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PD-L1 regulation in colorectal cancer and role of DNA damage induced by chemotherapies

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Abstract

For patients with metastatic colorectal cancer (CRC), first-line therapy is based on chemotherapeutic agents, such as oxaliplatin, 5-fluorouracil and irinotecan. These drugs increase the overall survival, but resistance to therapy appears in almost 90% of patients, and the 5-year survival rate for patients with metastatic CRC is only about 12%.

During the last few years, immune checkpoints blockade therapies have been developed and show good response in different cancers, including CRC with microsatellite instability (MSI). In this CRC subtype, the response rate to anti-PD-(L)1 antibodies is high thanks to the presence of neoantigens and tumor-infiltrating lymphocytes that are associated with the anti-tumor immune response. Nivolumab and pembrolizumab, two anti-PD-1 antibodies, have been approved for CRC MSI treatment. Moreover, it has been shown that the combination of chemotherapy and anti-PD-(L)1 molecules may convert cold tumors into hot tumors in which the immune system and T-cell infiltration are activated. In addition, recent studies found that DNA damage induces PD-L1 expression. ATM, ATR, DNA-PKcs and Chk1 are key sensors of the DNA damage response that regulate PD-L1 expression.

This review summarizes the current knowledge on PD-L1 regulation at the genetic, epigenetic, transcriptional and translational levels. It also describes PD-L1 activation in response to chemotherapy and DNA damage. Then, it summarizes the current clinical trials that assess anti-PD-(L)1 therapies in combination with kinase inhibitors or chemotherapeutic agents in CRC.

Introduction

Colorectal cancer (CRC) is the third most frequent cancer and a leading cause of cancer death worldwide [1]. Many potential curative treatments including surgery, chemotherapy, and targeting therapies have been developed for CRC. The currently used chemotherapies are based on 5-fluorouracile (5-FU), irinotecan and oxaliplatin [2]. These drugs, used in combination or as single agents, significantly improve the overall survival. However, the 5-year survival rate for metastatic CRC is only about 12% because almost 90% of patients will develop resistance to therapy [3].

Cancer immunotherapy is a novel approach that exploit the human immune system to treat cancer. Indeed, the T cell-based immune system destroys aberrant cells (including cancer cells) that are detected via the binding of T-cell receptors to antigenic peptides (in the form of major histocompatibility complex-peptide complexes) on target cells. This interaction is controlled by co-stimulatory and co-inhibitory receptors and ligands that are called immune checkpoint proteins. As tumors can exploit this immune response regulation mechanism to impair the anti-tumor response, checkpoint proteins are attractive therapeutic targets. For instance, antibodies that block cytotoxic T lymphocyte associated antigen 4 (CTLA4) and programmed cell death protein 1 (PD-1), two major immune checkpoint proteins, have been approved by the US Food and Drug Administration (FDA) for cancer treatment. Inhibition of CTLA-4 or PD-1 was first studied and approved for patients with metastatic melanoma, but they are now used for many solid cancers and have significantly improved patient survival [4].

PD-1 receptors are expressed on the surface of activated T cells. Programmed death ligand 1 (PD-L1), the main ligand of PD-1, is a co-inhibitory factor of the immune response that is expressed on myeloid, lymphoid, epithelial, dendritic cells and macrophages. The interaction between PD-1 and PD-L1 is essential for immune tolerance. PD-L1 is often overexpressed on tumor cells, and its expression is generally associated with poor prognosis [5]. Many studies suggest that PD-L1 expression may be a predictive biomarker of response to anti-PD-1/PD-L1 therapies. In CRC, anti-PD-(L)1 antibodies showed great success (response and survival rates) particularly in metastatic CRC with MSI compared with CRC characterized by microsatellite stability (MSS). MSI is detected in 5-10% of CRC and is due to impaired DNA mismatch-mediated repair (MMR). The better response to immune checkpoint blockade therapies of MSI CRC could be explained by their immunogenic nature. This includes the synthesis of neoantigens that promote a highly antigenic microenvironment with high density of tumor-infiltrating lymphocytes [6,7]. In the last few years, the US FDA approved four immune checkpoint inhibitors: the anti-PD1 monoclonal antibodies nivolumab and pembrolizumab and the anti-PD-L1 antibodies atezolizumab and durvalumab. Nivolumab in combination with ipilimumab (anti-CTLA4 antibody) has been validated for the treatment of patients with MSI High/deficient MMR (MSI-H/dMMR) metastatic CRC previously treated with conventional treatments, and pembrolizumab for the treatment of MSI CRC [8]. The challenge now is to use these anti-PD-(L)1 agents in combination with another treatment (e.g. chemotherapy or tyrosine kinase inhibitors) to convert cold MSS tumors into hot tumors with T-cell infiltration and immune activation [9].

Finally, several evidences indicate that DNA damaging agents can increase PD-L1 expression in tumors through the DNA damage checkpoint [10]. In this review, we will discuss the current knowledge on the correlation between DNA damage induced by

chemotherapy, expression of PD-L1 and activation of the innate immunity in CRC. For this purpose, we will first describe the mechanisms of PD-L1 regulation at different levels (genetic, epigenetic, transcriptional, translational, and post-translational). Then, we will summarize PD-L1 regulation and innate immunity activation by chemotherapy and DNA damage. Finally, we will describe the current clinical trials that use chemotherapy, DNA damage targets and immune checkpoint inhibitors in CRC.

PD-L1 regulation

TImmune checkpoint blockade with anti-PD-(L)1 molecules is beneficial in different cancer types. However, not all patients respond to these treatments, and it seems that PD-L1 expression level on tumor cells affects the clinical response. Thus, it is important to understand how PD-L1 expression is regulated in tumor cells. This regulation is complex and occurs at different levels [11,12] (Chen et al, 2016; Wang et al, 2016) (Table 1 and Figure 1).

Table 1. PD-L1 regulation

Level of regulation	Tumor type	Comments	WB or FACS	Reference
Genetic	Hodgkin's lymphoma and mediastinal large B cell lymphoma	9p copy number amplification	FACS	Green et al, 2010
	Mediastinal large B cell lymphoma	JAK2 modulates PD-L1 expression, and its amplification contributes to increase PD-L1 expression by enhancing IFN-g receptor signaling.		Twa et al, 2014
	NSCLC	PD-L1 is upregulated by PD-L1 and JAK2 amplification.	Western blotting (for JAK2) and FACS	Ikeda et al, 2016
	CRC	9p24.1 copy-number gain induces sensitivity to immune therapy.		Ree et al, 2019
	Adult T-cell leukemia/lymphoma, diffuse large B-cell lymphoma, and stomach adenocarcinoma	Truncation of PD-L1 3′UTR is associated with aberrant PD-L1 transcription.	Western blotting and FACS	Kataoka et al, 2016
Epigenetic	NSCLC cell lines	Azacytidine upregulates PD-L1 expression.	FACS	Wrangle et al, 2013
	Myelodysplastic syndrome and acute myeloid leukemia	In patients treated with epigenetic therapy, PD-L1 is upregulated. Exposure to decitabine leads to partial demethylation of PD-L1 promoter.	FACS	Yang et al, 2014
	Pancreatic cancer cells	MLL1 (H3K4me3) induces PD-L1 transcription.	Western blotting and FACS	Lu et al, 2017
	Prostate cancer cells	The histone methyltransferase MLL3 regulates PD-L1 expression.	Western blotting and FACS	Xiong et al, 2019
	CRC	PD-L1 promoter methylation is inversely correlated with PD-L1 mRNA expression.		Goltz et al, 2017
Transcriptional	T-cell lymphoma	PD-L1 upregulation, mediated by NF-κB activation via EBV-driven LMP1 signaling, correlates with poor prognosis.	Western blotting and FACS	Bi et al, 2016
	Ovarian cancer	Chemotherapy induces PD-L1 expression via NF-κB activation.	Western blotting and FACS	Peng et al, 2015
	Breast cancer cells	PTEN represses PD-L1 transcription and expression, suggesting a tumor suppressive function of PTEN.		Zhang et al, 2019
	Glioma and lung squamous cell carcinoma	Loss of PTEN results in PI3K activation and PD-L1 upregulation that leads to immune resistance and escape.		Peng et al, 2016
	Melanoma	PI3K activation induces PD-L1 expression through the transcription factor STAT3.	Western blotting and FACS	Jiang et al, 2013

	Tumor cells, myeloid-derived suppressor cells, dendritic cells, and macrophages	Hypoxia induces PD-L1 expression	Western blotting and FACS	Noman et al, 2014
	NSCLC	c-Myc expression significantly correlates with PD-L1 expression.	Western blotting	Kim et al, 2017
	Tumor cells, dendritic cells and macrophages	BET inhibitors significantly suppress PD-L1 expression.	FACS	Zhu et al, 2016
	TNBC	NPM1 binding to PD-L1 promoter activates PD-L1 transcription and inhibits T-cell activity. PARP1 suppresses PD-L1 transcription by interaction with NPM1.	Western blotting and FACS	Qin et al, 2020
	Melanoma	Activation of the MAPK pathway drives PD-L1 expression.	Western blotting and FACS	Atefi et al, 2014
	Melanoma cell lines	MAPK pathway increases PD-L1 expression by activating c-JUN.	Western blotting and FACS	Jiang et al, 2013
	EBV-positive gastric carcinoma	Upon IFN-γ stimulation, JAK-STAT signaling can activate IRF1 that binds to the PD-L1 promoter and induces PD-L1 transcription.	Western blotting	Moon et al, 2017
	Medullobastoma	IFN-γ-induced PD-L1 upregulation requires CDK5. CDK5 depletion downregulates PD-L1 expression.		Dixon Dorand et al, 2016
	Melanoma	HDAC inhibition reduces PD-L1 levels. PD-L1 is regulated by HDAC6 via STAT3.	Western blotting and FACS	Lienlaf et al, 2016
	NSCLC	The EGFR pathway increases PD-L1 expression mainly through the IL-6/JAK/STAT3 signaling axis.	Western blotting and FACS	Zhang et al, 2016
	CRC	FGFR2 induces PD-L1 expression via the JAK/STAT3 pathway	Western blotting	Li et al, 2019
Post-transcriptional	Human dermal lymphatic endothelial cells	By binding to the 3'UTR of PD-L1, TNF-a and IFN-g can induce miR-155 that suppresses PD-L1 protein expression.	Western blotting and FACS	Yee et al, 2017
	AML and NSCLC cells	miR-34a binds to the 3'UTR of PD-L1 and reduces PD-L1 mRNA levels.	Western blotting and FACS	Wang et al, 2015
	NSCLC	p53 suppress PD-L1 expression through miR-34.	Western blotting and FACS	Cortez et al, 2015
	CRC	miR-20, miR-21, and miR-130b increase PD-L1 expression by repressing PTEN.		Zhu et al, 2014
Translational and post-translational (phosphorylation)	Breast cancer	AMPK activation by metformin leads to PD-L1 phosphorylation, glycosylation, ubiquitination and degradation	Western blotting and FACS	Cha et al, 2018
	Breast cancer and colon cancer	GSK3β phosphorylates non-glycosylated PD-L1 and induces its degradation. EGFR inhibitors improve the anti-tumor immunity.	Western blotting and FACS	Li et al, 2016
	Hepatocellular carcinoma	IL-6–activated JAK1 phosphorylates PD-L1, inducing the recruitment of the N-glycosyltransferase STT3 that glycosylates and stabilizes PD-L1.	Western blotting and FACS	Chan et al, 2019

Translational and post-translational (glycosylation)	TNBC and androgen-independent prostate cancer cells	Inhibition of Sigma 1 downregulates PD-L1 by inducing its degradation	Western blotting and FACS	Maher et al, 2018
	Glioblastoma	FKBP51 catalyzes PD-L1 folding and induces its upregulation.	Western blotting and FACS	D'Arrigo et al, 2017
	Breast cancer	EMT promotes STT3 transcription through β-catenin and induces PD-L1 glycosylation and upregulation.	Western blotting and FACS	Hsu et al, 2018
	TNBC	EGR signaling activates B3GNT3 that catalyzes PD-L1 glycosylation. Targeting glycosylated PD-L1 promotes PD-L1 internalization and degradation.	Western blotting and FACS	Li et al, 2018
Translational and post-translational (ubiquitination)	Breast cancer and colon cancer	Cyclin D-CDK4 phosphorylates cullin 3-SPOP that ubiquitinates PD-L1.	Western blotting and FACS	Zhang et al, 2018
	Breast cancer	The ubiquitin E3 ligase HRD1 ubiquitinates abnormally glycosylated PD-L1.	Western blotting and FACS	Cha et al, 2018
	TNBC	β-TrCP polyubiquitinates non-glycosylated PD-L1, resulting in its degradation.	Western blotting and FACS	Li et al, 2016
	Oral squamous cell cancer	USP9X deubiquitinates PD-L1, leading to its upregulation.	Western blotting	Jingjing et al, 2018
	Breast cancer	CSN5 deubiquitinates PD-L1, resulting in its stabilization.	Western blotting	Lim et al, 2016
	Wide range of tumor cells and primary human dendritic cells	CMTM4/6 inhibits PD-L1 ubiquitination and enhances PD-L1 level.	Western blotting and FACS	Mezzadra et al, 2017
Translational and post-translational (palmitoylation)	CRC	ZDHHC3 silencing activates the antitumor immunity	Western blotting	Yao et al, 2019
	Breast cancer	ZDHHC9 palmitoylates PD-L1, allowing its stabilization and cell surface distribution	Western blotting	Yang et al, 2019

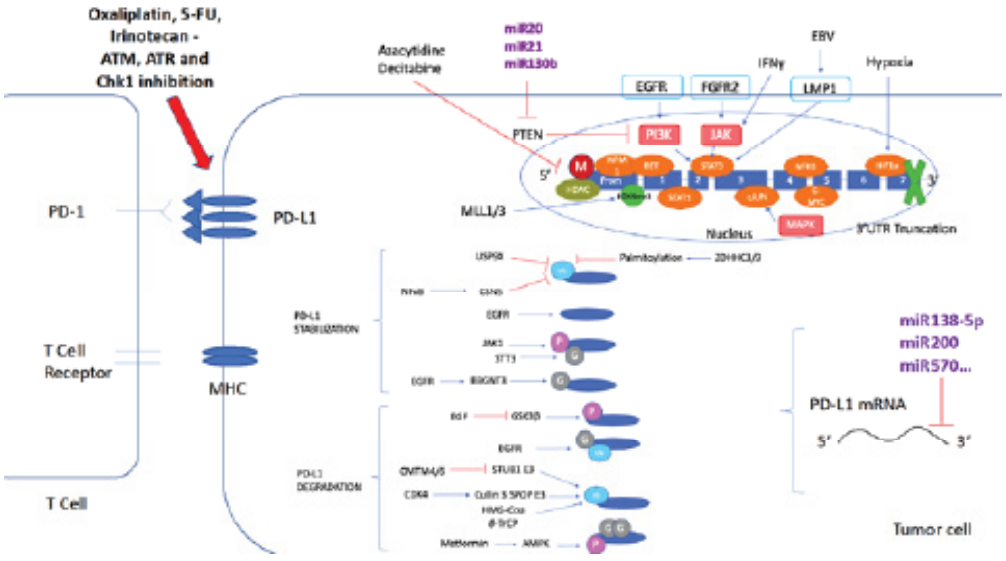

Figure 1. PD-L1 expression regulation. Schematic illustration of the genetic, epigenetic, transcriptional, post-transcriptional, translational and post-translational mechanisms that regulate PD-L1 expression in tumor cells.

Genetic mechanisms

PD-L1 is located on chromosome 9p24.1, 42 kb apart from programmed death ligand 2 (PD-L2). Amplifications and translocations are implicated in PD-L1 upregulation in several tumor types. The first descriptions of 9p copy number amplifications concerned patients with Hodgkin's lymphoma and primary mediastinal large B-cell lymphoma (PMBCL), and were correlated with increased expression of PD-L1 and PD-L2 [56]. Green [57] showed that Janus kinase 2 (JAK2), which is also located on chromosome 9p and is involved in the transduction of pro-inflammatory signals, modulates PD-L1 expression, and its amplification contributes to PD-L1 overexpression by enhancing interferon gamma (IFN-γ) receptor signaling. This 9p amplification correlated with PDL1 overexpression was confirmed in PMBCL and NSCLC patients [14,15]. Moreover, a rare 9p24.1 copy-number gain was identified in a patient with CRC who was refractory to the currently used therapies, but showed a partial but durable response upon treatment with pembrolizumab. Hence, anti-PD1 antibodies could be used in patients with CRC carrying this mutation [16].

Several studies showed that truncation of the 3′UTR (untranslated region) of the PD-L1 gene is associated with aberrant PD-L1 expression in various cancers, including adult T-cell leukemia/lymphoma, diffuse large B-cell lymphoma, and stomach adenocarcinoma. In agreement, deletion of the 3'UTR of the PD-L1 gene by CRISPR/Cas9 increases PD-L1 mRNA stability in human and murine cells. In mice, PD-L1 upregulation by 3'UTR loss promotes tumor growth and escape from immune surveillance mediated by cytotoxic T lymphocytes. On the other hand, PD-L1 blockade restores CD8+ cytotoxic T lymphocytes and the anti-cancer immune surveillance [17].

These findings suggest that genomic alterations that upregulate PD-L1 could potentially be used as a genetic marker to identify patients who may better respond to PD-(L)1 immune checkpoint blockade [58].

Epigenetic mechanisms

Epigenetic alterations influences cancer progression, immune surveillance, and tumor cell evasion. Aberrant DNA methylation is frequently observed in cancer. As in NSCLC cell lines, incubation with azacytidine, a DNA-demethylating agent, upregulates PD-L1 expression, Wrangle et al. [18] hypothesized that combining azacytidine and an immune checkpoint inhibitor might improve treatment response in NSCLC. In AML patients, Yang et al showed that decitabine treatment induce PD-L1 promoter demethylation associated with its upregulation. In addition, a recent international cohort study of 142 high-grade NSCLC samples discovered a DNA methylation signature that seems to predict which patients may benefit from anti-PD1 treatments. The BET (bromodomain and extra-terminal domain binding acetylated lysine motifs) inhibitor JQ1 suppresses PD-L1 expression on tumor cells, dendritic cells and macrophages. In a mouse model of ovarian cancer, suppression of PD-L1 expression by JQ1 delays tumor progression by enhancing the anti-tumor cytotoxic activity of T cells [30].

Lu et al. [20] demonstrated that the histone methyltransferase MLL1 binds to and catalyzes tri-methylation of histone H3 on lysine 4 on the PD-L1 promoter, and activates PD-L1 transcription in pancreatic cancer cells. Moreover, the histone methyltransferase MLL3 is also a regulator of PD-L1 expression in prostate cancer cells [21]. In 383 patients with CRC, Goltz et al. [22] showed that PD-L1 promoter methylation is inversely correlated with PD-L1 mRNA expression and is associated with reduced overall survival and recurrence-free survival. They suggested that inhibiting PD-L1 promoter methylation may be used to modulate PD-L1 expression and sensitize tumors to immunotherapy.

Transcriptional mechanisms

Various transcription factors, such as STAT3, NF-κB, hypoxia inducible factor 1α (HIF-1α) and c-Myc, regulate PD-L1 expression. These signaling pathways control PD-L1 expression by activating several key transcription factors that bind to PD-L1 promoter region to promote its expression.

NF-κB is involved in tumor development by promoting cell survival, proliferation, angiogenesis, and metastasis formation. Activation of NF-κB signaling upregulates PD-L1 to suppress the anti-tumor immunity.

In breast cancer cells, phosphatase and tensin homolog (PTEN) represses PD-L1 transcription and expression [25]. When PTEN is downregulated, PI3K may be activated. This process increases PD-L1 expression in glioma and lung squamous cell carcinoma, leading to immune resistance and escape [26]. Moreover, in human melanoma, PI3K activation can induce PD-L1 expression through the transcription factor STAT3 [27].

HIF-1α is a major cancer driver. It seems that hypoxic environments, which induce activation of HIF-1α and accumulation of lactate, contribute to evasion of tumor cells from the immune system. Several studies indicate that hypoxia induces PD-L1 expression in tumor cells, myeloid-derived suppressor cells, dendritic cells, and macrophages [28].

The transcription factor c-Myc, frequently amplified in cancer, induces the expression of many genes involved in cell proliferation, growth, differentiation, and apoptosis. Inactivation of c-Myc in mouse tumor models leads to complete tumor clearance and recruitment of CD4+ T cells. c-Myc can directly target PD-L1. Recent results also showed that c-Myc expression is significantly correlated with PD-L1 expression in patients with NSCLC with poor clinical outcome [29].

PD-L1 expression is higher in triple negative breast cancer (TNBC) than in the other subtypes. Qin et al. [31] discovered that nucleophosmin (NPM1) binds to the PD-L1 promoter in TNBC cells and activates PD-L1 transcription, thus inhibiting T cell activity in vitro and in vivo. They also found that PARP1 suppresses PD-L1 transcription through its interaction with NPM1. In agreement, olaparib, a PARP1 inhibitor, increases PD-L1 expression in TNBC.

Mitogen-activated protein kinase (MAPK) signaling pathways are frequently over-activated in human cancers. In melanoma cell lines, the MAPK pathway increases PD-L1 expression through the activation of the transcription factor c-JUN. Recent publications showed that the MAPK signaling pathway promotes tumor development and immunotherapy resistance and that its inhibition improves the anti-tumor response and decreases PD-L1 expression in tumor cells [27,32].

STAT3 is a transcription factor that upregulates PD-L1 expression. Upon IFN-γ stimulation, the JAK-STAT signaling pathway can activate interferon regulatory factor 1 (IRF1) that binds to the PD-L1 promoter and induces PD-L1 transcription in EBV-positive gastric carcinoma [33].

A recent study reported that in medulloblastoma, cyclin-dependent kinase 5 (CDK5) facilitates immune surveillance evasion. CDK5 is required for IFN-γ-induced PD-L1 upregulation, and CDK5 depletion downregulates PD-L1 expression in tumor cells [34].

In melanoma, inhibition of histone deacetylases (HDAC), which are epigenetic modifiers, reduce PD-L1 levels. PD-L1 expression is regulated by HDAC6 through STAT3. HDAC6 and STAT3 are recruited at the PD-L1 gene promoter. When HDAC are abrogated

genetically or pharmacologically, PD-L1 is downregulated [35].

In NSCLC, the EGFR pathway promotes PD-L1 expression through the IL-6/JAK/STAT3 signaling axis, and this effect can be limited by treatment with EGFR inhibitors [36].

In CRC, fibroblast growth factor receptor 2 (FGFR2) is frequently overexpressed and associated with poor survival. Li et al. showed that FGFR2 induces PD-L1 expression via the JAK/STAT3 signaling pathway in human CRC cell lines [37].

Post-transcriptional mechanisms

MicroRNAs (miRNAs) are post-transcriptional regulators of gene expression, including PD-L1. For instance, miR-513 suppresses PD-L1 translation by direct binding to its 3'UTR [38]. Similarly, miR-155, induced by TNFa and IFNg in human dermal lymphatic endothelial cells and miR-34a in AML and NSCLC suppress PD-L1 expression by direct binding to its 3'UTR [39]. In NSCLC, p53 seems to suppress PD-L1 expression through miR-34 [40]. Moreover, PD-L1 expression can be suppressed by miR-142-5p, miR-93, and miR-106b in pancreatic cancer [59,60], by miR-138-5p in CRC [61], by miR-217 in laryngeal cancer [62], by miR-200 in NSCLC [63,64] and gastric cancer, by miR-152 [65] and miR-570 in gastric cancer [66], by miR-17-5p in melanoma [67], and by miR-15a, miR-193a, and miR-16 in malignant pleural mesothelioma [68]. Finally, miRNAs can influence PD-L1 expression in an indirect manner. For instance, in CRC, miR-20, miR-21, and miR-130b increase PD-L1 expression by repressing PTEN [41].

Translational and post-translational modifications

Protein post-translational modifications (PTMs) include phosphorylation, glycosylation, ubiquitination, palmitoylation, methylation, SUMOylation, and acetylation. These modifications regulate protein activity, stability, localization, trafficking, and interaction with binding partners (protein, DNA, RNA and lipids) [50,69,70]. PD-L1 regulation by PTMs is summarized here but has been thoroughly described in two previous reviews [47,71].

Phosphorylation affects the protein conformation, activity and interactions. Activation of AMP-activated protein kinase (AMPK) by metformin induces PD-L1 phosphorylation at S195 that leads to its abnormal glycosylation and degradation in the endoplasmic reticulum [42]. GSK3β is a multifunctional switch that mediates the direct phosphorylation of many substrates and plays a role in cancer resistance to therapy [72]. Li and colleagues found that in breast cancer cells, GSK3β phosphorylates non-glycosylated PD-L1 and induces its degradation. Moreover, EGF signaling inhibits GSK3β and promotes PD-L1 stabilization. In agreement, gefitinib (an EGFR inhibitor) induces PD-L1 expression in CRC [50]. Interleukin-6 (IL-6) is one of the major cytokines linking inflammation and cancer [73]. In hepatocellular carcinoma, IL-6 activates the JAK1 kinase, which in turn phosphorylates PD-L1, leading to its stabilization [44].

N-glycosylation determines the protein structure and function. PD-L1 is N-glycosylated at N35, N192, N200, and N219 in most cells [43,74]. And this N-glycosylation is necessary for its interaction with PD-1 on T-cells. Most PD-L1 is glycosylated and more stable. By western blotting, glycosylated PD-L1 is detected as a 45-55 kDa band and the non-glycosylated form as a 33 kDa band. It has been proposed that PD-L1 expression level could be used as a biomarker of the response to anti-PD-(L)1 antibodies, but results are controversial. This might be explained by the fact that the antibodies used for PD-L1 detection recognize the non-

Table 2. Chemotherapies and PD-L1 expression.

Chemotherapeutic agents and combination	PD-L1 expression	Methods	Model	References
Oxaliplatin	Increases membrane PD-L1 expression. Sensitizes tumors to immune checkpoint blockade.	FACS	Various cancer cell lines including CRC	Park et al, 2019
Anti-PD-L1 antibody + oxaliplatin	Increases survival of mice. Inhibits tumor growth	FACS	CRC cell line	Golchin et al, 2019
5-FU	Enhances total and cell surface PD-L1 expression	Western blotting and FACS	CRC cell line	Goel et al, 2016
FOLFOX + anti-PD-1 antibody	Induces PD-L1 expression and high CD8+ T-cell infiltration in the tumor microenvironment	FACS and immunohistochemistry	CRC	Dosset et al, 2018
Irinotecan	Increases PD-L1 expression on tumor cells and sensitizes tumors to immunotherapy	FACS	CRC, melanoma, glioma cell lines	Derer et al, 2016
Anti-PD-L1 antibody + irinotecan	Increases the anti-tumor activity. Enhanced proliferation of CD8+ cells in tumors and lymph nodes.	FACS	Breast cancer	Iwai, 2018
Nivolumab after fluoropyrimidine and oxaliplatin or irinotecan	Durable response rates and prolonged survival (26%). Improvement in functioning, symptoms, and quality of life.		MSI CRC	Overman et al, 2018

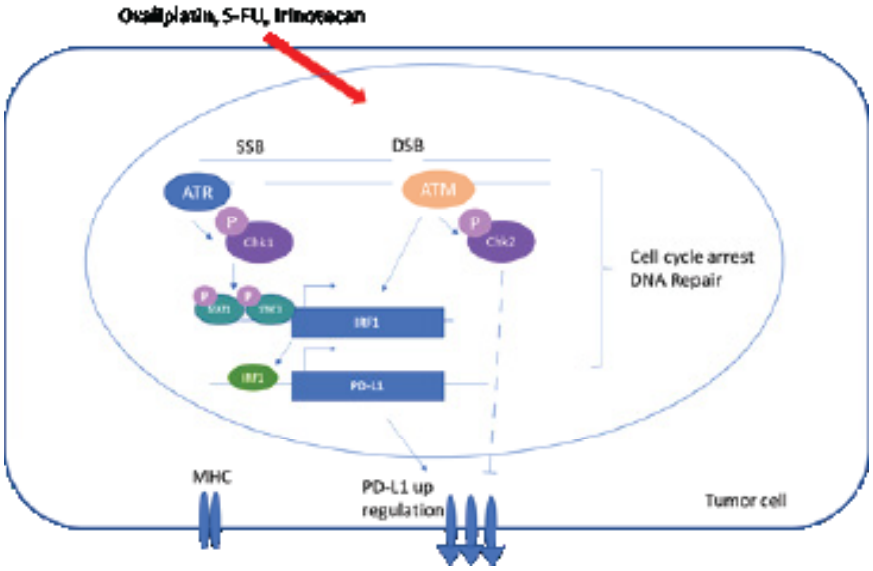

Figure 2. Schematic illustration of PD-L1 regulation through ATM and ATR pathways.

glycosylated form, and this can lead to false negative results. Conversely, an antibody against glycosylated PD-L1 might improve its detection and predictive potential [75]. Furthermore, targeting the glycosylated form promotes PD-L1 internalization and degradation, leading to eradication of TNBC cells [48]. Sigma 1, which plays a role in cellular protein homeostasis, prevents PD-L1 degradation by directly interacting with glycosylated PD-L1 in TNBC and androgen-independent prostate cancer cells [45]. In glioblastoma, FKBP51 participates in PD-L1 upregulation at the plasma membrane by allowing protein folding that is necessary for glycosylation [46]. PD-L1 phosphorylation by JAK 1 induces the recruitment of the N-glycosyltransferase STT3 that glycosylates and stabilizes PD-L1 [44]. This association between phosphorylation and glycosylation was also demonstrated by Cha et al. [44]. Furthermore, epithelial-mesenchymal transition (EMT) can induce STT3 transcription and PD-L1 upregulation [47]. EGR signaling activates b-1,3-N-acetylglucosaminyl transferase (B3GNT3) that catalyzes PD-L1 glycosylation and stabilization, and ultimately inhibits the anti-tumor immunity [48]. Thus, targeting glycosylated PD-L1 or glycosylation mechanisms is a good strategy to improve anti-tumor immunity.

PD-L1 metabolism is regulated by ubiquitination-dependent proteasomal degradation [76,77]. The E3 ubiquitin ligases CBL-B and C-CBL are involved in PD-L1 downregulation in NSCLC that express wild type EGFR [78,79]. Moreover, it was recently reported that the cyclin D-CDK4 complex destabilizes PD-L1 via the E3 cullin 3-SPOP, which is involved in PD-L1 ubiquitination. In agreement, in a mouse model of CRC, the CDK4/6 inhibitor palbociclib increases PD-L1 level [58]. Metformin-induced PD-L1 phosphorylation at S195 allows PD-L1 degradation via ubiquitination by the E3 ligase HMG-CoA reductase degradation protein 1 (HRD1) [42]. Li and colleagues found that β-TrCP, a subtype of E3 ligase, can polyubiquitinylate non-glycosylated PD-L1 after phosphorylation by GSK3β, leading to its degradation. EGFR signaling can reverse degradation of non-glycosylated PD-L1 by inhibiting GSK3β [43]. However, EGF treatment induces ubiquitination of glycosylated PD-L1 [77]. Thus, EGFR signaling seems to have different roles on PD-L1, depending on its glycosylation status.

PD-L1 de-ubiquitination and stabilization affect the inflammatory response. In oral squamous cell cancer, ubiquitin-specific peptidase 9 X-linked (USP9X) deubiquitinates PD-L1, resulting in its upregulation [51]. Similarly, COP9 signalosome 5 (CSN5) promotes PD-L1 de-ubiquitination in breast cancer. TNF-α activates NF-κB that induces CSN5 expression and consequently PD-L1 stabilization, while Curcumin, a CSN5 inhibitor, inhibits PD-L1 stabilization, particularly in CRC [52]. Finally, in a wide range of human tumor cells and in primary human dendritic cells, the type-3 transmembrane proteins CMTM4/6 enhance the PD-L1 protein pool by inhibiting the E3 ligase STUB1 [53]. Hence, targeting these ubiquitination/de-ubiquitination actors may represent a new therapeutic strategy.

Ubiquitination is linked to another PTM: palmitoylation, a lipid modification that regulates cell membrane proteins [80]. Recently, it was reported that two palmitoyl transferases, ZDHHC3 [54], and ZDHHC9 [55], palmitoylate PD-L1 and block its ubiquitination. This mechanism leads to PD-L1 stabilization, cell surface distribution, and immune escape of tumor cells. In in vitro and in vivo CRC models, ZDHHC3 silencing improves the antitumor immunity [55].

Chemotherapy and PD-L1 regulation

PD-L1 is overexpressed in many tumor types and this allows tumor cells to escape the host T-cell immunity [81]. Recent evidence indicates that chemotherapy may regulate PD-L1 expression on cancer cells, and thus influence the anti-tumor immunity and immune evasion [82]. Here, we will summarize the effect of the chemotherapeutic molecules used in CRC on PD-L1 expression. In addition, we will discuss how these molecules may be combined with anti-PD-(L)1 therapies (Table 2 and Figure 2).

Oxaliplatin is a platinum-based chemotherapeutic agent that reduces cancer cell proliferation and induces cancer cell death. This DNA alkylating drug can also promote the anti-tumor immune responses by converting an immune-suppressive tumor microenvironment into an immune-favorable environment [90]. Flow cytometry analysis showed that oxaliplatin increases membrane PD-L1 expression in different cancer cell lines, including CRC. By stimulating the anti-tumor activity, oxaliplatin may sensitize tumors to immune checkpoint blockade, including anti-PD-1/PD-L1 antibodies [83,91].

Golchin et al. [84] showed that an anti-PD-L1 antibody and oxaliplatin combination in BALB/c mice harboring CT26 CRC cell xenografts increased survival compared with monotherapy. Moreover, this combination inhibited tumor growth by enhancing T-cell activation, suppressing Treg cell development, and increasing tumor-infiltrating lymphocytes and the CD8+/Treg and CD4+/Treg cell ratios. The anti-PD-L1 antibody enhanced oxaliplatin-induced immune activity against the tumor [84].

5-FU is an anti-metabolite prescribed to patients with CRC, in combination with other cancer drugs, but resistance is frequent. This anti-metabolite promotes the proliferation and cytotoxicity of tumor-infiltrating CD8+ T cells. However, after repeated cycles, immunosuppressive factors, such as IL-10, are released and affect 5-FU anti-tumor efficacy [92]. Moreover, repeated 5-FU cycles repress the anti-tumor immune functions and upregulates PD-L1 on tumor cells [90]. Goel and colleagues show that in

Table 3. DNA damage and PD-L1 expression.

DNA damage sensors	Treatments	PD-L1 expression	Method to quantify PD-L1 expression	Model	References
ATR, ATM, CHK1	Ionizing radiation and etoposide	PD-L1 upregulation	Western blotting and FACS	Osteosarcoma, lung cancer, and prostate cancer cell lines	Sato et al, 2017
	Treatment with IFN-γ	The JAK-STAT-IRF1 pathway regulates PD-L1 expression	Western blotting and FACS	Melanoma	Gardia Diaz et al, 2017
ATR		ATR inhibitor down-regulates PD-L1 levels and enhances the anti-tumor immune responses	Western blotting and FACS	Breast, lung, cervical cancer	Sun et al, 2018
ATM		Increased INF type 1 and PD-L1 expression.	Western blotting	Pancreatic cancer	Zhang et al, 2019
Chk1	Anti-PD-L1 antibody + SRA737 + gemcitabine	Anti-tumor response, increased anti-tumorigenic CD8+ cytotoxic T cells, dendritic cells, and expression of IFN-β and chemokines	Western blotting and FACS	SCLC	Sen et al, 2019

CRC cell lines, 5-FU enhances the total and cell surface PD-L1 expression. Therefore, it would be interesting to combine 5-FU with anti-PD-(L)1 agent to limit 5-FU-induced chemoresistance and enhance its anti-cancer activity [85]. Moreover, in 2016, Wu et al. [93] suggested that a single cycle of 5-FU promotes the anti-tumor immune response, whereas repeated chemotherapy cycles impair the anti-tumor immune functions. This effect highlights the importance of using immunotherapy to improve the anti-tumor effect.

Dosset et al. [86] found that the combination of 5-FU and oxaliplatin (Folfox) associated with an anti-PD-1 antibody can cure Balb/c mice harboring CT26 CRC cell xenografts and C57BL/6 mice bearing MC38 CRC xenografts. In patients with CRC, Folfox induces PD-L1 expression and high CD8+ T-cell infiltration in the tumor microenvironment. Altogether, these results strongly support the use of immune checkpoint blockade in combination with chemotherapy agents, like Folfox [86,94].

Irinotecan is a topoisomerase 1 inhibitor used in many solid cancers. It stabilizes topoisomerase 1-DNA covalent complexes that lead to DNA double strand breaks (DSB) and cytotoxic damage [90]. Irinotecan may be used in combination with immunotherapy due to its ability to modulate the tumor immune microenvironment. Indeed, irinotecan reduces the abundance of regulatory T cells in lymph nodes and tumors and of myeloid-derived suppressor cells, leading to the production of IFN-γ by tumor-specific CD8+ T cells and their proliferation. Moreover, irinotecan increases PD-L1 expression on tumor cells [87]. These immunomodulating activities suggest that the combination of irinotecan with an anti-PD-L1 molecule may be effective in patients with CRC. Indeed, in a murine model of breast cancer [88], the combination of irinotecan with an anti-PD-L1 antibody showed a significantly higher anti-tumor activity than each agent alone. In an ongoing phase 2 clinical trial (NCT02060188) on MSI metastatic CRC, nivolumab is used in patients who are intolerant to first-line of treatment with 5-FU and oxaliplatin or irinotecan. Promising and durable response rates with prolonged survival have been observed in 26% of patients. The authors also reported clinically meaningful improvement in functioning (emotional, role, and social), symptoms (fatigue, pain, insomnia, appetite loss, constipation, and diarrhea), and quality of life [89].

DNA damage and PD-L1 regulation

Oxaliplatin, 5-FU and irinotecan exert their cytotoxic effects by inducing DNA damage, including DSBs. DNA DSBs are very toxic and may lead to cell cycle arrest and cell death [95]. The detection and repair of DNA damage is a key process for maintaining genomic integrity [96]. In cells, the DNA Damage Response (DDR) is a network of signaling cascades that detect DNA damage and transmit the damage signal to cell cycle checkpoints and DNA repair pathways. DDR may induce cell cycle arrest, cell death, or activation of DNA repair pathways, including homologous recombination (HR), MMR, or non-homologous end joining [97,98]. Hence, DDR ensures genomic stability and cell viability.

Table 4. Ongoing clinical trials that test the combination of anti-PD-(L)1 antibodies with other treatments.

clinical trial gov Identifier	Nom	Cancer	anti-PDL1	anti-PD1	anti-CTLA4	combinaison	MSI/MSS	patients	phase
NCT02777710	MEDIPLEX	CCR, pancreas, metastatic Cancer, advanced cancer	Durvalumab			CSF-1R (TKI) Pexidartinib		48	I
NCT03202758	MEDITREME	mCRC	Durvalumab			Tremelimumab (anti-CTLA4)			I/II
						FOLFOX			II
NCT03608046	AVETUXIRI	CRC	Avelumab			cetuximab irinotecan	MSS	59	
NCT03228667	QUILT-3.055	CCR + 12 different cancers	Avelumab Atezolizumab	Pembrolizumab Nivolumab		ALT-803 (IL15 superagonist)		611	II
NCT03993626	CAROSELL	mCRC		Nivolumab		CXD101 (histone deacetylase inhibitor)	MSS, MMR-proficient	55	I/II
NCT02437136		NSCLC, expansion NSCLC, Melanoma and CRC		Pembrolizumab		Entinostat (Class I HDAC inhibitor)	MMR proficient	202	I/II
NCT03891953		CRC (and 4 other cancers)		PDR-0001		DKY709	MSS	320	I
NCT03206073		CRC	Durvalumab		Tremelimumab	Pexa-Vec (oncolytic virus)		35	I/II
NCT03854799	AVANA	CRC	Avelumab			Capecitabine, external Beam irradiation		101	II
NCT02842125		Liver metastases of solid tumors (CRC)		Pembrolizumab Nivolumab		Capecitabine, Ad-p53		24	I/II
NCT03645876		mCRC		SHR1210		BP102 (Bavacizumab), axaliplatin, capecitabine		12	II
NCT02837263		mCRC		Pembrolizumab		Stereotactic body radiotherapy		18	I
NCT02837263	DAPPER		Durvalumab			Olaparib, Cediranib	MMR proficient	90	II
NCT04060342		pancreatic cancer, expansion phase on 7 cancer dont CCR		Pembrolizumab		GB1275 (CD11b modulator), Nab-paclitaxel, gemcitabine	MSS	202	I/II
NCT03642067		CRC		Nivolumab		Relatlimab LAG3 inhibitor)	MSS	64	II

NCT03085914	ECHO-207/KEY-NOTE-723	Solid tumors, dont CRC		Pembroli-zumab		Epacadostat (IDO1 inhibi-tor), FOLFOX		70	I/II
NCT03631407	MK-7690-046	CRC		Pembroli-zumab		vicriviroc (CCR5 inhib-itor)		40	II
NCT04001101		CRC		anti-PD1		Radiotherapy	MSI-High	140	II
NCT04140526	PRE-SERVE-001	8 cancers dont mCRC		Pembroli-zumab	ONC-392			91	I
NCT02903914		Solid tumors dont CRC		Penbroli-zumab		INCB001158 (arginase inhibitor)		424	I/II

DDR may also be involved in resistance to chemotherapy. Indeed, DDR-deficient tumors may acquire mutations in genes that confer therapy resistance [99]. For instance, HR deficiency can cause genomic instability that promotes the acquisition of additional genetic alterations that may induce resistance to therapy [100].

DDR is controlled by phosphatidyl-inositol 3-kinase-related kinases, such as ataxia telangiectasia-mutated (ATM) and DNA-dependent protein kinase catalytic subunit (DNA-PKcs) that are activated upon DNA DSB occurrence, and also Ataxia telangiectasia and Rad3 related (ATR) that responds to various types of DNA lesions by interacting with single-stranded DNA. These kinases play critical roles in the DDR and link DNA damage sensing to DDR effectors that regulate cell cycle progression and DNA repair [101,102].

ATR and ATM are serine threonine kinases that phosphorylate proteins involved mainly in DNA repair and cell cycle regulation [103]. The cellular responses to DNA damage are regulated by the ATM-Chk2 and ATR-Chk1 pathways that are activated upon DNA single-strand breaks (SSB) and DSBs. In response to DSBs, cells emit a signal that activates cell cycle checkpoints and stops the cell cycle. ATM participates in the ATR-Chk1 pathway activation by promoting the formation of single-stranded DNA through nucleolytic resection, which leads to initiation of DNA repair by HR [104].

Recent data established that DNA damage induces the expression of PD-L1 [10] (Table 3 and Figure 2).

Sato et al. [105] demonstrated that ionizing radiation and etoposide (a topoisomerase II inhibitor) induce PD-L1 upregulation through ATM, ATR and Chk1. This process depends on IRF1 signaling through the phosphorylation of the transcription factors STAT1 and STAT3. In melanoma cells, Gardia et al. [106] found that the JAK–STAT–IRF1 pathway regulates PD-L1 expression after incubation with IFN-γ. Based on these results, Sato et al. [105] found that DSB formation upregulates IRF1, STAT1 and STAT3 phosphorylation in osteosarcoma and NSCLC cell lines. They showed that DSB-dependent PD-L1 upregulation is mediated by the IRF1 pathway.

Sun and colleagues studied the effects of ATR inhibitors, such as AZD 6738 and VE-822, on the immune response in the tumor microenvironment. They confirmed that DNA damaging agents significantly induce cell surface PD-L1 expression in different cancer cell types and demonstrated that this effect is abolished upon ATR inhibition. These data suggest that ATR inhibitors downregulates PD-L1 levels and enhances anti-tumor immune responses. It would be interesting to combine ATR inhibitors with immune checkpoint blockade [69].

We recently performed a shRNA-based loss-of-function genetic screen using a kinome library to identify molecular targets involved in oxaliplatin resistance. We found that ATR silencing restored oxaliplatin sensitivity in oxaliplatin-resistant CRC cells. To overcome drug resistance, we used the ATR inhibitor VE-822 in combination with oxaliplatin (VOX) and observed a strong synergistic effect in different oxaliplatin-sensitive CRC cell lines and in their oxaliplatin-resistant clones. The VOX combination promoted DNA SSB and DSB formation, growth arrest, apoptosis, replicative stress, cytoplasmic DNA, and signals related to immunogenic cell death. In a syngeneic CRC mouse model, administration of VOX significantly increased survival by promoting anti-tumor T-cell responses. As oxaliplatin upregulates PD-L1 and ATR inhibitors downregulate PD-L1, it would be interesting to study VOX effect on PD-L1 expression [108].

In pancreatic cancer, Zhang et al. [25] evaluated the effect of ATM inhibition on tumor immunogenicity. ATM pharmacological and genetic inhibition increased IFN type 1 expression. Moreover, ATM silencing also increased PD-L1 expression and the tumor sensitivity to PD-L1 blocking antibodies. This suggests that the efficacy of immune checkpoint blockade in pancreatic cancer could be increased by inhibiting ATM.

In patients with Small Cell Lung Cancer (SCLC), treatment with the CHK1 inhibitor SRA737 in combination with an anti-PD-L1 antibody leads to an anti-tumor response. When low doses of gemcitabine were associated with this combination, anti-tumor CD8+ cytotoxic T cells, dendritic cells, and expression of IFN-β and chemokines were increased in a SCLC model. Thus, it would be interesting to test the combination of anti-PD-L1 antibody, CHK1 inhibitor and gemcitabine in patients with SCLC [107].

Altogether, these findings suggest that targeting the DDR molecular machinery with inhibitors may enhance the efficacy of the current cancer treatments that generate DNA damage.

PD-1/PD-L1 targeted therapies for colorectal cancer

Immune checkpoint inhibitors revolutionized the field of immuno-oncology [109]. These inhibitors disrupt co-inhibitory signaling pathways and promote the immune-mediated elimination of tumor cells. Nivolumab and pembrolizumab are the first two FDA-approved monoclonal antibodies against PD-1. These antibodies interfere with PD-1 and PD-L1 interaction that downregulates T cells, allowing cancer cells to evade immune surveillance. Anti-PD-1 therapies have shown great results in melanoma, NSCLC and TNBC [110]. Following these positive results, several clinical trials have been and are currently performed to test anti-PD-1 monoclonal antibodies in CRC.

As discussed earlier, CRC are characterized by their MSS or MSI status. Loss of function of MMR genes, such as MLH and MSH, and alterations in microsatellite size result in MMR deficiency and microsatellite instability. Moreover, loss of DNA repair fidelity

drives mutagenesis, the production of tumor-specific neoantigens, and the activation of the host anti-tumor immune response. For these reasons, the response to immune checkpoint inhibitors and survival rate are better in patients with MSI CRC (Darvin et al, 2018). On the other hand, immune checkpoint inhibitors show unsatisfactory results in MSS CRC that display very poor intra-tumoral immune cell infiltrate and are called cold tumors.

Chemotherapy upregulates PD-L1 and contributes to T-cell function suppression and immune escape. Chemotherapy can also activate immune cell death and the anti-tumor immunity. Thus, chemotherapy may render poorly immunogenic tumors more sensitive to anti-PD-(L)1 immunotherapy by transforming a cold immune environment into a hot one [110-113]. The challenge is now to convert cold tumors into hot tumors by using anti-PD-L1 molecules in combination with other drugs, such as chemotherapy or tyrosine kinase inhibitors. A search of the ClinicalTrials.gov database indicates that 24 ongoing clinical trials are testing the combination of anti-PD-(L)1 antibodies with other treatments, mostly in MSS CRC (Table 4), to identify the best drug combination(s) to turn a cold tumor into a hot one [114-120].

Nevertheless, more controlled clinical trials on anti-PD-(L)1 therapy are required to improve the long-term survival of patients with CRC. Given the large diversity of cytotoxic drugs and targeted small molecules, the possibilities of combinations are extremely diversified [90].

Conclusion

Immune checkpoint blockade therapy targeting PD-(L)1 shows promising results in different cancer types, including CRC. Understanding the regulation of PD-L1 expression may improve its efficacy. Moreover, different chemotherapeutic agents are currently evaluated to enhance the efficacy of immune checkpoint blockade therapy. The use of PD-L1 as a biomarker is still controversial. However, several studies suggest that PD-L1 is associated with bad prognosis. Currently, many cancer treatments induce PD-L1 expression, and many ongoing trials are exploring combination strategies. Hence, it would be interesting to identify and test treatments that do not enhance PD-L1 expression to limit cancer immune escape. Furthermore, targeting the DDR molecular machinery with inhibitors may enhance the efficacy of the current cancer treatments that generate DNA damage and increase the T-cell immune response.

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