Genetic, transcriptional and post-translational regulation of the programmed death protein ligand 1 in cancer: biology and clinical correlations

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Abstract
The programmed death protein 1 (PD-1) and its ligand (PD-L1) represent a well-characterized immune checkpoint in cancer, effectively targeted by monoclonal antibodies that are approved for routine clinical use. The regulation of PD-L1 expression is complex, varies between different tumor types and occurs at the genetic, transcriptional and post-transcriptional levels. Copy number alterations of PD-L1 locus have been reported with varying frequency in several tumor types. At the transcriptional level, a number of transcriptional factors seem to regulate PD-L1 expression including HIF-1, STAT3, NF-κB, and AP-1. Activation of common oncogenic pathways such as JAK/STAT, RAS/ERK, or PI3K/AKT/MTOR, as well as treatment with cytotoxic agents have also been shown to affect tumoral PD-L1 expression. Correlative studies of clinical trials with PD-1/PD-L1 inhibitors have so far shown markedly discordant results regarding the value of PD-L1 expression as a marker of response to treatment. As the indications for immune checkpoint inhibition broaden, understanding the regulation of PD-L1 in cancer will be of utmost importance for defining its role as predictive marker but also for optimizing strategies for cancer immunotherapy. Here, we review the current knowledge of PD-L1 regulation, and its use as biomarker and as therapeutic target in cancer.

Introduction
Cancer development and progression raises a strong anti-tumor immune response through which the immune system can eliminate cancer cells. This immunosurveillance theory describes the complex interactions between immune and cancer cells, divided in three distinct but often overlapping stages: elimination, equilibrium, and evasion. Thus, tumors can suppress immunity and escape eradication; evading immune destruction has been characterized as a hallmark of cancer [1, 2].

Programmed death protein 1 (PD-1) and its ligand (PD-L1) have been recognized as inhibitory molecules that cause impaired immune response against cancer cells. Therapeutic antibodies targeting PD-1/PD-L1 have been introduced into clinical practice, leading to better patient outcomes [3]. Immune checkpoint regulation has been under intense investigation over the last decades, however, the underlying mechanisms regulating the PD1 and PD-L1 expression are not fully understood; several oncogenic signaling pathways, epigenetic modifications, and genetic variations have been suggested. The aim of this review is to summarize the current knowledge on PD-L1 regulation, and its emerging role as a target in cancer immunotherapy.

Immune surveillance: the role of PD-1/PD-L1 axis as immune checkpoint

PD-1 (CD279) is a transmembrane protein, member of the CD28 family. It is mainly expressed on activated T cells but it can also be detected in other cells such as B- and natural killer (NK) cells upon induction [4]. PD-1 has two ligands, PD-L1 (CD274, B7-H1) and PD-L2 (CD273, B7-DC),
| Tumor type(s) | No. of cases | Method(s) | % of gains (n) | % Amplifications (n) | Association with IHC (PD-L1 expression) | Comments | Ref |
|---------------|--------------|-----------|----------------|---------------------|----------------------------------------|----------|-----|
| Solid tumors  |              |           |                |                     |                                        |          |     |
| NSCLC         | 221          | FISH      | 5 (11/221)     | NR                  | PD-L1 protein overexpression in all cases with gains | Slight predisposition of CNAs in SCCs | [13] |
| SCLC          | 210          | qPCR, SNP arrays | NR            | 1.9 (4/210)         | High PD-L1 expression in the cases with focal and high-level amplification | Susceptibility of this tumor subset to immune checkpoint blockade | [14] |
| SCC of vulva and cervix | 71 | FISH, NGS  | 12.5 (cervical-NGS) | 23 (cervical-FISH) | Highest PD-L1 expression in co-amplified cases, whereas lowest PD-L1 expression in cases with disomy | Detection of cogain or coamplification in both PD-L1 and PD-L2 genes | [15] |
| TNBC          | 183          | FISH      | 8.7 (16/183)   | NR                  | High PD-L1 protein expression in patients with copy number gains | Prolonged disease-specific OS in patients with high PD-L1 basal-like tumors or with gene copy number gains | [16] |
| BC            | 1980         | aCGH      | 3.3 (65/1980)  | 0.25 (5/1980)       | High PD-L1 protein expression in the three examined cases with amplification | Classification as IntClust 10 subtype: ✓ Four out of five (80%) cases with amplification ✓ 7/85 (57%) of the tumors with copy number gain ✓ Basal subtype: 74% of the amplified cases and more gains than other subtypes ✓ Losses: 4% (134/3145) ✓ Correlation of gains with elevated PD-L1 mRNA | [27] |
| BC            | 3145         | aCGH      | 5 (163/3145)   | 1 (39/3145)         |                          | In TNBC patients with the PDJ amplicon: worse DFS and OS and correlation of amplicon with high mRNA expression of PD-L1 and JAK2 | [17] |
| TNBC          | 41           | qPCR      | NR             | 29 (12/41)          |                          | Mostly HPV-negative SCCs 16/80 (20%) cases with polysomy 49/80 (61%) cases with disomy | [18] |
| Glioblastomas | 44           | FC        | NR             | 4.5 (2/44)          |                          | In TNBC patients with the PDJ amplicon: worse DFS and OS and correlation of amplicon with high mRNA expression of PD-L1 and JAK2 | [17] |
| Colon carcinomas | 68       | aCGH      | NR             | 2.9 (2/68)          |                          | Mostly HPV-negative SCCs 16/80 (20%) cases with polysomy 49/80 (61%) cases with disomy | [18] |
| SCC of the oral cavity | 80 | FISH      | Restriction to tumor cells: Absence in the inflammatory cell component | 15 (12/80): high-level amplification 4 (3/80): low-level amplification | PD-L1 positivity in 73% of the amplified cases | Mostly HPV-negative SCCs 16/80 (20%) cases with polysomy 49/80 (61%) cases with disomy | [18] |
| Pulmonary SCCs and ADCs | 159 | FISH      | 13.7 (21/159): high gains (mean ≥4) | 8.8 (14/159) | PD-L1 positivity (≥1%) in: 86% (12/14) of amplified cases 29.6% (16/54) of cases with gains | Identification of 9 (5.7%) JAK2 amplified cases, 7 of which with PD-L1 expression ✓ 11/14 (9%) of amplified tumors: ADC ✓ 3/14 (6%): SCC | [19] |
| Hematological and lymphoid tumors | |           |                |                     |                                        |          |     |
| DLBCL         | 190          | RNA-seq, FISH, WGS | 12 | 3 | Correlation with elevated PD-L1 expression in cases with cytogenetic changes | Detection of translocations (4%) in PD-L1/PD-L2 locus. Higher frequency of CNAs in the non-GCB subtype | [20] |
| cHL           | 108          | FISH      | 56 (61/108)    | 36 (39/108)         |                          | Detection of translocations (4%) in PD-L1/PD-L2 locus. Higher frequency of CNAs in the non-GCB subtype | [21] |
| Tumor type(s) | No. of cases | Method(s) | % of gains (n) | % Amplifications (n) | Association with IHC (PD-L1 expression) | Comments | Ref |
|--------------|--------------|-----------|----------------|---------------------|----------------------------------------|----------|-----|
| HL           | 10           | FISH      | 60             | 40                  | Correlation of genetic alterations with PD-L1 expression (especially in disomic cases) | Correlation of gene amplification with reduced PFS. Higher amplification frequency in patients with advanced stage disease | [22] |
| NSHL         | 16           | qPCR      | NR             | 38 (6/16)           | Association with PD-L1 protein expression in NSHL amplified cases | Association of JAK2 amplification with elevated PD-L1 transcription | [23] |
| PMBCCL       | 7            |           |                |                     |                                        | Correlation of PMBCL cases with increased PD-L1 transcript | [24] |
| MCHL         | 41           |           |                | 63 (26/41)          |                                        |          |     |
| Primary B-cell NHL | 67           | Oligonucleotide capture sequencing | NR           | NR                  | Significant association between rearrangements and PDL protein expression | Detection of 36 novel rearrangements (17 inversions/deletions/duplications and 19 translocations) | [25] |
| PMBCCL       | 125          | FISH      | 26             | 29                  | NR                                     | Increased PD-L1 expression in copy number gain(+) cases | [26] |
| PCNSLs       | 50           | HD-SNP    | 67/63 (EBV+/EBV-35) | 67/63 (EBV+/EBV-35) | Increased PD-L1 expression in copy number gain(+) cases | Translocations in 6% of EBV- PCNSLs and 4% of PTLs | [27] |
| PTLs         | 43           |           | NR             |                     |                                        |          |     |

NSCLC non-small cell lung carcinoma, SCLC small-cell lung carcinoma, SCC squamous cell carcinoma, BC breast cancer, TNBC triple-negative breast carcinoma, PDA pancreatic ductal adenocarcinomas, PDJ amplicon the loci for PD-L1, PD-L2, and JAK2, DLBCL diffuse large B-cell lymphoma, cHL classical Hodgkin lymphoma, NSHL nodular sclerosing Hodgkin lymphoma, NHL non-Hodgkin lymphoma, PMBC primary mediastinal B-cell lymphomas, PCNSL primary central nervous system lymphomas, PTL primary testicular lymphomas, EBV Epstein–Barr virus, IHC immunohistochemistry, NR not reported, OS overall survival, PFS progression-free survival, DFS disease-free survival, non-GCB non-germinal center B-cell-like cell, FISH fluorescent in-situ hybridization, qPCR quantitative polymerase chain reaction, SNP single-nucleotide polymorphism, NGS next-generation sequencing, FC flow cytometry, aCGH oligonucleotide array-based comparative genomic hybridization, RNA-seq RNA-sequencing, WGS whole-genome sequencing, MCHL mixed cellularity Hodgkin lymphoma, HD-SNP high-density single-nucleotide polymorphism arrays, CN copy number, ADC adenocarcinomas.
which belong to the B7-CD28 protein family [5]. PD-L1 is expressed on tumor cells but it can also be present on the surface of other cell types including T cells, B cells, dendritic cells, macrophages, mesenchymal stem cells, epithelial, endothelial cells, and as recently shown, brown adipocytes [6]. PD-L2 is typically expressed in antigen-presenting cells (APCs). PD-L1 is expressed upon stimulation of cytokine interferon-γ (IFNγ), secreted by activated T cells [7, 8].

PD-L1 and PD-L2 are encoded by the CD274 and PDCD1LG2 genes, respectively, located on chromosome 9p.24.1, whereas PD-1 is encoded by the PDCD1 gene located on chromosome 2q37.3 [4]. PD-1/PD-L1 axis plays an important role in the regulation of T-cell immunity and has been also implicated in autoimmunity and infection [9]. The PD-1/PD-L1 interaction has been characterized as an “immune checkpoint” due to its impact on the orchestration of immune response against tumor antigens. Along with cytotoxic T-lymphocyte-associated protein 4 (CTLA-4, CD152), they represent immunological “brakes” that modulate T-cell activation leading to an impaired immunosurveillance.

T-cell activation involves a two signal-model; APCs require a first signal from T-cell receptor (TCR), which recognizes the antigen along with the major histocompatibility complex (MHC) presented on the surface of APC. The second signal includes the co-stimulatory interaction between CD28 on the surface of T cells and CD80 (B7.1) or CD86 (B7.2) on the surface of APC [10, 11].

The engagement of PD-1 with its ligands leads to the inhibition of T-cell activation and response, via mechanisms that include blocking of proliferation, induction of apoptosis, and regulatory T-cell differentiation and therefore immune inhibition [11]. Blocking the PD-1/PD-L1 axis with potent monoclonal antibodies may reverse the impaired anticancer immunity and thus represents an appealing target of cancer immunotherapy [12].

The genetic basis of PD-L1 expression in cancer

The genetic aberrations of the PD-L1/PD-L2 gene loci represent a key mechanism of PD-L1 expression both in solid and hematologic tumors. Studies of copy number alterations (CNAs) have been reported in several tumor types (Table 1). The highest frequencies of CNAs have been seen in squamous cell carcinomas of vulva and cervix and triple-negative breast cancer (TNBC), as well as in classical Hodgkin lymphoma (cHL) and primary mediastinal B-cell lymphoma (PMBCL). Contrary, low or absent CNAs have been reported in small and non-small cell lung cancer (NSCLC) and in diffuse large B-cell lymphomas (DLBCL). In general, copy number gains and especially amplifications are well correlated with the protein levels of PD-L1. Given the challenges in determining the protein levels of PD-L1 as detailed below, detection of CNAs is an attractive alternative for identifying patients who could benefit from treatment with checkpoint inhibitors. Table 1 summarizes the current literature of the genetic regulation of PD-L1 [13–28]. In addition to these individual studies, a large in silico analysis of CNAs in PD-L1 has been conducted using the Cancer Genome Atlas datasets (22 cancer types, 9771 tumors). Interestingly, deletions of 9p24.1 were more common than gains in this analysis and were found mostly in melanoma and NSCLC, with gains occurring frequently in ovarian, head and neck, bladder, and cervical carcinomas [29].

Furthermore, a novel genetic regulatory mechanism of PD-L1 gene expression involving the disruption of its 3’ untranslated region (3′-UTR) has been shown in multiple tumor types including T-cell leukemia/lymphoma, DLBCL, and gastric adenocarcinoma. Through interruption of PD-L1 3′-UTR by structural variation, a deviant increase in PD-L1 transcripts occurs leading to immune escape in murine EG7-OVA cancer cells, which in turn can be reversed by PD-L1/ PD-1 inhibition [30].

PD-L1 regulation via oncogenic signaling pathways

RAS/RAF/MEK/MAPK-ERK pathway

The mitogen-activated protein kinase (MAPK) pathway is crucial for various functions in normal cells, including growth and differentiation. Its role is also important in carcinogenesis because its activation leads to cancer development [31]. The ERK-MAPK pathway has been shown to regulate PD-L1 expression in different cancer types. Both pharmacologic inhibition of mitogen-activated protein kinase (MEK) and small interfering RNA (siRNA) knockdown of ERK1/2 resulted in decreased levels of PD-L1 in melanoma cells resistant to BRAF inhibition [32]. Interestingly, in TNBC cells, MEK inhibition resulted in upregulation of MHC II and PD-L1 expression both in vitro and in vivo, whereas combined MEK/PD-1 inhibition increased the effectiveness of antitumor immunity [33]. MAPK signaling pathway was also responsible for the ectopic expression of PD-L1 in v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (KRAS)-mutant NSCLC cell lines, as revealed by the decrease in PD-L1 levels after both MEK and extracellular signal-regulated MAP kinase (ERK) abrogation [34]. In another study, Toll-like receptor 4 activation resulted in upregulation of PD-L1 in bladder cancer cells. The use of both ERK and JNK inhibitors
abrogated PD-L1 expression, further supporting the contribution of MAPK signaling in PD-L1 regulation [35]. Moreover, the interaction of tyrosine kinase receptor c-Met with its ligand hepatocyte growth factor (HGF) induced Ras activation. Ablation of Ras effect led to downregulation of c-Met-mediated expression of PD-L1 in renal cancer cells [36].

KRAS activation may also induce PD-L1 expression, as it resulted in stabilization of PD-L1 mRNA transcript assessed through Adenylate-uridylate-rich elements identification in its 3'-UTR in lung cancer cell lines. Additionally, MEK and Phosphoinositide 3-kinase (PI3K) inhibition led to decreased PD-L1 levels and enhanced effectiveness of antitumor immunity in vivo [37].

**PI3K/PTEN/Akt/mTOR pathway**

The PI3K/Akt/mTOR signaling represents another pathway that affects immune surveillance through the regulation of PD-L1. Its activation by either oncogenic PIK3CA mutations (catalytic subunit alpha of PI3K) or by loss-of-function mutations of its negative regulator, phosphatase and tensin homolog (PTEN) modulates immune responses contributing to a survival benefit of cancer cells [38]. In human gliomas, loss of PTEN and activation of PI3K pathway enhanced PD-L1 expression [39]. In TNBC, knockdown of PTEN by short hairpin RNA resulted in elevated levels of both PD-L1 protein expression and mRNA transcripts, whereas inhibition of Akt and mechanistic target of rapamycin (mTOR) decreased PD-L1 expression [40]. In a murine model of lung SCC, concurrent inactivation of PTEN and Lbk1 resulted in increased levels of PD-L1 [41]. PI3K inhibition, resulted in PD-L1 downregulation in different cancer types including renal cell carcinoma through HGF/c-Met [36], KRAS- or EGFR-mutated NSCLC [42] and melanoma [32]. Conversely, LY294002 did not abrogate PD-L1 expression in bladder cancer cells [35]. Moreover, mTOR inhibition with rapamycin reduced levels of PD-L1, both in human cell lines and in murine models of NSCLC and combined treatment with rapamycin and anti-PD-1 antibody inhibited tumor growth in mice [42].

**Epidermal growth factor receptor (EGFR)**

EGFR is commonly mutated in NSCLC and has been associated with PD-L1 upregulation in these tumors [43]. PD-L1 was overexpressed in EGFR-mutant murine lung cancer, whereas treatment with an anti-PD-1 antibody restrained tumor growth. Forced ectopic expression of mutant EGFR on bronchial epithelial cells resulted in PD-L1 upregulation, whereas this effect was abolished upon treatment with EGFR tyrosine kinase inhibitors [44, 45]. The EGFR-mediated regulation of PD-L1 in EGFR mutant NSCLC was dependent on MAPK pathway activation. Inhibition of ERK1/2/c-Jun resulted in reduced PD-L1 levels in PD-L1 overexpressing lung cancer cells [46]. In another more recent study, EGFR was shown to regulate the
expression of PD-L1 through the activation of Interleukin-6 (IL-6)/Janus Kinase (JAK)/signal transducer and activator of transcription 3 (STAT3) pathway in EGFR-driven NSCLC [47].

**EML4-ALK**

PD-L1 upregulation has been observed in patients with NSCLC harboring the anaplastic lymphoma kinase (ALK) and echinoderm microtubule-associated protein like-4 (EML4) chromosomal rearrangement. Activation of **EML4-ALK** was associated with increased PD-L1 expression; furthermore, treatment with either the ALK inhibitor alectinib or ALK gene silencing with siRNA abrogated this effect. Notably, PD-L1 upregulation was dependent on MAPK/ERK/MEK and PI3K/Akt signaling pathways [48]. In another study using pulmonary adenocarcinoma cell lines, EML4-ALK transcriptionally regulated PD-L1 via STAT3 and HIF-1a [49]. These studies indicate the different ways in which this chimeric protein can regulate the expression of PD-L1 and thus reveal the complexity of signaling pathways and their downstream targets. The various crosstalks in the cellular level can influence anticancer immunity and at the same time offer possible appealing therapeutic targets.

**Transcriptional control of PD-L1**

The transcriptional regulation of PD-L1 is summarized in Fig. 1.

**The JAK/STAT pathway**

STAT3 plays a key role in promoting cancer cell survival and proliferation, as well as creating immunosuppressive and thus pro-carcinogenic conditions in the tumor microenvironment (TME) [50]. Furthermore, STAT3 is involved in PD-L1 regulation in various cancer types. In nucleophosmin-anaplastic large-cell lymphoma kinase (NPM-ALK) positive anaplastic large-cell lymphoma (ALCL), STAT3 is activated by NPM-ALK oncprotein through JAK3 activation, binds physically to the PD-L1 gene promoter, and induces its expression in vitro and in vivo [51]. This STAT3-mediated transcriptional regulation of PD-L1 has been recently shown in another T-cell lymphoma, namely the ALK-negative ALCL. STAT3 gene silencing led to decreased PD-L1 levels in ALK-ALCL [52] and also in KRAS-mutant NSCLC cell lines [34]. By contrast, chromatin immunoprecipitation analysis did not show active binding of STAT3 directly on the promoter of PD-L1 in melanoma cells, despite the presence of putative binding sites of STAT3 on the promoter identified in silico. Abrogation of STAT3 resulted in enhancement of PD-L1 construct activity mediated by IFNg [53]. PD-L1 was also induced by latent membrane protein-1 in Epstein–Barr virus (EBV)-associated nasopharyngeal carcinomas (NPC) through JAK3/STAT3 activation [54].

Another STAT family member, STAT1, is considered to be a tumor suppressor that reduces proliferation, induces apoptosis, and enhances cancer immunosurveillance [55]. Accumulating evidence indicates the emerging role of STAT1 in tumor growth, immune suppression, and therapeutic resistance [56]. Upon stimulation with IFNg, STAT1 activation resulted in PD-L1 upregulation and in reduction of NK-cell activity against tumor cells in multiple myeloma, acute myeloid leukemia (AML), and acute lymphoblastic leukemia (ALL) [57]. Similarly, STAT1 inhibition led to decreased PD-L1 levels in myeloma cells and thus suppressed the antitumor function of cytotoxic T cells [58]. PD-L1 upregulation was JAK2/STAT1-dependent in head and neck cancer with wild-type EGFR, whereas JAK2 inhibition resulted in both basal and EGFR-mediated down-regulation of PD-L1. Moreover, knockdown of STAT1 gene abolished both IFNg- and EGFR-mediated upregulation of PD-L1. Of note, EGFR activation promotes phosphorylation of STAT1, which in turn binds to the promoter of PD-L1 and controls its expression [59]. Although putative binding sites for STAT1 on PD-L1 promoter have been postulated, active binding of STAT1 on PD-L1 gene promoter could not be demonstrated in melanoma cells [53].

Interferon regulatory factor 1 (IRF1) is a downstream effector of STAT1 upon IFNg stimulation. Its role is crucial in both constitutive and IFNg-mediated upregulation of PD-L1. Inhibition of IRF1 activity or expression resulted in decreased PD-L1 levels in human lung cancer cells [60]. The key role of IRF1 and interferon receptor pathway in the regulation of PD-L1 has also been implied in melanoma, where putative binding sites for IRF1 have been identified in the PD-L1 promoter and abrogation of IRF1 site resulted in reduced PD-L1 levels [53, 61]. Recently, another novel mechanism of PD-L1 regulation by DNA double-strand breaks (DSBs) was unveiled. This DSB-dependent PD-L1 upregulation was mediated by the activation of STAT1/STAT3 phosphorylation and IRF1 [62].

**Hypoxia-inducible factors (HIFs)**

Hypoxia signaling represents an important pathway in oncogenesis. HIF-1a and HIF-2a are the major components of a transcriptional complex, through which tumor cells adapt to hypoxic conditions. HIF stabilization leads to its binding to specific regions called hypoxia response elements (HRE) on certain gene promoters [63]. High levels of HIF-1 have been correlated with both worse outcomes and resistance to cytotoxic therapy [64]. Intriguingly, HIF-1...
expression by different cellular sub-populations of the innate and adaptive immunity can modify antitumor activity by repressing the effective T-cell response and alter TME to promote tumor cell survival [63]. A recent study revealed that HIF-1α guided CD8+ T-cell migration and function, whereas its depletion on T cells resulted in increased tumor growth and impaired antitumor control [65]. One of the mechanisms by which hypoxia signaling impairs T-cell functionality is the induction of PD-L1 on myeloid-derived suppressor cells under hypoxic conditions. Indeed, HIF-1α transcriptionally regulates PD-L1 expression by binding on HRE of its promoter [66]. Furthermore, PD-L1 may be a target of HIF2α in clear cell renal cell carcinoma (ccRCC) cells in which the tumor-suppressor pVHL was abrogated. Upon deficiency of pVHL increased PD-L1 levels, associated with HIF-2α activation, were observed in vitro [67]. Similar results were obtained from ccRCC patient samples with VHL loss-of-function mutations, where a positive correlation was seen between PD-L1 expression, HIF-2α expression and VHL mutations. Of note, HIF-2α tran-
ciptionally regulates PD-L1 by binding to the active HRE of its promoter [68]. Moreover, STAT3 can cooperate with HIF-1, but not HIF-2, in the regulation of HIF target genes in response to hypoxia. Inhibition of STAT3 expression or activity in breast and RCC cell lines reduced the expression of genes targeted by HIF-1 [69]. These findings support the idea of combining HIF-targeting therapies and immunotherapy.

The role of nuclear factor kappa B (NF-κB)

NF-κB is a master transcription factor activated in several cancer types, promoting inflammation, inhibiting apoptosis, and impairing effective antitumor immunity [70]. The NF-
κB family contains seven members, with the most representative being the p65 RelA/p50. This cytoplasmic heterodimer translocates to the nucleus and acts as a transcription factor of κB upon degradation of the IκB-α inhibitor [71, 72]. In melanoma cells, NF-κB mediated PD-L1 overexpression induced by IFN-γ. PD-L1 upregulation by NF-κB was independent of STAT3 and c-Jun, whereas targeting of MAPK and PI3K signaling pathways had a minor impact on PD-L1 expression [72]. Notably, STAT3 regulates and cooperates with NF-κB in additional cancer types [73]. For example, PD-L1 regulation may be dependent on p65/NF-κB and mediated by LMP1 in EBV-positive NPC, as inhibition of NF-κB activity resulted in decreased PD-L1 levels [54].

The Myc oncogene

Myc plays a pivotal role in carcinogenesis by controlling cell proliferation and survival in various cell systems. Tumor regression after Myc inactivation is associated with a not fully understood immune response, as reflected by the accumulation of CD4+ T cells [74–76]. Furthermore, a novel role of Myc was recently revealed in the context of avoiding effective cancer immunosurveillance. Using a Tet-off MYC-dependent mouse model of T-ALL (MYC T-ALL), Casey et al. showed that Myc transcriptionally regulates PD-L1 and CD47, an inhibitory regulator of the innate immune system [77]. Moreover, forced expression of PD-L1 and CD47 upon Myc inactivation was correlated with worse antitumor immune response as indicated by the reduction of macrophages and CD4+ T cells in TME, tumor progression, and maintenance of angiogenesis and senescence [78]. Elucidating the role of Myc in the regulation of immune-mediated antitumor response, the potential crosstalks with other oncogenic pathways and the immune infiltrate in TME may pave the way for the use of immune checkpoint inhibitors in patients with Myc-overexpressing tumors [79]. A recent work on ALK-negative ALCL also supports a Myc-mediated regulation of PD-L1, as forced expression of Myc led to PD-L1 upregulation in cell lines showing low baseline levels of PD-L1. Similarly, both inhibition and silencing of Myc resulted in PD-L1 downregulation in lymphoma cells [52].

The bromodomain and extraterminal (BET) protein BRD4

BET proteins modulate gene expression through enzymes that regulate chromatin and histone modification [80]. Specifically, the BET protein BRD4 acts through RNA polymerase II by binding to the acetyl-lysine region of histones [81]. Inhibition of BRD4 by the JQ1 inhibitor decreased PD-L1 expression and tumor growth. BRD4 gene silencing also resulted in decreased PD-L1 levels in mouse models and in ovarian cancer cell lines. Notably, BRD4 transcriptionally regulated PD-L1 by binding on its promoter [82]. Similarly, in a recent study on B-cell lymphoma, BET inhibitors enhanced effective antitumor immunity through regulation of PD-L1, whereas inhibition and genetic ablation of BRD4 resulted in suppression of PD-L1 expression in a transcriptional, Myc-independent, manner. Moreover, BRD4 synergized with IRF1 to regulate PD-L1 expression induced by IFN-γ [83]. Also, another BET inhibitor (I-BET151) was shown to abrogate NF-κB activity in melanoma, both in vitro and in vivo, thus indirectly affecting PD-L1 expression [84].

Histone deacetylases (HDACs)

The role of the epigenetic modifiers HDACs in the modification of non-histone targets, including those participating in tumor-host interactions, has recently been
investigated [85, 86]. In a study in melanoma, both inhibition and depletion of HDAC6 resulted in reduced PD-L1 levels in vitro and in vivo. PD-L1 regulation by HDAC6 was mediated by STAT3 and both HDAC6 and STAT3 were recruited to the PD-L1 gene promoter [87]. It should be noted that HDAC have pleiotropic effects within both the innate and adaptive immune response, and may thus affect PD-L1 levels via interferons [88].

The role of cell cycle

Cyclin-dependent kinases (CDKs) have a key role in cell cycle [89]. Cyclin-dependent kinase 5 (Cdk5) is a serine-threonine kinase important in central nervous system function [90] and other cellular functions [91, 92]. In a study of medulloblastoma, depletion of Cdk5 led to the upregulation of interferon regulatory factor 2 and interferon regulatory factor binding protein 2, which in turn, suppressed the expression of PD-L1. Cdk5 was thus necessary for PD-L1 upregulation after IFN-γ stimulation through STA1/IRF1 axis and its disruption led to tumor rejection in a CD4+ T-cell-dependent manner in medulloblastoma mouse models [93]. These data highlight Cdk5 as a novel target for interventions in combination with immune checkpoint blockade. Additionally, CDK4/6 inhibition has been recently shown to enhance antitumor immunity through increased T-cell cytotoxicity and Treg suppression [94]. This is discussed in detail in the post-translational regulation of PD-L1 hereunder.

The AP-1 transcription factors

c-Jun, the best known member of the AP-1 family, represents another transcription factor that is implicated in PD-L1 gene regulation. Knockdown of c-Jun resulted in decreased levels of PD-L1 in melanoma cells resistant to BRAF inhibitors [32], and co-activation of STAT3 and the subsequent formation of a transcriptional complex further enhanced these effects [95]. Similarly, combined knockdown of c-Jun and STAT3 genes in the same melanoma model showed a synergistic effect on PD-L1 downregulation [32]. Additionally, c-Jun and JUNB have been shown to bind AP-1 sites in the PD-L1 promoter in HL cells [96] and in KRAS-mutant NSCLC. In lung adenocarcinoma cell lines, the transcriptional activity was subjected to MAPK signaling pathway [34]. MAPK/AP-1 was also shown to contribute to LMP1-mediated upregulation of PD-L1 in EBV-associated NPC [54].

The ambivalent role of p53

The tumor-suppressor gene p53 has been implicated in antitumor immunity by regulating several genes involved in the immune system. Indeed, immune checkpoint regulation has been shown to represent a major target of p53 [97]. Paradoxically, activation of wild-type p53 using the small molecule Nutilin-3a resulted in increased expression of PD-L1 in human breast cancer [98] and in ALK-negative ALCCL cells [52]. In p53-mutated NSCLC, downregulation of miR-34 resulted in increased PD-L1 levels [99], whereas an inverse correlation between miR-34a and PD-L1 was also confirmed in AML [100].

MicroRNAs

MicroRNAs can bind to 3′-UTR of mRNAs and lead to their degradation or translational repression [101]. MiR-513 was shown to increase PD-L1 expression in cholangiocytes [102], whereas mutation in the 3′-UTR of PD-L1 mRNA led to overexpression of the protein by preventing miR-570 binding in gastric cancer [103, 104]. On the contrary, miR-197 downregulated PD-L1 by affecting STAT3 in platinum-resistant NSCLC [105], whereas miR-138-5p was associated with decreased levels of PD-L1 in colorectal cancer (CRC) [106]. Also in CRC, miR-20b, miR-21, and miR-130b caused PD-L1 upregulation through attenuation of PTEN [107].

Post-translational regulation of PD-L1

The role of ubiquitination

In a recent study by Lim et al., a novel regulatory mechanism involving the fifth protein element of COP9 signalosome complex (CSN5), also known as Jab1, was revealed in breast cancer. CSN5 has been associated with increased proliferation, decreased apoptotic rates, and survival of cancer cells [108]. Under chronic inflammatory conditions, tumor necrosis factor alpha (TNF-α), secreted mostly by macrophages, led to PD-L1 stabilization and therefore to an immunosuppressive profile of the tumor environment [61]. The stabilization of PD-L1 by TNF-α was shown to be mediated by NF-κB subunit RelA/p65, which binds on the promoter of CSN5 gene and has a direct effect on its regulation. CSN5 in turn, prevents the ubiquitination of PD-L1, hinders its degradation and as a result enhances tumor escape from immunosurveillance. Indeed, CSN5 inhibition or gene silencing abolished PD-L1 expression and tumor proliferation in vivo. Curcumin, a CSN5 inhibitor, induced better responses to anti-CTLA-4 treatment in vitro, indicating the potential of combinational administration of immune checkpoint with CSN5 inhibitors [61, 109, 110]. In another in vitro study, induction of both PD-L1 ubiquitination and PD-L1 protein levels was noted upon treatment with epidermal growth factor. An increase of
Table 2 Randomized phase 3 trials of PD-1 and PD-L1 inhibitors

| Trial [Ref] | N     | Clinical setting | Comparison | ORR (%) | PFS (months) | OS (months) |
|-------------|-------|------------------|------------|---------|--------------|-------------|
|             |       |                  |            |         |              |             |
| Non-small cell lung cancer |       |                  |            |         |              |             |
| KEYNOTE-024 [132] | 305   | First line       | Pembrolizumab vs platinum doublet | 44.8 vs 27.8 | 10.3 vs 6.0, p < 0.001 | HR = 0.60 (0.41–0.89), p = 0.005 |
| CheckMate 026 [133] | 541   | First line       | Nivolumab vs platinum doublet | 26 vs 33 (NS) | 4.2 vs 5.9, p = 0.25 | 14.4 vs 13.2 (NS) |
| KEYNOTE-010 [134] | 1034  | Second line      | Pembrolizumab (2 schedules) vs docetaxel | 18 and 18 vs 9, p = 0.0005 and p = 0.0002 | 3.9 and 4.0 vs 4.0 (NS) | 10.4 and 12.7 vs 8.5, p = 0.0008 and p < 0.0001 |
| CheckMate 017 [135] | 272   | Second line, squamous | Nivolumab vs docetaxel | 20 vs 9, p = 0.008 | 3.5 vs 2.8, p < 0.001 | 9.2 vs 7.3, p < 0.001 |
| CheckMate 057 [136] | 582   | Second line, non-squamous | Nivolumab vs docetaxel | 19 vs 12, p = 0.02 | 2.3 vs 4.2, p = 0.39 | 12.2 vs 9.4, p = 0.002 |
| OAK [137] | 850   | Second line       | Durvalumab vs placebo | 14 vs 13 (NS) | 2.8 vs 4.0, p = 0.49 | 13.9 vs 9.6, p = 0.0003 |
| PACIFIC [145] | 713   | Maintenance stage III | Durvalumab vs placebo | 28.4 vs 16.0, p < 0.001 | 16.8 vs 5.6, p < 0.001 | Not reported |
| Cutaneous melanoma |       |                  |            |         |              |             |
| KEYNOTE-006 [138] | 834   | First line       | Pembrolizumab (2 schedules) vs ipilimumab | 33.7 and 32.9 vs 11.9, p < 0.001 | HR = 0.58 (0.46–0.72), p < 0.001 | HR = 0.63 (0.47–0.83), p = 0.0005 |
| CheckMate 066 [139] | 418   | First line       | Nivolumab vs dacarbazine | 40.0 vs 13.9, p < 0.001 | 5.1 vs 2.2, p < 0.001 | HR = 0.42 (33.0–50.9), p < 0.001 |
| CheckMate 037 [140] | 405   | After ipilimumab | Nivolumab vs dacarbazine or carboplatin/paclitaxel | 31.7 vs 10.6 | 3.1 vs 3.7 (NS) | 16 vs 14 (NS) |
| CheckMate 067 [141] | 945   | First line       | Nivolumab + ipilimumab vs nivolumab vs ipilimumab | 58 vs 44 vs 19 | 11.5 vs 6.9 vs 2.9 | NR vs NR vs 20 |
| CheckMate 238 [146] | 906   | Adjuvant         | Nivolumab vs ipilimumab | HR for RFS 0.65 (97.56% CI, 0.51–0.83), p < 0.001 | HR for RFS 0.65 (97.56% CI, 0.51–0.83), p < 0.001 |
| Urothelial bladder cancer |       |                  |            |         |              |             |
| KEYNOTE-045 [142] | 542   | Second line       | Pembrolizumab vs paclitaxel or docetaxel or vinflunine | 21.1 vs 11.4, p = 0.001 | 2.1 vs 3.3, p = 0.42 | 10.3 vs 7.4, p = 0.002 |
| Imvigor 211b [147] | 931   | Second line       | Atezolizumab vs paclitaxel or docetaxel or vinflunine | 23.0 vs 21.6 (NS) | 2.4 vs 4.2 (NS) | 11.1 vs 10.6, p = 0.41 |
| Clear cell renal carcinoma |       |                  |            |         |              |             |
| CheckMate 025 [143] | 821   | After 1–2 TKIs   | Nivolumab vs everolimus | 25 vs 5, p < 0.001 | 4.6 vs 4.4, p = 0.11 | 25.0 vs 21.8, p ≤ 0.0148 |
| CheckMate 214c [148] | 1096  | First line       | Nivolumab + ipilimumab vs sunitinib | 42 vs 27, p < 0.0001 | 22.6 vs 8.4, p = 0.0331 | NR vs 32, p = 0.0003 |
| Head and neck squamous cell carcinoma |       |                  |            |         |              |             |
| CheckMate 141 [144] | 361   |                  | Nivolumab vs methotrexate or docetaxel or cetuximab | 13.3 vs 5.8 | 2.0 vs 2.3, p = 0.32 | 7.5 vs 5.1, p = 0.01 |

ORR objective response rate, PFS progression-free survival, OS overall survival, NS nonsignificant, NR not reached, HR hazard ratio, RFS relapse-free survival, CI confidence interval, TKI tyrosine kinase inhibitor

a The results presented here concern the primary endpoint of the study in the IC2/3 group of PD-L1 expression

b The results presented here concern the primary endpoint of the study in the intermediate and poor risk group
monovalent and multiquitilation of PD-L1 was seen, an effect that was abrogated upon inhibition of the EGFR pathway and/or ubiquitin E1 activating enzyme [111]. Furthermore, a recent study demonstrated a novel role of cyclin D-CDK4 and cullin 3-speckle-type POZ protein (SPOP) E3 ligase in regulating the expression of PD-L1. Cyclin D1-CDK4 was shown to phosphorylate SPOP and lead to ubiquitination-mediated PD-L1 destabilization. Thus, either inhibition of CDK4/6 or loss-of-function mutations of SPOP led to increased levels of PD-L1 and reduced tumor-infiltrating lymphocytes. Additionally, treatment with a CDK4/6 inhibitor and an anti-PD-1 antibody resulted in tumor regression and improved survival in vivo [112].

**Lysosomal-mediated degradation**

CKLF-like MARVEL transmembrane domain containing protein 6 (CMTM6) was recently identified as a novel regulator of PD-L1 [113, 114]. CMTM6—a tetraspanin protein—interacted with PD-L1 through its transmembrane domain and regulated PD-L1 expression in cancer and myeloid cells, both in vitro and in vivo [115]. Depletion of CMTM6 did not influence the CD274 transcript, but led to reduction of PD-L1 protein expression and augmentation of antitumor immunity. The mechanism of action of CMTM6 involves the avoidance of PD-L1 lysosome-mediated degradation, probably through prevention of its ubiquitination, as these two proteins are co-localized in the plasma membrane [116].

**The role of glycosylation**

N-glycosylation represents a crucial post-translational modification determining protein formation, functionality, and interaction with other proteins [117]. A novel association between procedure-glycosylation and ubiquitination in the regulation of PD-L1 has recently been unveiled. In basal-like breast cancer cells, N-glycosylation of PD-L1 (highly at sites N35, N192, N200, and N219) led to protein stabilization and avoidance of its degradation by 26S proteasome. In contrast, non-glycosylated forms interacted with Glycogen synthase kinase 3 beta (GSK3β), which in turn phosphorylated PD-L1 resulting in its degradation. Inhibition of GSK3β activity augmented immune suppression by tumor cells both in vitro and in vivo. Furthermore, EGFR promoted inactivation of GSK3β, and EGFR signaling blockade reversed stabilization of PD-L1 and led to enhanced antitumor responses [118]. In another study, N-linked glycosylation of PD-L1 (gPD-L1) was shown to increase PD-L1/PD-1 interaction, and consequently immunosuppression in TNBC. Its targeting with monoclonal antibodies or drug-conjugated gPD-L1 was thus proposed as a promising target of post-translational modifications of immune checkpoints [119].

**Effect of chemotherapy in PD-L1 expression**

Chemotherapeutic agents, apart from their direct cytotoxic effects on cancer cells, can also modulate immune responses against tumors [120, 121]. Treatment with paclitaxel, etoposide and 5-fluorouracil induced PD-L1 expression in breast cancer cell lines in a dose-dependent manner [122]. Paclitaxel was also associated with elevated levels of PD-L1 in human CRC and hepatocellular carcinoma cell lines. This regulation was dependent on MAPK activation [123]. Likewise, cisplatin induced PD-L1 expression in hepatoma cells in ERK1/2 phosphorylation-dependent manner [124]. In another study, doxorubicin led to PD-L1 downregulation on cell surface and a simultaneous PD-L1 upregulation in the nucleus of breast cancer cells. Nuclear PD-L1 expression was accompanied by nuclear AKT phosphorylation and proved to be dependent on PI3K/AKT pathway, whereas knockdown of PD-L1 was associated with enhanced doxorubicin-mediated apoptosis [125].

**Targeting immune checkpoint regulators: the era of immunotherapy in cancer**

The introduction of systemic cancer immunotherapy in clinical practice significantly predates the first randomized trials of immune checkpoint inhibitors. Despite the occurrence of rare, prolonged complete remissions in patients with metastatic melanoma and ccRCC [126, 127], the use of high-dose IL-2 was restricted by the significant, often fatal adverse events and the need for intensive monitoring and experience in its administration, whereas the use of IFNg in ccRCC was characterized by its perceived low efficacy [128]. The clinical application of cancer immunotherapy had remained stagnant until the first checkpoint inhibitor received regulatory approval for use in metastatic melanoma, the CTLA-4 inhibitor ipilimumab. Ipilimumab exhibits several recurring characteristics of immunotherapy: slow induction of response, a striking disassociation between imaging-assessed objective responses and survival, which led to the introduction of immune-related response criteria [129], unique patterns of toxicity termed “immune-related adverse events” [130] and robust, durable improvements in terms of patient survival [131].

Shortly after the approval of ipilimumab the first trials of PD-1 and later PD-L1 inhibitors were published. Their results have vastly changed the treatment landscape in multiple human malignancies, adding a new category of effective and, compared with cytotoxic chemotherapy, less toxic agents to the therapeutic armamentarium. The results of the published phase 3 trials are presented in Table 2 [132–148], whereas a selection of ongoing randomized trials in an ever-expanding list of indications, both at
| Disease                      | Trial          | Clinical setting                                                                 | Clinicaltrials.gov Identifier |
|------------------------------|----------------|-----------------------------------------------------------------------------------|--------------------------------|
| **Pembrolizumab**            |                |                                                                                   |                                |
| Breast cancer                | KEYNOTE-119    | Prior anthracycline/taxane, vs monochemotherapy                                  | NCT02555657                    |
| TNBC                         | KEYNOTE-522    | First line, chemotherapy ± pembrolizumab                                         | NCT03036488                    |
| TNBC                         |                | Adjuvant in residual disease after neoadjuvant chemotherapy                      | NCT02954874                    |
| HER2 + breast cancer         |                | First line, Paclitaxel/Trastuzumab/Pertuzumab ± pembrolizumab                    | NCT03199885                    |
| **Gastrointestinal cancer**  |                |                                                                                   |                                |
| Hepatocellular cancer        | KEYNOTE-394    | Pretreated (sorafenib or oxaliplatin), vs placebo                                | NCT03062358                    |
| Hepatocellular cancer        | KEYNOTE-240    | Prior sorafenib, vs placebo                                                      | NCT02702401                    |
| Gastric cancer               | KEYNOTE-063    | Second line, vs paclitaxel                                                        | NCT03019588                    |
| Esophageal cancer            | KEYNOTE-590    | First line, cisplatin/5FU ± pembrolizumab                                        | NCT03189719                    |
| Esophageal cancer            | KEYNOTE-181    | Second line, vs taxane or irinotecan                                              | NCT02564263                    |
| Colorectal cancer            | KEYNOTE-177    | First line, microsatellite instability-high or mismatch repair deficient, chemotherapy vs pembrolizumab | NCT02563002                    |
| **Genitourinary cancer**     |                |                                                                                   |                                |
| Renal cell carcinoma         | KEYNOTE-564    | Adjuvant, vs placebo                                                             | NCT03142334                    |
| Renal cell carcinoma         | KEYNOTE-426    | First line, pembrolizumab/axitinib vs sunitinib                                 | NCT02853331                    |
| Bladder cancer               | KEYNOTE-361    | First line, chemotherapy vs pembrolizumab vs combination                          | NCT02853305                    |
| **Lung and head and neck cancer** | | | | |
| NSCLC                        | KEYNOTE-091    | Adjuvant, vs placebo                                                             | NCT02504372                    |
| NSCLC                        | KEYNOTE-407    | First line, squamous cell, chemotherapy ± pembrolizumab                            | NCT02775435                    |
| NSCLC                        | KEYNOTE-189    | First line, non-squamous cell, chemotherapy ± pembrolizumab                       | NCT02578680                    |
| SCLC                         | KEYNOTE-604    | First line, chemotherapy ± pembrolizumab                                         | NCT03066778                    |
| Mesothelioma                 | PROMISE-Meso   | Second line, vs gemcitabine or vinorelbine                                       | NCT02991482                    |
| Head and neck cancer         | KEYNOTE-412    | After chemoradiation, vs placebo                                                 | NCT03040999                    |
| Head and neck cancer         | KEYNOTE-048    | Chemotherapy vs pembrolizumab vs combination                                       | NCT02358031                    |
| **Melanoma**                 |                |                                                                                   |                                |
| Melanoma                     | KEYNOTE-252    | First line, pembrolizumab ± epacadostat                                          | NCT02752074                    |
| Melanoma                     |                | Adjuvant, pembrolizumab vs ipilimumab vs interferon alfa-2B                      | NCT02506153                    |
| **Hematologic malignancies** |                |                                                                                   |                                |
| Hodgkin’s lymphoma           | KEYNOTE-204    | Relapsed/refractory disease, vs brentuximab vedotin                             | NCT02684292                    |
| Multiple myeloma             | KEYNOTE-183    | Relapsed/refractory disease, pomalidomide/dexamethasone ± pembrolizumab          | NCT02576977                    |
| Multiple myeloma             | KEYNOTE-185    | First line, lenalidomide/dexamethasone ± pembrolizumab                          | NCT02579863                    |
| **Nivolumab**                |                |                                                                                   |                                |
| Gastrointestinal cancer      |                |                                                                                   |                                |
| Hepatocellular cancer        |                |                                                                                   |                                |
| Gastric cancer               | CheckMate 649  | First line, vs sorafenib                                                         | NCT02576509                    |
| Esophageal and junction cancer | CheckMate 577 | Adjuvant, vs placebo                                                             | NCT02743494                    |
| Esophageal cancer            | CheckMate 648  | First line, nivolumab/ipilimumab vs nivolumab/chemotherapy vs chemotherapy        | NCT03143153                    |
| Mesothelioma                 |                | Second line, vs taxane                                                           | NCT02569242                    |
| **Genitourinary cancer**     |                |                                                                                   |                                |
| Bladder cancer               | CheckMate 274  | Adjuvant, vs placebo                                                             | NCT02632409                    |
| Bladder cancer               | CheckMate 901  | First line, nivolumab/ipilimumab vs chemotherapy                                 | NCT03036098                    |
| Disease                      | Trial                  | Clinical setting                                                                 | Clinicaltrials.gov Identifier |
|-----------------------------|------------------------|----------------------------------------------------------------------------------|--------------------------------|
| Renal cell carcinoma        | CheckMate 9ER          | First line, nivolumab/ipilimumab vs nivolumab/cabozantinib vs sunitinib          | NCT03141177                    |
| Lung and head and neck cancer |                        |                                                                                  |                                |
| NSCLC                       | ANVIL                  | Adjuvant, vs placebo                                                             | NCT02595944                    |
| NSCLC                       | CheckMate 816          | Neoadjuvant, nivolumab/ipilimumab vs chemotherapy                               | NCT02998528                    |
| NSCLC                       | CheckMate 227          | Stage III, after chemoradiation vs placebo                                        | NCT02768558                    |
| NSCLC                       | CheckMate 451          | Maintenance after first line, nivolumab/ipilimumab vs nivolumab vs placebo       | NCT02538666                    |
| Mesothelioma                | CheckMate 743          | First line, nivolumab/ipilimumab vs chemotherapy                               | NCT02899299                    |
| Mesothelioma                | CONFIRM               | Pretreated, vs placebo                                                           | NCT03063450                    |
| Head and neck cancer        | CheckMate 651          | First line, nivolumab/ipilimumab vs chemotherapy                               | NCT02741570                    |
| Melanoma                    | CheckMate 915          | Adjuvant, nivolumab/ipilimumab vs nivolumab vs ipilimumab                       | NCT03068455                    |
| Melanoma                    |                       | First line BRAF V600E, dabrafenib/trametinib → nivolumab/ipilimumab → dabrafenib/trametinib | NCT02224781                    |
| Hematologic malignancies    |                        |                                                                                  |                                |
| Hodgkin’s lymphoma          | CheckMate 812          | Relapsed/refractory disease, nivolumab/brentuximab vedotin vs brentuximab vedotin | NCT03138499                    |
| Multiple myeloma            | CheckMate 602          | Relapsed/refractory disease, pomalidomide/dexamethasone ± nivolumab vs nivolumab/pomalidomide/elotuzumab/dexamethasone | NCT02726581                    |
| Other tumors                |                        |                                                                                  |                                |
| Glioblastoma                | CheckMate 143          | Second line, nivolumab/ipilimumab vs nivolumab vs bevacizumab                   | NCT02017717                    |
| Glioblastoma                | CheckMate 498          | First line, radiation and temozolomide or nivolumab                              | NCT02617589                    |
| Atezolizumab                |                        |                                                                                  |                                |
| Breast cancer               | IMpassion 031          | Neoadjuvant, chemotherapy ± atezolizumab                                          | NCT03197935                    |
| TNBC                        | IMpassion 130          | First line, nab-paclitaxel ± atezolizumab                                        | NCT02425891                    |
| TNBC                        | IMpassion 131          | First line, paclitaxel ± atezolizumab                                            | NCT03125902                    |
| Gastrointestinal cancer     |                        |                                                                                  |                                |
| Colorectal cancer           | IMvigor 010            | Adjuvant, vs placebo                                                             | NCT02450331                    |
| Renal cell carcinoma        | IMmotion 010           | Adjuvant, vs placebo                                                             | NCT03024996                    |
| Renal cell carcinoma        | IMmotion 151           | First line, atezolizumab/bevacizumab vs sunitinib                                | NCT02420821                    |
| Prostate cancer             | IMbassador 250         | Castration-resistant, after anti-androgen and taxane, enzalutamide ± atezolizumab | NCT03016312                    |
| Ovarian cancer              | ATALANTE               | Relapsed, chemotherapy/bevacizumab vs atezolizumab                               | NCT02891824                    |
| Ovarian cancer              | IMagyn 050             | First line, Paclitaxel/Carboplatin/Bevacizumab ± atezolizumab                   | NCT03038100                    |
refractory disease, as well as in earlier lines of therapy or at the adjuvant setting is presented in Table 3. The results of these trials are eagerly awaited, because there are high unmet needs in many of the indications that these agents are being tested. Of interest are also hematologic malignancies; preliminary trials report impressive response rates in otherwise refractory disease [149], believed to be driven by both the inherent role of the PD-1/PD-L1 axis in the evasion of immunosurveillance in lymphoid tumors, particularly in those with a viral etiology [150], and by the presumed significance of PDL1 and PDL2 amplification in the biology of certain neoplasms such as Hodgkin lymphoma [22].

| Disease                        | Trial       | Clinical setting                                                                 | Clinicaltrials.gov Identifier |
|-------------------------------|-------------|----------------------------------------------------------------------------------|--------------------------------|
| Lung and head and neck cancer | IMpower 130 | First line, non-squamous, chemotherapy ± atezolizumab                              | NCT02367781                   |
| Lung and head and neck cancer | IMpower 131 | First line, squamous, chemotherapy ± atezolizumab                                  | NCT02409355                   |
| Lung and head and neck cancer | IMpower 132 | First line, platinum ineligible, vs monochemotherapy                            | NCT03191786                   |
| Melanoma                      | IMpower 133 | First line, chemotherapy ± atezolizumab                                            | NCT02763579                   |
| Genitourinary cancer          |             | First line BRAF V600E, vemurafenib/cobimetinib ± atezolizumab                     | NCT02908672                   |
| Bladder cancer                | MYSTIC      | First line, durvalumab/tremelimumab vs durvalumab vs chemotherapy                 | NCT02516241                   |
| Lung and head and neck cancer | NEPTUNE     | First line, durvalumab/tremelimumab vs chemotherapy                              | NCT02542293                   |
| Lung and head and neck cancer | CAURAL      | Second line, EGFR T790M +, osimertinib ± durvalumab                             | NCT02454933                   |
| Lung and head and neck cancer | Caspian     | Adjuvant, vs placebo                                                             | NCT02273375                   |
| Head and neck cancer          | KESTREL     | First line, durvalumab/tremelimumab/chemotherapy vs durvalumab/chemotherapy       | NCT02551159                   |
| Avelumab                      |             | First line, durvalumab/tremelimumab vs durvalumab vs chemotherapy                 | NCT02926196                   |
| Breast cancer                 |             | First line, durvalumab/tremelimumab vs durvalumab vs chemotherapy                 | NCT02453282                   |
| Gastric cancer                |             | Maintenance after first line, vs continuation chemotherapy                        | NCT02625610                   |
| Gastric cancer                |             | Third line, vs irinotecan or paclitaxan                                           | NCT02625623                   |
| Bladder cancer                | JAVELIN Bladder 100 | Maintenance after first line, vs placebo                                       | NCT02603432                   |
| Renal cell carcinoma          | JAVELIN Renal 101 | First line, avelumab/axitinib vs sunitinib                                      | NCT02684006                   |
| Ovarian cancer                | JAVELIN Ovarian 100 | First line, chemotherapy vs chemotherapy/avelumab vs chemotherapy with avelumab maintenance only | NCT02718417                   |
| Ovarian cancer                | JAVELIN Ovarian 200 | Platinum-resistant relapse, liposomal doxorubicin ± avelumab                      | NCT02580058                   |
| Lung and head and neck cancer | JAVELIN Lung 100 | First line, vs chemotherapy                                                       | NCT02576574                   |
| Lung and head and neck cancer | JAVELIN Lung 200 | Second line, vs docetaxel                                                         | NCT02395172                   |
| Head and neck cancer          | JAVELIN Head and neck 100 | Chemoradiotherapy ± avelumab                                                      | NCT02952586                   |
| Head and neck cancer          | REACH       | Chemoradiotherapy vs radiotherapy/cetuximab/avelumab                              | NCT02999087                   |

*NSCLC* non-small cell lung cancer, *SCLC* small cell lung cancer, *TNBC* triple-negative breast cancer, *HER2* human epidermal growth factor receptor 2
contrast, the recent discontinuation of the ongoing phase 3 trials in multiple myeloma due to an increased risk of death underscores the fact that better understanding of the underlying immune mechanisms is still needed.

Importantly, a new generation of clinical trials has been initiated and initial results are already available regarding a multi-faceted attempt to improve upon the efficacy of PD-1/ PD-L1 inhibitors as monotherapy: their combination with CTLA-4 inhibitors, already shown to improve outcomes in metastatic melanoma [141] and pursued in other malignancies including NSCLC and SCLC; their combination with cytotoxic chemotherapy, based upon the premise of the prevention of early disease progression due to the simultaneous administration of chemotherapy and the release of neoantigens due to the cytotoxic effects of the combinatory treatment, which may potentiate the activity of PD-1 inhibitors, an approach that has shown promising results in advanced NSCLC and at the neoadjuvant setting of TNBC [151, 152]; the combination of targeted agents and PD-1 axis blockade [153], with preliminary results showing that combining immunotherapy with inhibitors of known effectors of the axis, such as CDK4/6, results in promising activity [154]; and finally, the combination with inhibitors or stimulators of modulatory molecules such as indoleamine 2,3-dioxygenase (IDO) inhibitors, because IDO is a major negative feedback pathway regulated by IFNg. Preliminary results of the IDO inhibitor epacadostat with nivolumab in a variety of tumors and with pembrolizumab in melanoma are promising and phase 3 results are eagerly awaited [155, 156].

In short, the current era of cancer immunotherapy could be characterized as the “end of the beginning”. A variety of agents is available for use in multiple indications and clinical experience is accumulating. The next phase, namely the optimization of the use of the available agents and the exploration for novel combinations, has already begun.

**Immune checkpoint regulators as novel biomarkers: prognostic and predictive value**

Taking into account the significant clinical efficacy of PD-1/ PD-L1 blockade in a small subset of patients, the considerable costs and potential for devastating immune-related adverse events associated with the use of these inhibitors and the robust theoretical background explaining the biology of their mechanism of action, considerable efforts have been undertaken in order to identify putative predictive biomarkers. The best characterized biomarker is the immunohistochemistry (IHC)-assessed PD-L1 expression. The conflicting results of individual trials have been summarized in meta-analyses, which indicate that increased levels of PD-L1 expression are associated with an improved probability for objective response [157, 158]. Supporting these results are two recently published clinical trials in the first line of advanced NSCLC, KEYNOTE-024, and CheckMate 026. In the former, overall survival (OS) in patients selected for PD-L1 positivity ≥50% was improved with pembrolizumab compared with platinum-based chemotherapy [132]. Contrary, in the latter trial there were no OS gains in PD-L1 ≥5% patients treated with nivolumab versus chemotherapy [133]. As there are no perceived differences in the potency of these antibodies, the obvious discrepancy in the patient population could account for the different outcome. However, several observations hinder the routine selection of appropriate candidates according to PD-L1 expression. First, in addition to the modest concordance rates between the various antibodies used to assess PD-L1 expression reported in the literature, questions still remain regarding the uncontrolled pre-analytical conditions and the assay and inter-pathologist discrepancies [159], which can lead to PD-L1 status misclassifications despite the similar analytical performance of the available assays [160]. Second, PD-L1 expression exhibits significant intratumoral, intertumoral and temporal heterogeneity [161, 162], putting into question the widespread practice of assessing PD-L1 IHC expression on archival tissue. Third, as clearly shown in individual randomized trials such as the CheckMate 017 trial at the second line of lung SCC [135], characterizing patients as appropriate for anti-PD-1 therapy according to PD-L1 expression both includes patients who do not respond to treatment and also excludes potential responders. Fourth, in the aforementioned CheckMate 026 trial, nivolumab was not more effective than chemotherapy even in the subgroup of 50% or higher PD-L1 expression. As this was not a stratification factor, imbalances such as the sex of the patients could have confounded the results, implying that PD-L1 positivity by itself is not a strong predictive biomarker [133]. Finally, the association of objective response rates and PD-L1 expression in the trial-level meta-analyses is of unsure clinical importance, since checkpoint inhibitors can confer prolonged, clinically meaningful periods of disease stabilization and because their use beyond progression in patients deemed to derive clinical benefit has been found to improve outcomes in a diverse selection of solid malignancies [163–165].

Keeping in mind the shortcomings of PD-L1 expression, other biomarkers have been explored. Following the observation that smokers with NSCLC seem to derive improved benefit from anti-PD-1 agents [166], it was postulated that this effect may be a surrogate marker for an increased mutational load and subsequent increased neoantigen production and exposure and more effective immune response in patients chronically exposed to a strong mutagenic factor such as smoking. Indeed, mutational load has been found to be a predictive factor in NSCLC [167].
| Tumor type                          | PD-L1/L1 status | Correlation with outcome | Reference |
|------------------------------------|-----------------|--------------------------|-----------|
| **Breast cancer**                  |                 |                          |           |
| All                                | ↑ PD-L1 expression | Unfavorable              | [174, 178, 219] |
| All                                | ↑ PD-L1 expression | Favorable                | [175]     |
| HER2+                              | ↑ PD-L1 expression | Favorable                | [176]     |
| TNBC                               | ↑ PD-L1 expression | Favorable                | [177]     |
| TNBC                               | PD-L1 amplification | Unfavorable              | [16]      |
| Residual after neoadjuvant         | ↑ PD-L1 expression | Unfavorable              | [177]     |
| **Gastrointestinal cancer**        |                 |                          |           |
| All digestive tumors               | ↑ PD-L1 expression | Unfavorable              | [183]     |
| Hepatocellular cancer              | ↑ PD-L1/2 expression | Unfavorable              | [180, 181] |
| Colorectal cancer                  | ↑ PD-L1 expression | Favorable                | [186, 209] |
| Colorectal cancer                  | ↑ PD-L2 expression | Unfavorable              | [187]     |
| Gastric cancer                     | ↑ PD-L1 expression | Unfavorable              | [184, 185] |
| Cholangiocarcinoma                 | ↑ PD-L1 expression | Unfavorable              | [217]     |
| Esophageal cancer                  | ↑ PD-L1 expression | Favorable                | [214]     |
| Pancreatic cancer                  | ↑ PD-L1 expression | Unfavorable              | [182]     |
| **Genitourinary cancer**           |                 |                          |           |
| Clear cell renal                   | ↑ PD-L1/2 expression | Unfavorable              | [195–197] |
| Non-clear cell renal               | ↑ PD-L1 expression | Unfavorable              | [194]     |
| Papillary renal                    | ↑ PD-L1 expression | Unfavorable              | [193]     |
| Chromophobe renal                  | ↑ PD-L2 expression | Unfavorable              | [192]     |
| Bladder cancer                     | ↑ PD-L1 expression | Unfavorable              | [191, 218] |
| Prostate cancer                    | ↑ PD-L1 expression | Unfavorable              | [190]     |
| Prostate cancer                    | ↑ PD-L1 expression | Unfavorable              | [189]     |
| Ovarian cancer                     | ↑ PD-L1 expression | Favorable                | [188, 210] |
| **Lung and head and neck cancer**  |                 |                          |           |
| NSCLC                              | ↑ PD-L1 expression | Favorable                | [211, 213] |
| NSCLC                              | ↑ PD-L1 expression | Unfavorable              | [202–206] |
| NSCLC                              | ↑ PD-L1 expression | Not predictive           | [202]     |
| NSCLC                              | PD-L1 amplification | Unfavorable              | [200]     |
| SCLC                               | ↑ PD-L1 expression | Unfavorable              | [201]     |
| Pulmonary neuroendocrine           | ↑ PD-L1 expression | Unfavorable              | [220]     |
| Head and neck cancer               | ↑ PD-L1 expression | Favorable                | [199, 215] |
| Head and neck cancer               | ↑ PD-L1 expression | Unfavorable              | [198]     |
| **Melanoma and sarcoma**           |                 |                          |           |
| Melanoma                           | ↑ PD-L1 expression | Favorable                | [212]     |
| Melanoma                           | ↑ PD-L1 expression | Unfavorable              | [208]     |
| Soft tissue sarcoma                | ↑ PD-L1 expression | Unfavorable              | [207]     |
| **Hematologic malignancies**       |                 |                          |           |
| Hodgkin’s lymphoma                 | ↑ PD-1 expression | Unfavorable              | [222]     |
| Hodgkin’s lymphoma                 | PD-1/L1 co-expression | Unfavorable              | [225]     |
| Hodgkin’s lymphoma                 | PD-L1 amplification | Unfavorable              | [121]     |
| DLBCL                              | ↑ PD-L1 expression | Unfavorable              | [216, 227] |
| NK/T-cell lymphoma                 | ↑ PD-L1 expression | Unfavorable              | [226]     |
| Multiple myeloma                   | ↑ Soluble PD-L1  | Unfavorable              | [223, 224] |
| **All tumor types**                |                 |                          |           |
| Meta-analyses                      | ↑ PD-L1 expression | Unfavorable              | [221, 228, 229] |

HER2 human epidermal growth factor receptor, TNBC triple-negative breast cancer, NSCLC non-small cell lung cancer, SCLC small cell lung cancer, DLBCL diffuse large B-cell lymphoma, NK natural killer cells
Supporting this association is the observation that mismatch repair defective, and thus hypermutated tumors, are exquisitely sensitive to PD-1 blockade [168, 169]. In addition, NSCLC harboring driver molecular aberrations such as EGFR mutations, which exhibit lesser mutational loads have been shown to be relatively resistant to immune checkpoint inhibition [170], a finding supported by a recently published meta-analysis on the prediction of response in NSCLC patients. EGFR mutant and KRA5 wild-type status were associated with a lack of sensitivity to PD-1/PD-L1 inhibition, whereas clinical factors such as smoking status, histology, sex, performance status, and age did not affect the magnitude of benefit [171].

The quantitative and qualitative assessment of the host immune response has also been explored as a predictor in checkpoint inhibition. Factors such as the abundance of pre-existing CD8 (+) T cells, a restricted (clonal) TCR repertoire, a TH1-type response, increased levels of IFN-γ and IL-18 and decreased levels of IL-6, among others, have been correlated with improved responses [166, 172], but these results need to be evaluated prospectively in randomized trials. The implementation of multiparametric, high-throughput flow cytometry, and multiplex immunohistochemical staining techniques that vastly improve the T-cell population analysis [173] and of whole-exome sequencing for the evaluation of the mutational load and the presence of specific, predictive molecular alterations will aid in this respect.

On the other hand, PD-1 and PD-L1 expression both at the tissue level and on circulating tumor cells have been evaluated in a wide variety of malignancies for their prognostic impact (Table 4) [17, 21, 174–229]. The results have been thus far inconsistent among tumor types and somewhat confusing, with reports supporting both an improved and a decreased OS conferred by high expression, a phenomenon that resonates the previously mentioned shortcomings of the assessment of PD-L1. The biologic background of these observations is as of yet uncertain. Moreover, as the expansion of the indications of PD-1/PD-L1 blockade continues with the conduct and report of clinical trials, these associations could be affected due to the increasing use of these agents, making their clinical utility questionable at the moment.

**Open questions for future research**

Despite the progress in genetic and epigenetic regulation of PD-L1 expression, several gaps in the literature should be covered by intensive laboratory-based research. For instance, the signaling transduction pathways involved in PD-L1 regulation are only partially understood. Better understanding of the signaling mechanisms could provide the biologic rationale for combined targeted therapy with immunotherapy strategies in cancer. Furthermore, little is known about the post-translational modifications of PD-L1 protein including tyrosine or serine/threonine phosphorylation, acetylation, ubiquitination, and SUMOylation. It is also largely unknown how possible post-translational modifications not only regulate PD-L1 levels in the tumor cells, but also how they might affect its physiologic function or its interaction with the PD-1 receptor. In addition to PD-L1, the non-genetic mechanisms underlying PD-L2 expression and function in solid tumors and hematologic malignancies should be investigated, as both ligands compete for the same receptor, PD1, and therefore the relative levels of both proteins may impact certain immunotherapy approaches.

Regarding clinical practice, regulatory authorities both in Europe (European Medicine Agency), and the United States (Food and Drug Administration) have approved the use of PD-1/PD-L1 inhibitors for a variety of malignancies regardless of the presence or absence of predictive biomarkers. Exceptions include the use of pembrolizumab at the first and second line of NSCLC, which requires PD-L1 expression levels of ≥50% and ≥1% respectively, as well as the site agnostic indication for mismatch repair deficient tumors. In addition, the financial burden of the generalized use of these agents is considerable even in high-resource settings [230]. Overcoming this obstacle and achieving the personalized use of these agents requires a stepwise approach: first, taking into account the previously mentioned shortcomings of PD-L1 as a potential biomarker, it is important to retrospectively identify, in the large amount of collected tumor material from prospective studies, novel predictive biomarkers. These would ideally be prospectively validated, although the logistics of repeating single agent trials might be prohibitive. Instead, these biomarkers could form the basis of the next-generation combinatorial trials, of trials addressing the as yet unanswered question of the optimal duration of treatment or of trials in earlier disease settings where the overtreatment of already cured individuals in a massive scale could pose a significant public health burden.

**Summary**

Despite the clinical success of immune checkpoint inhibition in many tumors through PD-L1/PD-1 blockade, relatively little is known regarding the biology of these regulators of cancer immune surveillance. Many mechanisms have been demonstrated to regulate the expression of PD-L1 including signaling pathways, transcriptional factors, and post-transcriptional modulators. The oncogenic signaling pathways such as JAK/STAT, RAS/ERK, or...
PI3K/AKT/MTOR are activated by gene mutations and growth factors. At the transcriptional level, a number of transcriptional factors seem to regulate PD-L1 expression including HIF-1, STAT3, NF-kB, and AP-1. PD-L1 is subject to post-transcriptional regulation by several miRNAs, CSN5, CMTM6, CDK4 and possibly other, still unknown mechanisms. Better understanding of PD-L1 regulation may pave the way for combinational treatments with both immune checkpoint inhibitors and targeted therapies against kinases or transcription factors many of which are already available for clinical use.

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Compliance with ethical standards

Conflict of interest Dr. Foukakis has received research grants (institutional) from Pfizer and Roche; personal fees from Novartis, Pfizer, Roche and UpToDate outside the submitted work. The remaining authors declare that they have no conflict of interest.

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