nature portfolio

Peer Review File

A splicing isoform of PD-1 promotes tumor progression as a potential immune checkpoint

Corresponding Author: Professor Shan Gao

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

This is an interesting study from a group in China. This group has identified a novel splice variant of the negative costimulatory molecule, PD-1, and are characterizing its expression, gene regulation and function. They hypothesize that this splice variant of PD-1 will additionally mediate immune escape of cancer, in addition to the wildtype version. They first demonstrate the identification of the PD-1 splice variant, which seems to retain part of an intron. They then go on to focus on the splicing and function. The group even generated a specific antibody that specifically recognizes just the PD-1 splice variant protein. Then they go on to show that this splice variant of PD-1 does bind to PD-L1, which determines the inhibition of T cells. Finally, they over-express the human version of this PD-1 splice variant (PD-1^28) in mice and demonstrate faster tumor progression in these mice challenged with MC38 colon cancer tumors. Overall, there is some interesting data on the identification of this PD-1 splice variant. While significant and important for the field, significant issues exist with the story. These issues are outlined below:
Major Comments.
1. There is no context for the prevalence of this splice variant isoform with the other known variants, the relative expression compared to the wild type protein, or the negative costimulatory function in primary cells.
2. There is almost a complete dependence for all of the functional data on the use of a T cell line (Jurkat). The Jurkat T cell line is actually derived from a T cell leukemia. As such, it is not clear that PD-1 (or PD-1^28) is regulated in a normal fashion. It is also not clear that over-expression of knockdown of this PD-1^28 variant in Jurkats will give true information about its function. Such work needs to be done in primary T cells.
3. The transgenic expression of the human isoform in mice may not at all represent the true biology of this negative costimulatory molecule. As such, it is not clear how to interpret the murine studies.
4. The transcription factor studies (Figure 2) are interesting, but the regulation of the alternative mRNA splicing for this variant is not really related to the important aspects of the function. As such, the data here are not really integrated with the rest of the story.

Reviewer #2

(Remarks to the Author)
In this manuscript, Wang et al. has identified and characterized the function of a novel isoform of PDCD1, which is arising from a 28nt retention at intron 2, resulting in the expression of PDCD1^28 in activated T-cells. The claim behind the existence of PDCD1^28 is largely supported by experimental data. Functionally, Wang et al. showed that PDCD1^28 inhibits both T-cell proliferation and cytokine production and promotes tumour growth in vivo. Using their PDCD1^28 mouse models, the authors also suggested that PDCD1^28 might confer resistance to anti-PD-L1 therapy. Overall, this is an interesting study. However, there is a notable gap in understanding the mechanism behind the differential splicing of this isoform in activated T-cells, among other concerns.
Specific comments:
1) Can the authors determine the full-length sequence of this new isoform by 5' and 3'RACE? How abundant is it?
2) Antibody specificity: there seems to be a band (below 43KDa) detected in Jurkat PHA IP samples by WB, which questioned the specificity of the antibody used. As this antibody was used for all subsequent experiments, the authors must

provide more evidence to support its specificity.
3) Fig 1C: The starting material (input) for the immunoprecipitation experiment is missing. Please provide.
4) Is there a difference in cellular localization between PD-1 and PDCD1^28 upon T-cell activation (e.g., upon PHA treatment)? PD-1 is supposed to localize to the cell membrane, which will be a good control.
5) The authors used three classic methods to study whether PDCD1^28 can be induced upon T cell activation? However, in Fig. 1G-L, only the expression of PDCD1^28 was presented. It is essential for the authors to supplement this with additional data demonstrating whether the upregulation of PDCD1^28 is a result of PD1 upregulation, and/or if intron retention is more prominently activated?
6) A significant concern in this study lies in the omission of measuring splicing activity through a quantifiable readout such as PSI (percent splice in). In Figure 2, the authors sought to identify splicing factors responsible for intron 2 retention and PDCD1^28 production. However, the sole dependence on the expression of PDCD1^28 as a readout is inadequate for a comprehensive assessment of splicing activity. In Figure 2N, the authors concluded that based on the minigene reporter assay, TAF15 KD led to reduced splicing of PDCD1^28. However, is it plausible that TAF15 might affect the expression of PDCD1^28 through regulating PD-1, rather than exclusively through splicing alterations. All these concerns must be addressed.
7) The methodology employed for the specific detection of the PDCD1^28 isoform needs clarification. The authors should include a schematic diagram delineating the locations of the primers used.
8) Figure 3L: the changes are too small to support their claim.
9) Both PDCD-1 and PDCD-1^28 exhibit conservation in their N-terminal regions, with notable differences primarily in their C-terminal regions. Data shown in Figure 3F-3I reveals that canonical PDCD-1 and PDCD-1^28 exert nearly identical effects on the proliferation and IL-2 secretion of Jurkat cells, suggesting that their distinct C-terminal regions may not result in significant functional differences between these two isoforms. However, in Figure 3P, the PDCD1^28 cytoplasmic tail demonstrates a more potent inhibitory effect. If the authors were to fuse the PDCD-1 cytoplasmic tail with the CD28 extracellular domain and subsequently compare its impact on cell proliferation and IL2 production with that of CD28-PDCD1^28, what would you observe?
10) The authors demonstrated that PDCD-1^28 is primarily expressed in the cytoplasm, unlike PD1, which resides on the cell membrane. The interaction between PD-L1 and PDCD-1^28 within the cell, including whether and where such interaction occurs, needs clarification.

Reviewer #3

(Remarks to the Author)
In this study, Wang et al. characterize a splicing isoform of PD-1, termed as PD-1^28, as a potential immune checkpoint. They have found that PD-1^28 is expressed in activated T lymphocytes and is regulated by the RNA binding protein TAF15. While primarily located in the cytoplasm, this PD-1 splicing isoform exerts its repressive function by inhibiting T cell proliferation, cytokine production, and tumor cell killing. Additionally, PD-1^28 has been shown to confer resistance to anti-PD-L1 immunotherapy. Overall, the authors' findings shed light on an unexplored splicing isoform of PD-1, crucial for enhancing our understanding of the various ways in which PD-1 modulates immune responses. However, certain aspects of the study require strengthening to ensure the suitability of this work for publication.

Major comments:

1. The existence of PDCD1^28 transcripts in various human leukemia cell lines is verified by RT-PCR coupled with Sanger sequencing (Fig. S1). Based on band intensity, the mRNA levels of the PDCD1 and PDCD1^28 splicing isoform seems to be nearly equal. How about their ratio at the protein levels? It would be beneficial for the authors to perform a concurrent assessment of PD-1 and PD-1^28 in activated Jurkat cells and primary T cells, utilizing an antibody targeting their shared sequences within exon 1 and 2. Further, the authors have developed two monoclonal antibodies and one polyclonal antibody that target the cytoplasmic tail of PD-1^28 (Fig S1G). These antibodies are valuable tools. Can the authors also clarify their availability for flow cytometry analysis.

2. PD-1^28 mainly resides in the cytoplasm, in contrast to PD-1 which is located on the cell surface. It is crucial for the authors to elucidate how PD-1^28 exerts its immunosuppressive function in T cells. Investigating the effects of truncating the "extracellular" domain and cytoplasmic tail of PD-1^28 could provide valuable insights into its mechanism of action.

3. The PDCD1^28CKI mice are a valuable tool for elucidating the role of PD-1^28 in vivo. It is essential for the authors to detail the construction strategy used to creat this strain. Also, demonstrating PD-1 expression on T cells in these mice is necessary to determine whether it is influenced by the expression of PD-1^28.

Minor:
Fig S1H, the flow cytometry gates in need to be unified.
Fig S1J, is the PD-1 antibody used here targeting PD-1 or PD-1^28? Also, it would be helpful if the authors could simultaneously show the expression of both PD-1 and PD-1^28.

Version 1:

Reviewer comments:

Reviewer #2

(Remarks to the Author)
The authors have comprehensively addressed most of my concerns, except for one question shown below:

In response to one of the questions, the authors made the following conclusion: “To explore whether intron retention is more prominently activated, we compared the expression levels of PDCD1^28 and PDCD1, suggesting intron retention is less pronounced (Fig. 1G, I, and K)”.

I wondered how the authors came to this conclusion. The data presented in Fig. 1G, I, and K merely show that the mRNA expression of the canonical PDCD1 transcript and its intron-retained isoform (PDCD1^28) is upregulated in T-cells treated with CD3/CD28, PMA/Iono, and PHA. These datasets do not provide information about intron retention. The authors should measure the Percent Spliced In (PSI) values for this experiment. Therefore, is the increase in PDCD1^28 expression in activated T-cells merely due to the upregulation of the canonical PDCD1 transcript, or is splicing regulation also involved?

Reviewer #3

(Remarks to the Author)
The authors have performed extensive new experimental work to validate their findings and support their conclusion. These revisions addressed most of my concerns.

Reviewer #1 (Remarks to the Author):

We sincerely thank the reviewer's valuable comments and constructive criticisms, which were of great help in improving the manuscript. According to the reviewer's comments and suggestions, the manuscript has been systematically revised with the new data and additional interpretations. Reviewer's comments are responded point-by-point as below.

This is an interesting study from a group in China. This group has identified a novel splice variant of the negative costimulatory molecule, PD-1, and are characterizing its expression, gene regulation and function. They hypothesize that this splice variant of PD-1 will additionally mediate immune escape of cancer, in addition to the wildtype version. They first demonstrate the identification of the PD-1 splice variant, which seems to retain part of an intron. They then go on to focus on the splicing and function. The group even generated a specific antibody that specifically recognizes just the PD-1 splice variant protein. Then they go on to show that this splice variant of PD-1 does bind to PD-L1, which determines the inhibition of T cells. Finally, they over-express the human version of this PD-1 splice variant (PD-1^28) in mice and demonstrate faster tumor progression in these mice challenged with MC38 colon cancer tumors. Overall, there is some interesting data on the identification of this PD-1 splice variant. While significant and important for the field, significant issues exist with the story. These issues are outlined below:

Major Comments.

1. There is no context for the prevalence of this splice variant isoform with the other known variants, the relative expression compared to the wild type protein, or the negative costimulatory function in primary cells.

Response:

We sincerely appreciate the reviewer's constructive comments. Due to the lack of antibodies for other variants of *PDCD1*, we compared *PDCD1*, *PDCD1^28*,

and other variants at the mRNA levels. We have designed primers that could specifically recognize these different splicing forms respectively. The specific primer for *PDCD1* was designed at the junction of exon 2 and exon 3, which is the only difference between *PDCD1* and *PDCD1^28* at the mRNA level. The specific primer for *PDCD1^28* was designed against first unique 28bp of intron 2 and exon 3. In order to calculate the expression levels of other variants, as currently all splicing forms of *PDCD1* pre-mRNA retain exon 1, we designed primers specific to exon 1 and subtracted *PDCD1* and *PDCD1^28* from the expression of exon 1 in mature mRNA. Using this strategy, we have assessed the copy numbers of *PDCD1*, *PDCD1^28*, and other variants in $CD3^+$ T cells derived from PBMCs of three healthy individuals (Supplementary Fig. 1*Q*). Furthermore, we compared the expression of PD-1 and PD-1^28 in different populations of primary T cells with FACS (Fig. 1*M*). We have added these results in lines 113-119 (Supplementary Fig. 1*Q* and Fig. 1*M*) of the revised manuscript and corresponding methods in lines 120-122 of Supplementary information. The detailed protocol regarding the method to determine the exact copy number was shown below. We have made every effort to distinguish these different splicing forms and hope that the strategy is acceptable. About the negative costimulatory function of PD-1^28 in primary T cells, we described in the response to the second major comment.

lines 113-119

To evaluate the prevalence of *PDCD1^28* and other variants as well as *PDCD1*, we determined the copy numbers of *PDCD1*, *PDCD1^28*, and other variants of *PDCD1* in $CD3^+$ T cells derived from PBMCs of three healthy individuals (Supplementary Fig. 1*Q*), and further measured the expression of PD-1 and PD-1^28 in different populations of primary T cells with FACS (Fig. 1*M*), which revealed that PD-1 was relatively highly expressed compared to PD-1^28.

lines 120-122

Transcriptional copy number was measured by using standard curve method and the exact copy number was calculated by relating the CT value to

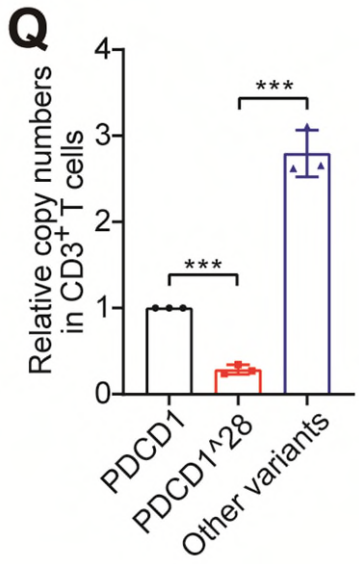
standard curve (Qu L, et al, 2016; Gu Y, et al, 2021).

In detail, this experiment was performed as the following three steps:

1) Establishing the Standard Curve: we prepared a series of standard solutions containing known concentrations of target genes, in which we used target gene plasmids as standard samples. Next, we perform qPCR on these standards to obtain the cycle threshold (Ct) values and then plot the Ct values with the logarithm of the known copy numbers to create a standard curve. The standard curve shows a linear relationship between the Ct values and the log of the initial copy number.

2) Sample Amplification: we run the qPCR reaction with unknown samples under the same conditions as the standards and obtain the Ct values for the unknown samples.

3) Data Analysis: using the standard curve, interpolate the Ct values of the unknown samples to determine their initial copy number.



Supplementary Fig. 1*Q*

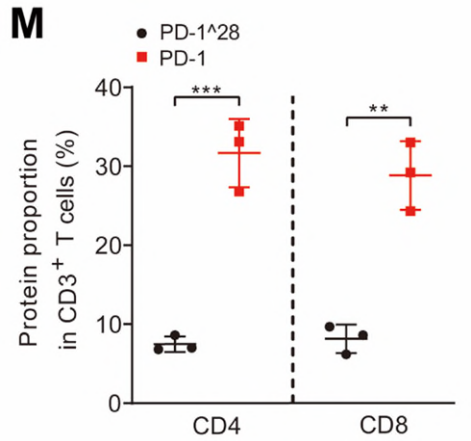


Fig. 1*M*

References:

Qu L, et al. Exosome-Transmitted lncARSR Promotes Sunitinib Resistance in Renal Cancer by Acting as a Competing Endogenous RNA. Cancer Cell 2016;29:653-68.

Gu Y, et al. DMDRMR-Mediated Regulation of m6A-Modified CDK4 by m6A Reader IGF2BP3 Drives ccRCC Progression. Cancer Res. 2021 Feb 15;81(4):923-934.

2. There is almost a complete dependence for all of the functional data on the use of a T cell line (Jurkat). The Jurkat T cell line is actually derived from a T cell leukemia. As such, it is not clear that PD-1 (or PD-1^28) is regulated in a normal fashion. It is also not clear that over-expression of knockdown of this PD-1^28 variant in Jurkats will give true information about its function. Such work needs to be done in primary T cells.

Response:

We apologize for the unclear description in functional experiments and isolation method of primary T cells in our original manuscript. Originally, we tested PD-1^28's function in Jurkat cells and primary T cells in parallel (Supplementary Fig. 3). Primary T cells were isolated from normal human PBMCs and further enriched with CD3 antibody followed by *PDCD1^28* KD or OE assays. Furthermore, we repeated the phenotype experiments of primary

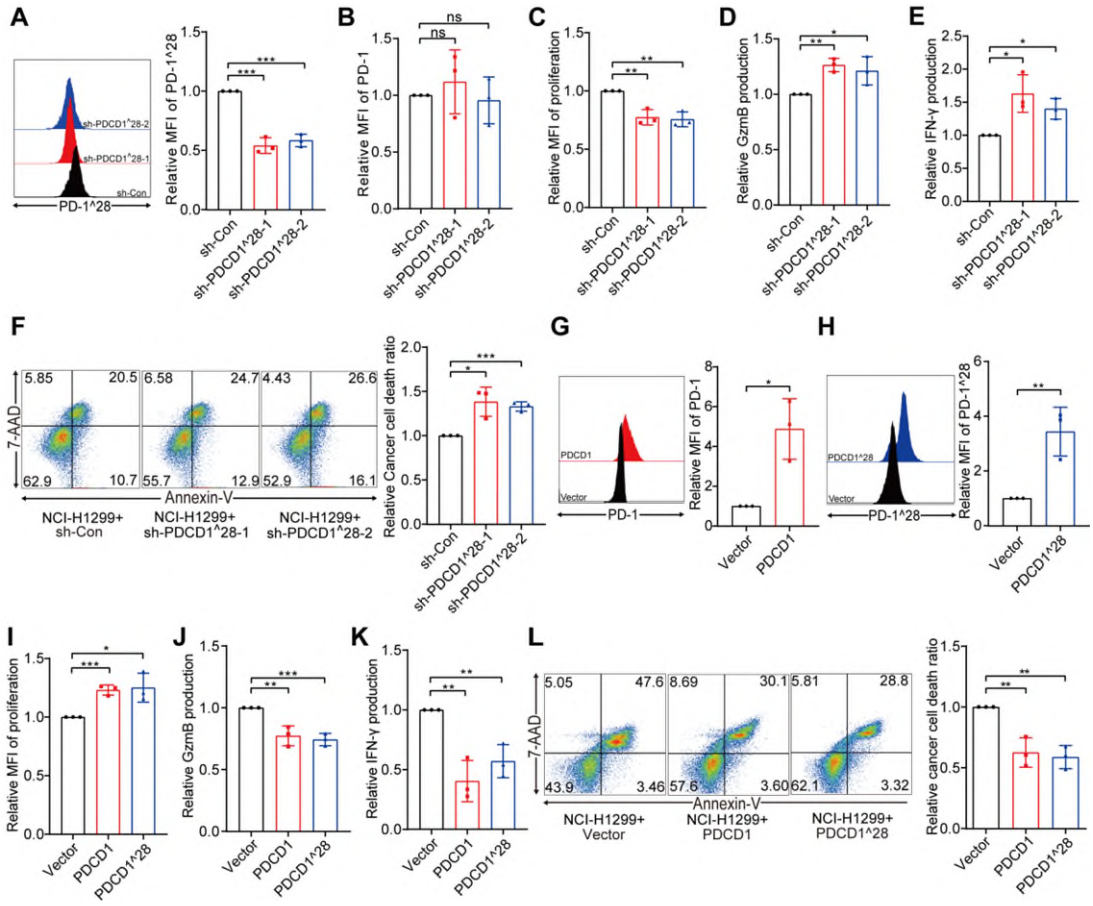
T cells and achieved better knockdown and overexpression efficiency, which led to better phenotypes. Phenotypic experiments showed that *PDCD1^28* KD enhanced cell proliferation and production of Granzyme-B (GzmB) and interferon gamma (IFN-γ) in primary T cells. As expected, *PDCD1^28* KD exhibited elevated killing ability against NCI-H1299 tumor cells. Conversely, *PDCD1^28* OE compromised cell proliferation, cytokine production, and killing ability against NCI-H1299 tumor cells compared to control cells. We have highlighted these results in lines 174-178 and replaced the results of primary T cells in Supplementary Fig. 3. We also added more details regarding primary T cell separation from human PBMCs in methods of Supplementary information (lines 98-99).

lines 174-178

Considering that the Jurkat T cell line derived from T cell leukemia, we conducted the same functional experiments in primary T cells derived from PBMCs to explore the true function of PD-1^28. As expected, we observed the similar immune function changes in primary T cells as Jurkat cells (Supplementary Fig. 3*A*-*L*).

lines 98-99

Peripheral blood mononuclear cells (PBMCs) were isolated by gradient centrifugation and enrichment of primary T cells with CD3.



Supplementary Fig. 3*A-L*

3. The transgenic expression of the human isoform in mice may not at all represent the true biology of this negative costimulatory molecule. As such, it is not clear how to interpret the murine studies.

Response:

We are very sorry for unclear descriptions in murine studies and many thanks for this concern. Firstly, we did not find an equivalent isoform similar to *PDCD1^28* in murine *Pdcd1*. Secondly, it has been reported that the amino acid sequence between human PD-L1 (hPD-L1) and murine Pd-l1 (mPd-l1) is very conserved, and their overall structure is almost identical (Magiera-Mularz et al., 2021). mPd-l1 and hPD-L1 have the same affinity when binding to human PD-1, and mPd-l1/hPD-1 can inhibit the activation of Jurkat cells (Magiera-Mularz et al., 2021). Importantly, the functional experiments in murine studies of our manuscript showed that PD-1^28/mPd-l1 axis indeed

engage and function effectively. We have rewritten the text as needed in lines 251-258 of the revised manuscript.

lines 251-258

To study the physiological function of PD-1^28, we did not find an equivalent isoform similar to *PDCD1^28* in murine *Pdcd1*. However, it has been well verified that the amino acid sequence between human PD-L1 and murine Pd-l1 is very conserved, and their overall structure is almost identical (Magiera-Mularz et al., 2021). Murine Pd-l1 and human PD-L1 have the same affinity when binding to human PD-1, and murine Pd-l1/human PD-1 axis can inhibit the activation of Jurkat cells (Magiera-Mularz et al., 2021). We speculated that PD-1^28 could bind to murine Pd-l1 to exert immune function.

References:

Magiera-Mularz, K. et al. Human and mouse PD-L1: similar molecular structure, but different druggability profiles. iScience 24, 101960 (2021).

4. The transcription factor studies (Figure 2) are interesting, but the regulation of the alternative mRNA splicing for this variant is not really related to the important aspects of the function. As such, the data here are not really integrated with the rest of the story.

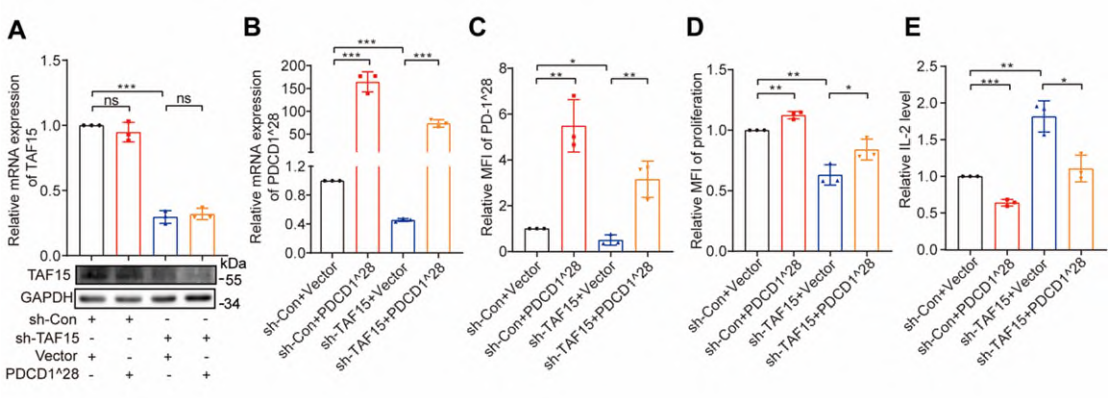
Response:

We thank the reviewer for the constructive suggestions. We have performed rescue assays to test whether the regulation of the alternative splicing for *PDCD1^28* is related to the immune function. Rescue experiments showed that *PDCD1^28* OE could rescue *TAF15* KD-mediated elevation of cell proliferation and IL-2 production. We have added these results in lines 210-213 (Supplementary Fig. 6*A*-*E*) of our revised manuscript.

lines 210-213

We next wondered whether the regulation of the alternative splicing for *PDCD1^28* is related to the immune function and performed rescue experiments. Strikingly, *PDCD1^28* OE rescued the increase of cell

proliferation and IL-2 production of the *TAF15* KD (Supplementary Fig. 6*A-E*).



Supplementary Fig. 6*A-E*

(A) qRT-PCR analysis for *TAF15* (top) and immunoblot analysis for the indicated proteins (bottom) in Jurkat cells transfected with the indicated plasmids (n = 3). (B and C) qRT-PCR analysis for *PDCD1^28* (B), and FACS analysis for PD-1^28 protein levels, quantified as MFI (C) (n = 3). (D and E) CFSE assay assessing the relative proliferation (D), and ELISA assessing the relative IL-2 production (E) (n = 3).

Reviewer #2 (Remarks to the Author):

We very much appreciated the reviewer's valuable comments and constructive criticisms, which were of great help in improving the manuscript. According to the reviewer's comments and suggestions, the manuscript has been systematically revised with the new data and additional interpretations. Reviewer's comments are responded point-by-point as below.

In this manuscript, Wang et al. has identified and characterized the function of a novel isoform of *PDCD1*, which is arising from a 28nt retention at intron 2, resulting in the expression of *PDCD1^28* in activated T-cells. The claim behind the existence of *PDCD1^28* is largely supported by experimental data. Functionally, Wang et al. showed that *PDCD1^28* inhibits both T-cell proliferation and cytokine production and promotes tumour growth in vivo. Using their *PDCD1^28* mouse models, the authors also suggested that *PDCD1^28* might confer resistance to anti-PD-L1 therapy. Overall, this is an interesting study. However, there is a notable gap in understanding the mechanism behind the differential splicing of this isoform in activated T-cells, among other concerns.

Specific comments:

1) Can the authors determine the full-length sequence of this new isoform by 5' and 3' RACE? How abundant is it?

Response:

Question 1:

We thank the reviewer for the constructive suggestions. According to the reviewer's comments, we have performed 5'- and 3'-RACE of *PDCD1^28* and successfully amplified 5' and 3' sequence based on nested PCR with three rounds (strategy shown in Supplementary Fig. 1*F*), in which we used primer against first unique 28bp of intron 2 and exon 3 in last round. Our results showed that *PDCD1^28* was a 2,124-nt transcript with five exons. We have

added these results in lines 80-83 (Supplementary Fig. 1*F* and *G*) of the revised manuscript. We also added corresponding methods in lines 126-133 and the sequence of *PDCD1^28* transcript in Supplementary information.

lines 80-83

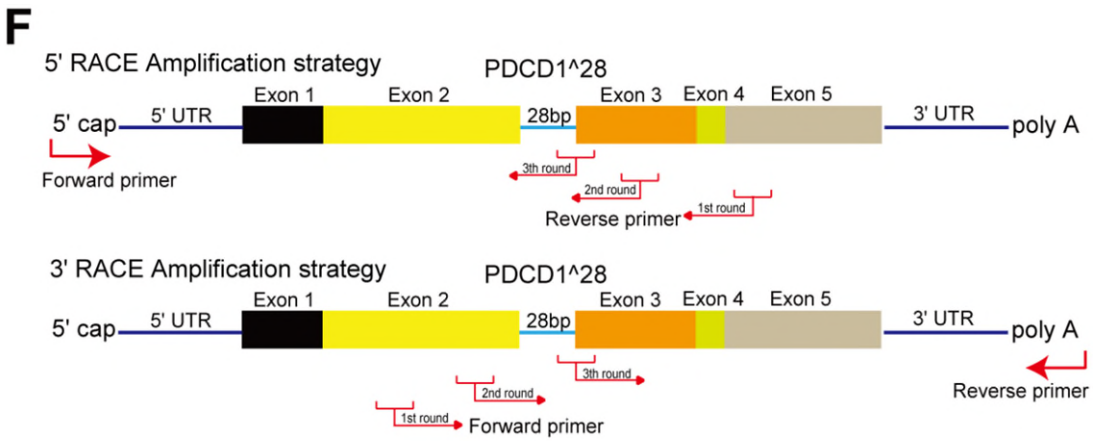
We further performed 5'-and 3'-rapid amplification of cDNA ends (RACE) and found that *PDCD1^28* was a 2,124-nt transcript with five exons (Supplementary Fig. 1*F* and *G*; Supplementary Table *S2*).

lines 126-133

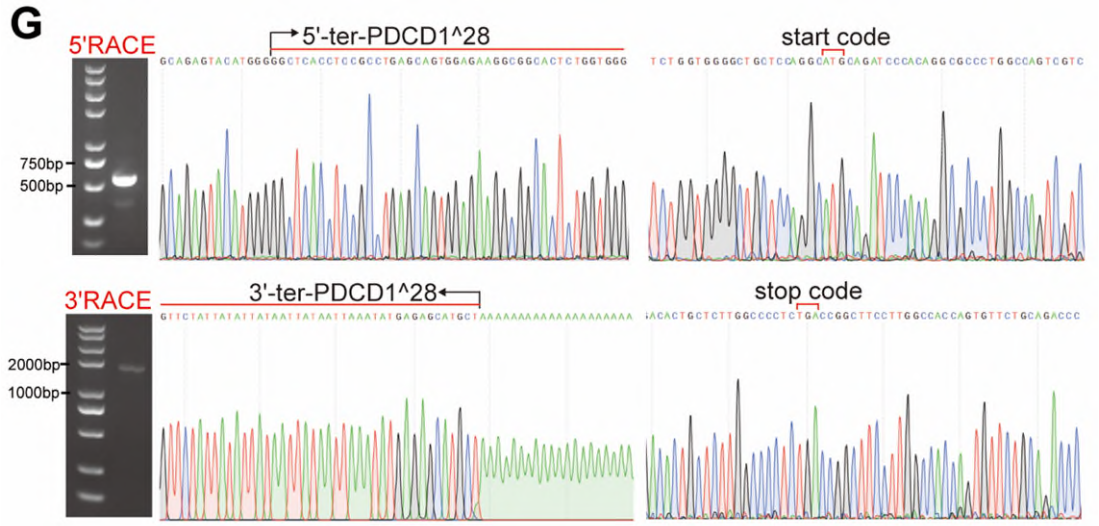
The 5'-and 3'-RACE analyses were performed to determine the transcriptional initiation and termination sites of *PDCD1^28* based on the nested PCR with three rounds. The primers of three rounds PCR were respectively as following:

5'-RACE: 5'-tccagctccccatagtccacag-3', 5'-cgacaccaaccaccaggg-3', and 5'-cttctctccctgccccgggg-3'.

3'-RACE: 5'-aactggtaccgcatgagccc-3', 5'-cttccacatgagcgtggtca-3', and 5'-ccccggggcagggagagaag-3'.



Supplementary Fig. 1*F*



Supplementary Fig. 1*G*

Question 2:

We greatly appreciate the reviewer's comments and suggestions. We have assessed the copy numbers of *PDCD1^28* in Jurkat cells stimulated by CD3 and CD28, representing the absolute expression levels. We have added these results in lines 104-107 (Supplementary Fig. 1*O*) of our revised manuscript, and also added the corresponding methods in lines 120-122 of Supplementary information. The detailed protocol regarding the method to determine the exact copy number was shown below.

lines 104-107

Both mRNA and protein levels of the PD-1^28 were elevated after Jurkat cell activation (Fig. 1*G-L*), and we also observed an increase in the copy numbers of *PDCD1^28* upon CD3 and CD28 stimulation (Supplementary Fig. 1*O*).

lines 120-122

Transcriptional copy-number was measured by using standard curve method and the exact copy number was calculated by relating the CT value to standard curve (Qu L, et al, 2016; Gu Y, et al, 2021).

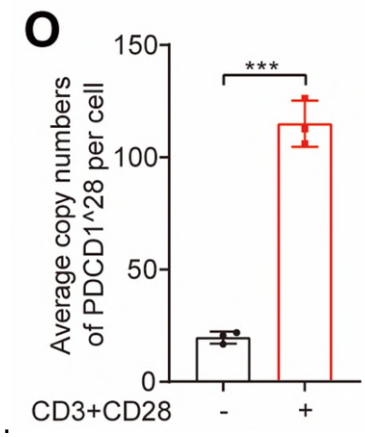
In detail, this experiment was performed as the following three steps:

1) Establishing the Standard Curve: we prepared a series of standard solutions containing known concentrations of target genes, in which we used target gene plasmids as standard samples. Next, we perform qPCR on these

standards to obtain the cycle threshold (Ct) values and then plot the Ct values with the logarithm of the known copy numbers to create a standard curve. The standard curve shows a linear relationship between the Ct values and the log of the initial copy number.

2) Sample Amplification: we run the qPCR reaction with unknown samples under the same conditions as the standards and obtain the Ct values for the unknown samples.

3) Data Analysis: using the standard curve, interpolate the Ct values of the unknown samples to determine their initial copy number.



Supplementary Fig. 1*O*

References:

Qu L, et al. Exosome-Transmitted lncARSR Promotes Sunitinib Resistance in Renal Cancer by Acting as a Competing Endogenous RNA. Cancer Cell 2016;29:653-68.

Gu Y, et al. DMDRMR-Mediated Regulation of m6A-Modified CDK4 by m6A Reader IGF2BP3 Drives ccRCC Progression. Cancer Res. 2021 Feb 15;81(4):923-934.

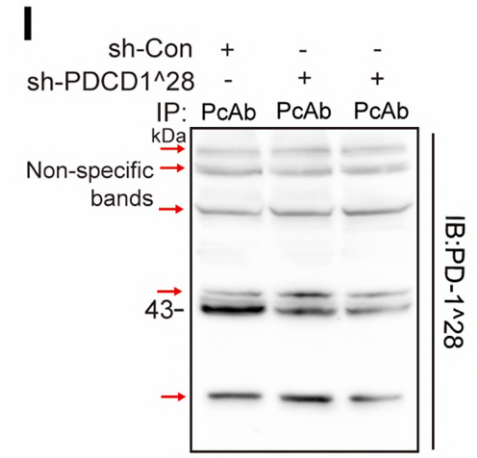
2) Antibody specificity: there seems to be a band (below 43KDa) detected in Jurkat PHA IP samples by WB, which questioned the specificity of the antibody used. As this antibody was used for all subsequent experiments, the authors must provide more evidence to support its specificity.

Response:

Thanks for pointing out this issue. Originally, we made three specific antibodies, including two monoclonal antibodies and one polyclonal antibody. After multiple tests, we found that polyclonal antibody worked better in IP and monoclonal antibody can recognize well the endogenous PD-1^28 with 43 kDa in WB after IP. To validate the specificity of these antibodies, we used two different shRNAs to knock down the endogenous PD-1^28 and performed an IP followed by WB in Jurkat cells activated by PHA. We only observed a reduced intensity of approximately 43 kDa size band in the whole membranes, while the remaining bands were non-specific and could serve as good controls to demonstrate that we equally loaded total protein. We conducted three biological replicates. We have added the results in Supplementary Fig. 1*I* and rewritten the descriptions in lines 87-90 of the revised manuscript. Additionally, the other two biological repeats were shown below (Fig. R*1*, communication only for review).

lines 87-90

We obtained monoclonal and polyclonal antibodies and verified that PD-1^28 could be specifically recognized through immunoprecipitation followed by immunoblot (Supplementary Fig. 1*H* and *I*).



Supplementary Fig. 1*I*

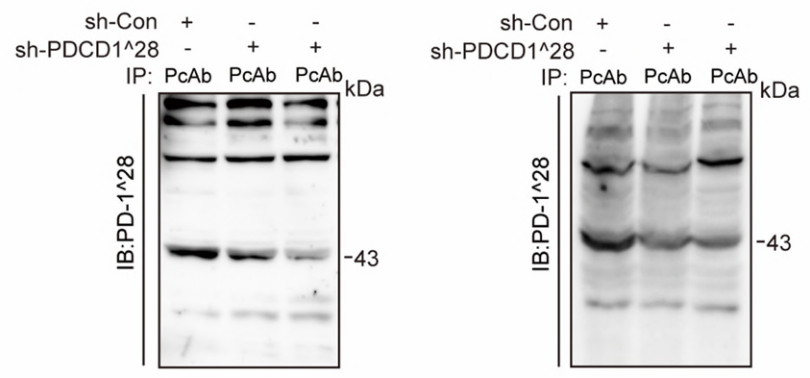


Fig. R*1* only for reviewer, another two biological repeats.

3) Fig 1C: The starting material (input) for the immunoprecipitation experiment is missing. Please provide.

Response:

We sincerely appreciate the reviewer's constructive comments. We are disappointed and apologize for the unsatisfactory performance of our antibodies. We failed to detect the endogenous PD-1^28 by direct WB and could only recognize endogenous PD-1^28 through an IP followed by WB. We only performed direct WB under the condition of overexpressing *PDCD1^28*. Therefore, we didn't show the input in Fig 1*C*. We are very sorry for this and hope that this is acceptable.

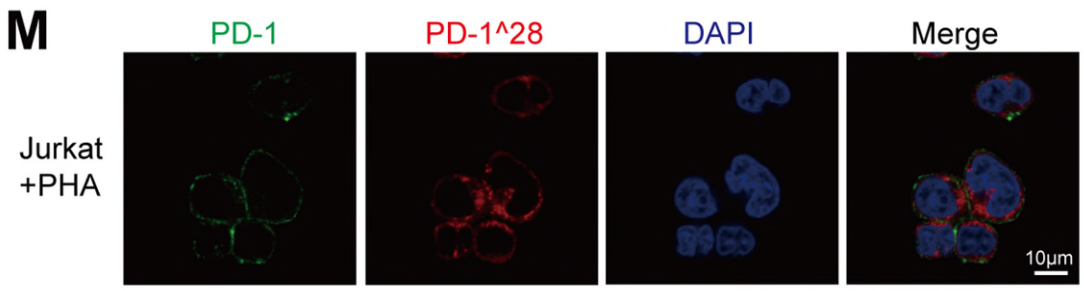
4) Is there a difference in cellular localization between PD-1 and PDCD1^28 upon T-cell activation (e.g., upon PHA treatment)? PD-1 is supposed to localize to the cell membrane, which will be a good control.

Response:

Many thanks for these nice suggestions. As suggested by the reviewer, we performed immunofluorescence experiment to simultaneously demonstrate the localization of PD-1 and PD-1^28 in Jurkat cells activated by PHA, suggesting that they have different cellular localization. We have added these results in lines 99-101 (Supplementary Fig. 1*M*) of our revised manuscript.

lines 99-101

We simultaneously detected the localization of PD-1 and PD-1^28 in Jurkat cells activated by PHA, suggesting that they have different cellular localization (Supplementary Fig. 1*M*).



Supplementary Fig. 1*M*

5) The authors used three classic methods to study whether *PDCD1^28* can be induced upon T cell activation? However, in Fig. 1G-L, only the expression of *PDCD1^28* was presented. It is essential for the authors to supplement this with additional data demonstrating whether the upregulation of PDCD1^28 is a result of PD1 upregulation, and/or if intron retention is more prominently activated?

Response:

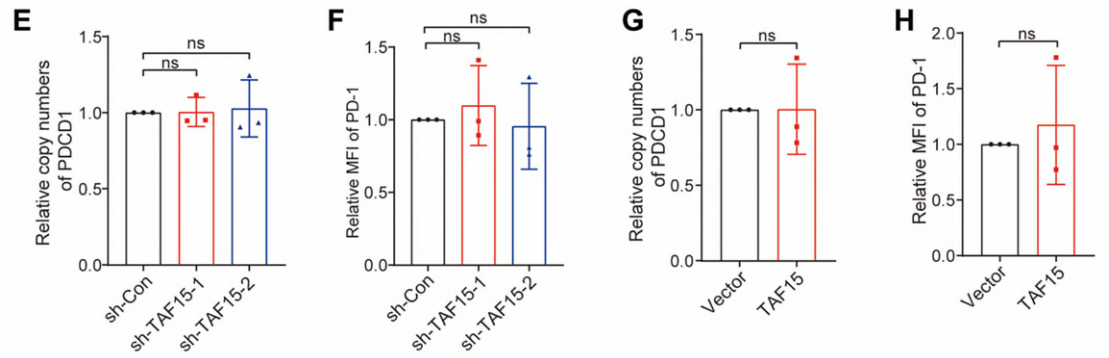
Question 1:

Thanks for the insightful comments and suggestions. In our previous manuscript, we showed that the splicing of *PDCD1^28* is regulated by TAF15. Therefore, to test whether the upregulation of *PDCD1^28* is a result of *PDCD1* upregulation, we knocked down and overexpressed *TAF15* in Jurkat cells and evaluated the expression changes of *PDCD1* upon PHA stimulation. Our new results showed that *TAF15* KD or OE did not affect the mRNA or protein expression levels of PD-1, indicating the upregulation of *PDCD1^28* and *PDCD1* is independent of each other. We have added these results in lines 143-146 (Supplementary Fig. 2*E-H*) of our revised manuscript. In addition, based on your comment, we have realized unclear drawing in our schematic

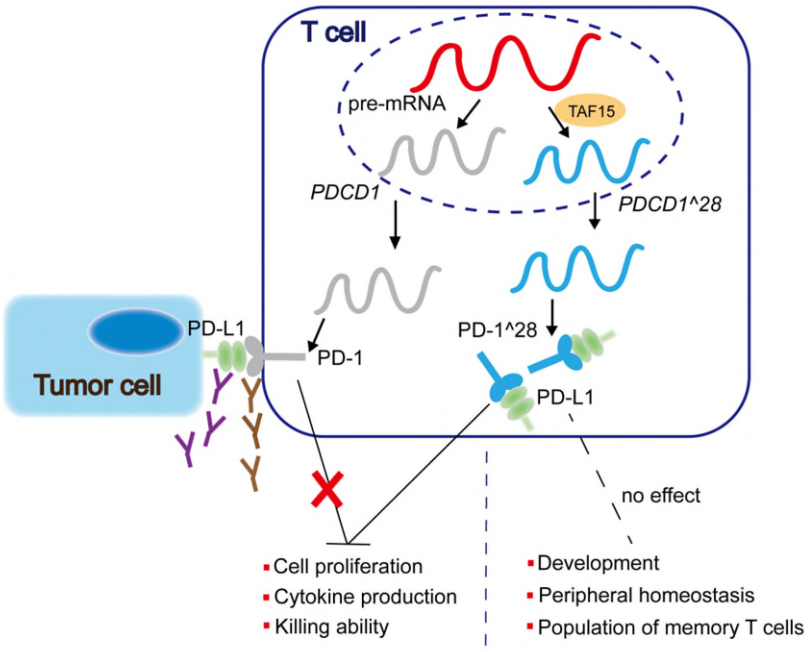
figure and have made modifications to clarify TAF15's function in our schematic figure. We apologize for any misunderstandings caused. We have replaced the new schematic figure in Supplementary Fig. 9.

lines 143-146

To further elucidate the activation mechanism of *PDCD1^28* with *TAF15*, we knocked down and overexpressed *TAF15* in Jurkat cells and found that the changes of *TAF15* did not affect the mRNA or protein expression levels of PD-1 (Supplementary Fig. 2*E-H*).



Supplementary Fig. 2*E-H*



Supplementary Fig. 9

Question 2:

To clarify whether intron retention is more prominently activated in Jurkat cells

under three classical stimulation conditions, we designed different primers targeting *PDCD1^28* and *PDCD1*, respectively. The specific primer for *PDCD1* was designed at the junction of exon 2 and exon 3, which is the only difference between *PDCD1* and *PDCD1^28* at the mRNA level. The specific primer for *PDCD1^28* was designed against the first unique 28bp of intron 2 and exon 3. We then compared the expression levels of *PDCD1^28* and *PDCD1*, and the results indicated that intron retention is less pronounced. We have replaced the results of the expression levels of both *PDCD1^28* and *PDCD1* in Fig. 1*G, I*, and *K*, and added the descriptions in lines 107-110 of our revised manuscript.

lines 107-110

To explore whether the intron retention is more prominently activated, we compared the expression levels of *PDCD1^28* and *PDCD1*, suggesting intron retention is less pronounced (Fig. 1*G, I,* and *K*).

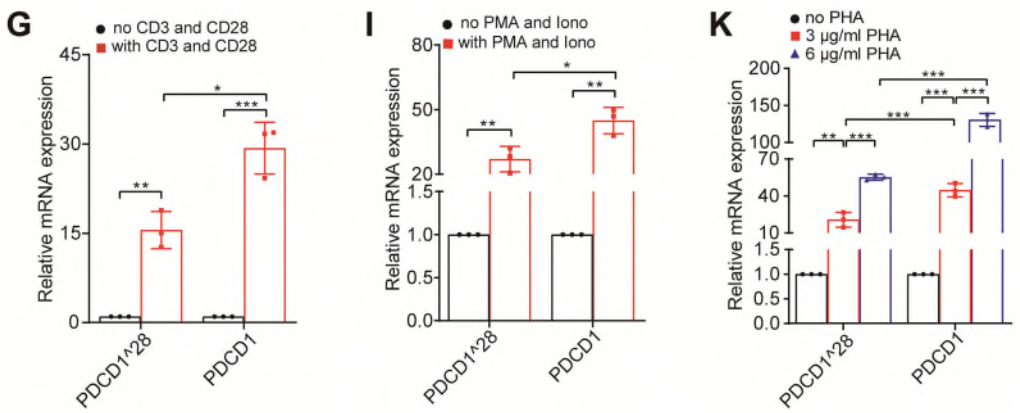


Fig. 1*G, I,* and *K*

6) A significant concern in this study lies in the omission of measuring splicing activity through a quantifiable readout such as PSI (percent splice in). In Figure 2, the authors sought to identify splicing factors responsible for intron 2 retention and *PDCD1^28* production. However, the sole dependence on the expression of *PDCD1^28* as a readout is inadequate for a comprehensive assessment of splicing activity. In Figure 2N, the authors concluded that based on the minigene reporter assay, *TAF15* KD led to reduced splicing of *PDCD1^28*. However, is it plausible that *TAF15* might affect the expression of

PDCD1^28 through regulating PD-1, rather than exclusively through splicing alterations. All these concerns must be addressed.

Response:

Question 1:

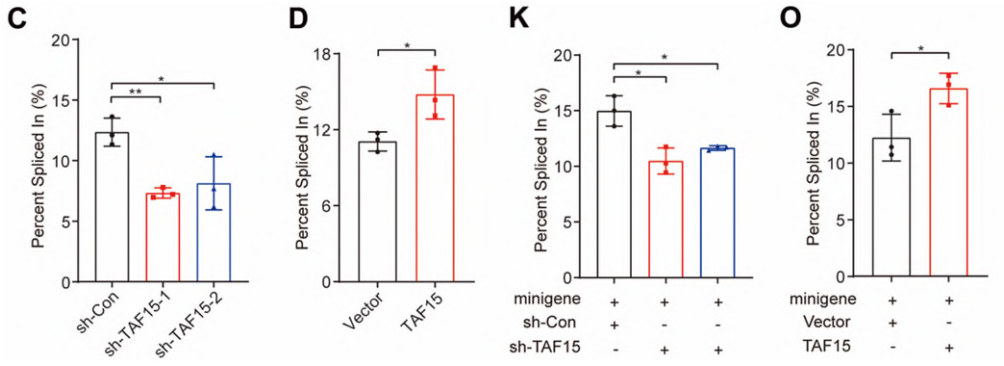
We greatly appreciate the reviewer's comments and suggestions. As suggested by the reviewer, we have measured the percent splice in (PSI) values for *PDCD1^28* splicing event in *TAF15* KD or OE cells and also detected changes of PSI in the *minigene* reporter assay. The data revealed that *TAF15* KD reduced the PSI of *PDCD1^28*, while *TAF15* OE increased it. We have added new descriptions in lines 138-142, and the new results were integrated in Supplementary Fig. 2*C* and *D*, *K* and *O* of the revised manuscript. We also added corresponding methods in lines 122-125 of Supplementary information.

lines 138-142

To evaluate whether *TAF15* affects the splicing activity of *PDCD1^28*, we measured the percent splice in (PSI) values for *PDCD1^28* splicing event in *TAF15* KD or OE cells and observed that *TAF15* KD reduced the PSI of *PDCD1^28*, while *TAF15* OE increased PSI (Supplementary Fig. 2*C* and *D*).

lines 122-125

To measure the value of percent splice in (PSI), we calculate using: $\mathrm{PSI} = \frac{\text{splice in}}{\text{splice in} + \text{splice out}}$. The method for determining exact copy numbers was described above.



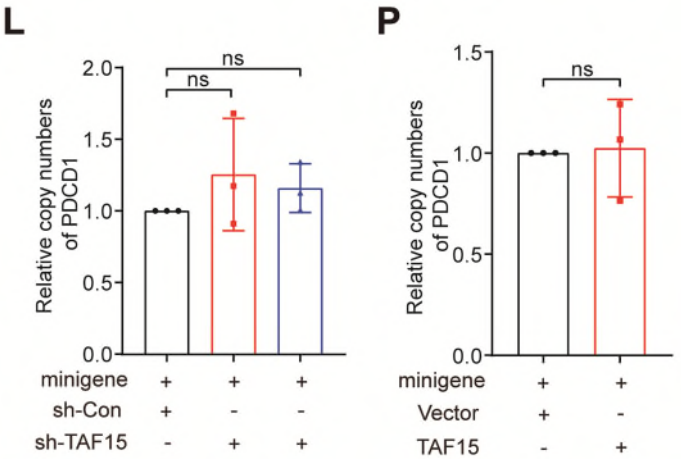
Supplementary Fig. 2*C* and *D*, *K* and *O*

Question 2:

To elucidated whether TAF15 affects the expression of *PDCD1^28* by regulating *PDCD1* in Figure 2N in which the reviewer mentioned in the comments, we detected copy numbers of *PDCD1* after *TAF15* KD or OE in the *minigene* reporter assay. The results indicated that the changes in *TAF15* did not affect the expression of *PDCD1*. We have added these results in lines 155-157 (Supplementary Fig. 2*L* and *P*) of our revised manuscript.

lines 155-157

However, the changes in *TAF15* did not affect the expression of *PDCD1* (Supplementary Fig. 2*L* and *P*).

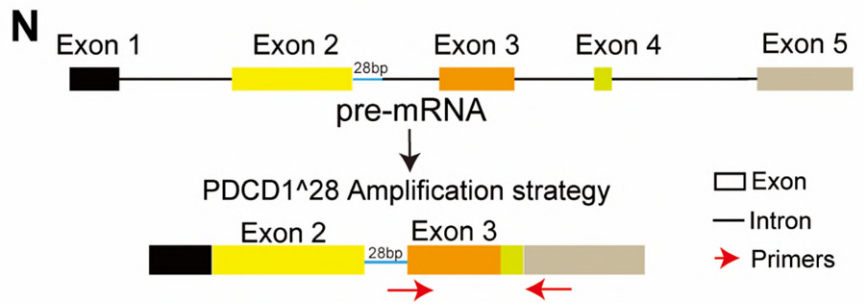


Supplementary Fig. 2*L* and *P*

7) The methodology employed for the specific detection of the *PDCD1^28* isoform needs clarification. The authors should include a schematic diagram delineating the locations of the primers used.

Response:

We sincerely appreciate the reviewer's comments and suggestions. As suggested by the reviewer, we added the schematic diagram for *PDCD1^28* amplification strategy in Supplementary Fig. 1*N* of our revised manuscript.



Supplementary Fig. 1*N*

8) Figure 3L: the changes are too small to support their claim.

Response:

We are sorry for this and many thanks for pointing this out. We increased the expression levels of *CD28* and *CD28-PDCD1^28*, which led to the increased attenuated cell proliferation and IL-2 production. We have replaced Fig. 3*K-M* with new results and hope that this is acceptable.

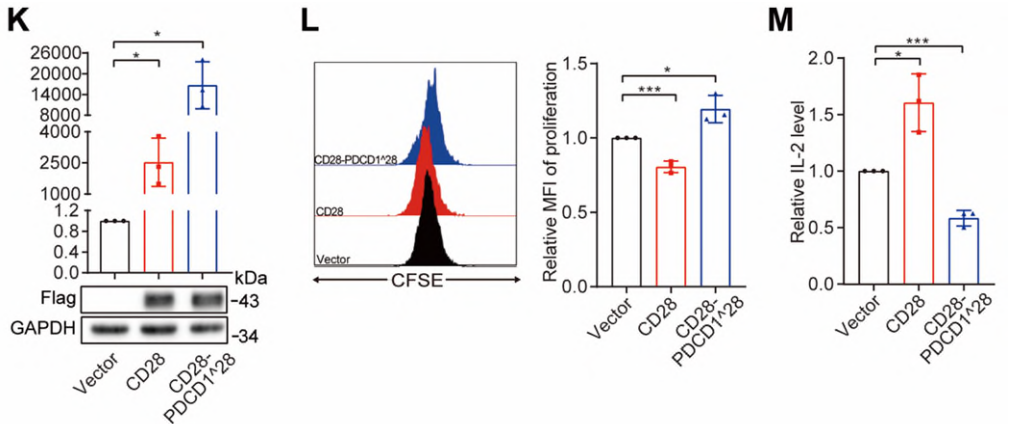


Fig. 3*K-M*

9) Both *PDCD-1* and *PDCD-1^28* exhibit conservation in their N-terminal regions, with notable differences primarily in their C-terminal regions. Data shown in Figure 3F-3I reveals that canonical *PDCD-1* and *PDCD-1^28* exert nearly identical effects on the proliferation and IL-2 secretion of Jurkat cells, suggesting that their distinct C-terminal regions may not result in significant functional differences between these two isoforms. However, in Figure 3P, the *PDCD1^28* cytoplasmic tail demonstrates a more potent inhibitory effect. If the

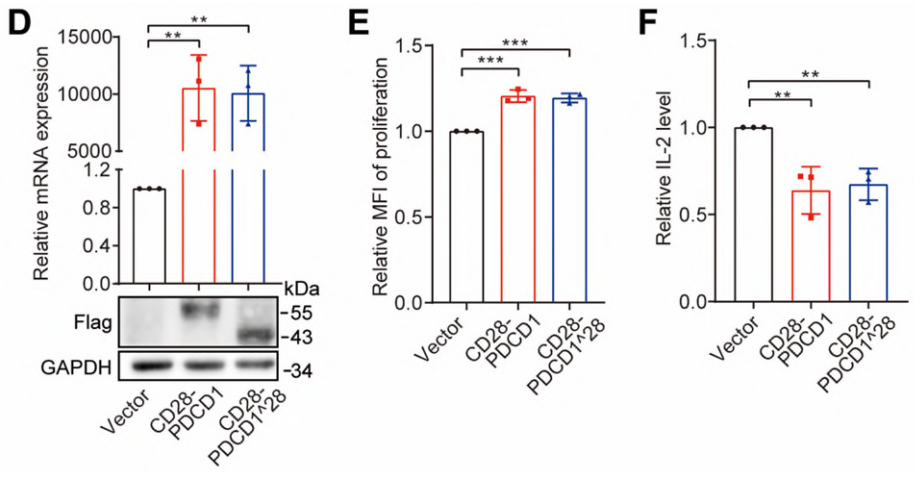
authors were to fuse the *PDCD-1* cytoplasmic tail with the *CD28* extracellular domain and subsequently compare its impact on cell proliferation and IL2 production with that of *CD28-PDCD1^28*, what would you observe?

Response:

We sincerely appreciate the reviewer's constructive comments. As suggested by the reviewer, we have fused the *PDCD1* cytoplasmic tail with the *CD28* extracellular domain (*CD28-PDCD1*) and compared its immune function with that of *CD28-PDCD1^28*. We overexpressed *CD28-PDCD1* and *CD28-PDCD1^28* in KO-1 cells, as indicated by the observed increases in the mRNA and protein levels and found that they have similar inhibitory effects on cell proliferation and IL-2 production. We have added these results in lines 205-209 (Supplementary Fig. 5*D-F*) of our revised manuscript.

lines 205-209

We further fused the *PDCD1* cytoplasmic tail with the *CD28* extracellular domain (*CD28-PDCD1*), and respectively overexpressed *CD28-PDCD1* and *CD28-PDCD1^28* (Supplementary Fig. 5*D*), which showed that both *CD28-PDCD1* and *CD28-PDCD1^28* inhibited cell proliferation and IL-2 production compared to the control cells (Supplementary Fig. 5 *E* and *F*).



Supplementary Fig. 5*D-F*

10) The authors demonstrated that PDCD-1^28 is primarily expressed in the cytoplasm, unlike PD1, which resides on the cell membrane. The interaction

between PD-L1 and PDCD-1^28 within the cell, including whether and where such interaction occurs, needs clarification.

Response:

We sincerely appreciate the reviewer's constructive comments. Firstly, it has been reported that PD-L1 exists not only on the cell membrane but also in the cytoplasm (Tu et al., 2019; Ying et al., 2021). Furthermore, we performed flow cytometry assay and the result revealed that the expression of PD-L1 was significantly higher in the permeabilized cells than the intact membrane cells, indicating PD-L1 is expressed in cytoplasm (Fig. 4*A* in revised manuscript). Secondly, we overexpressed *PDCD1^28* as well as *PDCD1* as a positive control in both KO-1 and KO-2 cells, followed by treatment with a recombinant PD-L1 Fc-fusion protein (PD-L1 immunoglobulin [Ig]) to elicit PD-1 signaling in T cells (Francisco et al., 2009). Exogenous addition of PD-L1 could not further inhibit IL-2 production in *PDCD1^28* OE cells (Fig. 4*B* in revised manuscript). However, in our original manuscript, we observed the inhibition of IL-2 production when simultaneous OE of *PDCD1^28* and *PDCD1LG1* in KO-1 and KO-2 cells (Fig. 4*F* in revised manuscript), indicating that PD-1^28 inhibits T cells activation by binding to cytoplasmic PD-L1. We have rewritten this part in lines 219-230 (Fig. 4*A* and *B*) of our revised manuscript.

lines 219-230

It has been reported that PD-L1 exists not only on the cell membrane but also in the cytoplasm (Tu et al., 2019; Ying et al., 2021), and the IgV domain responsible for PD-1 binding to PD-L1 is retained in PD-1^28 (Fig. 1*B*). Therefore, we hypothesized that PD-1^28 could engage with cytoplasmic PD-L1 to exert function. To verify this hypothesis, we first performed FACS, and the data showed that the expression of PD-L1 was significantly higher in the permeabilized cells than the intact membrane cells, indicating PD-L1 is expressed in cytoplasm (Fig. 4*A*). We overexpressed *PDCD1^28* as well as *PDCD1* as a positive control in both KO-1 and KO-2 cells, followed by treatment with a recombinant PD-L1 Fc-fusion protein (PD-L1 immunoglobulin

[Ig]) to elicit PD-1 signaling in T cells (Francisco et al., 2009). Exogenous addition of PD-L1 could not further inhibit IL-2 production in *PDCD1^28* OE cells (Fig. 4*B*).

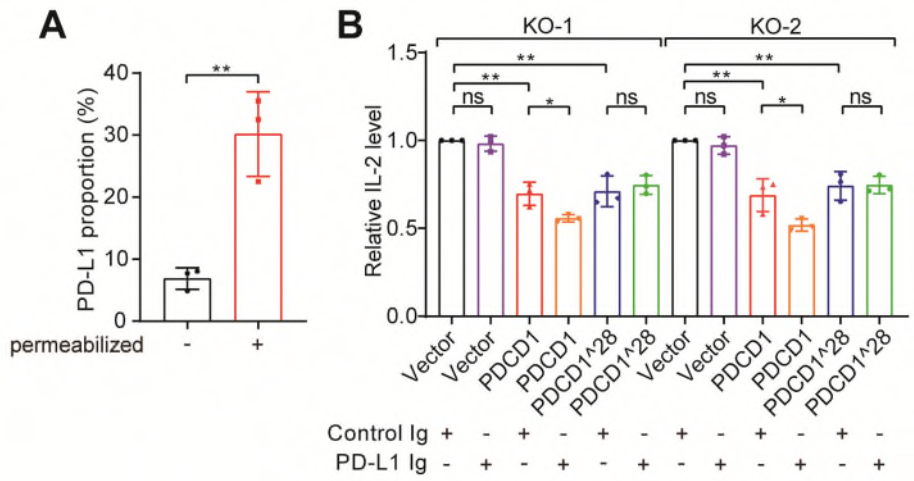


Figure 4 *A* and *B*

References:

Tu, X., et al. (2019). PD-L1 (B7-H1) competes with the RNA exosome to regulate the DNA damage response and can be targeted to sensitize to radiation or chemotherapy. Molecular cell 74, 1215-1226.e1214.

Ying, H., et al. (2021). Non-cytomembrane PD-L1: An atypical target for cancer. Pharmacol Res 170, 105741.

Francisco, L.M., et al. (2009). PD-L1 regulates the development, maintenance, and function of induced regulatory T cells. J Exp Med 206, 3015-3029.

Reviewer #3 (Remarks to the Author):

We sincerely thank the reviewer's valuable comments and constructive criticisms, which were of great help in improving the manuscript. According to the reviewer's comments and suggestions, the manuscript has been systematically revised with the new data and additional interpretations. Reviewer's comments are responded point-by-point as below.

In this study, Wang et al. characterize a splicing isoform of PD-1, termed as PD-1^28, as a potential immune checkpoint. They have found that PD-1^28 is expressed in activated T lymphocytes and is regulated by the RNA binding protein TAF15. While primarily located in the cytoplasm, this PD-1 splicing isoform exerts its repressive function by inhibiting T cell proliferation, cytokine production, and tumor cell killing. Additionally, PD-1^28 has been shown to confer resistance to anti-PD-L1 immunotherapy. Overall, the authors' findings shed light on an unexplored splicing isoform of PD-1, crucial for enhancing our understanding of the various ways in which PD-1 modulates immune responses. However, certain aspects of the study require strengthening to ensure the suitability of this work for publication.

Major comments:

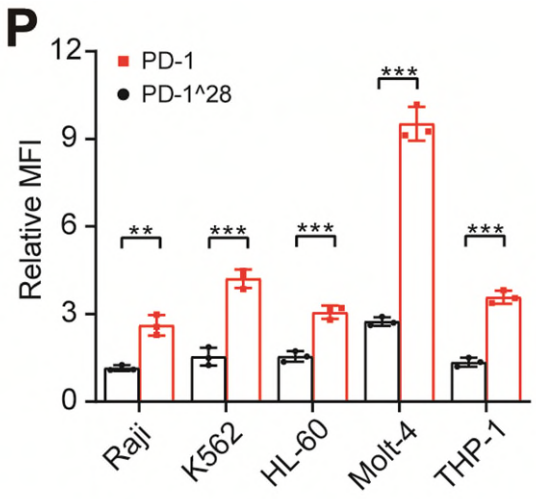
1. The existence of *PDCD1^28* transcripts in various human leukemia cell lines is verified by RT-PCR coupled with Sanger sequencing (Fig. S1). Based on band intensity, the mRNA levels of the *PDCD1* and *PDCD1^28* splicing isoform seems to be nearly equal. How about their ratio at the protein levels? It would be beneficial for the authors to perform a concurrent assessment of PD-1 and PD-1^28 in activated Jurkat cells and primary T cells, utilizing an antibody targeting their shared sequences within exon 1 and 2. Further, the authors have developed two monoclonal antibodies and one polyclonal antibody that target the cytoplasmic tail of PD-1^28 (Fig S1G). These antibodies are valuable tools. Can the authors also clarify their availability for flow cytometry

analysis.

Response:

Question 1:

We sincerely appreciate the reviewer's constructive comments. Because there are other isoforms of *PDCD1* also retain exon 1 and 2 (Nielsen et al., 2005), and we showed that PD-1 and PD-1^28 have different cellular localization as raised by Reviewer 2. To specifically detect classical PD-1 and PD-1^28, we used antibodies to detect membrane-bound PD-1, and PD-1^28 with PD-1^28 specific antibody. As suggested by the reviewer, we compared the protein expression levels of PD-1 and PD-1^28 in these six human leukemia cell lines. FACS analysis showed that there were differences between PD-1 and PD-1^28 at the protein level. The new results were integrated in Supplementary Fig. 1*P* of the revised manuscript, and we merged the description of this result with the result of the next question.



Supplementary Fig. 1*P*

References:

Nielsen, C., et al. Alternative splice variants of the human PD-1 gene. Cell Immunol 235, 109-116.

Question 2:

We thank the reviewer for the constructive suggestions. As suggested by the

reviewer, we have further performed a concurrent assessment of PD-1 and PD-1^28 in activated Jurkat cells (Fig. 1*H, J,* and *L*) and primary T cells (Fig. 1*M*) using FACS, and the expression in primary T cells is the same concern as Reviewer 1. We have replaced new images in Fig. 1*H, J, L* and *M*. We also added description in lines 110-119 which is integrated with the answers of Reviewer 1 in our revised manuscript.

lines 110-119

We also performed a concurrent assessment of PD-1 and PD-1^28 in activated Jurkat cells (Fig. 1*H, J,* and *L*) and human leukemia cell lines (Supplementary Fig. 1*P*), which showed that the protein expression level of PD-1 was higher. To evaluate the prevalence of *PDCD1^28* and other variants as well as *PDCD1*, we determined the copy numbers of *PDCD1*, *PDCD1^28*, and other variants of *PDCD1* in $CD3^+$ T cells derived from PBMCs of three healthy individuals (Supplementary Fig. 1*Q*), and further measured the expression of PD-1 and PD-1^28 in different populations of primary T cells with FACS (Fig. 1*M*), which revealed that PD-1 was relatively highly expressed compared to PD-1^28.

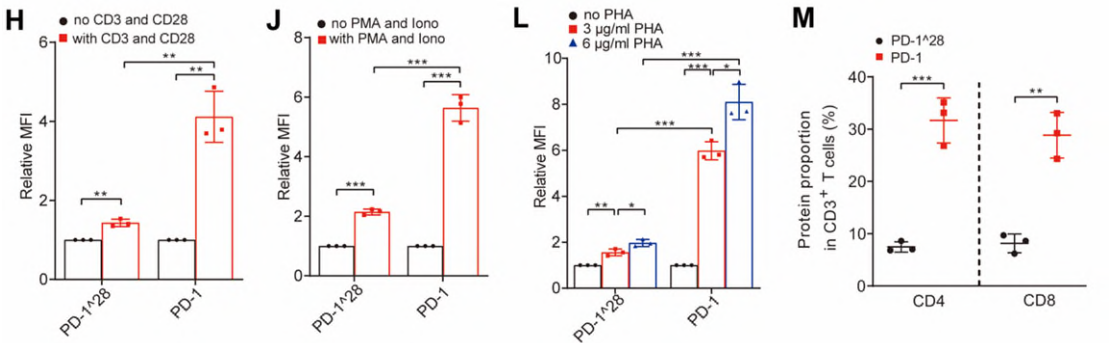


Fig. 1*H, J, L* and *M*

Question 3:

We apologize for the unclear description regarding to experiments using customized antibodies. In our previous manuscript, we showed that the monoclonal and polyclonal antibodies could specifically recognize PD-1^28 through immunoblot (Supplementary Fig. 1*H* in revised manuscript). After multiple tests, we found that only one monoclonal antibody worked well in the

flow cytometry experiments (Supplementary Fig. 1*J* in revised manuscript). In subsequent experiments, we only used this monoclonal antibody for flow cytometry assay. We rewritten the descriptions of antibody usage in lines 87-91 of the revised manuscript.

lines 87-91

We obtained monoclonal and polyclonal antibodies, and verified that PD-1^28 could be specifically recognized through immunoprecipitation followed by immunoblot (Supplementary Fig. 1*H* and *I*). One of the monoclonal antibodies was used in flow cytometry (FACS) (Supplementary Fig. 1*J*).

2. PD-1^28 mainly resides in the cytoplasm, in contrast to PD-1 which is located on the cell surface. It is crucial for the authors to elucidate how PD-1^28 exerts its immunosuppressive function in T cells. Investigating the effects of truncating the “extracellular” domain and cytoplasmic tail of PD-1^28 could provide valuable insights into its mechanism of action.

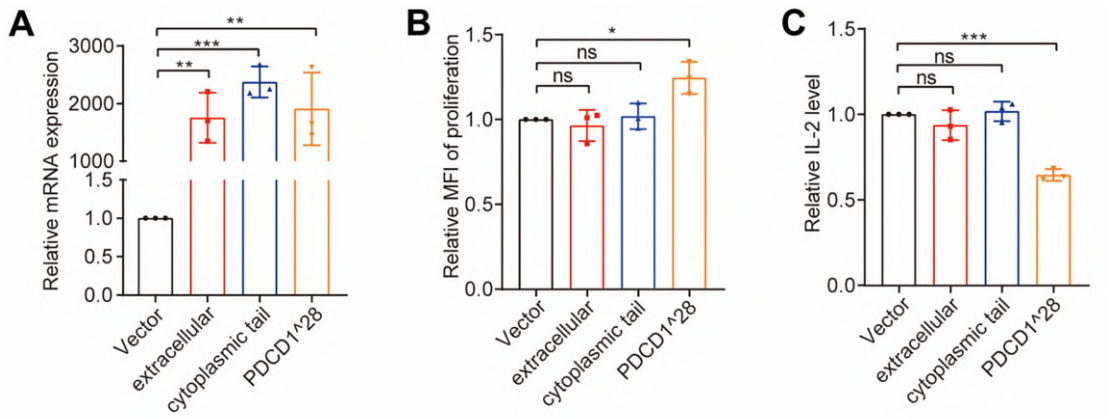
Response:

We thank the reviewer for the constructive suggestions. According to the reviewer’s comments, we constructed truncated extracellular domain and cytoplasmic tail of *PDCD1^28* separately, and fused Flag-tag at C-terminal. We separately overexpressed extracellular domain, cytoplasmic tail, and *PDCD1^28* in KO-1 cells. The mRNA levels of these three molecules were correspondingly elevated in OE cells compared to control cells (Supplementary Fig. 5*A*). Immunoblot of whole cell lysate revealed that only PD-1^28 was expressed within cells, and we were unable to detect protein expression of extracellular domain and cytoplasmic tail. Therefore, we further detected whether extracellular domain and cytoplasmic tail present in the culture supernatant, and Immunoblot results showed that protein of extracellular domain were highly secreted, but the cytoplasmic tail was still undetectable (Fig. R*2*, communication only for review), which may be due to post-translational modifications or instability of the protein. *PDCD1^28* OE

cells showed attenuated cell proliferation and IL-2 production, but there were no immune function changes in extracellular or cytoplasmic tail OE cells (Supplementary Fig. 5*B* and *C*). We have added these results in lines 187-192 (Supplementary Fig. 5*A*-*C*) of our revised manuscript.

lines 187-192

To further elucidate how PD-1^28 exerts its immunosuppressive function in T cells, we evaluated truncated extracellular domain and cytoplasmic tail of *PDCD1^28* separately (Supplementary Fig. 5*A*). *PDCD1^28* OE cells showed attenuated cell proliferation and IL-2 production, but there were no immune function changes in extracellular or cytoplasmic tail OE cells (Supplementary Fig. 5*B* and *C*).



Supplementary Fig. 5*A*-*C*

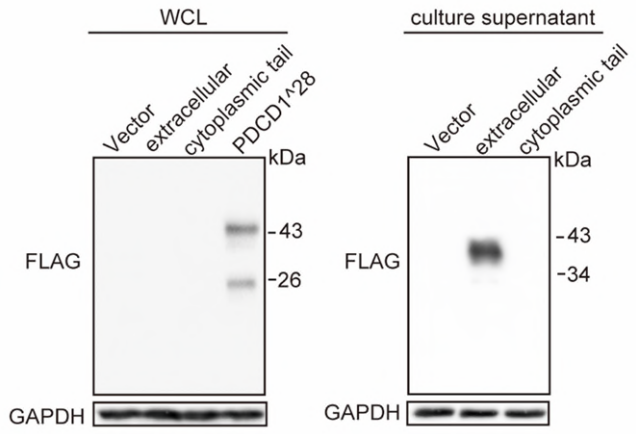


Fig. R2 only for reviewer

3. The *PDCD1^28*^{CKI} mice are a valuable tool for elucidating the role of

PD-1^28 in vivo. It is essential for the authors to detail the construction strategy used to creat this strain. Also, demonstrating PD-1 expression on T cells in these mice is necessary to determine whether it is influenced by the expression of PD-1^28.

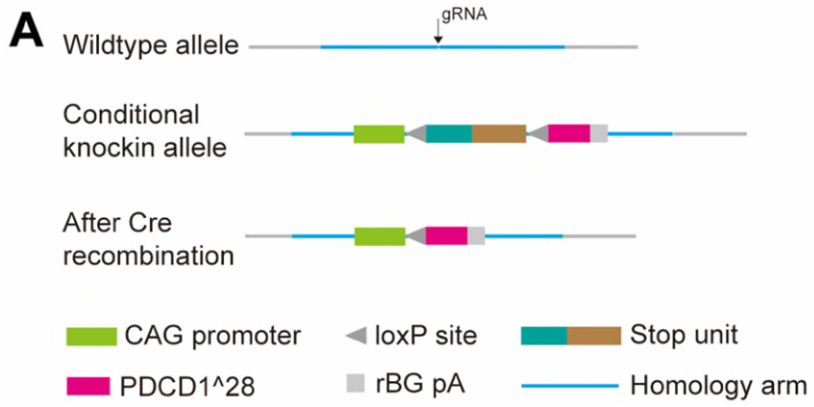
Response:

Question 1:

We sincerely appreciate the reviewer's comments and suggestions. We have added the strategy diagram for *PDCD1^28*^{CKI} mice production in Supplementary Fig. 7*A* of our revised manuscript and corresponding methods in lines 65-70 of Supplementary information.

lines 65-70

The knock-in mice were created at the locus of ROSA26 which is located on mouse chromosome 6 in C57BL/6J mice. The CAG promoter-loxP-stop unit-loxP-*PDCD1^28*-rBG pA cassette was inserted between exon 1 and 2 of ROSA26. Homology arms generated by PCR using BAC clone as template. Cas9 and gRNA co-injected into fertilized eggs with targeting vector for mice production.



Supplementary Fig. 7*A*

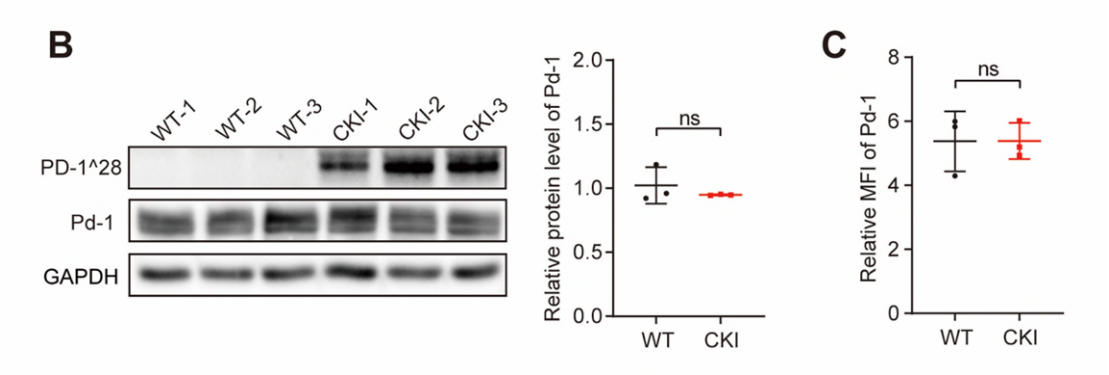
Question 2:

We measured the expression of Pd-1 in *PDCD1^28*^{CKI} mice. Immunoblot and quantification results showed that PD-1^28-knockin did not alter the expression of mus-Pd-1 in *PDCD1^28*^{CKI} mice, and FACS assay showed the same results as immunoblot. We have replaced new representative images of

the knock-in efficiency of PD-1^28 in Supplementary Fig. 7*B* and added new results in lines 261-262 (Supplementary Fig. 7*B* and *C*) of our revised manuscript.

lines 261-262

Immunoblot and FACS showed that PD-1^28-knockin did not alter the expression of Pd-1 in *PDCD1^28^{CKI}* mice (Supplementary Fig. 7*B* and *C*).



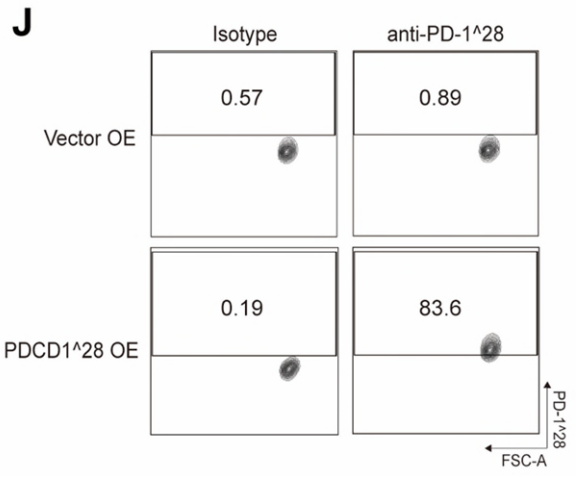
Supplementary Fig. 7*B* and *C*

Minor:

Fig S1H, the flow cytometry gates in need to be unified.

Response:

Many thanks for this nice advice and we have added the isotype control of these two types of cells. We have replaced new representative images in Supplementary Fig. 1*J* of our revised manuscript.

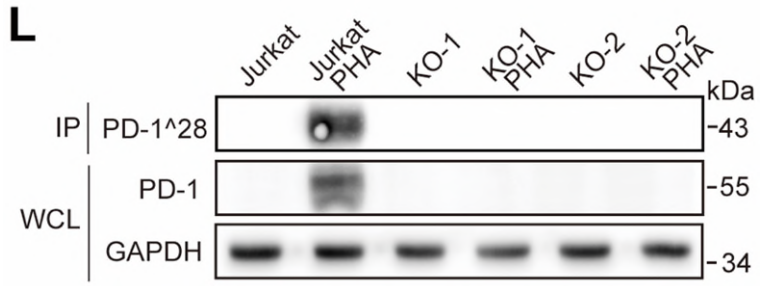


representative images of Supplementary Fig. 1*J*

Fig S1J, is the PD-1 antibody used here targeting PD-1 or PD-1^28? Also, it would be helpful if the authors could simultaneously show the expression of both PD-1 and PD-1^28.

Response:

We thank the reviewer for the constructive suggestions. Indeed, the antibody used in original Fig S1J could simultaneously recognize the IgV domain of both PD-1 and PD-1^28. We have tried our best but unfortunately failed to observe the expected 43 kDa band. The possible reason may be that the band of wild type PD-1 is relatively too strong to observe the band of PD-1^28. To validate the KO efficiency through the expression of PD-1^28, we utilized specific antibodies against PD-1^28. In addition, we could only recognize endogenous PD-1^28 through an IP followed by WB as we described in the response to Reviewer 2. We have replaced new representative images in Supplementary Fig. 1*L* of our revised manuscript.



Supplementary Fig. 1*L*

Reviewer #2 (Remarks to the Author):

The authors have comprehensively addressed most of my concerns, except for one question shown below:

In response to one of the questions, the authors made the following conclusion: “To explore whether intron retention is more prominently activated, we compared the expression levels of PDCD1^28 and PDCD1, suggesting intron retention is less pronounced (Fig. 1G, I, and K)”.

I wondered how the authors came to this conclusion. The data presented in Fig. 1G, I, and K merely show that the mRNA expression of the canonical PDCD1 transcript and its intron-retained isoform (PDCD1^28) is upregulated in T-cells treated with CD3/CD28, PMA/Iono, and PHA. These datasets do not provide information about intron retention. The authors should measure the Percent Spliced In (PSI) values for this experiment. Therefore, is the increase in PDCD1^28 expression in activated T-cells merely due to the upregulation of the canonical PDCD1 transcript, or is splicing regulation also involved?

Response:

We sincerely appreciate the reviewer’s constructive comments and are sorry for inaccurate descriptions. According to the reviewer's suggestions, we have made revisions to the conclusion in line 105-108.

line 105-108

To determine whether *PDCD1^28* is the primary expression form compared to *PDCD1*, we compared the expression levels of *PDCD1^28 and PDCD1*, suggesting *PDCD1^28* is less pronounced (Fig. 1G, I, and K).