Review Article
Emerging role of PD-L1 modification in cancer immunotherapy

Xiaoli Hu1, Zixia Lin1, Zhiwei Wang2, Qiangyong Zhou1

1Department of Gynecology, The First Affiliated Hospital of Wenzhou Medical University, Wenzhou 325027, Zhejiang, China; 2Department of Obstetrics and Gynecology, The Second Affiliated Hospital of Wenzhou Medical University, Wenzhou 325027, Zhejiang, China

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Abstract: Accumulating evidence demonstrates that the expression levels of programmed cell death protein 1 (PD-1) and programmed death ligand 1 (PD-L1) are regulated at the various levels, including transcription, post-transcriptional modification and post-translational modifications (PTMs). The PTMs of PD-1/PD-L1 contain phosphorylation, ubiquitination, methylation, glycosylation and palmitoylation. Recently, PD-L1 was reported to be acetylated at Lys263 site by p300 and was deacetylated by histone deacetylase 2 (HDAC2). Acetylation of PD-L1 prevented its translocation to the nucleus and led to a reduction of the nuclear portion of PD-L1, resulting in evading immune surveillance of tumor cells. In this review article, we briefly describe the PTMs of PD-1/PD-L1 and mainly summarize the novel findings of PD-L1 acetylation in tumor cells. Moreover, we discuss the associations of PD-L1 acetylation and ubiquitination, phosphorylation and methylation. Furthermore, we highlight that targeting acetylation of PD-L1 by HDAC inhibitors might be useful for enhancing tumor immunotherapy.

Keywords: Acetylation, PD-1, PD-L1, ubiquitination, immunotherapy

Introduction
Programmed cell death protein 1 (PD-1, also known as CD279), one of coinhibitory receptors, is expressed on the surfaces of multiple types of immune cells, including B cells, natural killer (NK) T cells, CD4+ T cells, CD8+ T cells, dendritic cells and tumor infiltrating lymphocytes [1, 2]. There are two ligands of PD-1: programmed death ligand 1 (PD-L1, also named CD274 or B7-H1) and PD-L2 (also called CD273 or B7-DC) [3]. PD-L1 is expressed on hematopoietic cells, including T cells, B cells, dendritic cells, macrophages and mast cells, and non-hematopoietic healthy tissue cells such as keratinocytes, vascular endothelial cells, astrocytes, islet cells, placental syncytiotrophoblasts, endothelial cells and corneal epithelium. Both PD-L1 and PD-L2 can be expressed in tumor cells and tumor stroma [4]. The interaction of PD-1 and PD-L1 transmits inhibitory signals to T cells so that the tissue can maintain self-tolerance and avoid immune-mediated tissue damage [5]. PD-1 is a 288 amino acid type 1 transmembrane protein that is composed of extracellular domain, a transmembrane domain and a cytoplasmic domain containing immunoreceptor tyrosine-based switch motif (ITSM) and immunoreceptor tyrosine-based inhibitory motif (ITIM), while PD-L1, a 290 amino acid type 1 transmembrane protein, consists of a short cytoplasmic tail without typical signal motif [4, 5]. The interaction between the extracellular domain of PD-1 and PD-L1 leads to the conformational change of PD-1 and tyrosine phosphorylation in the cytoplasmic domain of PD-1, resulting in an increased connection between tyrosine phosphatase of SHP-2 and ITSM. SHP-2 recruitment results in a reduction of phosphorylation of TCR molecules, decreasing stimulation of downstream signals of TCR and inhibiting T cell responses. The PD-1/PD-L1 pathway eventually reduces the production of cytokines, such as IFN-γ, and cell survival proteins, such as Bcl-xl, leading to dysfunction or apoptosis of T cells [4-6]. PD-L2 is also a negative regulator of T cell activation and is highly expressed on tumor cells and antigen presenting cells [7].
PD-L1 post-translational modifications

Evidence has demonstrated that PD-1/PD-L1 expression is regulated at different levels, such as transcription, post-transcriptional modification and post-translational modifications (PTMs) [8, 9]. Regarding PTMs, PD-1/PD-L1 has been reported to be modulated by phosphorylation, ubiquitination, glycosylation, methylation, palmitoylation and acetylation [9, 10]. In the following paragraphs, we will briefly describe these PTMs of PD-1/PD-L1 in human cancers.

**Phosphorylation of PD-L1**

AMP-activated protein kinase (AMPK) is activated by metformin, leading to PD-L1 phosphorylation at Ser195 site [11]. Knockout of AMPKα abrogated this phenotype of PD-L1 phosphorylation. Moreover, PD-L1 Ser195 phosphorylation might occur in the ER lumen and block its ER-to-Golgi translocation, leading to ER-mediated PD-L1 degradation [11]. The Janus kinase 1 (JAK1) could phosphorylate PD-L1 at Tyr112 site, resulting in enhancement of STT3A association with PD-L1 and promotion of PD-L1 glycosylation to stabilize the PD-L1 stability [12]. In addition, glycogen synthase 3 beta (GSK3β) binds and phosphorylates PD-L1 at T180 and S184 sites [13].

**Glycosylation of PD-1/PD-L1**

PD-L1 N-glycosylation at N192, N200 and N219 maintains stability of PD-L1 via preventing GSK3β-involved degradation of PD-L1 and inhibits T cell activity [13]. Tunicamycin, an N-linked glycosylation inhibitor, can remove the glycosylation of PD-L1 in cells. Moreover, EGF signaling pathway promoted PD-L1 glycosylation [13]. Targeting glycosylated PD-L1 prevents the interaction between PD-1 and PD-L1, and enhances PD-L1 degradation [14]. PD-1 is also N-glycosylated and maintains stability and localization of PD-1 in T cells [15]. TCR activation induces PD-1 glycosylation, especially at the N58 site, leading to enhanced PD-1 stability and membrane expression, and mediating the interaction between PD-1 and PD-L1 [15].

**Ubiquitination of PD-L1**

Several E3 ligases have been reported to target PD-L1 for ubiquitination and degradation, including beta-transducin repeats-containing protein (β-TrCP) [13, 16], SPOP (speckle-type POZ protein) [17-19], STIP1 homology and U-box containing protein 1 (STUB1) [20], and HMG-CoA reductase degradation protein 1 (HRD1) [11, 21]. F-box protein 38 (FBXO38) targets the PD-1 for ubiquitination and degradation, leading to regulating cancer immunotherapy [22]. Kelch like family member 22 (KLHL22) also participates into PD-1 degradation and regulates antitumor function of T cells and tumor progression [23]. In addition, ubiquitin specific peptidase 22 (USP22) and ubiquitin specific peptidase 9 X-linked (USP9x) induce deubiquitination of PD-L1 and maintain its stabilization [24-26].

**Palmitoylation of PD-L1**

Multiple investigations demonstrate that Zinc finger DHHC-type palmitoyltransferase 3 (ZDHHC3) and ZDHHC9 induce PD-L1 palmitoylation and stabilize its protein activity, leading to tumor growth promotion [27, 28]. 2-bromo-palmitate, a palmitoylation inhibitor, decreases the PD-L1 protein level, indicating that PD-L1 could have a palmitoylation modification. Moreover, Cys272 site is validated as a key palmitoylation site of PD-L1, contributing to PD-L1 stability and blockade of the immune surveillance of T cells [28]. In addition, PD-L1 palmitoylation is observed in cisplatin-resistant bladder cancer cells [29]. Inhibition of fatty acid synthase (FASN) repressed PD-L1 palmitoylation and its expression [29]. Targeting PD-L1 palmitoylation increases the sensitivity of tumor cells to T-cell killing and retards tumor growth [27, 28].

**Acetylation of PD-L1 at Lys263**

Acetylation is an important modification of PTMs in which protein residues are added the acetyl group by acetyltransferases from acetyl coenzymes A [30, 31]. The acetyltransferases include histone acetyltransferases (HATs), lysine acetyltransferases (KATs) and Nα-acetyltransferases (NATs) [32]. The deacetylases that can catalyze the removal of acetyl group from the acetylated proteins have histone deacetylases (HDACs) and Sirtuins (SIRTs) [33]. In the following sections, we will describe the role of PD-1/PD-L1 acetylation in regulation of its stabilization and tumor immunotherapy (*Figure 1*).

One study revealed that EGF stimulation increased tyrosine phosphorylation and acetylation of PD-L1 in A431 cells [34]. Another study
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reported that overexpression of p300 enhanced PD-L1 acetylation, whereas depletion of p300 or p300 inhibitor A485 reduced the acetylation of PD-L1 [35]. Moreover, Lys263 site was identified as the major acetylation site on PD-L1 by p300. Lys263 acetylation mainly blocked the translocation of PD-L1 into the nucleus from the plasma membrane, but did not reduce half-lives or dimerization of PD-L1. Nuclear PD-L1 upregulation could promote cancer cells to evade immune surveillance [35]. Huntington interacting protein-1 related (HIP1R) protein targeted PD-L1 for lysosomal degradation to regulate T cell-involved cytotoxicity [36]. HIP1R interacted with PD-L1 via its C-tail, whereas Lys263 acetylation of PD-L1 blocked this interaction.

HIP1R was connected to PD-L1 and was also linked to Adaptin-β2 (AP2B1), leading to clathrin-dependent endocytosis [35]. Deacetylation of PD-L1 on the plasma membrane could bind with HIP1R and AP2B1 for endocytosis and bind with vimentin to traffic via the cytoskeleton, and enter into the nucleus by importin-α1. PD-L1 might bind to DNA and alter gene transcription, including interferon (IFN) signaling, NF-κB pathway, MHC class-I genes [35]. Moreover, this study identified that only HDAC2 deacetylase interacted with PD-L1 and reduced p300-mediated acetylation of PD-L1. HDAC2 inhibitor in combination with anti-PD-1 antibodies enhanced tumor growth repression and improved the survival in MC38 syngeneic mo-

Figure 1. A schematic diagram showing how PD-L1 is acetylated and deacetylated in tumor cells. PD-L1 is acetylated at Lys263 site by p300, leading to preventing the translocation of PD-L1 into the nucleus from the plasma membrane. HIP1R interacts with PD-L1 and AP2B1, resulting in clathrin-dependent endocytosis. Acetylation of PD-L1 blocks the binding between PD-L1 and HIP1R. HDAC2 can reduce p300-mediated acetylation of PD-L1 and increase the nuclear portion of PD-L1, leading to regulation of immune surveillance of tumor cells.
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use model [35]. Therefore, combining HDAC2 inhibitor with PD-1/PD-L1 blockade is a novel strategy for cancer immunotherapy. One HDAC2 inhibitor, santacruzamate A (SCA), and the HDAC1/2 inhibitor ACY957 upregulated PD-L1 acetylation and decreased the nuclear portion of PD-L1, alleviated the transcription of immune checkpoints, including VISTA and B7-H3, resulting in increased infiltration of CD8+ T cells in tumor microenvironment [35].

HDACs regulate histone acetylation of PD-L1 promoter region

HDACs are critically involved in regulating acetylation via removing acetyl groups from the N-acetyl lysine amino acid of histones [37]. HDACs have been classified as I, Ila, IIb, III and IV. HDAC inhibitors, including LBH589 (panobinostat), MS275 (etinostat) and MGCD0103 (mocetinostat), elevated the expression of PD-L1 in melanoma cell lines [38]. Cells obtained from patient melanomas were treated with HDAC inhibitors and demonstrated that HDAC inhibitors, especially class I HDACs inhibitors, promoted PD-L1 and PD-L2 expression [38]. LBH589 treatment increased the expression of PD-L1 and PD-L2 in C57BL/6 mice [38]. Moreover, LBH589 treatment led to higher histone acetylation at the promoter region of PD-L1 and PD-L2 in WM983A melanoma cells [38]. Furthermore, LBH589 mediated histone 3 acetylation and subsequently upregulated PD-L1 expression at mRNA, protein and gene acetylation levels. Combining LBH589 and PD-1 blocking antibody retarded tumor progression and increased survival in mice [38].

HDAC3 suppression increased PD-L1 expression and enhanced the efficacy of anti-PD-L1 treatment in B-cell lymphomas [39]. HDAC3 inhibitor (RGFP966) and SAHA elevated histone acetylation and recruitment of bromodomain-containing protein 4 (BRD4) by B-cell lymphoma 6 protein (BCL6) at the PD-L1 gene promoter, resulting in the activation of PD-L1 transcription [39]. HDAC3 inhibitors decreased DNA methyltransferase 1 (DNMT1) expression and caused activation of PD-L1 transcription [39]. HDAC3 inhibitor in combination with anti-PD-L1 treatment promoted tumor regression in murine lymphoma model [39]. Moreover, HDAC3 inhibitor reduced the mRNA and protein levels of PD-L1 via regulation of signal transducer and activator of transcription 3 (STAT3) in pancreatic cancer cells, indicating that HDAC3 inhibitors could enhance immunotherapy [40]. Moreover, HDAC3 overexpression inhibited PD-L1 expression in non-small cell lung cancer (NSCLC) cells. The lower expression of constitutive photomorphogenic 1 (COP1) elevated the accumulation of c-Jun and consequently repressed HDAC3 expression and led to promoting histone H3 acetylation of the PD-L1 promoter, resulting in high expression of PD-L1 in drug-resistant NSCLC cells [41]. A HDAC3 inhibitor, romidepsin, upregulated PD-L1 expression via enhancing the acetylation of histones H3 and H4 and elevating BRD4 expression, leading to suppression of cellular immune function in colon cancer cells [42]. Taken together, HDACs regulate histone acetylation of PD-L1 promoter region.

PD-L1 acetylation and other PTMs

The associations of PD-1/PD-L1 PTMs are also needed to be clarified. Epidermal growth factor (EGF) stimulation induced upregulation of phosphorylation, acetylation and ubiquitination of PD-L1, but not SUMOylation, in A431 cells [34]. PD-L1 palmitoylation enhanced PD-L1 stability via preventing its ubiquitination and repressing its subsequent degradation by lysosomes [27]. PD-L1 activity is regulated by ubiquitination and N-glycosylation. GSK3β binds with PD-L1 and activates β-TrCP-mediated degradation of PD-L1, whereas glycosylation (at N192, N200 and N219) of PD-L1 antagonizes GSK3β interaction. EGF led to enhanced PD-L1 stability via inactivation of GSK3β in breast cancer, resulting in reduction of antitumor T cell immunity and efficacy of anti-PD-1 therapy in syngeneic mouse models [13]. N-glycosylation (at N35, N192, N200 and N219) and ubiquitylation (at K178) of PD-L1 cannot affect its interaction with the biphenyl drug BMS-202 and did not change PD-L1 dimer stability [43]. PD-L1 phosphorylation is linked to its glycosylation and stabilization. Metformin treatment led to PD-L1 phosphorylation at Ser195 site that was phosphorylated by AMPK (AMP-activated protein kinase), resulting in abnormal PD-L1 glycosylation and subsequent accumulation of PD-L1 in the ER (endoplasmic reticulum) and ERAD (ER-associated protein degradation)-mediated destruction [11]. Moreover, IL-6 stimulated JAK1 to cause PD-L1 phosphorylation at Tyr112,
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leading to PD-L1 glycosylation by N-glycoltransferase STT3A and promotion of PD-L1 stability [12]. Interestingly, PD-L1 glycosylation did not affect its acetylation and nuclear translocation [13]. PD-L1 glycosylation by B3GNT3 blocked cell surface internalization and GSK3β-involved degradation of PD-L1 [14].

Lysine specific demethylase (LSD) is recruited to the PD-L1 gene locus by B lymphocyte induced maturation protein 1 (Blimp-1) and inhibits the expression of PD-L1 via removing H3K4 methylation of PD-L1 following ex vivo stimulation, such as acute viral infection [44]. LSD1, an H3K4 and H3K9 demethylase, can govern eomesoderin (EOMES) nuclear dynamics through switching demethylation and acetylation of EOMES residues in PD-1+CD8+ T cells [45]. This observation suggests that demethylation and acetylation can be switched in special conditions. HDAC3 inhibitors alleviated DNA methyltransferase DNMT1 expression, leading to activation of PD-L1 transcription [39]. Therefore, studies are underway to explore whether PD-L1 acetylation is correlated with its methylation.

Protein acetylation has been reported to antagonize its ubiquitination to regulate stability and subcellular localization of proteins [46, 47]. For example, S-phase kinase associated protein 2 (Skp2) was acetylated by p300 and acetylation of Skp2 blocked its proteolysis by CDH1, leading to enhanced oncogenic activity [46]. USP7 regulated the degradation of Tip60, one of HATs, and forkhead box P3 (Foxp3) expression [48]. Tip60 promoted Foxp3 acetylation and dimerization [49, 50]. In the absence of acetylation, several lysines on Foxp3 could be ubiquitinated and caused its destruction [51]. Hyperacetylation of Foxp3 blocked its ubiquitination and degradation and increased Foxp3 protein levels, providing a rapid temporal regulation of Foxp3 expression levels [51]. It is necessary to determine whether PD-L1 acetylation prevents its ubiquitination in cancer cells. Entinostat, class I HDAC inhibitor, increased STAT3 acetylation and subsequently suppressed STAT3 phosphorylation and activity, and resulted in inhibited Foxp3 expression in Foxp3+ Treg cells [52]. Similarly, entinostat enhanced STAT3 acetylation in myeloid-derived suppressor cells (MDSCs)-like cells [53]. It is necessary to define the relationship between PD-L1 acetylation and its phosphorylation.

Targeting acetylation pathway for enhancing PD-1/PD-L1 therapy

Antibodies targeting PD-1/PD-L1 pathway have been used and exhibited impressive outcomes in several types of cancers [54]. In the past decade, Food and Drug Administration (FDA) has approved several antibodies targeting cytotoxic T-lymphocyte antigen-4 (CTLA-4), PD-1 or PD-L1 for the treatment of a wide spectrum of cancers, including melanoma, hepatocellular carcinoma, renal cell carcinoma (RCC), NSCLC, head and neck cancer, and bladder cancer [55-60]. Anti-PD-1 antibodies have pembrolizumab and nivolumab, while anti-PD-L1 antibodies have atezolizumab and durvalumab, and Ipilimumab is a monoclonal antibody against CTLA-4 [61, 62]. Unfortunately, the response rate is low in some tumors, such as the prostate and colon, and nearly 1/3 of respondents will relapse [6, 63]. Some patients develop resistance to immune checkpoint blockade, leading to poor prognosis [64]. Therefore, targeting PD-1/PD-L1 PTMs might be useful to improve the efficacy of anti-PD-1/PD-L1 therapy.

Selenium nanoparticles inhibited the expression of PD-1 and upregulated the expression of cytotoxicity factors such as CD16, interferon-γ (IFN-γ) and natural killer group 2, member D (NKG2D), and increased tubulin-α acetylation in γδ cells, leading to potentiation against tumor cytotoxicity [65]. HDAC6 upregulated PD-L1 expression via regulation of STAT3 pathway, and suppression of HDAC6 inhibited tumor progression in mice [66]. MPT0G612, an inhibitor of HDAC6, blocked IFN-γ-mediated upregulation of PD-L1 and stimulated apoptosis via inhibition of autophagy [67]. Several studies have shown that HDAC inhibitors, including VPA, nexturastat A, increased the efficacy of anti-PD-L1 antibody via activation of immune surveillance [68, 69]. HDAC3 inhibitor romidepsin in combination with anti-PD-1 therapy enhanced antitumor effects on colon cancer cells [42]. SCA and ACY957 elevated PD-L1 acetylation and the expression of immune checkpoints, leading to enhancement of immunotherapy [35]. It is important to mention that not all HDAC inhibitors can target acetylation of PD-L1. Moreover, HDAC inhibitors cannot specifically target acetylation of PD-L1. Targeting HDAC might be an effective approach to improve immune checkpoint blockade in cancer cells.
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Conclusions and perspectives

To the end, this review article proposes a combination therapy of HDAC inhibition and PD-1/PD-L1 blockade, which makes up for the deficiency of PD-1/PD-L1 blockade resistance, and provides a theoretical basis for this new tumor immunotherapy. However, a couple of critical concerns still need to be addressed in follow-up studies. The expression of nuclear PD-L1 in metastatic tumors is higher than that in primary tumors, but it is not clear how nuclear PD-L1 increases tumor invasiveness [35]. Without a doubt, further investigations are wanted to determine whether PD-L1 nuclear translocation inhibitors can be used as a complementary therapy for malignant tumors. Lys263 site was reported as the key acetylation site on PD-L1 by p300. Whether there are other acetylation sites on PD-L1 needs to be determined. Do other acetyltransferases involve in regulation of PD-L1 acetylation? It is also necessary to define whether other deacetylases, besides HDAC2, could reduce acetylation of PD-L1. High expression of PD-L1 was associated with cisplatin resistance and ADR resistance in non-small cell lung cancer [41]. It is unclear whether PD-L1 acetylation is associated with drug resistance, which is required to be fully explored. Since PD-L1 has multiple types of PTMs, it is pivotal to determine the associations among PTMs of PD-L1. Discovery of the regulatory mechanisms how PD-L1 acetylation is involved in regulating other PTMs might be useful to find the new approaches for targeting PD-L1 acetylation other than HDACs inhibitors. One group showed that knockdown of transcription factor PU.1 decreased the expression and H3K27 acetylation of PD-L2 via interacting with p300 in dendritic cells [70]. Depletion of p300 reduced the expression and PD-L2 acetylation in dendritic cells [70]. Moreover, PU.1 could bind to IRF4 on an Ets-IRF composite element (EICE) sequence of PD-L2 to regulate p300-mediated PD-L2 acetylation in dendritic cells [70]. It is elusive whether targeting PD-L2 acetylation is helpful to improve the tumor immunotherapy. In conclusion, it is required to further investigate the role and molecular mechanism of PD-1/PD-L1 acetylation in tumor cells for improving immunotherapy.

Disclosure of conflict of interest

None.
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