nature portfolio

Peer Review File

Type I interferon signaling induces melanoma cell-intrinsic PD-1 and its inhibition antagonizes immune checkpoint blockade

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author): with expertise in cancer immunology, IFNs

In this manuscript, Holzgruber et al., report a striking finding that IFN-I induces PD-1 expression in melanoma cells to regulate melanoma response to PD-1 blockade therapy. The authors first analyzed human melanoma scRNA-seq datasets, as well as melanoma (human and mouse) cell lines, and observed a correlation between IFNAR1 and R2 with PD-1 expression level. Treatment of melanoma cell lines with IFNa/b induces PD-1 expression in melanoma cells in vitro. ATAC-seq was then performed in IFNa/b-treated melanoma cells to determine Pdcd1 promoter chromatin accessibility, which is validated by pSTAT1 and IRF9-ChIP. The authors then used IFNAR1 blocking antibody and determined that blocking IFNAR1 diminishes IFNa induction of PD-1 expression in melanoma cells. The authors also determined that loss of tumor cell IFN-I intrinsic signaling abolishes melanoma response to anti-PD-1 in mouse tumor models.

Overall, the findings that IFN-I tumor intrinsic signaling activates PD-1 expression in tumor cells (melanoma) is a very interesting one since the current notion is that PD-1 is primarily expressed and functions in T cells and myeloid cells. The authors used complementary approaches to validate the findings. The author also determined that the IFN-I-activated pSTAT1/IRF9 binds to the Pdcd1 promoter region in melanoma cells. Furthermore, the findings that anti-PD-1 efficacy in mice depends on tumor cell intrinsic IFN-I has high translational potential.

One weakness is the lack of sufficient human cancer relevance. Including human melanoma scRNA-seq data showing correlation between PD-1 expression and IFN-1 signaling pathway activation (ISG signature) will significantly strength the conclusion. Alternatively, isolating melanoma cells from patient tumors (depleting CD45+ cells) and perform analysis, such as RNA-Seq and or ATAC-Seq, to correlate PD-1 expression to ISG signature will also strength the conclusion.

Major comments:

Figure 1 shows the IFN receptor expression profiles in melanoma. Panel A shows various gene expression in human melanoma (scRNA-seq datasets). Is PD-1 mRNA detectable in tumor cells in this dataset? Panel g shows IFNAR1 and IFNAR2 expression level in PD-1+ vs PD-1- cell subsets in human and mouse melanoma cell lines. Are these cell lines treated with IFN-I? Does this correlation also exist in the human melanoma scRNA-seq dataset as shown in A?
IFNAR1 is the rate-limiting receptor for IFN-I. IFNAR2 level does not control IFN-I signaling and can be omitted since this figure is very crowded.

Figure 3 shows the ATAC-seq analysis of genome-wide and Pdcd1 locus chromatin accessibility.
a) Panel b will be more informative if the authors label some of the key genes/loci or chromatin regions that show IFNa/b-induced change of chromatin openness.
b) Panel c should indicate genome locations (at least approximately) of the predicted STAT1:STAR2 and IRF9 binding site (relative to Pdcd1 transcription start site). Also enhancers such as E0446806/enhD, E0446805/enhD need to be clarified or cited with reference.
c) Panel e: including a diagram showing the locations of the PCR primers at the pdcd1 promoter region will help the readers understand the ChIP results.

Figure 4 shows that blocking IFNAR1 diminishes PD-1 induction by iFNa in melanoma cells, which further strengths the findings that PD-1 is induced by IFN-I. Both mRNA and protein of PD-1 were analyzed. For the PD-1 protein level, it appears that all cells are PD-1+ after IFNa treatment. However, only a small fraction of cells is PD-1+ in Fig. 2d. This needs to be clarified.

Figure 5. The in vivo tumor models convincedly demonstrated the role of the IFN-I pathway in melanoma response to anti-PD-1. Use of NSG mice rules out involvement of IFN-I signaling in immune cells. Analysis of tumor cell PD-1 expression and IFN-I signaling (pSTAT1) in the tumor tissues will further strength the conclusion.

Minor points:

Figure 1 legend: f, I1 should be IFNAR1?

Reviewer #2 (Remarks to the Author): with expertise in melanoma, cancer immunology, IFNs

Holzgruber et al. demonstrate the type I IFN enhances/induces PD-1 expression on a subpopulation of murine and human melanoma cells. Using JAK/TYK2 inhibitors and STAT1/STAT2 shRNA they provide evidence for involvement of downstream IFNAR1/IFNAR2 signaling in the regulation of PD-1 expression in tumor cells, confirmed by analysis of chromatin accessibility via ATAC-Seq. In vivo studies in NSG mice demonstrate that anti-tumor effects of anti-PD-1 antibody treatment are abrogated when mice received additional treatment with anti-INFAR1 antibodies.

Comment of the reviewer: Previous studies by the authors demonstrated constitutive expression of PD-1 by murine and human melanoma cells and provided evidences for direct anti-tumor effects of anti-PD-1 antibodies on melanomas in NSG mice. The present study reveals a role for type I IFN in PD-1 expression in murine/human melanoma cells. This is an interesting finding however, major questions remain regarding the underlying mechanisms.

Statement, authors: „… Ab-mediated blockade of IFNAR1 on murine B16-F10, YUMM1.7, or YUMMER1.7D4 melanoma cells significantly reversed IFN-α-induced PD-1 gene and protein expression down to baseline levels (Fig. 4a). Blockade of human IFNAR1 with the FDA-approved inhibitor, anifrolumab27, also significantly suppressed IFN-α-dependent PD-1 expression by A2058 human melanoma cells (Fig. 4b). Consistently, inhibition with the clinically approved pharmacologic antagonists targeting JAK1, ruxolitinib or upadacitinib, and TYK2, deucravacitinib, significantly attenuated the marked induction of PD-1 transcript and protein by IFN-α in both murine (Fig. 4c) and human (Fig. 4d) melanoma cell lines ...“

Reviewer: This statement is correct but only partially describes the effects of pharmacologic JAK1/TYK2 inhibition or anti-IFNAR1 blockade on PD-1 expression. The inhibitors clearly

decrease PD-1 expression in melanoma cells below control levels (Fig. 4c, 4d). Is there any evidence for constitutive activation of JAK or TYK2 kinases in melanoma cells that could trigger constitutive PD-1 expression? The effects of inhibitor treatment on constitutive PD-1 are stronger at the RNA level, compared to the protein level, most likely due higher protein stability. Therefore, PD-1 surface expression should be measured after extended inhibitor treatment (or anti-IFNAR1 treatment, as shown for A2058 cells in Fig. 4b.). Do the indicated melanoma cells constitutively express low type I IFN levels?

Reviewer: Along this line, the blockade of anti-IFNAR1 completely abrogates the anti-PD1 antibody effect for A2058 in NSG mice. Could this be due to the fact that constitutive and cytokine-inducible PD-1 expression are completely blocked by anti-IFNAR1.

Reviewer: This leads to the following critical question: what is the source of type I IFN in vivo, in particular in human melanoma in NSG mice. Is there an autocrine type I IFN signaling due to tumor cell-intrinsic activation of innate immune receptors? Or does murine type I bind to human IFNAR1?

Reviewer, minor:
Data from page 1 and page 2 could be fused and partly included in the supplements in particular data related to PD-1 expression in T cells.

The very detailed one-page summary of results at the end of the introduction section should be shortened.

Reviewer #3 (Remarks to the Author): with expertise in cancer immunology
The manuscript by Holzgruber, et al. aims to understand how expression of PD-1 on melanoma cells is regulated and proposes a role for type I interferon. According to the authors and the title of the manuscript, this finding is relevant because PD-1 expression on melanoma cells compromises the therapeutic response to immune checkpoint inhibition.

General comments

1. The biological concept or hypothesis underlying the work is unclear to this reviewer. The beneficial effect of immune therapy in general and PD-1 blockade as used here relies on CD8+ T-cells. The tumor-promoting effect of PD-1 when expressed on melanoma cells, however, seems independent of immunity as the authors show similar effects in immunocompetent and -deficient mice. Nevertheless, PD-1 expression on melanoma cells seems to hamper the efficacy of PD-1 blockade, suggesting that PD-1 expression on melanoma cells makes the latter intrinsically resistant to immune control. How this supposedly works was not addressed here. Taken together, the manuscript contains quite a few circular arguments, and the experimental approaches are insufficiently rigorous.
2. The title is an overstatement because the authors only used PD-1 blockade. Generalizing the conclusion to immune checkpoint inhibition is misleading and incorrect. This must be changed.
3. The authors refer to anti-PD-1 as “immune checkpoint therapy (ICT)”, which is an unusual designation and probably not correct in the strictest sense. The community generally refers to this kind of treatments as immune checkpoint blockade or immune checkpoint inhibition. To improve readability of the manuscript, I strongly recommend that the authors stick to the widely used nomenclature and abbreviations instead of inventing their own.
4. In the first paragraph of the introduction, the authors state “In patients and preclinical tumor models, PD-1:PD-L1 axis blockade reinvigorates … suppressing neoplastic progression.” This is an obsolete oversimplification that is probably incorrect. During the last couple of years our knowledge of the different subsets of tumor-associated CD8+ T-cells greatly increased. For example, we know that PD-1 blockade mainly targets a small population of stem-like CD8+ T-cells and that their presence essentially determines the clinical response in mice and humans. Also, stem-like cells are not functionally exhausted and thus, the term reinvigoration is misleading in this context. The authors must revise the introduction according to our current knowledge and recent literature.
5. The authors should correlate PD-1 expression by melanoma cells in vivo with the immunoscore of the tumor in a sufficiently large patient cohort and discuss the findings in the context of the frequently described type I IFN signature of hot tumors. Also, the findings must be discussed with the commonly accepted opinion that hot tumors (ie., those with a type I IFN signature) respond better to immune checkpoint blockade.
6. Too few cell lines are used in this study.

7. The authors mention multiple times that the work described here systematically investigates the regulation of PD-1 expression by melanoma cells. “Systematically” is an overstatement, because very few melanoma cell lines were used, and a limited number of cytokines and growth factors were tested. Please, rephrase.
8. The way the data are presented insufficiently meets the common standards of transparency. Therefore, authors must improve data presentation as follows. First, the authors must show raw flow cytometry data including gating strategies and dot plots. Second, displaying data as mean fluorescence intensity (MFI) bar graphs in cases where the expression is obviously bimodal (for example in figure 2d) is incorrect. Third, data in figure 1g must be expressed as dot plots showing PD-1 on one axis and IFNAR1 on the other axis after gating on live singlets.

Specific comments
a. The experiments are exclusively performed in female mice without justification. The use of one sex only is not state-of-the-art anymore. Male mice must be included in the studies, because male and female mice oftentimes respond differently.
b. The amount of IFN-I used in vitro (60 ng/ml) seems unphysiologically high. The authors must provide evidence that such concentrations indeed are found in ex vivo tumor homogenates using ELISA to determine the concentration.
c. Cells were often (always?) serum-starved before in vitro manipulations. The authors must justify why this is done.
d. The authors describe that serum-starved cells were incubated with cytokines for 3-6 h for RT-qPCR analysis without justifying the variation in incubation time. Using a variation of 3 h seems unacceptable for studying immediate responses.
e. The authors must justify why they opted for short-hairpin RNA instead of CRISPR/Cas9 technology to manipulate cell lines. Only the latter method results in clean knock-out of the target gene.
f. Data should be displayed using SD instead of SEM, because the use of SEM is misleading (even when it is declared in some but not all legends).
g. The authors must include data on ex vivo expression of IFNAR1 protein on melanoma cells both in a sufficient number of human samples and mouse models.
h. References 19 and 20 refer to publications describing loss-of-function mutations in the

interferon signaling pathway, and therefore, seem inappropriate in the context used here.

i. In figure 1c and 1e the authors describe co-culture experiments of melanoma cells and T-cells. Crucial information about T-cells is lacking and must be provided. For example, did the authors use CD4+ or CD8+ T-cells; were the T-cells naïve, memory, effector (defined how), terminally differentiated? This reviewer is concerned by the fact that the authors didn't think this information is worth mentioning. Even if this information were given, the experimental set up remains probably irrelevant, because the state of T-cells in the tumor microenvironment can't be mimicked in vitro.

j. Data shown in figure 2 don't convince this reviewer. The data shown in figure 2b must be displayed as dotplots overlaying induced and uninduced conditions (instead of induced with relevant Ab and isotype control Ab). See also comment above under point 8.

k. The authors used only a single cell line (YUMM4.1) in figure 3 without justifying why this cell line was chosen or showing that this YUMM4.1 is representative of something. Further, the authors must justify why YUMM4.1 is not used for the experiments shown in in figures 4 and 5.

Reviewer 1: ***Holzgruber et al. report a striking finding that IFN-I induces PD-1 expression in melanoma cells to regulate response to PD-1 blockade therapy. The authors analyzed human melanoma scRNA-seq datasets, as well as melanoma (human and mouse) cell lines, and observed a correlation between IFNAR1 and R2 with PD-1 expression level. Treatment of melanoma lines with IFNα/β induces PD-1 expression in vitro. ATAC-seq performed in IFNα/β-treated melanoma cells determined Pdcd1 promoter chromatin accessibility, validated by p-STAT1 and IRF9-ChIP. The authors then determined that ab blocking of IFNAR1 diminishes IFNα induction of PD-1 expression in melanoma cells and that loss of tumor cell IFN-I intrinsic signaling abolishes melanoma response to anti-PD-1 in mouse tumor models. Overall, the findings that IFN-I tumor intrinsic signaling activates PD-1 expression in tumor cells (melanoma) is very interesting since the current notion is that PD-1 is primarily expressed and functions in T-cells and myeloid cells. The authors used complementary approaches to validate findings and determined that IFN-I-activated p-STAT1/IRF9 binds the Pdcd1 promoter region in melanoma cells. The findings that anti-PD-1 efficacy in mice depends on tumor cell intrinsic IFN-I has high translational potential.***

Point 1: *One weakness is the lack of sufficient human cancer relevance. Including human melanoma scRNA-seq data showing correlation between PD-1 expression and IFN-1 signaling pathway activation (ISG signature) will significantly strength the conclusion.*
Response: We have now incorporated additional human patient melanoma scRNA-seq analyses, including of a second independent dataset (Bierman et al., *Cell*, 2022) with higher numbers of melanoma cells compared to the first (Tirosh et al., *Science*, 2016), to confirm PD-1 expression by melanoma cells, co-expression with IFNAR1/2, and correlation with IFN I pathway activation (i.e. ISG signature) as shown in NEW Extended Data Figure 5.

Point 2: *Figure 1, Panel a shows gene expression in human melanoma (scRNA-seq datasets). Is PD-1 mRNA detectable in tumor cells in this dataset?*
Response: PD-1 mRNA is indeed detectable in this dataset. Accordingly, we have generated a new figure revealing that patient melanoma cells positive for PD-1 gene expression robustly co-express both IFNAR1 and IFNAR2 (NEW Extended Data Fig. 5a). In addition, we have incorporated a more recent scRNA-seq dataset from Biermann et al (*Cell*, 2022), which contains a larger cohort of PD-1-positive human patient melanoma cells. This second dataset independently confirms results from the first dataset in that melanoma cells positive for the PD-1 gene transcript co-express high levels of IFNAR1 and IFNAR2 (NEW Extended Data Fig. 5b) and tend to overexpress ISG signature genes (NEW Extended Data Fig. 5c).

Point 3: *Figure 1, Panel g shows IFNAR1 and IFNAR2 expression level in PD-1$^+$ vs PD-1$^-$ cell subsets in human and mouse melanoma cell lines. Are these cell lines treated with IFN-I?*
Response: These cell lines are *not* treated with IFN-I and thus depict basal, constitutive protein co-expression levels of IFNAR1 and IFNAR2 on PD-1$^+$ vs PD-1$^-$ human melanoma subsets. We have better clarified this in the results section describing Fig. 1h to read "native" cells. Of note, Fig. 1g has now become NEW Fig 1h.

Point 4: *Does this correlation (above) also exist in the human melanoma scRNA-seq dataset as shown in A?*
Response: Both scRNA-seq datasets show that *PDCD1*$^+$ melanoma cells highly co-express both *IFNAR1* (~50-80% of cells) and *IFNAR2* (~40-60%), though were not enriched in these receptors compared to *PDCD1*$^-$ melanoma cells (NEW Extended Data Fig. 5a,b). Gene levels do not always reflect protein expression due to protein stability regulated by posttranslational modifications, among other reasons. Nevertheless, our new data co-expression of PD-1 with type I IFN receptors and preferential enrichment of ISG signature genes (NEW Extended Data Fig. 5c), thus independently corroborating PD-1 regulation by the type I IFN pathway.

Point 5: *IFNAR1 is the rate-limiting receptor for IFN-I. IFNAR2 level does not control IFN-I signaling and can be omitted since this figure is crowded.*
Response: We appreciate the reviewer's biological insights and recommendations and have thus clarified in the results describing Fig. 1c that "IFNAR1 is the rate-limiting subunit for the type I IFN receptor complex". While we would have liked to transfer IFNAR2 data out of this main figure into the Extended Data, we have

unfortunately reached the maximum allowable number of Extended Data Figures because we have incorporated substantial new data to rigorously address all reviewers' comments. We respectfully hope that the reviewer understands.

Point 6: *Figure 3 shows ATAC-seq analysis of genome-wide and Pdcd1 locus chromatin accessibility. Panel b will be more informative if the authors label some of the key genes/loci or chromatin regions that show IFNα/β-induced change of chromatin openness.*
Response: We have now created a new table, Supplementary Table 1, listing the top 100 genes with the greatest fold change in chromatin opening induced by both IFN-α and IFN-β treatment of melanoma cells as determined by ATAC-seq analysis. This table lists the gene symbol, gene name, Ensembl ID, Chromosome number, base numbers for the chromatin accessible region opened by IFN-α and IFN-β treatment, corresponding genomic motif, and the accompanying fold changes and p-values.

Point 7: *Figure 3, Panel c should indicate genome locations (at least approximately) of the predicted STAT1:STAT2 and IRF9 binding site (relative to Pdcd1 transcription start site). Also, enhancers such as E0446806/enhD, E0446805/enhD need to be clarified or cited with a reference.*
Response: Based on the reviewer's very insightful suggestion, we have now expanded Fig. 3c to show the genome base locations of STAT1:STAT2 and IRF9 binding sites relative to the *Pdcd1* transcriptional start site (TSS,+1), clarified locations of enhancers E0446806/enhD, E0446805/enhD, and E0446812/enhP in the expanded portion of Fig. 3c and in Fig. 3d by both illustrating and labeling their positions.

Point 8: *Figure 3, Panel e: including a diagram showing the locations of the PCR primers at the Pdcd1 promoter region will help the readers understand the ChIP results.*
Response: We now include an illustration in Fig. 3c showing the locations of PCR primers and their product size along with the ChIP amplicon (blue) position relative to the *Pdcd1* promoter transcriptional start site (TSS).

Point 9: *Figure 4 shows that blocking IFNAR1 diminishes PD-1 induction by IFNα in melanoma cells, which further strengthens the findings that PD-1 is induced by IFN-I. Both mRNA and protein of PD-1 were analyzed. For the PD-1 protein level, it appears that all cells are PD-1$^+$ after IFNα treatment. However, only a small fraction of cells is PD-1$^+$ in Fig. 2d. This needs to be clarified.*
Response: Though PD-1 is highly expressed by a subset of melanoma cells with high fluorescence intensity, the majority of cells also express PD-1, albeit at lower levels. The shift of the main population was not as obvious in the previous iteration of the manuscript, because the x axis had a much larger logarithmic scale in previous Fig. 2d compared to Fig. 4a. We have now replaced the histograms previously shown in Fig. 2 with dot plot overlays (as requested by reviewer 3) to more clearly show the main population and the shift in response to IFN treatment. The dot plots also more clearly depict the smaller melanoma subpopulation expressing high PD-1 levels (NEW Figs. 2b, d).

Point 10: *Figure 5. The in vivo tumor models convincingly demonstrated the role of the IFN-I pathway in melanoma response to anti-PD-1. Use of NSG mice rules out involvement of IFN-I signaling in immune cells. Analysis of tumor cell PD-1 expression and IFN-I signaling (p-STAT1) in the tumor tissues will further strength the conclusion.*
Response: We have now analyzed tumor cell-PD-1 and p-STAT1 protein expression in all mouse models used by FACS analysis and found that IFNAR1 blockade significantly reduced PD-1 protein expression in YUMM1.7 cells grown in both C57BL/6 mice (NEW Extended Data Fig. 9d) and NSG mice (NEW Extended Data Fig. 9h) as well as in A2058 tumor xenografts (NEW Extended Data Fig. 10a). STAT1 phosphorylation was also reduced in YUMM1.7 (NEW Extended Data Fig. 9h) and A2058 tumors (NEW Extended Data Fig. 10b) grown in NSG mice treated with either IFNAR1 ab or ruxolitinib, though levels in immunocompetent mice were not significantly altered, likely owing to a lesser tumor cell-directed effect due to the presence of adaptive immune cells in this model. Nevertheless, we found significant reductions in ISG signature genes in both immunocompetent (NEW Extended Data Fig. 9e) and particularly immunocompromised mice (NEW Extended Data Fig. 9i) in response to

both IFNAR1 blockade and ruxolitinib administration. Finally, *PDCD1* expression by human A2058 melanoma xenografts also correlates with ISG signature genes (NEW Extended Data Fig. 10f), thus validating *in vivo* effects of type I IFN signaling on melanoma cell-intrinsic PD-1 expression.

Point 11: *Minor point, Figure 1 legend f: I1 should be IFNAR1?*
Response: We have now corrected this error.

Reviewer 2: ***Holzgruber et al. demonstrate that type I IFN enhances/induces PD-1 expression on a subpopulation of murine and human melanoma cells. Using JAK/TYK2 inhibitors and STAT1/STAT2 shRNA they provide evidence for involvement of downstream IFNAR1/IFNAR2 signaling in the regulation of PD-1 expression in tumor cells, confirmed by analysis of chromatin accessibility via ATAC-Seq. In vivo studies in NSG mice demonstrate that anti-tumor effects of anti-PD-1 antibody treatment are abrogated when mice received additional treatment with anti-IFNAR1 antibodies. Previous studies by the authors demonstrated constitutive expression of PD-1 by murine and human melanoma cells and provided evidence for direct anti-tumor effects of anti-PD-1 antibodies on melanomas in NSG mice. The present study reveals a role for type I IFN in PD-1 expression in murine/human melanoma cells. This is an interesting finding.***

Point 1: *The inhibitors clearly decrease PD-1 expression in melanoma cells below control levels (Fig. 4c, 4d). Is there any evidence for constitutive activation of JAK or TYK2 kinases in melanoma cells that could trigger constitutive PD-1 expression?*
Response: Indeed, consistent with basal PD-1 expression by unstimulated murine and human melanoma cells, we found some degree of constitutive phosphorylation of JAK1, TYK2, STAT1, and STAT2 (NEW Extended Data Figs. 8c,d).

Point 2: *The effects of inhibitor treatment on constitutive PD-1 are stronger at the RNA level, compared to the protein level, most likely due higher protein stability. Therefore, PD-1 surface expression should be measured after extended inhibitor treatment (or anti-IFNAR1 treatment, as shown for A2058 cells in Fig. 4b.).*
Response: We have now measured PD-1 surface protein expression on A2058 cells after extended IFNAR1 ab and ruxolitinib treatment for 3 days (as before) and 14 days. As suspected by the reviewer, prolonged inhibitor treatment does indeed reduce PD-1 protein expression more substantially over time versus shorter incubation (NEW Extended Data Fig. 8a).

Point 3: *Do the indicated melanoma cells constitutively express low type I IFN levels?*
Response: The reviewer raises an excellent point. Indeed, melanoma cells constitutively express type I IFN levels, particularly *in vivo* (NEW Extended Data Figs. 9f, 10d; NEW Supplementary Figs. 5, 6a,b). We now also discuss the significance of autocrine type I IFN in PD-1 regulation.

Point 4: *Along this line, the blockade of anti-IFNAR1 completely abrogates the anti-PD-1 antibody effect for A2058 in NSG mice. Could this be due to the fact that constitutive and cytokine-inducible PD-1 expression are completely blocked by anti-IFNAR1.*
Response: We have now examined PD-1 protein expression in melanoma grafts obtained from IFNAR1 ab or ruxolitinib-treated mice. In NSG mice devoid of adaptive immune cells and therefore enriched in cancer cell-PD-1 both treatments, and particularly IFNAR1 ab blockade, significantly reduced PD-1 expression on melanoma cells (NEW Extended Data Figs. 9h, 10a), albeit not completely at the experimental endpoint. Both treatment regimens additionally reduced expression of type I IFN pathway effectors, including p-STAT1 and ISGs (NEW Extended Data Figs. 9e,h,i 10b), supporting the substantially diminished PD-1 therapeutic efficacy. PD-1 and ISG levels might be further decreased at earlier timepoints. While this scenario cannot be examined due to the lack of sufficient tissue, our data nevertheless supports diminished PD-1 ab efficacy due to reduced target expression. Indeed, we now report that prolonged (14 day) treatment off A2058 cells with ruxolitinib or anifrolumab *in vitro* more robustly reduces PD-1 expression compared to shorter treatment durations (3 days, NEW Extended Data Fig. 8a).

Point 5: *This leads to the following critical question: what is the source of type I IFN in vivo, in particular in human melanoma in NSG mice. Is there autocrine type I IFN signaling due to tumor cell-intrinsic activation of innate immune receptors? Or does murine type I bind to human IFNAR1?*

Response: There are multiple cellular sources of type I IFN production *in vivo*, as shown in NEW Extended Data Fig. 9f and Supplementary Fig. S5. The predominant producers of IFN-α and IFN-β were DCs, macrophages, NK cells and melanoma cells. Murine IFN-α and IFN-β did not induce PD-1 on human A2058 melanoma cells (NEW Extended Data Fig. 10c). However, human A2058 cells produced substantial amounts of both IFN-α and IFN-β *in vivo* (NEW Extended Data Fig. 10d). Additionally, *PDCD1* frequency in A2058 tumor xenografts correlated with both *IFNA2* and *IFNB* expression (NEW Extended Data Fig. 10e), and showed correlative trends for the ISG genes, *CMPK2, IFIT2, IRF7, ISG15,* and *ISG20* (NEW Extended Data Fig. 10f).

Point 6: *Minor point, Data from pages 1 and 2 could be fused and partly included in the Supplement, particularly data related to PD-1 expression in T-cells.*

Response: We appreciate the reviewer's insightful recommendation. While we would have liked to transfer some data from these initial pages out of the main mansucript, we have unfortunately reached the maximum allowable number of Extended Data Figures because we have had to incorporate substantial new data to rigorously address all reviewers' comments. We respectfully hope that the reviewer understands.

Point 7: *The very detailed one-page summary of results at the end of the introduction section should be shortened.*

Response: We have now substantially shortened the summary of results within the introduction section.

Reviewer 3: ***The manuscript by Holzgruber, et al. aims to understand how expression of PD-1 on melanoma cells is regulated and proposes a role for type I interferon. According to the authors and the title of the manuscript, this finding is relevant because PD-1 expression on melanoma cells compromises the therapeutic response to immune checkpoint inhibition.***

Point 1: *The biological concept or hypothesis underlying the work is unclear to this reviewer. The beneficial effect of immune therapy in general and PD-1 blockade as used here relies on CD8+ T-cells. The tumor-promoting effect of PD-1 when expressed on melanoma cells, however, seems independent of immunity as the authors show similar effects in immunocompetent and -deficient mice. Nevertheless, PD-1 expression on melanoma cells seems to hamper the efficacy of PD-1 blockade, suggesting that PD-1 expression on melanoma cells makes the latter intrinsically resistant to immune control. How this supposedly works was not addressed here. Taken together, the manuscript contains quite a few circular arguments, and the experimental approaches are insufficiently rigorous.*

Response: We agree with the referee that PD-1 checkpoint blockade efficacy relies on CD8+ T-cells and have better clarified this important fact in our introduction. The reviewer is also correct that tumor cell-intrinsic PD-1 promotes tumorigenesis independent of immunity. Consequently, its blockade suppresses tumor growth via the inhibition of tumor cell-intrinsic pathways in both immunocompromised and immuncompetent mice (e.g. Kleffel et al. *Cell* 2015, Martins et al. *Sci Adv* 2024, Li et al. *Hepatol* 2017, Mirzaei et al. *Sci Adv* 2021). As such, PD-1 expression on melanoma cells does not hamper the efficacy of PD-1 blockade, as is the case for other cancers such as NSCLC (e.g. Du et al. *Oncoimmunol* 2018, Wang et al. *PNAS* 2020). In fact, it enhances melanoma growth inhibition by boosting tumor cell-directed PD-1 ab targeting. Conversely, downmodulation of PD-1 target expression (NEW Extended Data Figs. 9 and 10) on melanoma cells via IFNAR1 ab or ruxolitinib treatment hampered efficacy of PD-1 blockade (NEW Fig. 5), as newly found herein. In further agreement, hot tumors commonly known to respond to PD-1 therapy showed higher frequency of PD-1-expressing melanoma cells in published scRNA-seq datasets compared to cold tumors (NEW Extended Data Fig. 5d). Together, these results are thus consistent in that they clearly delineate IFN-dependent PD-1 target level, not only on CD8+ T-cells but also on melanoma cells as an important variable in response to PD-1 checkpoint blockade.

Point 2: *The title is an overstatement because the authors only used PD-1 blockade. Generalizing the conclusion to immune checkpoint inhibition is misleading and incorrect.*
Response: In response to the reviewer's valid point, we have changed our title from immune checkpoint "therapy" to "blockade".

Point 3: *The authors refer to anti-PD-1 as "immune checkpoint therapy (ICT)", which is an unusual designation and probably not correct in the strictest sense. The community generally refers to this kind of treatment as immune checkpoint blockade or immune checkpoint inhibition. To improve readability of the manuscript, I strongly recommend that the authors stick to the widely used nomenclature and abbreviations instead of inventing their own.*
Response: Based on the reviewer's feedback, with have now changed immune checkpoint therapy (ICT) to immune checkpoint blockade (ICB) throughout the manuscript. Nonetheless, we respectfully note that several experts in the immune checkpoint field, including nobel laureate, Dr. Jim Allison, and Dr. Padmanee Sharma published a seminal review article in *Cell* (Sharma et al. 2023, 86(8):1652-1669) using the abbreviation "ICT". We had therefore used it in the previous iteration of our manuscript.

Point 4: *In the first paragraph of the introduction, the authors state "In patients and preclinical tumor models, PD-1:PD-L1 axis blockade reinvigorates…suppressing neoplastic progression." This is an obsolete oversimplification that is probably incorrect. During the last couple of years our knowledge of the different subsets of tumor-associated $CD8^+$ T-cells greatly increased. For example, we know that PD-1 blockade mainly targets a small population of stem-like $CD8^+$ T-cells and that their presence essentially determines the clinical response in mice and humans. Also, stem-like cells are not functionally exhausted and thus, the term reinvigoration is misleading in this context. The authors must revise the introduction according to our current knowledge and recent literature.*
Response: We have now removed the term "reinvigoration" and edited this statement to read "PD-1:PD-L1 axis blockade stimulates antitumor immunity by increasing activation and proliferation of tumor-reactive T-cells, including of self-renewing stem-like $CD8^+$ T-cells". We also newly reference Gill et al. 2023 *Sci Immunol*.

Point 5: *The authors should correlate PD-1 expression by melanoma cells in vivo with the immunoscore of the tumor in a sufficiently large patient cohort and discuss the findings in the context of the frequently described type I IFN signature of hot tumors. Also, the findings must be discussed with the commonly accepted opinion that hot tumors (those with a type I IFN signature) respond better to immune checkpoint blockade.*
Response: We have now correlated PD-1 expression by melanoma cells with tumor immunoscore in NEW Extended Data Fig. 5d and found a significantly elevated melanoma cell-PD-1 frequency with increasing immunoscore, consistent with our cell line and melanoma mouse model data. We also elaborate on this finding in the discussion section.

Point 6: *Too few cell lines are used in this study.*
Response: This study includes six different human (A2058, A375, G361, MeWo, MDA-MB-435S, SK-MEL-2) and six distinct mouse melanoma cell lines (B16-F10, YUMM1.7, YUMMER1.7D4, YUMM1.G1, YUMM4.1, YUMM5.2) as well as respective human and murine T-cell controls, which we believe represents an adequate number of independent lines spanning a wide range of clinically relevant tumor phenotypes. To further expand on this cell line cohort, we have newly analyzed single cell suspensions from $n = 5$ independent melanoma patients (NEW Fig. 1c) and an additional patient scRNA-seq dataset (NEW Extended Data Fig. 5) and have conducted extensive additional experimentation involving the above cell lines.

Point 7: *The authors mention multiple times that the work described here systematically investigates the regulation of PD-1 expression by melanoma cells. "Systematically" is an overstatement, because very few melanoma cell lines were used, and a limited number of cytokines and growth factors were tested. Please, rephrase.*
Response: We have now removed the term "systematically" from these statements.

Point 8: *The way the data are presented insufficiently meets the common standards of transparency. Authors must improve data presentation as follows. First, they must show raw flow cytometry data including gating strategies and dot plots. Second, displaying data as mean fluorescence intensity (MFI) bar graphs in cases where the expression is obviously bimodal (for example in figure 2d) is incorrect. Third, data in figure 1g must be expressed as dot plots showing PD-1 on one axis and IFNAR1 on the other axis after gating on live singlets.*

Response: We now include gating strategies and dot plots for all flow cytometry data (NEW Supplementary Figs. 1,5,6). All data depicted in previous Fig. 2, which showed a bimodal distribution, have now been replaced with bar graphs showing percent PD-1 positivity (as opposed to MFI, NEW Figs. 2b,d). We have also prepared dot plots showing co-expression of PD-1 with IFNAR1 after gating on live singlets (NEW Supplementary Figs. 1d,e).

Point 9: *The experiments are exclusively performed in female mice without justification. The use of one sex only is not state-of-the-art anymore. Male mice must be included in the studies, because male and female mice oftentimes respond differently.*

Response: We thank the referee for raising this important point. Indeed, several reports have uncovered important immunological differences between male and female mice (e.g. divergent Treg abundance). Therefore, we have now repeated all tumorigenicity studies using male immunocompetent C57BL/6 mice and found similar trends across both genders in terms of effects of IFNAR1 ab blockade and ruxolitinib treatment on PD-1 ICB outcomes. Accordingly, we have now combined tumor growth curves of both male and female mice (NEW Fig. 5a) and incorporated male mice into all new analyses reported in NEW Extended Data Figs. 9 and 10.

Point 10: *The amount of IFN-I used in vitro (60 ng/ml) seems unphysiologically high. The authors must provide evidence that such concentrations indeed are found in ex vivo tumor homogenates using ELISA to determine the concentration.*

Response: We have now measured and detected both IFN-α2 and IFN-β in *ex vivo* murine YUMM1.7 and human A2058 tumor homogenates using LegendPlex and ELISA-based assays. Individual IFN cytokine amounts averaged across whole tumor lysates reached up to 70 pg/ml (not shown) and were thus approximately one log-fold lower than the amount used *in vitro*, as correctly noted by the referee. Similarly, tumor patient serum contained approximately 125 pg/ml of IFN-β (not shown), thus mirroring approximate *in vivo* concentrations reached in our models. While the reviewer is correct that IFN concentrations used *in vitro* do indeed substantially exceed levels of distinct type I IFNs in tumor tissue or patient serum, we would like to point out that 13 distinct IFN-α subtypes (+IFN-β) exist, all of which act in concert on the IFNAR receptor complex and are constantly being produced to maintain biological activity (in contrast to *in vitro* one time addition). Moreover, the combined concentration of all 14 type I IFNs in tumor tissue would thus reach nanogram levels, consistent with the herein used *in vitro* concentrations, albeit at higher constant biological activity as elaborated above. Average cytokine amounts in serum or across tissues do not accurately reflect substantially elevated localized concentrations at immunological synapses or cell-cell contact points enriched for cytokine amounts due to IFN immobilization on cell surface glycosaminoglycans. For these known reasons, techniques have been developed for more sensitive quantification of localized cytokine concentrations (e.g. ELISPOT) otherwise not captured by ELISA or other standard assays. Finally, we would also like to point out that the cytokine concentrations used *in vitro* herein are consistent with those recommended in the manufacturer's product sheets and also used by many other groups. Our study also includes experiments that do not use exogenously added IFNs (e.g. *in vivo* experimentations shown in Fig. 5 and NEW Extended Data Figs. 9,10). In these models, melanoma cell-intrinsic PD-1 expression does indeed significantly correlate with intratumoral human IFN-β amounts as well as IFN-α2 levels (NEW Extended Data Fig. 10e). We are therefore confident that our conclusions reflect physiologic type I IFN functions in melanoma cells.

Point 10: *Cells were often (always?) serum-starved before in vitro manipulations. The authors must justify why this is done.*

Response: As noted above, serum contains IFNs, which might influence experimental results. Accordingly, serum starvation is widely used to eliminate such potential confounders on biological readouts.

Point 11: *The authors describe that serum-starved cells were incubated with cytokines for 3-6 h for RT-qPCR analysis without justifying the variation in incubation time. Using a variation of 3 h seems unacceptable for studying immediate responses.*
Response: We now include a time course for all human and murine cell lines used in IFN induction experiments (NEW Supplementary Fig. 2). These results demonstrate that 3-6h represents the optimal window for type I IFN-based PD-1 induction in a cell line-dependent manner, of which 3h were an optimal timepoint for YUMMER, YUMM1.G1, YUMM4.1, and YUMM5.2, whereas 6h were more potent with respect to PD-1 induction in A2058, A375, B16-F10, and YUMM1.7 cells.

Point 12: *The authors must justify why they opted for short-hairpin RNA instead of CRISPR/Cas9 technology to manipulate cell lines. Only the latter method results in clean knock-out of the target gene.*
Response: While the referee raises a valid point that CRISPR/Cas9-based KO approaches completely eliminate gene and protein expression, our use of these two particularly independent STAT1 and STAT2 hairpins each resulted in highly efficient gene (NEW Extended Data Fig. 8e) and near complete protein (NEW Extended Data Fig. 8f) knockdown, thus justifying the validity of our approach. An advantage of RNAi over CRISPR is that the latter requires single cell cloning, which might not accurately reflect overall IFN effects in the tumor cell line at large. In contrast, shRNA-based knockdown does not necessitate single cell cloning.

Point 13: *Data should be displayed using SD instead of SEM, because the use of SEM is misleading (even when it is declared in some but not all legends).*
Response: While we acknowledge there are several ways to represent data, both SD and SEM are widely used in scientific publications. This is because SD and SEM are closely related, can be derived from one another, and thus do not affect calculations of statistical significance. Moreover, on page 2 of the "Nature Portfolio Editorial Policy Checklist" the use of both SD and SEM is clearly in line with journal guidelines as specified in the "data presentation" subsection. We have made sure to declare error bars in all legends in response to the reviewer's comment.

Point 14: *The authors must include data on ex vivo expression of IFNAR1 protein on melanoma cells both in a sufficient number of human samples and mouse models.*
Response: We now include IFNAR1 protein expression by tumor cells in single cell suspensions obtained from $n = 5$ patients (NEW Fig. 1c), as determined by flow cytometry. We also newly show representative dot plots of IFNAR1 expression in *ex vivo* murine YUMM1.7 and human A2058 tumors (NEW Supplementary Figs. 5, S6a,b).

Point 15: *References 19 and 20 refer to publications describing loss-of-function mutations in the interferon signaling pathway, and therefore, seem inappropriate in the context used here.*
Response: We included these references on loss-of-function mutations in the IFN pathway because they represent an alternative and independent mode of inactivation of this signaling cascade to the IFNAR1 ab and small molecule inhibitors used herein. They thus reflect respective biological effects of IFN inhibition and place them into a clinically relevant context. Multiple prior studies of IFN regulation, including of PD-L1 (Ribas and colleagues, 2017, *Cell Rep*), referenced and discussed these IFN inactivating mutations in a similar context. We have now incorporated the terminology use by this referee, "loss-of-function", in the introduction, to better clarify the relevance of these publications to our work.

Point 16: *In Figure 1c and 1e the authors describe co-culture experiments of melanoma cells and T-cells. Crucial information about T-cells is lacking and must be provided. For example, did the authors use $CD4^+$ or $CD8^+$ T-cells; were the T-cells naïve, memory, effector (defined how), terminally differentiated? This reviewer is concerned by the fact that the authors didn't think this information is worth mentioning. Even if this information were given, the experimental set up remains probably irrelevant, because the state of T-cells in the tumor microenvironment can't be mimicked in vitro.*
Response: We would like to clarify that Figs. 1c and 1e (now NEW Figs. 1d and 1f) were not co-culture experiments. They simply show gene expression of IFNAR1/2 across several melanoma cell lines, with human

or murine T-cells simply used as respective controls. We have now clarified in the figure legend and methods that these T-cells were activated $CD3^+$ cells. More detailed analyses not included in this manuscript revealed that IFNAR1 expression predominates on $CD4^+$ T-resident memory (TRM) cells, while IFNAR2 is over-represented on $CD8^+$ central memory (CM) and effector memory (EM) T-cells. We believe these data are beyond the scope of this study given the absence of co-culture assays, but we nevertheless included them here in our response to the referee for further clarification.

Point 17: *Data shown in Figure 2 don't convince this reviewer. The data shown in Figure 2b must be displayed as dot plots overlaying induced and uninduced conditions (instead of induced with relevant Ab and isotype control Ab). See also comment above under point 8.*
Response: We now display NEW Figs. 2b and d as dot plot overlays of IFN-induced vs. uninduced conditions.

Point 18: *The authors used only a single cell line (YUMM4.1) in Figure 3 without justifying why this cell line was chosen or showing that this YUMM4.1 is representative of something. Further, the authors must justify why YUMM4.1 is not used for the experiments shown in in Figures 4 and 5.*
Response: We justified the use of YUMM4.1 cells in the ATAC-seq analysis by stating that this cell line "demonstrated the greatest fold induction of *Pdcd1* transcription by IFN-α and IFN-β among all lines examined" (Fig. 2c and NEW Supplementary Fig. 2b). Because ATAC-seq and ChIP-qPCR are technically challenging and require robust levels of transcription factor DNA binding for sufficient detection, we selected this cell line. We now include an additional widely used and highly tumorigenic line, YUMM1.7, which independently corroborated results from YUMM4.1 showing p-STAT1/2 and IRF9 binding to the Pdcd1 enhancer element identified as a type I IFN target by ATAC-seq (NEW Fig. 3e). YUMM4.1 cells, in our hands, did not consistently produce tumors. We therefore opted for YUMM1.7 as our representative *in vivo* model of choice having established similar IFN-dependent PD-1 regulatory mechanisms.

REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

The authors added a 2nd human patient scRNA-Seq dataset to strengthen the human melanoma relevance of their findings, added new data for PD-1 expression correlation with IFN-I/R in human patient tumor. Other comments are addressed by clarifications, revision and new analysis.

My comments have been addressed.

Reviewer #2 (Remarks to the Author):

The authors addressed the points that I raised, although some experimental settings were not meaningful. To demonstrate higher levels of interferon signaling in PD-1 positive melanoma cells it would have been more conclusive to directly compare sorted PD-1pos and PD-1neg tumor cell subpopulations from bulk cultures for phosphorylation levels of different IFN signaling components.

Reviewer #1: ***The authors added a 2nd human patient scRNA-Seq dataset to strengthen the human melanoma relevance of their findings, added new data for PD-1 expression correlation with IFN-I/R in human patient tumor. Other comments are addressed by clarifications, revision and new analysis. My comments have been addressed.***

Response: We thank the reviewer for the insightful review and are pleased to have addressed all of his/her comments.

Reviewer #2: ***The authors addressed the points that I raised, although some experimental settings were not meaningful. To demonstrate higher levels of interferon signaling in PD-1 positive melanoma cells it would have been more conclusive to directly compare sorted PD-1pos and PD-1neg tumor cell subpopulations from bulk cultures for phosphorylation levels of different IFN signaling components.***

Response: We thank the reviewer for confirming that we have addressed all points raised. To address the additional point raised by the referee pertaining to phosphorylation of type I IFN signaling components in PD-1(+) vs. PD-1(-) tumor cell subpopulations from bulk cultures, we now provide new data in Supplementary Figures 10e-f. Indeed, expression levels of phosphorylated (p)-JAK1 and p-STAT1 proteins are significantly elevated in PD-1(+) versus PD-1(-) (S10e) murine B16-F10 and YUMM1.7 and (S10f) human A2058 and A375 melanoma cell subpopulations as determined by multicolor flow cytometry. These results thus further corroborate the type I IFN signaling pathway as a critical regulator of melanoma cell-intrinsic PD-1 expression.