Dear Editor,

Programmed cell death-ligand 1 (PD-L1) on tumor cells can inhibit CD8+ cytotoxic T lymphocyte (CTL)-mediated antitumor response by trans-engagement with programmed death protein 1 (PD-1). Besides tumor cells, PD-L1 is expressed on T cells. However, the intrinsic role of PD-L1 in T cells has not been widely studied. PD-L1 expression is essential for the survival of activated CD8+ T cells, and PD-L1 blockade at the contraction stage reduced the number of effector T cells [1]. CD8+ T cell responses to influenza virus infection were also impaired in PD-L1-deficient mice [2]. In contrast, a recent study showed that PD-L1 engagement by PD-1 induced inhibitory signaling in T cells [3]. PD-L1+ T cells may also suppress the effector function of neighboring macrophages and effector T cells via the canonical PD-L1-PD-1 axis to dampen antitumor immunity [3]. These conflicting results suggest that the role of PD-L1 in T cells may depend on the microenvironment and immune response stage. Here, we aimed to elucidate the role of PD-L1 in CD8+ CTLs, which may provide new insights into PD-L1-based immunotherapy.

First, we assessed the PD-L1 expression pattern during CTL activation. PD-L1 was rapidly induced in CTLs upon antigen stimulation, peaked on days 1 and 2, and then declined slowly (Supplementary Figure S1A). In mice bearing B16 melanoma tumors, CD4+ and CD8+ T cells from the spleen and xenograft tumors showed high PD-L1 expression. Interestingly, tumor-infiltrating T cells had reduced levels of PD-L1 compared to peripheral T cells from the spleen (Figure 1A; Supplementary Figure S1B), suggesting a possible intrinsic role of PD-L1 in T cell-mediated antitumor immunity.

To assess the role of PD-L1 in CD8+ CTLs, we transduced primary CTLs from OT-I mice, in which T cells express the T cell receptor (TCR) specific for ovalbumin peptide 257-264 (SIINFEKL, OVA257-264), with retroviral vectors containing short hairpin RNAs (shRNAs) targeting PD-L1 (RV-shPD-L1 #1 and RV-shPD-L1 #2) or targeting firefly luciferase (RV-shFF) as a control (Supplementary Figure S2A). We then stimulated CTLs with antigen-pulsed EL4 lymphoma cells and found that PD-L1 knockdown greatly inhibited the expression of tumor necrosis factor-α (TNF-α), interleukin-2 (IL-2), and interferon-γ (IFN-γ) in CD8+ CTLs (Figure 1B; Supplementary Figure S2B-C).

Next, we stably transduced Jurkat cell line with a nuclear factor of activated T cell (NFAT) response element-driven luciferase reporter to construct a Jurkat NFAT reporter cell line (Jurkat-NFAT-Luc). Artificial antigen-presenting cells (aAPCs) based on 293T cells, which express a membrane-associated anti-human CD3 single-chain variable fragment (OKT3-scFv), were used to stimulate Jurkat-NFAT-Luc cells and induce luciferase expression (Figure 1C). Given that PD-L1 was constitutively expressed on Jurkat cells (Supplementary Figure S3A), we knocked out PD-L1 in Jurkat-NFAT-Luc cells using the CRISPR-Cas9 system (Supplementary Figure S3B). PD-L1 knockout greatly downregulated the NFAT activity in Jurkat-NFAT-Luc cells upon aAPC stimulation (Figure 1D) and notably decreased IL-2, IFN-γ, and TNF-α mRNA levels in Jurkat-NFAT-Luc cells stimulated with anti-CD3 and anti-CD28 antibodies (Figure 1E). In addition, PD-L1 deficiency decreased the proliferation of Jurkat-NFAT-Luc cells (Figure 1F).

Jurkat-NFAT-Luc cells were stably transduced with control or PD-L1-overexpressing vector (Supplementary Figure S4A). PD-L1 overexpression enhanced NFAT activity in Jurkat-NFAT-Luc cells cocultured with 293T cells or aAPCs (Figure 1G-H). The qPCR results showed that overexpression of PD-L1 markedly upregulated IL-2, IFN-γ, and TNF-α mRNA levels in Jurkat-NFAT-Luc cells.
FIGURE 1 PD-L1 on CD8+ CTLs promotes antitumor activity via in cis interaction with CD80. (A) Flow cytometric analysis of PD-L1 expression on the tumor-infiltrating T cells and splenic T cells from mice bearing B16F10 melanoma on day 25 post inoculation (n = 4). (B) Cytokine production of OT-I CD8+ CTLs transduced with control (RV-shFF) or PD-L1 shRNA (RV-shPD-L1) retrovirus. CTLs were
upon anti-CD3/28 stimulation (Figure 1J). We then retrovirally transduced OT-1 CTLs with control or PD-L1-overexpressing vector (Supplementary Figure S4A). Upon stimulation with antigen-pulsed EL4 cells, PD-L1 overexpression notably enhanced IFN-γ, TNF-α, and IL-2 production in CTLs (Figure 1J; Supplementary Figure S4B). To further assess the role of PD-L1 in vivo, the control and PD-L1-overexpressing OT-1 CTLs were transferred to ovalbumin-expressing B16 melanoma tumor (B16-MO5)-bearing mice (Figure 1K). Strikingly, PD-L1 overexpression enhanced the antitumor activity of CTLs in vivo (Figure 1L). Further flow cytometric analysis revealed that PD-L1 overexpression did not affect infiltration of CTLs into tumor tissues; however, increased accumulation of CTLs in the spleen and lymph nodes (Figure 1M) and enhanced cytokine production by CTLs in the tumor microenvironment were observed (Figure 1N; Supplementary Figure S5). Together, these results showed that PD-L1 intrinsically promotes the antitumor activity of CD8+ CTLs.

To verify whether PD-L1 promotes T cell function by transducing signals via the intracellular domain, a vector expressing PD-L1 lacking the intracellular domain (PD-L1-ΔIC) was used to transfect Jurkat-NFAT-Luc cells. Luciferase assay was performed using Jurkat-NFAT-Luc cells expressing an empty vector, PD-L1, or PD-L1-ΔIC (Supplementary Figure S6). At the basal level, PD-L1-ΔIC increased NFAT activity to a level similar to that of PD-L1 (Figure 1O). After coculturing with aAPCs, PD-L1-ΔIC showed higher NFAT luciferase readings compared

Abbreviations: aAPC, artificial antigen-presenting cell; ANOVA, analysis of variance; CTL, cytotoxic T lymphocyte; IFN-γ, interferon-γ; IL-2, interleukin-2; KO, knock out; mRNA, messenger RNA; NFAT, nuclear factor of activated T cells; OD, optical density; PD-L1, programmed cell death ligand 1; PD-1, programmed death protein 1; SEM, standard error of mean; sgRNA, small guide RNA; shRNA, short hairpin RNA; TIL, tumor infiltrating lymphocyte; TCR, T cell receptor; TNF-α, tumor necrosis factor-α.
with the control vector, and showed a significant decrease in NFAT activation compared with the full-length PD-L1 (Figure 1O). As expected, PD-L1-ΔIC notably increased IL-2 mRNA levels in Jurkat-NFAT-Luc cells upon anti-CD3/28 stimulation (Figure 1P). Interestingly, PD-1 overexpression on aAPCs disrupted the enhancement of NFAT activity induced by PD-L1 or PD-L1-ΔIC overexpression (Figure 1O). Similar to PD-L1, PD-L1-ΔIC overexpression increased cytokine production in OT-I CTLs (Figure 1Q; Supplementary Figure S7). These data indicate that the intracellular domain of PD-L1 is dispensable for promoting CTL effector functions.

Ruling out the signal transduction role of PD-L1 in CTLs, we speculated that PD-L1 might regulate T cell function via its binding partners. CD80 has been reported to interact with PD-L1 only in cis, but not in trans [4]. In addition, in cis CD80-PD-L1 interaction on APC cells inhibits PD-L1-PD-1 interaction [5, 6]. CD80 blockade abrogated cytokine upregulation (Figure 1R; Supplementary Figure S8) and the augmented in vivo antitumor response (Figure 1S) induced by PD-L1 overexpression in OT-I CTLs. Furthermore, overexpression of mouse PD-L1 with the Y56A mutation (PD-L1-Y56A), which completely disrupts in cis PD-L1-CD80 interaction but still binds PD-1 [6], failed to enhance the in vivo antitumor activity of CTLs (Figure 1T). This suggests that PD-L1 promotes CTL function in a CD80-dependent manner. To further test whether in cis CD80-PD-L1 interaction promotes T cell activation, we constructed human PD-L1 with the N63D/G119S mutation (PD-L1-Y56A), which completely disrupts in cis PD-L1-CD80 interaction but still binds PD-1 [6], failed to enhance NFAT activity (Figure 1U). Consistent with this finding, PD-L1-N63D/G119S did not alter IL-2 mRNA levels in Jurkat-NFAT-Luc cells (Figure 1V). Therefore, intrinsic PD-L1 interacts with CD80 in cis to promote T cell activation.

Collectively, we found that intrinsic PD-L1 promoted cytokine secretion by CTLs upon tumor stimulation and positively regulated NFAT activity and cytokine production in Jurkat-NFAT-Luc cells. Importantly, PD-L1 overexpression notably potentiated the antitumor activity of CD8+ CTLs. Mechanistically, PD-L1 promotes CTL function in an intracellular domain-independent manner, whereas the in cis interaction between PD-L1 and CD80 was critical for the positive regulatory role of PD-L1 in T cells. Our study offers new molecular insights into the PD-L1 pathway and may reconcile the contradictory findings on PD-1 ligation, which did not consider the in cis PD-L1-CD80 interaction. On the basis of our results and those of previous studies, we propose that the in cis binding site between PD-L1 and CD80 should be carefully considered when designing therapeutic PD-L1 antibodies against tumors. Moreover, PD-L1 antibodies that block PD-1-PD-L1 interaction, but not CD80-PD-L1 interaction, should show better efficacy on tumor. Further studies are required to clarify how in cis PD-L1-CD80 interaction regulates CD8+ CTL function and provide a reference for clinical immunotherapy.

DECLARATIONS

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ETHICS APPROVAL AND CONSENT TO PARTICIPATE

All animal studies were performed in strict accordance with the ethical guidelines and were approved by the Animal Care and Use Committee of Shandong University (Jinan, Shandong, China).

CONSENT FOR PUBLICATION

All authors read and approved the final manuscript for publication.

CONFLICT OF INTEREST

The authors declare no competing interests.

DATA AVAILABILITY STATEMENT

All data in this study are available from the corresponding author upon reasonable request.

AUTHOR CONTRIBUTIONS

CL conceived the project and designed experiments; YD and CC performed experiments and acquired data; YD, CC and CL analyzed and interpreted data; SM, JP, HD, FZ and XS participated in the experiments or contributed to technical or material support; YD, CC and CL wrote the paper;
CL and CM revised the manuscript and supervised the study. All authors read and approved the final manuscript.

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SUPPORTING INFORMATION
Additional supporting information can be found online in the Supporting Information section at the end of this article.

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