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LETTER TO THE EDITOR

Palmitoylation stabilizes PD-L1 to promote breast tumor growth

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Dear Editor,

PD-L1 is a well-known transmembrane protein, which is highly expressed on many types of cancer cells. By binding to its receptor PD-1 on T cells, PD-L1 significantly inhibits T cells activation and activity, and thus plays a pivotal role in driving the escape of tumor cells from immune surveillance.[1] Antibody blockade of PD-L1/PD-1 interaction has revolutionized cancer therapy with promising clinical outcomes in many cancer types, including melanoma, lung cancer, bladder cancer, colorectal cancer, and renal-cell cancer.[2] However, in some others such as prostate cancer, ovarian cancer, and breast cancer, the response rate of PD-L1/PD-1 antibody therapy is less satisfactory.[3] Recent studies have shown that PD-L1 can be regulated by post-translational regulations such as ubiquitination,[4] phosphorylation and glycosylation,[5] providing opportunity for marker-guided effective combinational therapy with immune checkpoint therapy.

Palmitoylation is one of the co- and post-translational modifications of proteins in which a palmitate is covalently linked to a cysteine residue as vast majority via a thioester linkage (also known as S-palmitoylation).[6] By affecting protein membrane anchoring, trafficking, interaction and degradation, palmitoylation plays important roles in human physiological and pathological processes, including cancers.[7,8] For instance, several cancer-related proteins, such as EZH2,[9] TEAD,[10] and c-Met,[11] are palmitoylated for stabilization, and knockdown of ZDHHC5, a palmitoyltransferase of EZH2, significantly inhibits glioma tumor growth.[9] Thus, palmitoylation or palmitoyltransferases could be novel targets for cancer therapy. In the current study, we searched for other possible metabolic-related modifications of PD-L1 such as lipid modification, and unexpectedly discovered that palmitoylation occurred on PD-L1 and played an important role in PD-L1 stability. Targeting PD-L1 palmitoylation sensitized tumor cells to T-cell killing and inhibited tumor growth.

Previously, we reported that saccharide regulates PD-L1 stability via glycosylation.[5] Recently, lipid modification has also been shown to play an important role in regulation of cell membrane proteins,[12] we were curious whether PD-L1 might also be regulated by lipid modification. Since palmitoylation is an important and broadly studied post-translational lipid modification of proteins, we first explored the possibility whether PD-L1 is regulated by palmitoylation. We treated breast cancer cell lines MDA-MB231 and BT549 with a general palmitoylation inhibitor, 2-bromopalmitate (2-BP). As shown in Fig. 1a, b, PD-L1 protein level significantly decreased upon 2-BP treatment in a dose- and time-dependent manner, suggesting that PD-L1 expression is regulated by protein palmitoylation. On the basis of prior studies,[9,11] which indicated that palmitoylation could regulate protein stability, we speculated that PD-L1 itself undergoes palmitoylation to maintain stability. To validate this hypothesis, we subjected PD-L1^{Flag}-expressing MDA-MB-231 (MB231-PD-L1^{Flag}) and BT549 (BT549-PD-L1^{Flag}) breast cancer cells to acyl-biotin exchange (ABE) assays in which free cysteine thiol groups of the proteins are irreversibly blocked by N-ethylmaleimide (NEM), whereas palmitoylated cysteines are later cleaved by hydroxylamine (HAM) and biotinylated. Palmitoylation of PD-L1 was detected using streptavidin-HRP, following immunoprecipitation with α-Flag beads (Fig. 1c). Consistently, by using ABE assay, the palmitoylation of PD-L1 was confirmed in primary human tumor samples (Fig. 1d). These results unveiled a novel post-translational modification of PD-L1.

To further investigate the role of palmitoylation on PD-L1, we used an online software CSS-Palm (csspalm.biocuckoo.org) to predict the palmitoylation site(s) on PD-L1. The result revealed a single palmitoylation site at Cys272 located in cytosolic domain of PD-L1 that is highly conversed among different species (Supplementary information, Fig. S1a). On the basis of the above results, endogenous PD-L1 in MDA-MB231 and BT549 cells was knocked out and substituted with PD-L1^{Flag} or PD-L1$^{C272A\text{-}Flag}$ (termed as MB231^{KO}-PD-L1^{Flag}, MB231^{KO}-PD-L1$^{C272A\text{-}Flag}$, BT549^{KO}-PD-L1^{Flag}, and BT549^{KO}-PD-L1$^{C272A\text{-}Flag}$). Results of ABE assay revealed that mutation of Cys272 to Ala substantially abolished PD-L1 palmitoylation (Fig. 1e), suggesting that this cysteine residue is the major palmitoylation site of PD-L1. Furthermore, in line with 2-BP treatment, which blocks palmitoylation, PD-L1$^{C272A\text{-}Flag}$ exhibited a faster turnover rate as well as less cell surface distribution than PD-L1^{Flag} in both MB231 and BT549 cells treated with protein synthesis inhibitor cycloheximide (CHX) (Fig. 1f; Supplementary information, Fig. S1b). Given that PD-L1 induces T-cell exhaustion through binding to its receptor PD-1, we next investigated whether palmitoylation of PD-L1 affects T-cell killing activity. In order to adequately mimic the in vivo circumstance, we first constructed 4T1^{KO}-mPD-L1^{Flag} and 4T1^{KO}-mPD-L1$^{C272A\text{-}Flag}$ mouse cell lines with endogenous mPD-L1 knocked out and substituted with mPD-L1^{Flag} or mPD-L1$^{C272A\text{-}Flag}$. Consistent with the results in MB231 and BT549 cells (Fig. 1e, f; Supplementary information, Fig. S1b), mutation of Cys272 to Ala dramatically abolished mPD-L1 palmitoylation (Fig. 1g), resulting in much less protein level of mPD-L1^{C272A} (Fig. 1h) as well as its cell surface distribution (Supplementary information, Fig. S1c). It is worth noting that compared with that observed in MB231 and BT549 cells, mPD-L1^{C272A} was substantially less stable in 4T1 cells (Fig. 1f *vs.* 1h; Supplementary information, Fig. S1b vs. S1c), which could be attributed to faster degradation of PD-L1^{C272A} in 4T1 cells (Supplementary information, Fig. S1d). Next, we isolated CD8^{+} T cells from 4T1 tumor infiltrating lymphocytes (TILs) and performed T-cell killing assay with 4T1^{KO}-mPD-L1^{Flag} and 4T1^{KO}-mPD-L1$^{C272A\text{-}Flag}$ cells. As shown in Fig. 1i, 4T1^{KO}-mPD-L1$^{C272A\text{-}Flag}$ cells were more sensitive to T-cell killing than 4T1^{KO}-mPD-L1^{Flag} cells, with results that were similar to mPD-L1 antibody blockade. Together, our data suggested that palmitoylation at Cys272

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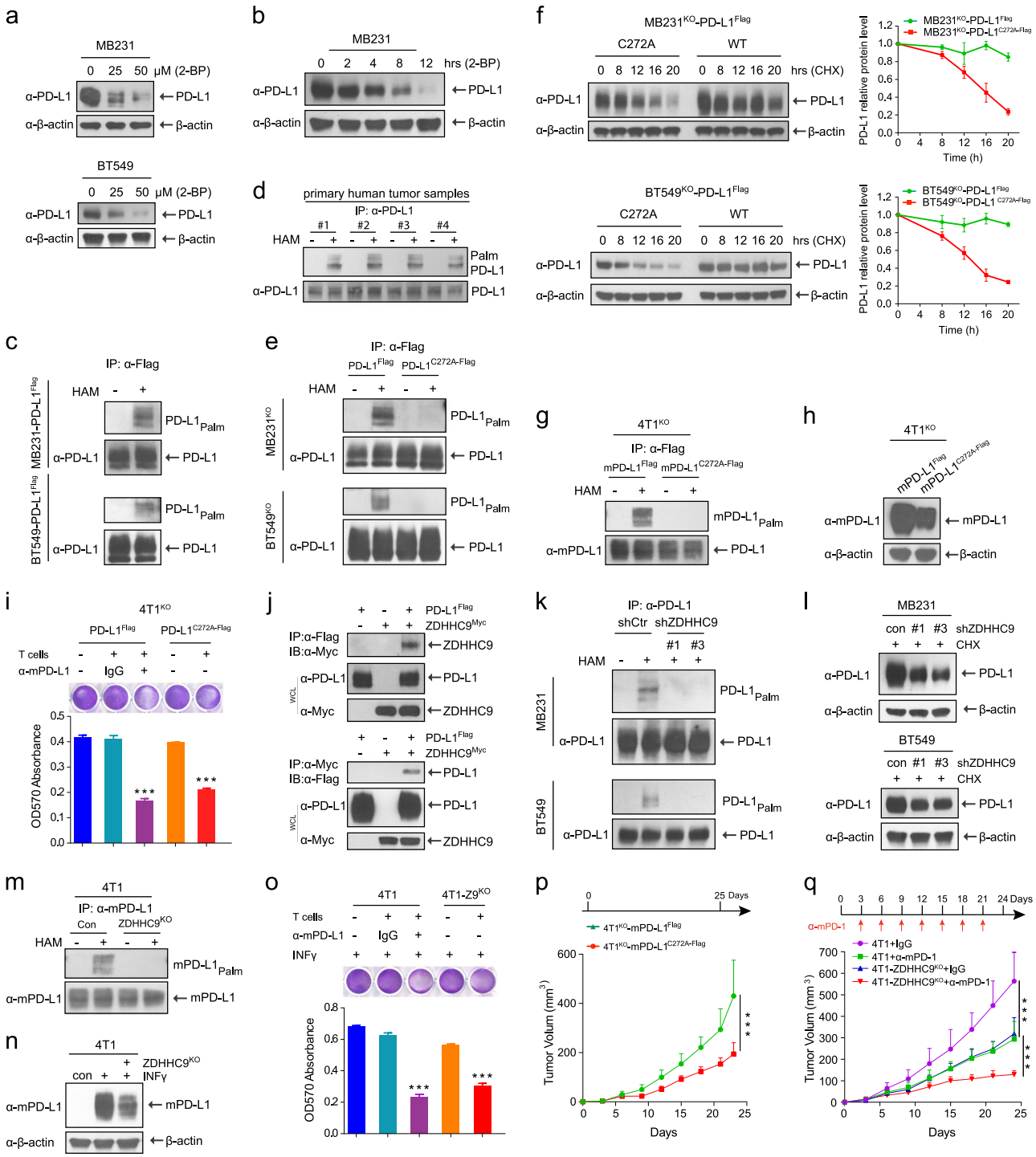

contributes to PD-L1 protein stability, and thus protects tumor cells from the immune surveillance of T cells.

Protein S-palmitoylation is mediated by a family of cysteine-rich zinc finger proteins containing a conserved Asp-His-His-Cys (DHHC) catalytic domain, and there are a total of 23 DHHC proteins in human.[13] To determine which palmitoyltransferases may regulate PD-L1, we searched our previous MS/MS tandem mass spectrometry data[5] of PD-L1 and found palmitoyltransferase ZDHHC9 was one of the associated proteins in breast cancer cells. To validate the interaction between PD-L1 and ZDHHC9, we co-transfected PD-L1^{Flag} and ZDHHC9^{myc} expression vectors into HEK293T cells, followed by immunoprecipitation with α-Flag or α-Myc beads. The results showed that PD-L1 bound to ZDHHC9 and *vice versa* (Fig. 1j). To further identify the function of ZDHHC9 on PD-L1 palmitoylation, we generated *ZDHHC9* knockdown MDA-MB-231 and BT549 stable cell lines by multiple shRNAs (Supplementary information, Fig. S1e). Next, we chose #1 and #3 clones which have the best *ZDHHC9* knockdown efficiency and subjected them to the ABE assay. The results revealed that knocking down *ZDHHC9* substantially decreased PD-L1 palmitoylation (Fig. 1k). Consistent with C272A mutation of PD-L1 (Fig. 1f), sh*ZDHHC9* reduced PD-L1 protein level in MB231 and BT549 cells as well as its cell surface distribution upon CHX treatment (Fig. 1l; Supplementary information, Fig. S1f). However, there was no

Fig. 1 Palmitoylation stabilizes PD-L1 to protect breast cancer cells from T-cell killing and promote tumor growth. **a** Western blot of PD-L1 in MDA-MB-231 (MB231) and BT549 cells treated with 2-BP as indicated for 12 h. **b** Western blot of PD-L1 in MB231 cells treated with 2-BP at 50 μM and harvested at the indicated time points. **c** PD-L1 palmitoylation was detected in MB231 and BT549 cells using streptavidin-HRP after ABE assay and immunoprecipitation with α-Flag beads. The same membranes were then re-probed with α-PD-L1 antibody. **d** PD-L1 palmitoylation was detected in primary human tumor tissues using streptavidin-HRP after ABE assay and immunoprecipitation with α-PD-L1 beads. One percent of tissue lysates served as input and were probed with α-PD-L1 antibody. **e** Detection of palmitoylation of PD-L1 and its C272A mutant in MB231 and BT549 cells (as $MB231^{KO}$-$PD\text{-}L1^{Flag}$, $MB231^{KO}$-$PD\text{-}L1^{C272A\text{-}Flag}$, $BT549^{KO}$-$PD\text{-}L1^{Flag}$, $BT549^{KO}$-$PD\text{-}L1^{C272A\text{-}Flag}$) using streptavidin-HRP after ABE assay and immunoprecipitation with α-Flag beads. The same membranes were then re-probed with α-PD-L1 antibody. **f** Western blot of PD-L1 in $MB231^{KO}$-$PD\text{-}L1^{Flag}$, $MB231^{KO}$-$PD\text{-}L1^{C272A\text{-}Flag}$ and $BT549^{KO}$-$PD\text{-}L1^{Flag}$, $BT549^{KO}$-$PD\text{-}L1^{C272A\text{-}Flag}$ cells treated with CHX and harvested at the indicated time points (left). The relative protein level of PD-L1 was quantified (right). Data represent mean ± SD of three independent experiments, $^{***}P < 0.001$, Student's *t* test. **g** Detection of palmitoylation of mouse PD-L1 and its C272A mutant in 4T1 cells (as $4T1^{KO}$-$mPD\text{-}L1^{Flag}$ and $4T1^{KO}$-$mPD\text{-}L1^{C272A\text{-}Flag}$) using streptavidin-HRP after ABE assay and immunoprecipitation with α-Flag beads. The same membranes were then re-probed with α-mPD-L1 antibody. **h** Western blot of mPD-L1 in $4T1^{KO}$-$mPD\text{-}L1^{Flag}$ and $4T1^{KO}$-$mPD\text{-}L1^{C272A\text{-}Flag}$ cells. **i** Top, T-cell-meditated tumor cell-killing assay in $4T1^{KO}$-$mPD\text{-}L1^{Flag}$ and $4T1^{KO}$-$mPD\text{-}L1^{C272A\text{-}Flag}$ cells along with mPD-L1 antibody or IgG treatment as indicated. Bottom, quantification of cell viability. Data represent mean ± SD of three independent experiments, $^{***}P < 0.001$, Student's *t* test. **j** $PD\text{-}L1^{Flag}$ and $ZDHHC9^{Myc}$ expression plasmids were co-transfected into 293T cells, the interaction between PD-L1 and ZDHHC9 was determined by immunoprecipitation with α-Flag beads or α-Myc beads followed by immunoblotting with α-Myc or α-Flag antibody. One percent of whole cell lysates served as input. **k** PD-L1 palmitoylation was detected in MB231 and BT549 cells with *ZDHHC9* knockdown as indicated using streptavidin-HRP after ABE assay and immunoprecipitation with α-PD-L1 beads. One percent of cell lysates served as input and were probed with α-PD-L1 antibody. **l** Western blot of PD-L1 in MB231 and BT549 cells with *ZDHHC9* knockdown as indicated following CHX treatment at 100 μg/mL for 12 h. **m** mPD-L1 palmitoylation was detected in 4T1 and 4T1-$ZDHHC9^{KO}$ cells using streptavidin-HRP after ABE assay and immunoprecipitation with α-mPD-L1 beads. One percent of cell lysates served as input and were probed with α-mPD-L1 antibody. **n** Western blot of mPD-L1 in 4T1 and 4T1-$ZDHHC9^{KO}$ cells following INFγ treatment at 50 μg/mL for 12 h. **o** Top, T-cell-meditated tumor cell-killing assay in 4T1 and 4T1-$ZDHHC9^{KO}$ cells along with mPD-L1 antibody or IgG treatment as indicated. Bottom, quantification of cell viability. Data represent mean ± SD of three independent experiments, $^{***}P < 0.001$, Student's *t* test. **p** Tumor growth of $4T1^{KO}$-$mPD\text{-}L1^{Flag}$ and $4T1^{KO}$-$mPD\text{-}L1^{C272A\text{-}Flag}$ cells in BALB/c mice. Tumors were measured at the indicated time points ($n = 10$ mice per group). **q** Tumor growth of 4T1 and 4T1-$ZDHHC9^{KO}$ cells in BALB/c mice treated with mPD-1 antibody or IgG as indicated. Tumors were measured at the indicated time points ($n = 8$ mice per group). Data represent mean ± SD. $^{**}P < 0.01$, $^{***}P < 0.001$, Student's *t* test

significant decrease in PD-L1 protein level without CHX treatment (Supplementary information, Fig. S1g). This may be attributed to a much slower degradation rate of PD-L1 without palmitoylation in MB231 and BT549 cells than that in the mouse 4T1 cells (Supplementary information, Fig. S1d). PD-L1 degradation may have been compromised by the de novo protein synthesis in the absence of CHX. Therefore, the differences in degradation rates between non-palmitoylated PD-L1 and wild-type PD-L1 could not be distinguished well in the absence of CHX in MB231 and BT549 cells. However, in mouse 4T1 cells, non-palmitoylated PD-L1 degraded at a faster rate than wild-type PD-L1 and could be detected even in the absence of CHX (Fig. 1h). Consistent with *ZDHHC9* knockdown in MB231 and BT549 cells, *mZDHHC9* knockout (Supplementary information, Fig. S1h) distinctly ablated mPD-L1 palmitoylation in 4T1 cells (Fig. 1m) and reduced mPD-L1 protein level and its cell surface distribution upon INFγ treatment (Fig. 1n; Supplementary information, Fig. S1i). In line with the results of the T-cell killing assay of $4T1^{KO}$-$mPD\text{-}L1^{Flag}$ and $4T1^{KO}$-$mPD\text{-}L1^{C272A\text{-}Flag}$ cells (Fig. 1i), 4T1-$mZDHHC9^{KO}$ cells exhibited significant sensitivity to the specific T-cell killing effect similar to that of mPD-L1 antibody blockade (Fig. 1o). Collectively, our data suggested that ZDHHC9 palmitoylates PD-L1 to maintain its protein stability and cell surface distribution, leading to immune escape of the tumor cells.

To determine the role of PD-L1 palmitoylation *in vivo*, we injected mice with $4T1^{KO}$-$mPD\text{-}L1^{Flag}$ and $4T1^{KO}$-$mPD\text{-}L1^{C272A\text{-}Flag}$ cells. Consistent with the T-cell killing results (Fig. 1i), C272A mutation of mPD-L1 greatly impaired 4T1 tumor growth (Fig. 1p), supporting that palmitoylation of PD-L1 plays a vital role in attenuating anti-tumor immunity to promote breast tumor growth. In addition, we evaluated the effects of ZDHHC9 on 4T1 tumor growth by injecting mice with 4T1 or 4T1-$mZDHHC9^{KO}$ cells along with mPD-1 antibody or IgG treatment. Consistent with the T-cell killing results (Fig. 1o), *mZDHHC9* knockout significantly inhibited 4T1 tumor growth and improved anti-mPD-1 therapeutic efficacy (Fig. 1q). Furthermore, we explored the clinical relevance of ZDHHC9 in cancers through bioinformatic analyses using the cBioportal (www.cbioportal.org) and The Human Protein Atlas (www.proteinatlas.org) databases. The results indicated that *ZDHHC9* is amplified in several types of human cancers including breast cancer with varied frequencies from 0.44% to 21.4% (Supplementary information, Fig. S2a). Moreover, high ZDHHC9 expression in tumor tissues appeared to correlate with poor survival of cancer patients (Supplementary information, Fig. S2b). Since ZDHHC9 has been reported as the palmitoyltransferase of NRAS[14] and HRAS[15] to play important roles in leukemogenesis and neuronal development, it is conceivable that ZDHHC9 could serve as an oncoprotein to promote RAS-activated tumor growth through regulating distinct substrates in addition to facilitating PD-L1 stabilization.

Collectively, our current findings revealed a role of palmitoylation in regulating PD-L1 stability. Disruption of PD-L1 palmitoylation by site-specific point mutation or inhibiting the expression of its palmitoyltransferase ZDHHC9 sensitized breast cancer cells to T-cell killing and thus repressed tumor growth. Furthermore, ZDHHC9 could be a potential target to boost anti-tumor response of immunotherapy. The current study indicates that lipid metabolism can contribute to tumor evasion of immune surveillance through facilitating PD-L1 palmitoylation and that targeting PD-L1 palmitoylation by small molecules against lipid metabolism may provide a new alternative approach to improve anti-PD-L1/PD-1 therapeutic efficacy.

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ADDITIONAL INFORMATION

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