoate	3861164		VISTA	Virtual screening, molecular docking, molecular dynamics simulation, free energy estimation, ADME	In silico	Interaction with Arg54 and Arg127 in the active site of VISTA	NA	[184]
Paratocarpin K	14187087		VISTA	Virtual screening, molecular docking, molecular dynamics simulation, free energy estimation, ADME	In silico	Interaction with Arg54 and Arg127 in the active site of VISTA	NA	[184]
Cosmosiin	5280704		PD-L1	Pair ELISA assay SPR binding assay Molecular docking and dynamics simulation	Molecular interaction studies <i>In vitro</i> assays, no cellular or animal models	PD-L1 binding with molecular displacement in the dimer interface (hydrogen bond interactions with polar residues, e.g., Tyr56)	10–100 μM (Elisa) 1.56–25 μM (SPR); binding affinity to PD-L1: K_D 3.32 × 10 ⁻⁶ M	[189]
Tannic acid	16129778		PD-1 PD-L1	Pair ELISA assay SPR binding assay	Molecular interaction studies <i>In vitro</i> assays, no cellular or animal models	Non-selective binding to PD-1/PD-L1 with higher affinity for PD-1, resulting in PD-1/PD-L1 interaction block	10–100 μM (Elisa) 0.06–0.5 μM (SPR); binding affinity: PD-1: K_D 1.46 × 10 ⁻⁶ M; PD-L1: K_D 1.21 × 10 ⁻⁶ M	[189]
Kaempferol	5280863		PD-1 PD-L1	Pair ELISA assay SPR binding assay	Molecular interaction studies <i>In vitro</i> assays, no cellular or animal models	Non-selective binding to PD-1/PD-L1 with higher affinity to PD-1	10–100 μM (Elisa) 1.56–25 μM (SPR) Binding affinity to PD-1: K_D 3.04 × 10 ⁻⁷ M; PD-L1: K_D = 3.3 × 10 ⁻⁵ M	[189]
Monoterpenoid indole-alkaloid-like compounds	NA		CTLA-4	Diversity-enhanced extracts, flow cytometric analysis of CTLA-4 expression	Human HTLV-I-infected CD4 ⁺ T-cell leukemia MT2 cells; THP-1	Inhibition of CTLA-4 expression	10–20 μM	[199] [200]
o-(Biphenyl-ylmethoxy)nitrophenyl derivatives (PD-1/PD-L1-IN-10)	156807419		PD-1	Time-resolved fluorescence binding assay, molecular docking, and dynamics simulation; <i>in vitro</i> and <i>in vivo</i> (xenografts) assays	Murine LLC cells and LLC allograft C57BL/6 mice	PD-L1 binding and block of PD-1/PD-L1 interaction by potential PD-L1 dimerization; IFNγ secretion; <i>in vivo</i> anti-cancer activity	IC ₅₀ values: 2.7 (B2) – 87.4 nM <i>In vivo</i> : 5 mg/kg i.g.	[193]

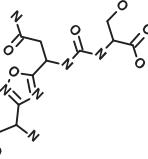
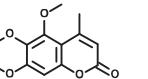
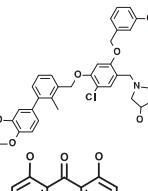
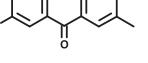
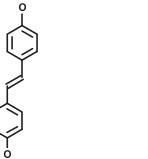
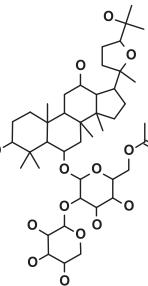
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Table 2 (continued)

Compound	PubChem ID	Chemical structure	Target	Methodology	Experimental model	Mechanism of action	Doses	Refs
Benzo[c][1,2,5] oxadiazole derivatives	NA		PD-L1	Time-resolved fluorescence binding assay, SPR assay, molecular docking, cell-based assays, co-culture assays and <i>in vivo</i> (xenografts) assays	PD-1 ⁺ /NFAT-luc/Jurkat; PD-L1 ⁺ : aAPC/ CHO-K1 cocultures; pharmacokinetic (Sprague-Dawley rats) syngeneic/PD-L1 humanized C57BL/6 mice (MC38; B16-F10); immune cell profiling	Inhibition of PD-1/PD-L1 interaction by potential PD-L1 dimerization;	EC50: ≈ 375 nM (co-culture assays) <i>In vivo</i> : range from 15 to 300 mg/kg acute toxicity: up to 2000 mg/kg (oral gavage) Binding affinity to PD-L1: K_D : 3.34 (SPR)	[194]
TPFS-201 (amide analogue of brefelamide)	155195802	L7: R=H L24 (Orally available): R=Et (HCl salt) 	PD-L1	Analysis of PD-L1 expression (mRNA expression, PCR; immunophenotyping, FACS; luminescence-reporter assays); co-culture assays (Jurkat T-cells/ A549)	Prostate PC-3; NSCLC A549 with luciferase reporter vectors (WT/mutated PD-L1 promoter) Monocytic AML THP-1 Murine B16F10 (melanoma) and RAW.264.7 (macrophage)	Transcriptional PD-L1 inhibition with potential involvement of the TEAD/TAZ binding site on the PD-L1 promoter (Hippo pathway)	Human cell models: 0.5–3 μM Murine cell models: 2–8 μM	[201]
TPFS-202 (amide analogue of brefelamide)	155195813		CTLA-4	Diversity-enhanced extracts, flow cytometric analysis of CTLA-4 expression	Human HTLV-I-infected CD4 ⁺ T-cell leukemia MT2 cells; THP-1	Inhibition of CTLA-4 expression	10–20 μM	[201]
Resveratrol	445154		PD-L1	Pair ELISA assay SPR binding assay	Molecular interaction studies <i>In vitro</i> assays, no cellular or animal models	Selective binding to PD-L1	10–100 μM (Elisa) 1.56–25 μM (SPR); Binding affinity to PD-L1: $K_D = 3.79 \times 10^{-5}$ M 25 mg/kg/day (orally) for four weeks	[189]
Resorcinal diphenyl ether-based molecules PROTAC-like	146673162		CAFs	<i>In vivo</i> assays (tumour mass weight, angiogenesis, cell proliferation/death, autophagy, infiltrating macrophages/CAFs)	C57BL/6 J bearing murine Lung Lewis carcinoma (LLC) cell line	Infiltrating immune cell decrease CAF maturation inhibition potentially mediated by autophagy	Cell Lines: 25 μM Mice: 40 mg/kg (oral gavage) 1–10 μM	[202] [205] [222]
			TAMs	Macrophage differentiation and polarization; cytokine assay (Elisa); <i>in vivo</i> (xenografts) combinations	MDA-MB-231, T-47D, and THP-1 cells; NOD/SCID xenograft mice	M1 macrophage polarization via IL6 production inhibition		

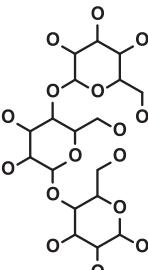
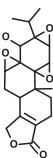
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Table 2 (continued)

Compound	PubChem ID	Chemical structure	Target	Methodology	Experimental model	Mechanism of action	Doses	Refs
CA-170	NA		PD-L1 VISTA	PD-L1/L2 functional assays Syngeneic mice models	CKO/CKO-K1/PD-L1, PBMCs Syngeneic Balb/c, C57B6 mice (CT26, MC38, B16F10) In clinical trial	Binding to PD-L1/VISTA active site with formation of a defective ternary complex CA-170/PD-L1/PD-L1.	Range of concentrations between 10 and 1000 nM depending on the assay	[198]
5,6,7-trimethoxy-4-methyl-2-H-chromen-2-one AK087	156807419		PD-L1	Engineered human melanoma cells Coculture assays (M238R:H358 cells:Jurkat T-cells Subcutaneous syngeneic melanoma tumor models (mass cytometry of murine tissues)	H293T, M238R H358, MDA-MB-231, YUMM1.7ER, NILER1-4, mSK-Mel254, KPC, CT26 in C57BL/6, BALB/c, NSG mice PC-9, Jurkat T, A375 (WT and PD-L1 stably expressing) cells; SW116-RFP; H1975; 293 cells	Downregulation of tumor cell-surface PD-L1/L2 via ubiquitin-directed lysosomal degradation and suppression of MAPKi resistance in vivo	Cell lines: 20–80 μM Mice: 10 mg/kg/day	[197]
BMS-1166	118434635		PD-L1	Coculture assays (PC9/PD-L1:Jurkat/PD-1) PD-L1 glycosylation analysis	PD-L1 glycosylation inhibition leading to PD-L1 accumulation into the ER and preventing PD-L1 cell surface exposure	Range of concentrations between 0.01 and 50 μM depending on the assay	[192]	
Emodin	3220		TAMs	Co-culture assays (breast EO771 or 4T1 cancer cells/mouse peritoneal macrophages); cell culture in macrophage-conditioned medium; tumor mammosphere (EO771, 4T1, MCF7, or MDA-MB-231); migration/invasion assays; in vivo studies	Breast EO771, 4T1, MCF7, and MDA-MB-231 cancer cells C57BL/6, BALB/c and NOD-SCID mice	Inhibition of macrophage migration, invasion and macrophage-induced EMT formation via inhibition of both canonical and non-canonical pathways of TGF-β1	Cell lines: 25 μM Mice: 40 mg/kg i.p. injected daily when tumor was palpable	[206]
4,4'-Dihydroxystilbene	5282363		CAFs	<i>In vivo</i> assays (tumour mass weight, angiogenesis, cell proliferation and death, autophagy, infiltrating macrophages and CAFs)	C57BL/6 J bearing murine Lung Lewis carcinoma (LLC) cell line	Infiltrating immune cell decrease CAF maturation inhibition potentially mediated by autophagy	25 mg/kg/day (oral) for four weeks	[202]
Ginsenosides	44593678		TAMs	Macrophage culture and polarization co-culture assays (NSCLC A549 or H1299 cells/macrophages); LLC-bearing allograft female C57BL/6 DNA transfection and luciferase reporter assays (pNF-AT-Luc and pNF-κB-Luc)	Murine RAW264.7 cells and human THP-1 differentiated in M1/M2 macrophages; NSCLC A549 or H1299 EL-4 T-cells	VEGF, MMP2, and MMP9 expression <i>in vivo</i> with conversion of M2 to M1 macrophages T-cell function modulation via NF-AT-mediated IL-2 production	Co-culture assays: 100 μM. Mice: 40 mg/kg i.p. daily for 21 days. 2.5–10 μM	[212] [223]

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Table 2 (continued)

Compound	PubChem ID	Chemical structure	Target	Methodology	Experimental model	Mechanism of action	Doses	Refs
β -glucans	46173706		M-MDSCs	<i>Ex vivo</i> mono/co-culture assays (M-MDSCs/CD4 ⁺ T-cells and CD8 ⁺ T-cells from wild-type C57BL/6 mice spleens); immunophenotyping	C57BL/6 J bearing LLC cells and MDSCs from the spleens of LLC tumor-bearing mice	Differentiation and maturation of M-MDSCs via NF- κ B/dectin-1 pathway	10 μ g/ml of particulate β -glucans	[207]
				C57BL/6 WT/dectin-1 KO mice bearing murine LLC or mammary cell carcinoma E0771	Trials on NSCLC patients	M-MDSC suppression activity PMN-MDSC apoptosis; M-MDSC differentiation to APCs via dectin-1 and ERK1/2 pathways	Mice: daily oral gavage of 100 μ l particulate β -glucan	[208]
				Co-culture assays (CD14 ⁺ cells from the PBMC of NSCLC patients treated with β -glucans/ CD3 ⁺ T-cells from healthy donors		Humans: 500 mg particulate β -glucan, daily, for 10/14 days		
Sap-derivatives extracellular vesicles from <i>Dendropanax morbifera</i>	NA	NA	CAFs	3D microfluidic cancer metastasis model	CAF differentiated from HUVEC cells in presence of melanoma-derived exosomes and sap-derived extracellular vesicles	CAF decrease associated with growth factors and ECM-related proteins integrins and collagens potentially promoting ECM stiffness	1–10 μ g/ml of EVs	[203]
Triptolide	107985		CAFs	Chip-Seq, superenhancer analysis, whole transcriptome RNA-Seq on cell models; in vivo studies	PDAC (MIA PaCa-2, PANC-1, P4057, PSN1, AsPC1, Capan-1, BxPC-3, PA-TU8902, PA-TU8988S) cells; CAFs from PDAC patient tumor transgenic mice; PDAC PDX	CAF epigenetic reprogramming by targeting of super-enhancers Sensitization to chemotherapy in combinational regimens	0.42 mg/kg daily for 7 days of the triptolide pro-drug minnelide (i.p.)	[204]

Abbreviations: ADME: absorption, distribution, metabolism, and excretion; APC: antigen-presenting cell; CAF: cancer-associated fibroblast; MDSC: myeloid-derived suppressor cell; SPR: surface plasmon receptor; TAM: tumor-associated macrophage; TME: tumor microenvironment.

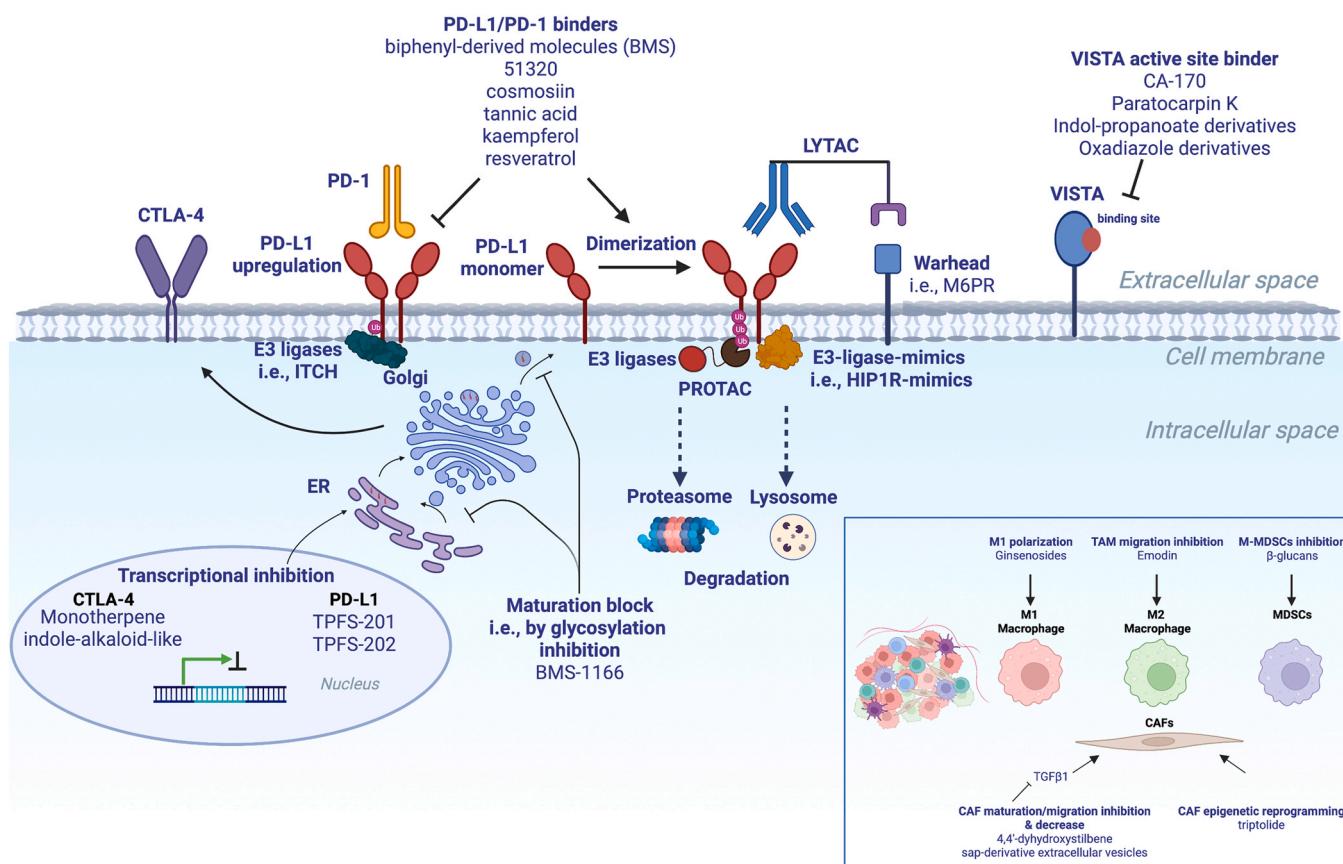


Fig. 3. Indirect and direct targeting of ICB by naturally occurring or synthetic modulators. This is a visualization of the molecular mechanisms that are hypothesized or proven to be involved in the modulatory effects of naturally occurring or derived compounds, as well as selected synthetic compounds, on ICIs, as discussed in Section 5. Abbreviations: BMS: biphenyl-derived molecules; CTLA-4: cytotoxic T-lymphocyte-associated protein 4; HIP1R: huntingtin interacting protein 1 related; LYTAC: lysosome-targeting chimera; M6PR: mannose 6-phosphate receptors; MDSC: myeloid-derived suppressor cell; PD-1: programmed death 1; PD-L1/2: programmed death-ligand 1/2; PROTAC: proteolysis-targeting chimera; TAM: tumor-associated macrophage; TGF β : tumor growth factor β ; VISTA: V-domain Ig suppressor of T-cell activation.

compounds also promoted IFN γ secretion and suppressed cancer cell growth and viability *in vivo* in a dose-dependent manner. Similarly, benzo-oxadiazole derivatives were characterized as PD-L1 inhibitors [194]. The compound L7 that was chosen had 20 times more potency in binding to PD-L1 compared to the reference compound BMS-1016 (1.8 nM IC₅₀ value in the HTRF assay); Consistently, it was found to be more effective than BMS-1016 in blocking the interaction between PD-L1 and PD-1 in cell-based co-culture assays, with an EC₅₀ value of 375 nM vs. 2075 nM. The induction of PD-L1 dimerization is hypothesized as the main mechanism of action. Of note, L24, the orally available ester prodrug of L7, showed significant anti-cancer effects in syngeneic and PD-L1 humanized tumor models. This effect was significant at low and medium dosages but not at high dosages (300 mg/kg or 50 mg/kg, respectively, in the MC38 and the PD-L1 humanized mouse models). Further studies will allow to understand this discrepancy between the dose and the expected results. The bioavailability of hydrophobic biphenyl compounds remains an Achille's heel for the translation of these compounds to therapy. Based on their overall safety profile and improved bioavailability, further testing of these novel compounds in preclinical trials is encouraged.

The hydrophobic nature of molecules with PD-1/PD-L1 inhibitory activity leads to poor pharmacokinetics, low solubility, and off-side target effects. This characteristic limited the design of clinical trials and the rapid translation into clinical practice for several small molecules inhibiting PD-1/PD-L1. Alternative approaches to overcome these limitations are based on PD-L1 internalization and degradation via the proteasome/lysosome pathway. The proteolysis-targeting chimera

(PROTAC)-like molecules rely on the conjugation of a putative PD-L1 binder (the protein of interest) to an E3 ubiquitin ligase with a linker to promote the ubiquitination and degradation of the target [195]. The lysosome-targeting chimera (LYTAC)-like molecules conjugate the putative PD-L1 binder to a warhead recognizing another surface molecule (for example, the mannose 6-phosphate receptors, M6PR) to promote clathrin-dependent endocytosis and subsequent lysosome-dependent degradation [196]. Additional molecules promoting PD-L1 internalization/degradation are the PD-LYSO HIP1R (huntingtin interacting protein 1 related)-mimics [191] and the E3 ligase ITCH inhibitor AK087 [197]. These recent methods show potential for being translated into clinical use, but further studies are needed to validate their effectiveness in the near future.

The orally available small molecule CA-170 was created to target the PD-L1/PD-1 interface. This molecule can selectively inhibit both PD-L1 and VISTA. [198]. CA-170 binding does not prevent PD-L1/PD-1 interaction but leads to the formation of a defective ternary complex that blocks the PD-L1 pathway. The inhibitory effect is dose-dependent in PD-L1-overexpressing cells. The authors hypothesized that the minimum pharmacophore of CA-170 allows its interaction with other conserved sites on proteins belonging to the same immunoglobulin superfamily, like VISTA. CA-170 is currently being evaluated in clinical trials for its effectiveness in treating advanced solid tumors and lymphomas (NCT02812875).

As alternatives to ICB, some classes of compounds show potential as IC expression inhibitors (recently reviewed in [181]). The screening of a library of synthetic indole-alkaloid-type compounds produced by

diversity-enhanced extracts of Japanese cornelian cherry identified a pentacyclic compound as a CLTA-4 expression inhibitor [199]. The chemical modification of this compound generated several derivatives with increased inhibitory potency at the mRNA and protein levels, acting as dual CLTA-4 and PD-1 inhibitors [200]. These modulatory effects are not selective and have been tested using a limited range of concentrations (between 10 and 20 μ M), requiring further experimental validation to test the relevance of the dual inhibitory activity of CLTA-4/PD-L1 expression.

Analogs of brefelamide downregulate PD-L1 expression by modulating the human-specific transcriptional coactivator TAZ [201]. The lead compound TPFS-202 inhibits dose-dependently PD-L1 expression; the disruption of the putative TAZ/TEA domain (TEAD)-binding motif robustly but not fully abrogated this modulation, suggesting the involvement of additional mechanisms. This effect is species-specific as mice lack PD-L1 modulation by TAZ.

5.3. Natural derivatives with modulatory activities on TME cellular components

Several natural compounds destabilize the TME, limiting CAF infiltration and maturation. Resveratrol and its analog 4,4'-dihydroxystilbene counteracted the stromal component in xenografted mice bearing lung Lewis carcinoma (LLC) [202]. The extracellular vesicles derived from sap of *Dendropanax morbifera* affected CAFs. They modulated the expression level of ECM-related genes, including integrins and collagens, in a 3D microfluidic system mimicking the TME [203]. The natural compound triptolide acts as an anti-CAF agent by promoting epigenetic reprogramming in PDAC. The mechanism of action involved disrupts the function of superenhancer elements [204].

Several compounds are also able to revert TAM polarization and M2 differentiation. Breast cancer models showed a particular propensity for this type of modulation. Resveratrol inhibits IL-6 production and STAT3 activation while increasing the levels of M1 macrophage chemokine (C-X-C motif) ligand 10 (CXCL10). These modulations were accompanied by tumor regression *in vivo* [205]. Emodin reduced breast cancer metastasis by suppressing TAM-induced EMT by suppressing canonical and noncanonical TGF β 1 signaling pathways [206]. The reprogramming and differentiation of TAMs toward immunosuppressive phenotypes is a critical factor in lung cancer. The transcription factor c-Maf is a crucial modulator of immunosuppressive macrophage polarization. β -glucans show reprogramming and modulatory activities on different myeloid subsets, including macrophages, MDSCs, and dendritic cells [207–209]. The yeast-derived natural compound β -glucan downregulates c-Maf expression and macrophages, thereby increasing anticancer immunity in murine models [210]. Of note, a clinical trial on a cohort of NSCLC patients taking β -glucan daily for 10–14 days increased nonclassical monocytes ($CD14^{\text{dim}}CD16^+$), a finding compatible with an increased patrolling of nonclassical monocytes, which control tumor metastasis [210,211]. The glycosylated triterpene ginsenoside Rh2, a compound extracted from *Panax ginseng*, decreased the expression of factors associated with M2 TAM differentiation (VEGF and metalloproteinase-2 and –9 (MMP2 and MMP9)). The authors used different cytokine combinations to achieve M1/M2 polarization of murine and human macrophages. The results were further confirmed in NSCLC xenografts [212].

6. Conclusions and perspectives

Immunotherapy holds great potential as a curative therapy for cancer. However, several challenges remain in the search for a cure for cancer. The next decade will be essential to overcome limitations and offer new perspectives.

First, we need effective predictive biomarkers allowing patient stratification and therapy response. TMB, MSI, DNA damage, TIL infiltration, and the expression of specific checkpoints, i.e., PD-1, are not, per se, good indicators. The fast-growing knowledge about TME cellular

heterogeneity is expected to suggest innovative holistic biomarkers, which need to integrate consolidated features with emerging characteristics of tumor immunity. The stratification of the patient's immune landscape into archetypes or through TME and/or immune cell classification will allow the future personalization of immunotherapies. An atlas mapping the diversified T-cell profiles will identify the predominant infiltrating T-cell subsets and predict ICI response [132]. Scoring the spatiotemporal distribution of T-cells within the tumor tissue will inspire new therapeutic schemes. Approved agents might be administered in a specific sequential way, potentially resulting in more efficient TME targeting.

Second, we need to elucidate further the multiple checkpoints (redundant and not) co-opted by cancer cells to understand resistance better. Innovative combinatorial approaches are being tested to target corrupted modulators of the different steps of the cancer-immunity cycle. One strategy involves triggering the formation of neoantigens and an immune response through neoantigen vaccines or CAR-T methods. Then, administering ICIs to prevent T-cell exhaustion [213–215]. Another exciting approach aims to increase immunogenicity within the TME by combining immunogenic cell death inducers and ICIs to facilitate TIL recruitment [216].

Third, we expect to develop noninvasive, sensitive, and large-scale applicable diagnostic methods, further helping TME classification and patient stratification. Digital and machine learning methods will allow the tracking of spatiotemporal patterns within the TME. Liquid biopsies will enable the analysis of cell-free DNA in patients affected by glioblastoma [217,218], one of the most immune-refractory cancer types. The current studies testing similarities and differences in immune cells within the tumor vs. circulating in the peripheral blood might provide information about immune system activity and the predominant type of immune cell infiltration into the tumor. Furthermore, this approach may offer a kinetic analysis during therapy to timely monitor patient responses.

Fourth, we must find a way to extend immunotherapy to hematological malignancies. This review focuses on solid tumors, the most suitable candidates for ICIs. Leukemias are generally considered poor candidates as tumors with low TMB and scarce ability to generate neoantigens. Nevertheless, checkpoints such as TIM-3 and VISTA are also crucial for the prognosis [219,220]. The anti-CD47 magrolimab, approved against myelodysplastic syndrome (MDS) and AML, target don't eat me signals, promoting recognition and engulfment of cancer cells by macrophages [221]. This approach is being investigated with azacytidine and venetoclax, showing favorable response rates in TP53-mutated AML (NCT04435691, NCT04778397, and NCT05079230).

Excluding gentuzumab ozogamicin, modern anticancer immunotherapy started its journey just a decade ago. Along the way, we are learning the translational challenges of this new approach. The great wave of discoveries and technological advancements in recent years encourages us to look at destinations with promising perspectives.

CRediT authorship contribution statement

Claudia Cerella and Marc Diederich wrote and edited the manuscript. **Mario Dicato** edited the manuscript.

Declaration of Competing Interest

None.

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Appendix A. Supporting information

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