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LETTER TO THE EDITOR

Intrinsic PD-L1 promotes antitumor activity of CD8+ cytotoxic T lymphocytes via *in cis* interaction with CD80

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.
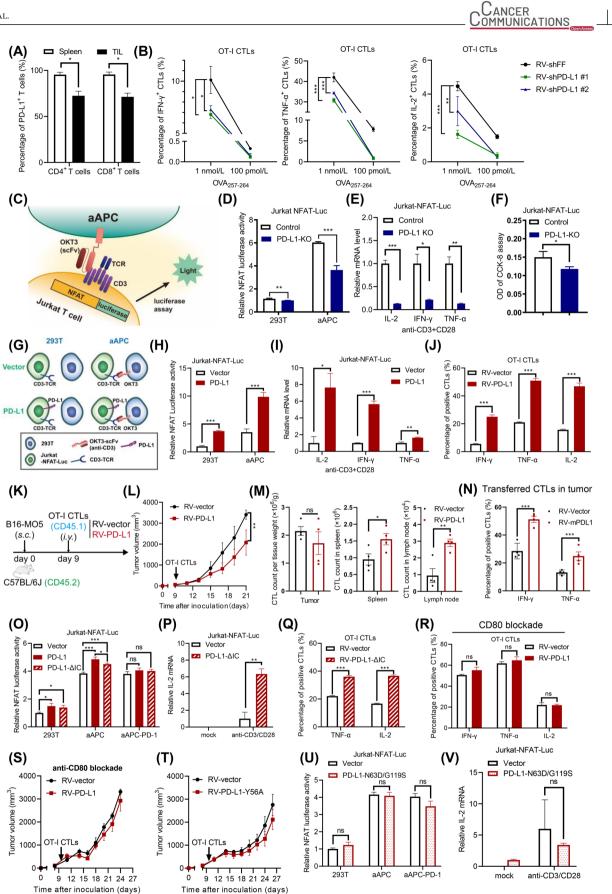
To assess the role of PD-L1 in CD8+ CTLs, we transduced primary CTLs from OT-I mice, in which T cells express the T cell receptor (TCR) specific for ovalbumin peptide 257-264 (SIINFEKL, $OVA_{257-264}$), with retroviral vectors containing short hairpin RNAs (shRNAs) targeting PD-L1 (RV-shPD-L1 #1 and 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 1I). We then retrovirally transduced OT-I 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-I 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

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stimulated with 1 nmol/L or 100 pmol/L OVA$_{257-264}$-pulsed EL4 cells for 4 h, followed by flow cytometric analysis of IFN-γ, TNF-α, and IL-2 ($n = 3$). **(C)** Schematic diagram of NFAT luciferase reporter assay system. An artificial antigen-presenting cell line (aAPC) was designed based on the 293T cell line, which expresses membrane-associated anti-human CD3 single-chain variable fragment (OKT3-scFv) to stimulate T cell receptors. The luciferase gene, driven by NFAT responsive elements which can indicate T cell activation, was stably expressed in Jurkat cells (Jurkat-NFAT-Luc). **(D)** Small guide RNA (sgRNA)/Cas9 was used to knock out PD-L1 in Jurkat-NFAT-Luc cells. Control and PD-L1-deficient Jurkat-NFAT-Luc cells were cocultured with 293T cells or aAPCs for 6 h, followed by luciferase detection ($n = 3$). **(E)** Control and PD-L1-KO Jurkat-NFAT-Luc cells were stimulated with anti-CD3 and anti-CD28 antibodies, and relative mRNA levels of IL-2, IFN-γ, and TNF-α were detected by qPCR ($n = 3$). **(F)** Cell-Counting Kit 8 assay was used to determine cell proliferation of control and PD-L1-deficient Jurkat-NFAT-Luc cells ($n = 3$). **(G)** Schematic diagram of the NFAT luciferase reporter assay with vector control and PD-L1-overexpressing Jurkat-NFAT-Luc cells. **(H)** Jurkat-NFAT-Luc cells transduced with empty or PD-L1-overexpressing vector were cocultured with 293T cells or aAPCs for 6 h, followed by detection of luciferase activity ($n = 3$). **(I)** Control and PD-L1-overexpressing Jurkat-NFAT-Luc cells were stimulated with anti-CD3 and anti-CD28 antibodies, and relative mRNA expression levels of IL-2, IFN-γ, and TNF-α were detected using qPCR ($n = 3$). **(J)** Control and PD-L1-overexpressing OT-I CTLs were stimulated with EL4 cells pulsed with 100 pmol/L OVA$_{257-264}$. The expression levels of IFN-γ, TNF-α and IL-2 were detected using flow cytometry ($n = 3$). **(K–N)** Schematic diagram of adoptive transfer of control or PD-L1-overexpressing CD45.1$^+$ CTLs to B16-MO5 tumor-bearing CD45.2$^+$ wild-type mice on day 9 post inoculation (K). Tumor growth was measured every 2 days ($n = 4$) (L). At 7 days post CTL transfer, the number of transferred control or PD-L1-overexpressing CD45.1$^+$ OT-I CTLs in the tumor, spleen, and lymph node of recipient mice were assessed using flow cytometry (M), and the percentages of IFN-γ^+ and TNF-α^+ transferred CTLs in tumor were estimated by intracellular staining (N) ($n = 4$). **(O)** Jurkat-NFAT-Luc cells transduced with empty vector, PD-L1- or PD-L1-ΔIC (PD-L1 lacking intracellular domain)-overexpressing vector were cocultured with 293T cells, aAPCs or PD-1-overexpressing aAPCs (aAPC-PD-1s) for 6 h, followed by detection of luciferase activity ($n = 3$). **(P)** Control and PD-L1-ΔIC-overexpressing Jurkat-NFAT-Luc cells were stimulated with anti-CD3 and anti-CD28 antibodies, and relative mRNA expression of IL-2 was detected using qPCR ($n = 3$). **(Q)** OT-I CTLs transduced with control vector or PD-L1-ΔIC were stimulated with EL4 cells pulsed with 100 pmol/L OVA$_{257-264}$, and TNF-α and IL-2 expression was detected by flow cytometry ($n = 3$). **(R)** Control and PD-L1-overexpressing OT-I CTLs were treated with anti-CD80 antibody (5 μg/mL), then stimulated with EL4 cells pulsed with 1 nmol/L OVA$_{257-264}$. The expression of IFN-γ, TNF-α, and IL-2 was detected using flow cytometry ($n = 3$). **(S)** Control and PD-L1-overexpressing OT-I CTLs were pre-treated with anti-CD80 antibody (5 μg/mL) during in vitro culturing and transfection, then transferred into wild-type B16-MO5 tumor-bearing mice on day 10 post tumor inoculation. Recipient mice were intraperitoneally injected with 100 μg anti-CD80 antibody every 3 days. Tumor growth was measured every 2 days ($n = 5$). **(T)** Control and PD-L1-Y56A-overexpressing CTLs were transferred into B16-MO5 tumor-bearing wild-type mice on day 10 post tumor inoculation. Tumor growth was measured every 2 days ($n = 6$). **(U)** Jurkat-NFAT-Luc cells transduced with empty vector or mutant PD-L1 (PD-L1-N63D/G119S)-overexpressing vector were cocultured with 293T cells, aAPCs or aAPC-PD-1 for 6 h, followed by detection of luciferase activity ($n = 3$). **(V)** Control and PD-L1-N63D/G119S-overexpressing Jurkat-NFAT-Luc cells were stimulated with anti-CD3 and anti-CD28 antibodies, and relative mRNA expression of IL-2 was detected by qPCR ($n = 3$). All results are representative of at least three independent experiments. Data are presented as mean ± standard error of mean (SEM) and were analyzed by unpaired Student's t-test (A, D, E, F, H, I, J, M, N, O, P, Q, R, U, V) or two-way ANOVA (B, L, S, T). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$; ns, no significance.
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 over-expression 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 inter-act 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. Further-more, 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-N63D/G119S), which corresponds to PD-L1-Y56A [6]. Luciferase assay revealed that PD-L1-N63D/G119S 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). There-fore, 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 pro-duction 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 find-ings 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 consid-ered 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

ETHICS APPROVAL AND CONSENT TO PARTICIPATE
All animal studies were performed in strict accordance with the ethical guidelines and were approved by the Ani-mal 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 tech-nical 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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