Peer Review File

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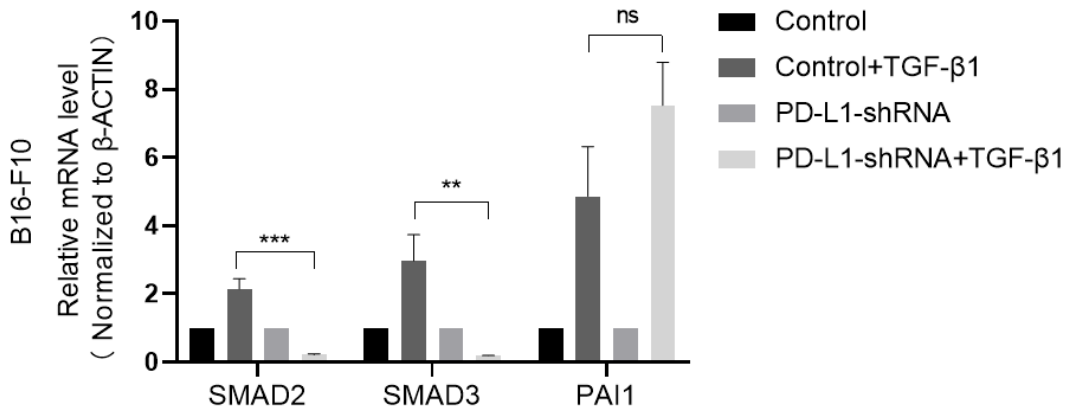
Reviewer A

Comment 1/2: Dose PD-L1 signalling finally affect Smad2/3 signalling as downstream of TGF-β? How is phosphorylation Smad2/3, PAI-1 expression?

Reply 1/2: In response to your comment, we detected the mRNA levels of Smad2, Smad3 and PAI-1 by RT-qPCR of PD-L1-shRNA B16-F10 cells with or without 48h of TGF-β treatment (5ng/mL). The data (Response Figure 1) showed that the comparative mRNA levels of Smad2 and Smad3 increased in PD-L1-shRNA B16-F10 cells in compared to controls, however TGF-β induced PAI-1 expression didn't decrease in PD-L1-shRNA B16-F10 cells comparing control group. Control was defined as cells transducted with NC-shRNA. Due to the impact of COVID-19, we performed these only on the B16F10 cells. But we believed it would suffice because B16F0 and B16F10 cells are of the same origin, and B16F10 cell line was more invasive and malignant like real-world clinical melanoma behaviors.

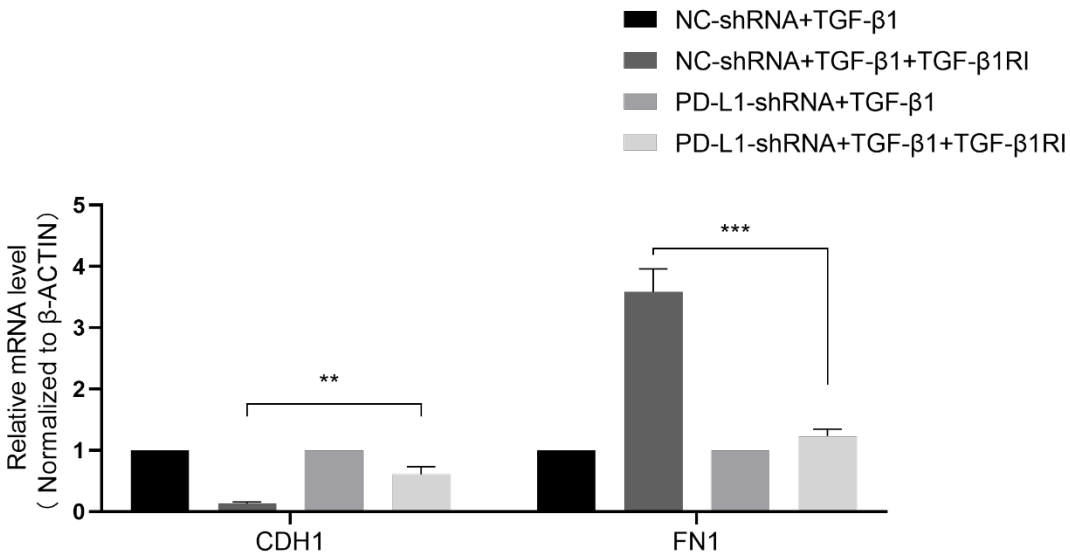


Response Figure 1. PD-L1 induced the expression of Smad2/3 in B16F10 melanoma cell lines. Control was defined as cells transducted with NC-shRNA. The data was normalized to β-actin. Two-sided unpaired *t*-test was used to evaluated the significant differences. Each column represents the mean ± SD of n = 3 experiments. □□p<0.01, □□□p<0.001, ns not significant. The primer sequences were as follows: SMAD2 forward, CAGCTTGGATTTGCAGCCAG; SMAD2 reverse, CTGTGACGCATGGAAGGTCT, SMAD3 forward, TCTCCCCGAATCCGATGTCC; SMAD3 reverse, GCTGGTTCAGCTCGTAGTAGG;

PAI1forward, AGCTTTGTGAAGGAGGACCG; PAI1 reverse, CTGATGGGCTGTGTGGGATT.

Comment 3: Does the inhibitor of TGF-β signalling cancel the effect of PD-L1 on EMT?

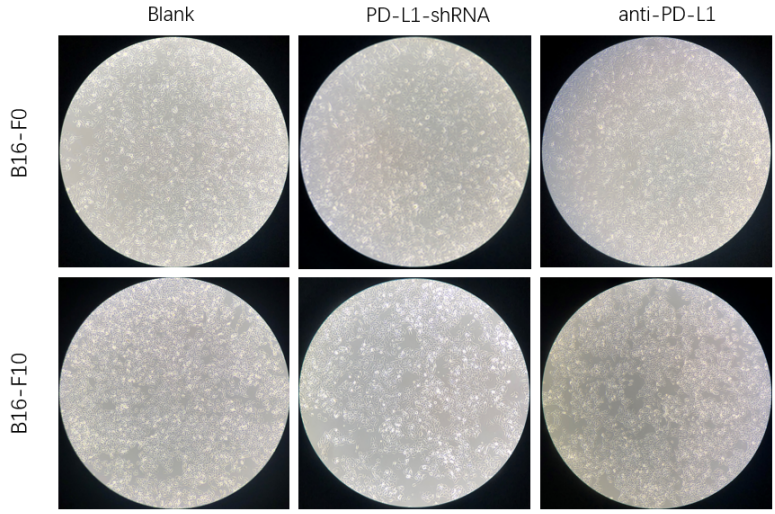
Reply 3: PD-L1-shRNA B16-F10 cells were treated with an TGF-β inhibitor (SB431542, 10uM) to inhibit the TGF-β signalling for 48h, then the mRNA levels of EMT makers were detected. We found that SB431542 could cancel the effect of PD-L1 on EMT as showed in Response Figure 2, manifesting that the TGF-β induced CDH1 downregulation and FN1 upregulation could be cancelled by TGF-β inhibitor, which further demonstrated that TGF-β signalling played a pivotal role in the PD-L1 induced EMT.



Response Figure 2. TGF-β inhibitor attenuated the effect of PD-L1 on TGF-β induced EMT in B16F10 melanoma cell lines. Control was defined as cells transducted with NC-shRNA. The data was normalized to β-actin. Two-sided unpaired *t*-test was used to evaluated the significant differences. Each column represents the mean ± SD of n = 3 experiments. □□p<0.01, □□□p<0.001.

Comment 4: Does PD-L1 change the morphology of cells?

Reply 4: We observed the cell morphology after PD-L1-shRNA transfection and after anti-PD-L1 treatment (with purified anti-mouse CD274, 5ug/ml, 48h) respectively. And under the light microscope, the cell showed no obvious change in morphology.



Response Figure 3. The cell morphology of B16F0 and B16F10 cells in different groups. Blank represented the regular cells, and anti-PD-L1 represented cells treated with purified anti-mouse CD274 (5ug/ml) for 48h.

Comment 5: How is use other melanoma cells to confirm the author's data?

Reply 4: We will verify our data and conclusions in our following researches using other melanoma cells including human melanoma cells.

Reviewer B

Comment : In my opinion, the section indicating that PD-L1 enhanced the intracellular TGF-β1 mRNA levels and induced the secretion of TGF-β1 needs clarification based on obtained results.

Reply /Changes in the text: In response to your comments, we revised the 3.2 section as follows. "In order to determine whether a bidirectional regulation existed between PD-L1 and TGF-β1 in melanoma cells, we explored the effect of PD-L1 on the expression and secretion of TGF-β1. Cell-surface PD-L1 of B16-F0 and B16-F10 cells was blocked with purified anti-mouse CD274 (anti-PD-L1, 5ug/mL) for 48h. The cultured supernatant was then collected and the total RNA of B16-F0 and B16-F10 cells were extracted. The concentration of TGF-β1 in the supernatant was detected by ELISA kit and the mRNA level of intracellular TGF-β1 was measured by RT-qPCR. The results showed that anti-PD-L1 could significantly inhibit the secretion of TGF-β1 was significantly suppressed to 1.90-fold in B16-F0 cells ($p<0.0001$, Figure 2A) and 1.49-fold in B16-F10 cells ($p<0.0001$, Figure 2A) in anti-PD-L1 group compared with those from control group. Importantly, anti-PD-L1 downregulated intracellular TGF-β1 mRNA levels to 2.20-fold in B16-F0 cells ($p<0.0001$, Figure 2B), and to 1.45-fold in B16-F10 cells ($p=0.0011$, Figure 2B), respectively. Together with above results, it was indicated that cell intrinsic PD-L1 could induced the expression and secretion of TGF-β1 in melanoma cells.'' (see Page 11-12)

Reviewer C

Introduction

The introduction lacks perspective. Some findings seem there just to fill the space, while some more important papers are not even mentioned (e.g. Mariathasan et al, Nature 2018).

Reply:

We have revised the introduction section to include more recent and important papers in this filed (See Line 30-66), and the paper you mentioned (e.g. Mariathasan et al, Nature 2018) was already included in our citations (See citation [8]).

Materials and Methods

The TGFB inhibitor and TGFB molecule are not described. Reference number, lot number, concentration and timing for each experiment should be described. Concentration and timing is a recurrent problem throughout the papers also in other experiments.

It seems that in the qPCR the authors used only one reference gene (GAPDH), whereas it is common practice to use three different genes as a reference (e.g. ACTB, HPRTI, SDHA, RPL13A, etc). Using only one reference gene might lead to big biases in the downstream analysis. Primer sequences for qPCR should be included.

Introduction of foreign DNA into a cell by viral vectors is called transduction and not transfection.

Statistical tests should be described per each experiment and not left to the reader to guess if it is a t-test or an ANOVA.

Reply:

We have revised the methods section, mainly added the concentrations and time of each reagent used. We also presented the primer sequences used for qPCR (See Line 103-110). GAPDH was a well-known reference gene and many articles used only one reference gene to analyse the relatively expression of mRNA (e.g. Chen, G., et al. Nature 2018), so we think it was reasonable to use GAPDH to normalize the results. We have revised ‘transfection’ to ‘transduction’ (See method section 2.5 and figure legend 3). Specific statistical tests for each experiment used were described in each figure legend (See Figure legend 1-5)

Results

In Figure 1 in the flow cytometry analysis, blank and control are not defined, not in the results chapter, nor in the materials and methods, nor in the figure legend. I assume blank is an unstained sample, though is not clear why control should differ from the DMSO sample. The shift on the PD-L1 signal intensity axis between blank and control makes the following gates very suspicious and most likely all the samples are PD-L1 negative. On a similar topic, it is not clear why an in vitro model should show such heterogeneity in the baseline expression of PD-L1. The authors should have shown and described the full analysis strategy for the flow cytometry experiment. It would also have been interesting to see the same data presented in the format Relative cell number/PD-L1 expression. Furthermore in the Figure 1C it is shown the expression level of the blank and not of the control.

Reply:

Blank in Figure 1 indicated an unstained sample, while DMSO group indicated PD-L1 stained in an DMSO treated sample and control indicated PD-L1 stained in an untreated sample. To avoid ambiguity, we revised Figure 1 as showed below and in manuscript. We deleted the blank sample, and cells treated with DMSO (DMSO) or without (Control) were defined as controls for anti-TGF-β1 and TGF-β1 group respectively because TGF-β1 inhibitor was dissolved in DMSO. We revised the method and figure legend 1 (See page 6 and 21). In the Figure 1C, relative expression of PD-L1 mRNA was showed in control, TGF-β1, DMSO, anti-TGF-β1 group was showed, indicating the induction of TGF-β1 on PD-L1 was on both mRNA and cell surface protein level.

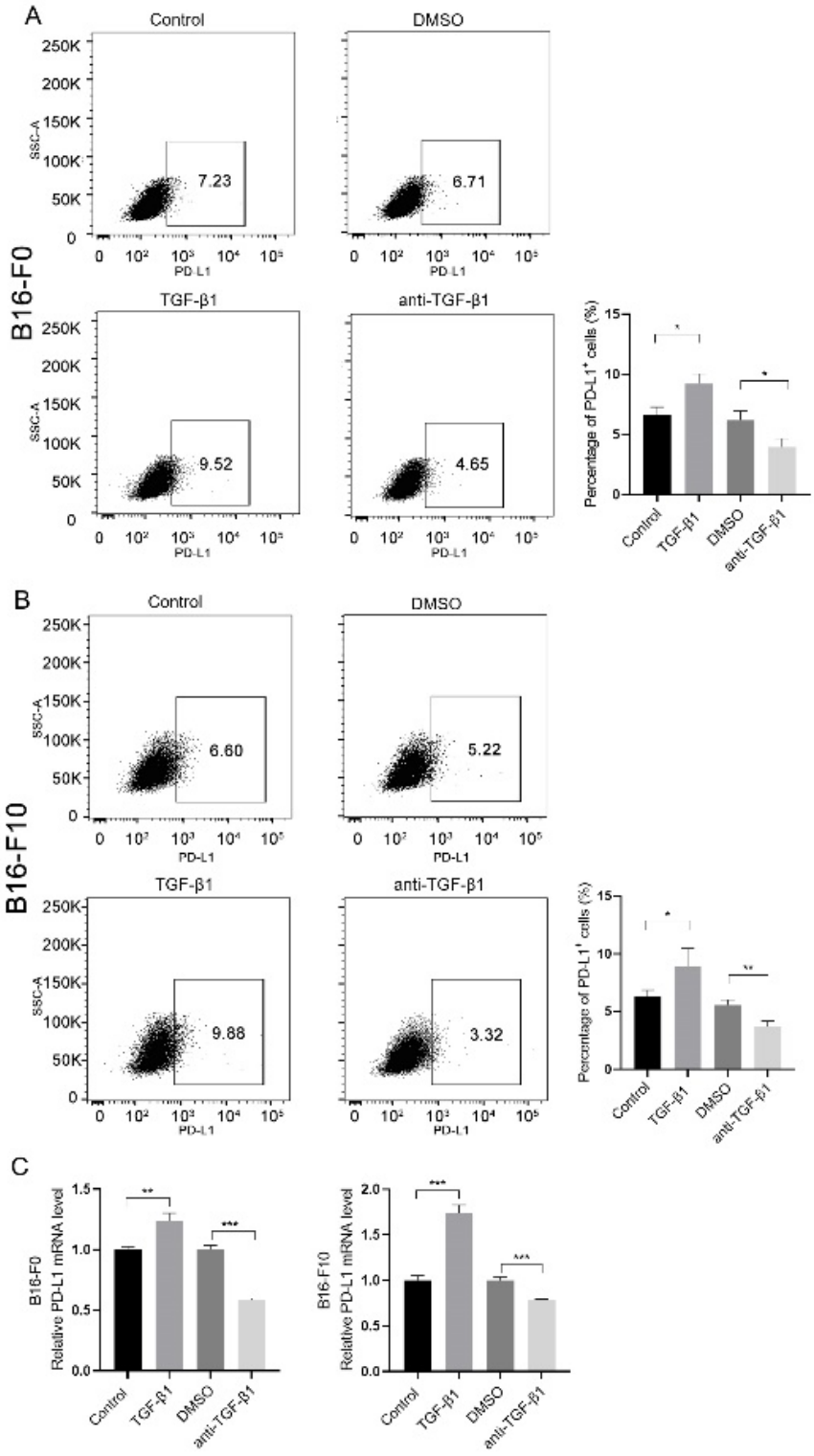


Figure 1. TGF-β1 upregulated PD-L1 expression in melanoma cell lines. The protein expression of PD-L1 were identified by flow cytometry in B16-F0 cells (A) and B16-F10 cells (B) treated with TGF-β1 or TGF-β1 inhibitor (anti-TGF-β1). Cells treated with DMSO (DMSO) or without (Control) were defined as controls for anti-TGF-β1 and TGF-β1 group respectively because TGF-β1 inhibitor (SB431542) was dissolved in DMSO. Two-sided unpaired t-test was used to

evaluated the significant differences. Each column represents the mean ± SD of n = 3 experiments. □p<0.05, □□p<0.01. C, Relative PD-L1 mRNA expression in B16-F0 and B16-F10 cells stimulated with TGF-β1 or TGF-β1 inhibitor (anti-TGF-β1). Cells treated with DMSO (DMSO) or without (Control) were defined as controls for anti-TGF-β1 and TGF-β1 group respectively because TGF-β1 inhibitor (SB431542) was dissolved in DMSO. Two-sided unpaired t-test was used to evaluated the significant differences. Each column represents the mean ± SD of n = 3 experiments. □□p<0.01, □□□p<0.001.

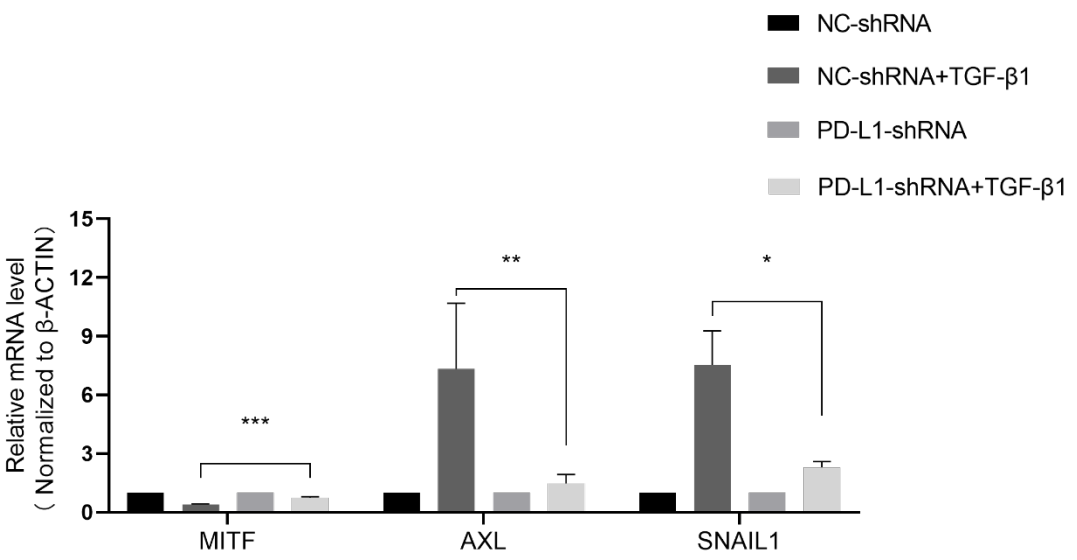
In the text α-smooth muscle actin is used as an EMT marker. α-SMA is not a recognized marker of EMT in melanoma. Furthermore, the manuscript suggests a co-localization of PDL1 positive cells and α-SMA. This does not seem to be the case in Figure 3. Authors should stain for both markers on the same slides, not separately in different slides. Furthermore, co-localization should be assessed with quantification via ImageJ or similar.

Reply:

α-SMA was also used as an EMT in melanoma in other papers. (e.g. Han-En Tsai, et.al; Mol Cancer Ther (2013)). In the immunohistochemistry experiments, we cut our tumor tissue successively into 5-μm-thick sections, so basically PD-L1 positive cells and α-SMA positive cells could be viewed as on the same layer.

The last two panels of Figure 3C are DUPLICATED. The statistical analysis in this panel is incorrect and inconclusive. A much bigger panel of markers (melanocytic and mesenchymal) should be used (AXL, PRRX1, ZEB1, MITF, etc).

Reply: We have revised the Figure 3C in manuscript. In response to your query of a bigger panel of EMT markers, AXL, SNAIL1 and MITF mRNA as EMT makers were measured to verify our results, using another reference β-actin as reference gene. As showed in Response Figure 1 below, the TGF-β1 induced upregulation of AXL and SNAIL1 (mesenchymal makers) decreased and the TGF-β1 induced downregulation of MITF (melanocytic maker) increased in PD-L1-shRNA+ TGF-β1 group compared with NC-shRNA+ TGF-β1. Due to the impact of COVID-19, we performed these only on the B16F10 cells. But we believed it would suffice because B16F0 and B16F10 cells are of the same origin, and B16F10 cell line was more invasive and malignant like real-world clinical melanoma behaviors.



Response Figure 4. PD-L1 promoted the TGF-β1 induced EMT in bigger panel. B16F10 PD-L1-shRNA cells were cultured in RPMI 1640 (Gibco, USA) containing 10% fetal bovine serum (FBS) (NQBB, China) with recombinant mouse TGF-β1 (catalog 7666-MB, R&D Systems, USA, 5ng/mL) for 48h, total RNA was extracted and the RT-qPCR was performed as described in Section 2.3 Real-time quantitative PCR. Two-sided unpaired *t*-test was used to evaluated the significant differences. Each column represents the mean ± SD of n = 3 experiments. □p<0.05, □□p<0.01, □□□p<0.001. The primer sequences were as follows: MITF forward, CAAATGGCAAATACGTTACCCG; reverse, CAATGCTCTTGCTTCAGACTCT; SNAIL1 forward, CACACGCTGCCTTGTGTCT; reverse GGTCAGCAAAAGCACGGTT; AXL forward, GGAACCCAGGGAATATCACAGG; reverse, AGTTCTAGGATCTGTCCATCTC

In figure 4, panel C is AGAIN duplicated. In materials and methods it is stated that cells for this assay were imaged at 6 different POV, but only one is presented. Multiple time points should have been imaged.

Reply:

After careful review of our data and figure, the Figure 4C in manuscript was not duplicated. The wound healing assay were imaged at six different points of view under the light microscope after 24h of TGF-β1 treatment. We didn't image multiple timepoints because results after 24h was most significant.

Overall the in vitro part needs much more solid data, as well as KO, KI and rescue validation experiments.

Control for the in vivo model has not been defined.

Reply:

We have revised the method section 2.8 (See Page 9). Specifically, the B16-F0 and B16-F10 cells were infected with shRNA lentiviruses encoding mouse PD-L1 (PD-L1-shRNA) or meaningless negative control sequence (NC-shRNA) as control coupled with green fluorescent protein (GFP), respectively. All animals were randomly divided into two groups (B16F0 and B16F10 group), 6 in each group. And the mice in each group were randomly divided to be inoculated PD-L1-shRNA melanoma cells and NC-shRNA melanoma cells as control subcutaneously with 200ul suspension containing 5×10^5 cells, respectively. We plan to complete the KO, KI and rescue validation experiments next to verify our results and to further investigate the relationship of tumor-intrinsic PD-L1 and TGF-β in melanoma.

Discussion

The discussion mostly repeats the introduction and adds very little context and future perspectives to the work.

Typos

There are different typos in the text. Some examples:

Line 33, remove comma before “and”

Line 36, remove comma before “and”

Line 156 “Ki-67”

Line 161 “diaminobenzidine”

Line 198 “is expressed”

Line 230 “weighted”

Line 266 “What’s more”

Reply: The language of our manuscript has been reviewed and edited by a senior English speaker again. The discussion section has been revised hopefully to better explain our results (see Page 14-18).