EMBO reports

MLLT6 Maintains PD-L1 Expression and Mediates Tumor Immune Resistance

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1st Editorial Decision 3rd Mar 2020

Dear Dr. Theis,

Thank you for the submission of your research manuscript to EMBO reports. We have now received reports from the three referees that were asked to evaluate your study, which can be found at the end of this email.

As you will see, all referees think that the findings are of interest, but they also have several comments, concerns and suggestions, indicating that a major revision of the manuscript is necessary to allow publication in EMBO reports. As the reports are below, and I think all points need to be addressed, I will not detail them here.

Given the constructive referee comments, we would like to invite you to revise your manuscript with the understanding that all referee concerns must be addressed in the revised manuscript and/or in a detailed point-by-point response. Acceptance of your manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

Revised manuscripts should be submitted within three months of a request for revision; they will otherwise be treated as new submissions. Please contact me if a 3-months time frame is not sufficient so that we can discuss the revisions further.

When submitting your revised manuscript, please also carefully review the instructions that follow below.

PLEASE NOTE THAT upon resubmission revised manuscripts are subjected to an initial quality control prior to exposition to re-review. Upon failure in the initial quality control, the manuscripts are sent back to the authors, which may lead to delays. Frequent reasons for such a failure are the lack of the data availability section (please see below) and the presence of statistics based on n=2 (the authors are then asked to present scatter plots or provide more data points).

When submitting your revised manuscript, we will require:

1) a .docx formatted version of the final manuscript text (including legends for main figures, EV figures and tables), but without the figures included. Please make sure that the changes are highlighted to be clearly visible. Figure legends should be compiled at the end of the manuscript text.

2) individual production quality figure files as .eps, .tif, .jpg (one file per figure), of main figures and EV figures. Please upload these as separate, individual files upon re-submission.

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Please follow the nomenclature Appendix Figure Sx, Appendix Table Sx etc. throughout the text, and also label the figures and tables according to this nomenclature.

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3) a .docx formatted letter INCLUDING the reviewers' reports and your detailed point-by-point responses to their comments. As part of the EMBO Press transparent editorial process, the point-by-point response is part of the Review Process File (RPF), which will be published alongside your paper.

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5) that primary datasets produced in this study (e.g. RNA-seq, ChIP-seq and array data) are deposited in an appropriate public database. This is now mandatory (like the COI statement). If no primary datasets have been deposited in any database, please state this in this section (e.g. 'No primary datasets have been generated and deposited').

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Please remember to provide a reviewer password if the datasets are not yet public.

The accession numbers and database should be listed in a formal "Data Availability " section (placed after Materials & Methods) that follows the model below. Please note that the Data Availability Section is restricted to new primary data that are part of this study.

Data availability

The datasets produced in this study are available in the following databases:

- RNA-Seq data: Gene Expression Omnibus GSE46843 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE46843)
- [data type]: [name of the resource] [accession number/identifier/doi] ([URL or identifiers.org/DATABASE:ACCESSION])

*** Note - All links should resolve to a page where the data can be accessed. ***

Moreover, I have these editorial requests:

6) We strongly encourage the publication of original source data with the aim of making primary

data more accessible and transparent to the reader. The source data will be published in a separate source data file online along with the accepted manuscript and will be linked to the relevant figure. If you would like to use this opportunity, please submit the source data (for example scans of entire gels or blots, data points of graphs in an excel sheet, additional images, etc.) of your key experiments together with the revised manuscript. If you want to provide source data, please include size markers for scans of entire gels, label the scans with figure and panel number, and send one PDF file per figure.

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8) Regarding data quantification and statistics, can you please specify, where applicable, the number "n" for how many independent experiments (biological replicates) were performed, the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values in the respective figure legends. Please provide statistical testing where applicable, and also add a paragraph detailing this to the methods section. See:
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9) Please add scale bars to all microscopic images (presently, some images do not have scale bars). Please refrain from writing the size on or near the scale bars. Please add the size information to the respective figure legend.

Finally, please note that all corresponding authors are required to supply an ORCID ID for their name upon submission of a revised manuscript. Please find instructions on how to link your ORCID ID to your account in our manuscript tracking system in our Author guidelines:
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I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

Yours sincerely

Achim Breiling
Editor
EMBO Reports

Referee #1:

Although immunotherapy is effective against several malignancies a considerable number of patients either do not respond or show disease progression despite receiving treatment. It is thus important to understand why certain tumors are refractive and to identify additional targets and increase treatment efficacy. In the present work Sreevalsan et al show that the transcription factor

MLLT6 regulates PD-L1 expression in tumor cell lines and also confers resistance to CTL mediated tumor cell killing. This work contributes new insight into a possible mechanism of immunotherapy resistance.

I think this work will benefit by addressing the following issues:

Protein expression evidence for MLLT6 is missing:
> The authors set up a CRISPR screen and identify MLLT6 as an important factor regulating PD-L1 expression both in the presence and absence of IFNg treatment. They go on to generate MLLT6 KO lines and show that PD-L1 expression is decreased in these cells. However, the paper does not contain any evidence at the protein level that MLLT6 expression is absent in KO lines. The authors need to include a Western Blot image showing the absence of MLLT6 protein in targeted lines.

MLLT6 KO lines:
> The authors don't mention whether multiple MLLT6 KO clones were generated and tested for PD-L1 expression. Results from different clones need to be presented to make a strong case.

> When comparing the data between MLLT6 KO from U2OS and MLLT6 lines (Fig 2 A vs B panels), there is a clear difference in the percentage of PD-L1 expressing cells between the 2 lines. While the data in U2OS cells is convincing, the effect in RKO line is consistent but modest. Is this difference explained by the levels of MLLT6 expression and perhaps an incomplete KO phenotype of these cells? Additionally, from the flow panels in figure 2B, it seems as if MLLT6 is important to maintain high-level expression of PD-L1 as opposed to being necessary or required for PD-L1 expression. Thus the authors may make a clearer case if they also present mean fluorescence intensity (MFI) data for the MLLT6 KO clones from both lines.

General applicability of the study:
> This study could benefit from collecting more evidence for the ability of MLLT6 to regulate PD-L1 expression. More cancer cell lines where MLLT6 and PD-L1 are co-expressed can be tested to confirm that their co-expression implies MLLT6 plays a role in the regulation of PD-L1. Negative results will also be meaningful as they will indicate that additional factors play a role in regulating PD-L1 expression even when MLLT6 is expressed.

Referee #2:

The authors present a screen for genes involved in expression of PDL1. They identify MLLT6 as being important for the expression of PDL1 in RKO cells and in its upregulation in response to IFNg. Subsequently they show that MLLT6 knockout cells are resistant to CD8 T cell mediated killing but that this was independent of the effect on PDL1. They suggest that this is due to a more general effect that MLLT6 has on IFNg signaling and that knocking out MLLT6 leads to a blunted response to IFNg due to a shift in phosphorylation of STAT1 from the alpha to the beta isoform.

The authors present an interesting screen and convincingly demonstrate that MLLT6 knockdown leads to a decrease in PDL1 expression. However, there are several issues with this paper and its conclusions that I believe would preclude publication in its current form and would require significant revisions.

Major concerns

1) The authors are unclear as to whether they generated clonal or pooled knockout populations of cells. While replacement of MLLT6 did lead to an increase in PDL1 within the resulting cells it is unclear whether other effects of MLLT6 knockdown are due to clonal selection effects or off target effects - especially since the resistance to CD8 T cell mediated killing was independent of PDL1. It would be good to see the changes in sensitivity to T cell mediated killing and the changes in responsiveness to IFNg in multiple cell lines and, if clonally selecting knockouts, in multiple clones of each cell line.

2) It is unclear how MLLT6 knockdown leads to the cells becoming resistant to T cell mediated killing. While a feasible model is proposed, that other putative immune suppressive factors are blocked by knockdown of MLLT6, this is just a model and needs to be tested. Knockdown of some of the proposed factors e.g. IDO would be good ways to test whether this hypothesis is correct.

3) The conclusion that MLLT6 knockdown leads to resistance to T cell mediated killing due to effects on responses to IFNg are unsupported. Although it is unclear the figure legend would suggest that the knockout cells were tested for their sensitivity to T cell mediated killing without pre-treatment with IFNg. This suggests that whatever effect MLLT6 is having is mediated at homeostasis rather than in response to IFNg and so the proposed mechanism, that of blunted response to IFNg due to a shift in STAT1 phosphorylation, is unlikely to be the true cause of the response.

4) The final conclusion that ratio of STAT1a:STAT1b phosphorylation levels is the cause of any effects found is problematic. Firstly this shift should be quantified and shown to be robust since it is the final mechanistic conclusion. Secondly the assertion that this shift is towards the phosphorylation of an inactive STAT1b isoform is not sufficiently supported by the literature to allow this to form a conclusion in and of itself. The review cited in support of this conclusion cites Semper et al. 2014 which showed that in mice deficient in STAT1a, STAT1b still responded to IFNg and drove transcription of IFNg responsive genes. As such this final observation generates a hypothesis which should be tested but cannot be presented as a conclusion.

Minor concerns

1) The statistical tests used, the number of replicates and the individual values are not explained within the text, legends or methods

2) The flow plots shown as representative would represent outliers when examined compared to the bar graphs sitting comfortably outside of the error bars making them dramatic examples of the phenomena described but exaggerating the conclusions

3) When examining a population of cells' expression of PDL1 and its shift in response to IFNg the authors see a monotonic shift in their population. It would be more representative to present readouts such as MFI or gMFI as opposed to %PDL1+ as this would give a more accurate description of the data

4) The model in figure 4 shows MLLT6 blocking phosphorylated STAT1 from interacting with DNA but the outlined model in the text suggests that the effect is upstream at STAT1 phosphorylation

5) Interferon g and IFNg are used interchangeably and should be made consistent once the abbreviation has been defined

6) Figure 2F is referenced after 2C and before 2D putting the panels out of order

7) Figure 3A is used to assert that cells lacking MLLT6 show a blunted response to IFNg. The first panel shows the response to IFNg in wildtype cells but the second shows a comparison of wildtype and knockout cells both responding to IFNg rather than showing the response of MLLT6 cells to IFNg. As such the claim that this shows a blunted response is incorrect and suggests that the cells have lower expression of these genes in the context of IFNg but without the baseline expression of these genes the responsiveness cannot be ascertained. While this is partly addressed by figure 4C the text should be amended to reflect what this initial screen shows.

Referee #3:

Sreelvasan et al perform a CRISPR-Cas9 screen using an sgRNA library targeting a subset of genes (1,572 total) to identify negative regulators of PD-L1 expression in a colorectal cancer cell line (RKO). They identify a requirement for MLLT6 to maintain PD-L1 expression and loss of MLLT6 leads to reduced basal and IFN-induced PD-L1 mRNA expression. Using co-culture experiments, they demonstrate that MLLT6 KO tumour cells are killed more effectively in the presence of bi-specific T-cell engager antibodies such as EpCAM-CD3, HER2-CD3 and EGFR-CD3. In addition, they demonstrate that MLLT6 knockout cells exhibit impaired interferon-induced gene expression and have low levels of STAT1.

The regulation of PD-L1 expression is an area of prime importance for cancer therapeutics and is of broad general interest. In addition, the identification of a potential specific, and non-redundant function for MLLT6 in IFN-induced gene expression is an interesting finding. However, I have several concerns about some of the data presented and conclusions drawn:

1. According to figure 1B and supplementary table 3 only 1 of 7 MLLT6 sgRNA were enriched in the screen, making this a very low confidence 'hit' on the basis of the screen data. Importantly the screen fails to identify many of the positive control sgRNA e.g. in the screen for interferon-induced PD-L1 expression only 1 of 7 sgRNA targeting STAT1 is enriched and no sgRNA targeting other essential components of the IFN-response pathway e.g. JAK1 and JAK2 are enriched. Furthermore, only 3 (no IFN screen) or 5 (IFN screen) of 12 sgRNA targeting PD-L1 itself are enriched. Together, this suggests that representation of a large proportion of the sgRNA in the library has been lost. The performance of the screen is particularly poor given that a relatively small sgRNA library has been used. Whilst the subsequent validation data supports a genuine role for MLLT6 in PD-L1 regulation, the relative importance of MLLT6 as a PD-L1 regulator cannot be ascertained from this screen. If the authors are unable to provide any additional screening data, the first section of the results should be modified to highlight the limitations and uncertainty of these data - in most screens of this type enrichment of only one of seven sgRNA would have a high probability of being spurious. I believe it is misleading based on the dropout of essential genes to suggest that this validates 'the effectiveness of the screening process' (line 111) given that the rest of the data suggests that the PD-L1 screen was largely ineffective. The authors should state in the text that only a single sgRNA targeting STAT1 (line 100) was enriched in the IFN screen and similarly explicitly state that only one of seven sgRNA targeting MLLT6 (line 112) was enriched.

2. The complementation experiments in figure 2B/C provide important support for the author's proposal that the effects of the MLLT6 sgRNA are on-target and that MLLT6 is an important

regulator of PD-L1. However, I am concerned that all the remaining experiments in the paper appear to use a single MLLT6 knockout clone that was generated using the sgRNA sequence of the only sgRNA that was enriched in the screen. Including the MLLT6 BAC complemented MLLT6 KO in the qRT-PCR in figure 2F would help support the proposal that MLLT6 is a transcriptional regulator of PD-L1. In addition, it is particularly important to confirm that the effects of MLLT6 in the in vitro co-culture assays are MLLT6-dependent on-target effects. The authors could use the MLLT6 BAC complemented cells to confirm this +/- test independent MLLT6 sgRNA. Sequences for a number of different MLLT6 sgRNA are listed in the methods but I can't see data relating to these different sgRNA in the manuscript?

3. With respect to interpreting the BITE experiments in figure 3, it is also important to demonstrate whether MLLT6 knockout modulates expression of the target antigen. How does EpCAM, EGFR and HER2 cell surface expression and mRNA expression compare in MLLT6 KO vs control cells?

4. In appendix S4 it looks as though expression of a large number of genes may be altered following MLLT6 knockout therefore it would be helpful to include a heatmap in the supplementary information to demonstrate all the genes that are significantly up- or down-regulated in control IFN treated vs MLLT6 KO IFN treated cells. This would allow evaluation of the broader function and specificity of MLLT6 as a transcriptional regulator and of the significance of IFN-pathway regulation in this context.

5. Given the potential broad functions of MLLT6, the authors make substantial assumptions in attributing the increased sensitivity of MLLT6 KO cells to T-cell killing to impaired interferon-induced gene expression. This is particularly relevant given that several other large scale CRISPR screens (e.g. PMID: 28783722, 28723893, 29301958) and data in cancer patients treated with immune checkpoint inhibitors have suggested the converse i.e. that impaired interferon signaling leads to resistance to T-cell killing. Clearly, factors modulating T-cell killing in the presence of a bi-specific T cell engager antibody may be different to those regulating T-cell cytotoxicity mediated by TCR recognition of peptide-MHC. However, given that enhanced T-cell killing of tumour cells in the context of impaired tumour interferon-signalling is counter to what would be predicted the authors should more directly demonstrate that the enhanced killing of MLLT6 KO cells in their assays is due to impaired interferon-induced gene expression e.g. does STAT1 KO phenocopy MLLT6 KO and lead to enhanced tumour cell killing in the assays in figure 3 and does restoring IFN-signalling through STAT1 overexpression in MLLT6 KO cells restore immune resistance?

6. The authors suggest that MLLT6 KO 'only mildly changed MHC class I gene expression', however, IFN-induced HLA-B expression shows a greater relative reduction than PD-L1 expression in MLLT6 knockout cells (log2 fold change -1.4 for HLA-B vs -1.0 for PD-L1, lines 225-229) plus from the gene-list in appendix tab S4, expression of immunoproteasome components PSMB8/PSMB9 is substantially reduced which could adversely impact MHC-I peptide generation. The statement that 'loss of MLLT6 in tumor cells leaves MHC-I expression largely unaltered' (line 334) is not justified on the basis of the data presented.

7. Given that MLLT6 KO also substantially impairs IFN-induced expression of a number of pro-inflammatory genes e.g. key T-cell attracting chemokines CXCL9, CXCL10 and CXCL1, it is difficult to predict what the net effect of MLLT6 on anti-tumour immunity in vivo would be. The discussion is currently heavily biased towards potential effects of IFN-γ (and hence MLLT6) in promoting immune evasion. However, in the absence of any in vivo data and in vitro data limited to assays with bi-specific antibodies, a more balanced view of the potential pro- and anti-tumourigenic effects of blocking IFN-γ induced gene expression by targeting MLLT6 should be presented.

Response to referees:

Referee #1:

Although immunotherapy is effective against several malignancies a considerable number of patients either do not respond or show disease progression despite receiving treatment. It is thus important to understand why certain tumors are refractive and to identify additional targets and increase treatment efficacy. In the present work Sreevalsan et al show that the transcription factor MLLT6 regulates PD-L1 expression in tumor cell lines and also confers resistance to CTL mediated tumor cell killing. This work contributes new insight into a possible mechanism of immunotherapy resistance. I think this work will benefit by addressing the following issues:
Protein expression evidence for MLLT6 is missing:
> The authors set up a CRISPR screen and identify MLLT6 as an important factor regulating PD-L1 expression both in the presence and absence of IFNg treatment. They go on to generate MLLT6 KO lines and show that PD-L1 expression is decreased in these cells. However, the paper does not contain any evidence at the protein level that MLLT6 expression is absent in KO lines. The authors need to include a Western Blot image showing the absence of MLLT6 protein in targeted lines.

Reply:
We thank the referee for raising this important point. To quantify MLLT6 protein levels, we analyzed cell lysates utilizing three MLLT6 antibodies from different vendors (Bethyl, Abcam, GeneTex). However, all antibodies failed in detecting a band at the expected molecular size or did not deliver a signal at all. Notably, the vendors product sheets report different molecular sizes of MLLT6 protein in Western Blot and meanwhile, the antibody from Abcam has been withdrawn. Based on this data, we are not sure of the quality of these antibodies and have discontinued their use. Alternatively, we present *MLLT6* mRNA expression data generated by qRT-PCR (Fig EV4B, Appendix Fig S2B,E, S4A). The qRT-PCR data show reduced expression of *MLLT6* mRNA for all *MLLT6* knockout cell lines and validate the results from genotyping (Appendix Fig S1B,C, S2A,D, EV4A) on a transcriptional level.

MLLT6 KO lines:
> The authors don't mention whether multiple MLLT6 KO clones were generated and tested for PD-L1 expression. Results from different clones need to be presented to make a strong case.

Reply:
We are grateful to the referee for the suggestion and apologize for not being sufficiently clear in the manuscript. The data presented on the RKO cells were obtained from experiments performed on a monoclonal *MLLT6* knockout cell line. To avoid basing all conclusions on a single clone, we

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conducted all experiments in U2OS cells on a polyclonal line. However, we agree with the referee that a single knockout clone used for the experiments on RKO cells is not sufficient to make a strong case. Therefore, we generated nine additional monoclonal *MLLT6* knockout lines in RKO cells and found *PD-L1* expression reduced (Fig EV4C) in all clones, corroborating the results presented earlier. All clones were genotyped (Fig EV4A) and validated for reduced *MLLT6* expression by qPCR (Fig EV4B). The data has been added to the revised version of the manuscript. Furthermore, we generated polyclonal *MLLT6* knockout lines in the cervical carcinoma cell line HeLa and the colon carcinoma cell line SW480 (Appendix Fig S2). We observed a correlation of *MLLT6* and *PD-L1* expression in HeLa cells (Appendix Fig S2C). In contrast, we observed a mild but not significant reduction in PD-L1 cell surface presentation in SW480 cells in the absence of IFN-γ (Appendix Fig S2F).
We conclude that *MLLT6* is required for the expression of *PD-L1* in different cancer types such as colon carcinoma (RKO), osteosarcoma (U2OS), and cervical carcinoma (HeLa) in multiple single clones and in polyclonal mixture. However, the colon carcinoma cell line SW480 failed in showing an MLLT6 dependency of *PD-L1* cell surface presentation (Appendix Fig S2 F).

> When comparing the data between MLLT6 KO from U2OS and MLLT6 lines (Fig 2 A vs B panels), there is a clear difference in the percentage of PD-L1 expressing cells between the 2 lines. While the data in U2OS cells is convincing, the effect in RKO line is consistent but modest. Is this difference explained by the levels of MLLT6 expression and perhaps an incomplete KO phenotype of these cells?

Reply:
We checked the expression of *MLLT6* in diverse cancer cell lines from the Cancer Cell Line Encyclopedia (CCLE) repository and found *MLLT6* widely expressed at comparable levels (Fig EV3A). Accordingly, *MLLT6* mRNA is expressed at a percentile rank of 84.1 % (FPKM: 11.4) and 96.9 % (FPKM: 13.1) in RKO and U2OS cells, respectively. Consistently, we found *MLLT6* mRNA expression at higher levels in U2OS cells than in RKO cells when measuring by qRT-PCR (Fig R1) providing a plausible explanation for the observed differences in phenotypic strength (Fig 2A and B).

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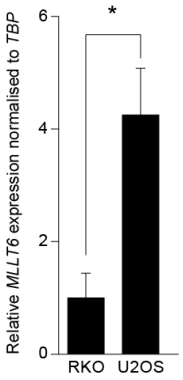

Figure R1. MLLT6 transcript expression.
MLLT6 transcript levels in RKO and U2OS wild type cells normalized to expression in RKO (* $p < 0.05$).

Additionally, from the flow panels in figure 2B, it seems as if MLLT6 is important to maintain high-level expression of PD-L1 as opposed to being necessary or required for PD-L1 expression. Thus the authors may make a clearer case if they also present mean fluorescence intensity (MFI) data for the MLLT6 KO clones from both lines.

Reply:
Many thanks for this helpful suggestion. We have included the mean fluorescence intensities (MFI) in the paper.

General applicability of the study:
> This study could benefit from collecting more evidence for the ability of MLLT6 to regulate PD-L1 expression. More cancer cell lines where MLLT6 and PD-L1 are co-expressed can be tested to confirm that their co-expression implies MLLT6 plays a role in the regulation of PD-L1. Negative results will also be meaningful as they will indicate that additional factors play a role in regulating PD-L1 expression even when MLLT6 is expressed.

Reply:
We agree that the manuscript benefits from testing more cancer cell lines. We now included data for two more cell lines derived from colon (SW480) and cervical carcinomas (HeLa). We observed a decrease in *PD-L1* cell surface presentation in HeLa cells devoid of *MLLT6* in the presence and absence of IFN-γ (Appendix Fig S2C) and a mild but not significant reduction of PD-L1 cell surface presentation in SW480 cells in the absence of IFN-γ (Appendix Fig S2F).

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Referee #2:

The authors present a screen for genes involved in expression of PDL1. They identify MLLT6 as being important for the expression of PDL1 in RKO cells and in its upregulation in response to IFNg. Subsequently they show that MLLT6 knockout cells are resistant to CD8 T cell mediated killing but that this was independent of the effect on PDL1.

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The authors present an interesting screen and convincingly demonstrate that MLLT6 knockdown leads to a decrease in PDL1 expression. However, there are several issues with this paper and its conclusions that I believe would preclude publication in its current form and would require significant revisions.

Major concerns

1) The authors are unclear as to whether they generated clonal or pooled knockout populations of cells. While replacement of MLLT6 did lead to an increase in PDL1 within the resulting cells it is unclear whether other effects of MLLT6 knockdown are due to clonal selection effects or off target effects - especially since the resistance to CD8 T cell mediated killing was independent of PDL1. It would be good to see the changes in sensitivity to T cell mediated killing and the changes in responsiveness to IFNg in multiple cell lines and, if clonally selecting knockouts, in multiple clones of each cell line.

Reply:

We apologize for not being clear on the clonal status of the knockout cell lines utilized in the manuscript. The experiments in RKO cells were done on a monoclonal knockout cell line. To avoid basing all conclusions on this line, we conducted all experiments in U2OS cells on a polyclonal line. However, we agree with the referee that more clones are required on RKO cells to raise meaningful conclusions. We generated nine additional clonal *MLLT6* knockout cell lines and found *PD-L1* expression reduced in all clones (Fig EV4C), corroborating results presented earlier. Furthermore, in the revised manuscript, we included data on pooled *MLLT6* knockout lines in the colon carcinoma cell line SW480 and in the cervical carcinoma line HeLa. We observed decreased levels of *PD-L1* cell surface presentation in HeLa cells devoid of *MLLT6* in the presence and absence of IFN-γ (Appendix Fig S2C) corroborating the results presented in RKO and U2OS cells. However, no significant correlation between *MLLT6* levels and *PD-L1* surface presentation was observed in SW480 cells (Appendix Fig S2F). We agree with the referee that off-targeting might lead to misinterpretations and should carefully be ruled out. In addition to the BAC data that shows a partial rescue of the knockout phenotype (Fig 2B,C, Fig EV4D), we

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generated polyclonal knockout lines using four sgRNAs targeting different regions of the *MLLT6* genomic locus (Appendix Fig S1A, S4). All four sgRNAs led to a knockout of *MLLT6* (Appendix Fig S4A) and show reduced *PD-L1* expression levels (Appendix Fig S4B) excluding the involvement of a potential off-target gene. The data has been added to the revised version of the manuscript.

2) It is unclear how MLLT6 knockdown leads to the cells becoming resistant to T cell mediated killing. While a feasible model is proposed, that other putative immune suppressive factors are blocked by knockdown of MLLT6, this is just a model and needs to be tested. Knockdown of some of the proposed factors e.g. IDO would be good ways to test whether this hypothesis is correct.

Reply:
We are grateful for this suggestion and agree that the *MLLT6* knockout affects multiple immune suppressive factors beyond *PD-L1* (Fig 4) such as *IDO1*, *GBP5* or *CD74* (Fig 4C). Although many more genes are affected in their expression by MLLT6 depletion (Fig 4A and Appendix Table S4), we tested IDO1, GBP5 and CD74 for their contribution to tumor immune resistance by measuring CTL-mediated cytotoxicity on polyclonal knockout cell lines after proving depletion of target proteins by Western Blotting (Fig R2A). We observed a mild increase in CTL-mediated lysis for all tested gene knockouts in the absence of IFN-γ (Fig R2B) with GBP5 knockout sensitizing cells the most for CTL-mediated lysis. However, none of the genes showed an effect as strong as the *MLLT6* knockout (Fig 3). Therefore, we hypothesize that the simultaneous depletion of these genes - eventually along with other genes (Appendix Table S4) – is required for the full penetrance of the MLLT6 phenotype.

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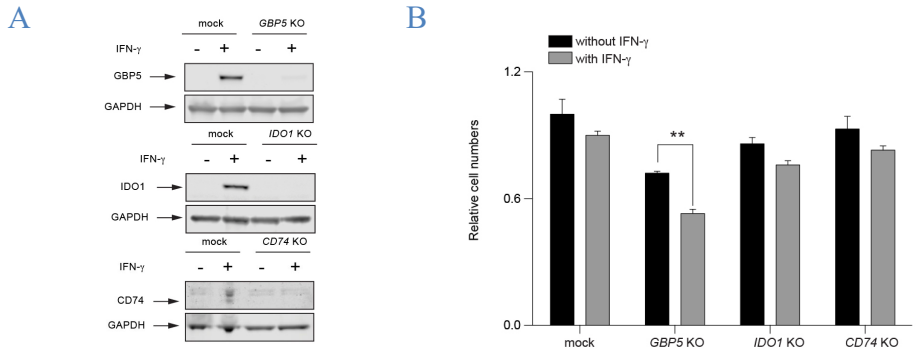

Figure R2. T cell activity in *GBP5*, *IDO1* and *CD74* knockout cells.
A Total cellular protein levels of GBP5 (top), IDO1 (middle) and CD74 (bottom) in polyclonal U2OS knockout cells.
B Relative cell numbers (± SD (n=3)) of U2OS mock or *GBP5*, *IDO1* and *CD74* knockout cells treated (grey) or untreated (black) with IFN-γ (**$p < 0.01$) normalized to mock untreated cells.

3) The conclusion that MLLT6 knockdown leads to resistance to T cell mediated killing due to effects on responses to IFNg are unsupported. Although it is unclear the figure legend would suggest that the knockout cells were tested for their sensitivity to T cell mediated killing without pre-treatment with IFNg. This suggests that whatever effect MLLT6 is having is mediated at homeostasis rather than in response to IFNg and so the proposed mechanism, that of blunted response to IFNg due to a shift in STAT1 phosphorylation, is unlikely to be the true cause of the response.

Reply:
We agree with the interpretation of the referee that the effects on immune resistance by MLLT6 is mediated at homeostasis. However, we also would like to point out that the stimulation of cancer cells with IFN-γ reduces immune resistance in *MLLT6* knockout cells (Fig 3E) but leaves immune resistance unaltered in *MLLT6* expressing cells (Fig 3E).
Additionally, we show that the signal transduction of IFN-γ is perturbed by *MLLT6* knockout (Fig 4). We believe these results justify our claims that *MLLT6* plays an important role in both homeostasis and IFN-γ signaling. However, we agree with the referee that the blunted response to IFN-γ due to a shift in STAT1 phosphorylation is unlikely to be the true cause of the response in homeostasis. We changed the Results and Discussion parts of the manuscript in that respect.

4) The final conclusion that ratio of STAT1a:STAT1b phosphorylation levels is the cause of any effects found is problematic. Firstly this shift should be quantified and shown to be robust since it is the final mechanistic conclusion. Secondly the assertion that this shift is towards the phosphorylation of an inactive STAT1b isoform is not sufficiently

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supported by the literature to allow this to form a conclusion in and of itself. The review cited in support of this conclusion cites Semper et al. 2014 which showed that in mice deficient in STAT1a, STAT1b still responded to IFNg and drove transcription of IFNg responsive genes. As such this final observation generates a hypothesis which should be tested but cannot be presented as a conclusion.

Reply:
We are grateful to the referee for raising this issue. We now provide data dissecting the effects of the STAT1 isoforms STAT1α and STAT1β on the expression of IFN-γ inducible genes such as *IDO1*, *CD74* and *GBP5* (Fig EV5). Specific depletion of STAT1β or STAT1α utilizing RNA interference enhanced or reduced gene induction by IFN-γ, respectively (Fig EV5). The data is in accordance with a function of STAT1α as a transcriptional activator and STAT1β as a transcriptional repressor. However, we also acknowledge that the functional role of both STAT1 isoforms in transcription is controversially discussed in the literature. We therefore now included the study of Semper et al., 2014 in the manuscript.

Minor concerns

1) The statistical tests used, the number of replicates and the individual values are not explained within the text, legends or methods

Reply:
Many thanks for pointing this out. We provide this information in the revised manuscript in the Materials and Methods under the subheading "Statistical Analysis" or in the figure legends.

2) The flow plots shown as representative would represent outliers when examined compared to the bar graphs sitting comfortably outside of the error bars making them dramatic examples of the phenomena described but exaggerating the conclusions

Reply:
Many thanks for pointing this out. We replaced the flow plot with a more representative one.

3) When examining a population of cells' expression of PDL1 and its shift in response to IFNg the authors see a monotonic shift in their population. It would be more representative to present readouts such as MFI or gMFI as opposed to %PDL1+ as this would give a more accurate description of the data

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Reply:
Thank you for this helpful suggestion. We included the MFI in the manuscript.

4) The model in figure 4 shows MLLT6 blocking phosphorylated STAT1 from interacting with DNA but the outlined model in the text suggests that the effect is upstream at STAT1 phosphorylation

Reply:
We are grateful to the referee for pointing this out and we have corrected the Fig 4 accordingly.

5) Interferon g and IFNg are used interchangeably and should be made consistent once the abbreviation has been defined

Reply:
Thank you for pointing this out. We have changed the manuscript accordingly.

6) Figure 2F is referenced after 2C and before 2D putting the panels out of order

Reply:
Thank you for picking this up. We have corrected the figure referencing.

7) Figure 3A is used to assert that cells lacking MLLT6 show a blunted response to IFNg. The first panel shows the response to IFNg in wildtype cells but the second shows a comparison of wildtype and knockout cells both responding to IFNg rather than showing the response of MLLT6 cells to IFNg.
As such the claim that this shows a blunted response is incorrect and suggests that the cells have lower expression of these genes in the context of IFNg but without the baseline expression of these genes the responsiveness cannot be ascertained. While this is partly addressed by figure 4C the text should be amended to reflect what this initial screen shows.

Reply:
We are grateful to the referee for picking this. The baseline expression of the genes has now been included in Appendix Table S4 and visualized in Appendix Fig S8. The Results part of the manuscript was amended.

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Referee #3:

Sreelvasan et al perform a CRISPR-Cas9 screen using an sgRNA library targeting a subset of genes (1,572 total) to identify negative regulators of PD-L1 expression in a colorectal cancer cell line (RKO). They identify a requirement for MLLT6 to maintain PD-L1 expression and loss of MLLT6 leads to reduced basal and IFN-induced PD-L1 mRNA expression. Using co-culture experiments, they demonstrate that MLLT6 KO tumour cells are killed more effectively in the presence of bi-specific T-cell engager antibodies such as EpCAM-CD3, HER2-CD3 and EGFR-CD3. In addition, they demonstrate that MLLT6 knockout cells exhibit impaired interferon-induced gene expression and have low levels of STAT1.

The regulation of PD-L1 expression is an area of prime importance for cancer therapeutics and is of broad general interest. In addition, the identification of a potential specific, and non-redundant function for MLLT6 in IFN-induced gene expression is an interesting finding. However, I have several concerns about some of the data presented and conclusions drawn:

1. According to figure 1B and supplementary table 3 only 1 of 7 MLLT6 sgRNA were enriched in the screen, making this a very low confidence 'hit' on the basis of the screen data. Importantly the screen fails to identify many of the positive control sgRNA e.g. in the screen for interferon-induced PD-L1 expression only 1 of 7 sgRNA targeting STAT1 is enriched and no sgRNA targeting other essential components of the IFN-response pathway e.g. JAK1 and JAK2 are enriched. Furthermore, only 3 (no IFN screen) or 5 (IFN screen) of 12 sgRNA targeting PD-L1 itself are enriched. Together, this suggests that representation of a large proportion of the sgRNA in the library has been lost. The performance of the screen is particularly poor given that a relatively small sgRNA library has been used. Whilst the subsequent validation data supports a genuine role for MLLT6 in PD-L1 regulation, the relative importance of MLLT6 as a PD-L1 regulator cannot be ascertained from this screen. If the authors are unable to provide any additional screening data, the first section of the results should be modified to highlight the limitations and uncertainty of these data - in most screens of this type enrichment of only one of seven sgRNA would have a high probability of being spurious.

Reply:

We thank the referee for pointing this out. We agree that a large proportion of the sgRNAs in the library got lost throughout the screen. This is due to the rigorous selection of sgRNAs by three consecutive rounds of sorting that removed 99.9 %, > 98.9 % and > 89.0 % of the PD-L1-EGFP^{high} cells (Fig EV2). Thereby, sgRNAs that provide robust but weak phenotypes were lost. This procedure reduced redundancy and biased the selection

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towards sgRNAs with high knockout efficiency and strong phenotypes. This is illustrated by the observation that only the positive controls that directly target the reporter genes (eGFP and PD-L1) - providing large phenotypic penetrance - were enriched with multiple sgRNAs (Fig 1A,B and Appendix Table S3). In contrast, sgRNAs targeting regulatory genes that indirectly modulate reporter gene expression such as STAT1 led to weaker phenotypes and failed in enriching multiple sgRNAs (Fig 1A,B and Appendix Table S3). Competition of direct and indirect positive controls with sample genes for enrichment reduced the dynamic range. Due to this low dynamic range, conclusions on the relative importance of *MLLT6* cannot be drawn and for that reason, we refrained from comparing primary hit genes in the manuscript. However, we still observed strong enrichment of particular sgRNA in addition to the positive controls and identified *MLLT6* as gene necessary for the maintenance of *PD-L1* expression. We included these considerations in the manuscript and modified the discussion part (line 300-303) accordingly.

I believe it is misleading based on the dropout of essential genes to suggest that this validates 'the effectiveness of the screening process' (line 111) given that the rest of the data suggests that the PD-L1 screen was largely ineffective.

Reply:
We agree with the referee and removed the statement given in line 111.

The authors should state in the text that only a single sgRNA targetg STAT1 (line 100) was enriched in the IFN screen and similarly explicitly state that only one of seven sgRNA targeting MLLT6 (line 112) was enriched.

Reply:
This important information was added to the revised manuscript (line 102-104).

2. The complementation experiments in figure 2B/C provide important support for the author's proposal that the effects of the MLLT6 sgRNA are on-target and that MLLT6 is an important regulator of PD-L1. However, I am concerned that all the remaining experiments in the paper appear to use a single MLLT6 knockout clone that was generated using the sgRNA sequence of the only sgRNA that was enriched in the screen.

Reply:
This is an important point and we apologize for not being sufficiently clear in the manuscript. The experiments in RKO cells were done on a monoclonal knockout cell line. To avoid basing all conclusions on a single clone, we conducted all experiments in U2OS cells on a polyclonal line.

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However, we agree with the referee that a single knockout clone used for the experiments on RKO cells is not sufficient to make a strong case. Therefore, we generated nine additional monoclonal *MLLT6* knockout lines in RKO cells. All clones were genotyped (Fig EV4A) and validated for reduced *MLLT6* expression by qPCR (Fig EV4B). We found *PD-L1* expression reduced in all clones (Fig EV4C), corroborating the results presented earlier.
To exclude effects resulting from the sgRNA used for CRISPR/Cas9 mediated knockout, we generated polyclonal knockout lines using three alternative sgRNAs targeting different sites of the *MLLT6* genomic locus (Appendix Fig S1A). All four sgRNAs led to an efficient knockout of *MLLT6* (Appendix Fig S4A) and showed reduced *PD-L1* expression (Appendix Fig S4B). Furthermore, we generated polyclonal *MLLT6* knockout lines in colon (SW480) and cervical carcinoma cells (HeLa). We observed that MLLT6 depletion reduced *PD-L1* expression in the presence and absence of IFN-γ in HeLa cells (Appendix Fig S2C) but not in SW480 cells (Appendix Fig S2F).
We conclude that *MLLT6* is required for the expression of *PD-L1* in different cancer types such as colon carcinoma (RKO), osteosarcoma (U2OS), and cervical carcinoma (HeLa) in multiple single clones and polyclones, corroborating the results presented earlier.

Including the MLLT6 BAC complemented MLLT6 KO in the qRT-PCR in figure 2F would help support the proposal that MLLT6 is a transcriptional regulator of PD-L1. In addition, it is particularly important to confirm that the effects of MLLT6 in the in vitro co-culture assays are MLLT6-dependent on-target effects. The authors could use the MLLT6 BAC complemented cells to confirm this +/- test independent MLLT6 sgRNA. Sequences for a number of different MLLT6 sgRNA are listed in the methods but I can't see data relating to these different sgRNA in the manuscript?

Reply:
We thank the referee for this helpful suggestion. We measured *PD-L1* transcript levels in both the *MLLT6* knockout cell line and after stable transfection of the *MLLT6*-BAC construct. We found that the *PD-L1* mRNA expression recovered in the BAC line (Fig EV4D) corroborating earlier findings on the role of *MLLT6* in *PD-L1* transcription.

3. With respect to interpreting the BITE experiments in figure 3, it is also important to demonstrate whether MLLT6 knockout modulates expression of the target antigen. How does EpCAM, EGFR and HER2 cell surface expression and mRNA expression compare in MLLT6 KO vs control cells?

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Reply:
This is an important point and we are grateful to the referee for bringing this up. We now include flow cytometry measurements of the expression of *EpCAM*, *EGFR* and *HER-2* in *MLLT6* knockout and control cells in the revised version of the manuscript (Appendix Fig S5).

4. In appendix S4 it looks as though expression of a large number of genes may be altered following MLLT6 knockout therefore it would be helpful to include a heatmap in the supplementary information to demonstrate all the genes that are significantly up- or down-regulated in control IFN treated vs MLLT6 KO IFN treated cells. This would allow evaluation of the broader function and specificity of MLLT6 as a transcriptional regulator and of the significance of IFN-pathway regulation in this context.

Reply:
We agree that displaying the RNA-Seq data in form of a heat map is helpful to illustrate the observed transcriptional changes. We included a heat map in Appendix Fig S7.

5. Given the potential broad functions of MLLT6, the authors make substantial assumptions in attributing the increased sensitivity of MLLT6 KO cells to T-cell killing to impaired interferon-induced gene expression. This is particularly relevant given that several other large scale CRISPR screens (e.g. PMID: 28783722, 28723893, 29301958) and data in cancer patients treated with immune checkpoint inhibitors have suggested the converse i.e. that impaired interferon signaling leads to resistance to T-cell killing.
Clearly, factors modulating T-cell killing in the presence of a bi-specific T cell engager antibody may be different to those regulating T-cell cytotoxicity mediated by TCR recognition of peptide-MHC. However, given that enhanced T-cell killing of tumour cells in the context of impaired tumour interferon-signalling is counter to what would be predicted the authors should more directly demonstrate that the enhanced killing of MLLT6 KO cells in their assays is due to impaired interferon-induced gene expression e.g. does STAT1 KO phenocopy MLLT6 KO and lead to enhanced tumour cell killing in the assays in figure 3 and does restoring IFN-signalling through STAT1 overexpression in MLLT6 KO cells restore immune resistance?

Reply:
We are grateful to the referee for this suggestion. We now present a more balanced view on the pro- and anti-tumorigenic effects of IFN-γ (line: 345-356) and cite the work by Pan et al., 2018, Patel et al., 2017 and Manguso et al. 2017 in the manuscript.

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Besides these considerations on the role of IFN-γ, we would like to point out that the effects of *MLLT6* on tumor immune resistance are not exclusively mediated through *STAT1* signaling and the IFN-γ pathway. Pronounced effects on T cell mediated cytolysis by MLLT6 depletion were also observed in the absence of IFN-γ (Fig 3D, E and Fig 4C). Additionally, the gene sets regulated by MLLT6 and STAT1 are not identical, hence *STAT1* knockout is unlikely to phenocopy the effects of *MLLT6* knockout. Consistently, we did not observe striking effects on CTL-mediated cytolysis in *STAT1* knockout cells in the presence or absence of IFN-γ (Fig R3). Consequently, we do not expect a rescue by expression of *STAT1* in *MLLT6* knockout cells. Nevertheless, *MLLT6* knockout impairs IFN-γ signaling (Fig 1B, 2D-F, 4A-E) involving STAT1 (Fig 4E, EV5) and reduces the expression of genes typically induced by IFN-γ such as *PD-L1* (Fig 4A,B, Appendix Fig S7, S8, Table S4). Although the effects on CTL activity observed in cells devoid of *MLLT6* are not exclusively mediated by STAT1, our results corroborate a functional model of MLLT6 modulating the transcriptional activity of STAT1. We have added these results to the revised manuscript.

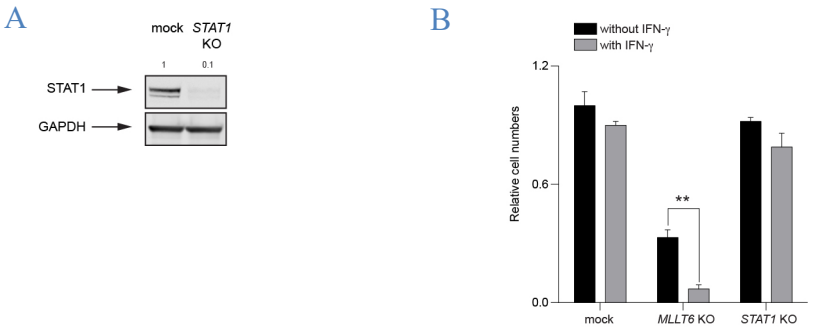

Figure R3. T cell activity on *STAT1* knockout cells.
A Total cellular protein levels of STAT1 in polyclonal U2OS knockout cells. Numbers indicated band intensities normalized to GAPDH expression.
B Relative cell numbers (± SD (n=3)) of U2OS mock, *MLLT6* or *STAT1* knockout cells treated (grey) or untreated (black) with IFN-γ (**$p < 0.01$).

6. The authors suggest that MLLT6 KO 'only mildly changed MHC class I gene expression', however, IFN-induced HLA-B expression shows a greater relative reduction than PD-L1 expression in MLLT6 knockout cells (log2 fold change -1.4 for HLA-B vs -1.0 for PD-L1, lines 225-229) plus from the gene-list in appendix tab S4, expression of immunoproteasome components PSMB8/PSMB9 is substantially reduced which could adversely impact MHC-I peptide generation. The statement that 'loss of MLLT6 in tumor cells leaves MHC-I expression largely unaltered' (line

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334) is not justified on the basis of the data presented.

Reply:
We agree with the referee's conclusions. We have removed the statement in line 334. Furthermore, we have included a comment on the expression of PSMB8 and PSMB9 and its participation in MHC-I peptide generation (line 242-244). Notably, in cells devoid of *MLLT6* we observed no changes in MHC class I or PSMB8 and PSMB9 expression in the absence of IFN-γ (Appendix Table S4). This observation has been added to the manuscript (line 242-244 and 359-361).

7. Given that MLLT6 KO also substantially impairs IFN-induced expression of a number of pro-inflammatory genes e.g. key T-cell attracting chemokines CXCL9, CXCL10 and CXCL1, it is difficult to predict what the net effect of MLLT6 on anti-tumour immunity in vivo would be. The discussion is currently heavily biased towards potential effects of IFN-γ (and hence MLLT6) in promoting immune evasion. However, in the absence of any in vivo data and in vitro data limited to assays with bi-specific antibodies, a more balanced view of the potential pro- and anti-tumourigenic effects of blocking IFN-γ induced gene expression by targeting MLLT6 should be presented.

Reply:
We thank the referee for pointing this out. Our study identifies a previously unknown regulator of immune checkpoint protein expression. However, the conclusions are indeed limited to bi-specific antibodies or T cell engagers. Beronja et al., Nature, 2013 [51] presents data highlighting the importance of *MLLT6* to oncogenic growth in mice providing *in vivo* data, although not characterizing the functional role of *MLLT6* in immune resistance. We added these limitations (line: 380-382) and a more balanced view on the pro- and anti-tumorigenic effects of IFN-γ (line: 345-356) in the revised version of the manuscript.

1st Revision - Editorial Decision 7th Sep 2020

Dear Dr. Theis,

Thank you for the submission of your revised manuscript to our editorial offices. We have now received the reports from the three referees that were asked to re-evaluate your study, you will find below. As you will see, the referees now support the publication of your study in EMBO reports. Nevertheless, referee #3 has two remaining concerns, we ask you to address in a final revised manuscript. Please also provide a point-by-point response addressing these remaining points.

Moreover, I have these editorial requests:

- Please provide the abstract written in present tense throughout.

- We have recently changed our reference format. Please change the format in the final manuscript text accordingly:
http://www.embopress.org/page/journal/14693178/authorguide#referencesformat

- As most of the Western blots shown are significantly cropped, could you to provide the source data for all the blots (main, EV and Appendix figures). The source data will be published in a separate source data file online along with the accepted manuscript and will be linked to the relevant figure. Please submit the source data (scans of entire blots) together with the final revised manuscript. Please include size markers for the scans of entire blots, label the scans with figure and panel number, and send one PDF file per figure.

- Please make sure that the funding information added in the online submission system is complete and similar to the one in the manuscript. Please add all the grant numbers.

- Please provide the legend for the movie as separate text file and upload this ZIPed together with the movie file. Then remove the movie legend from the main manuscript text.

- You uploaded 6 tables as datasets. Please name these (and upload these as) Dataset EVx (1-6) and change their call outs in the manuscript text accordingly (presently, they are still called out as Appendix tables).

- The synopsis image provided has not the correct size and format. Please provide this in jpeg or tiff format with the exact width of 550 pixels and a height of not more than 400 pixels. The writing is also a bit small. Could you provide the image with bigger fonts?

- Finally, please find attached a word file of the manuscript text (provided by our publisher) with changes we ask you to include in your final manuscript text, and some queries, we ask you to address. I think you already addressed this, but please check this once more. Please provide your final manuscript file with track changes, in order that we can see any modifications done.

I look forward to seeing the final revised version of your manuscript when it is ready. Please let me know if you have questions regarding the revision.

Kind regards,

Achim

Achim Breiling
Editor
EMBO Reports

Referee #1:

In the revised manuscript, the authors have included additional data, edited the manuscript to address key issues and provided explanation for questions indicated in my initial review. Upon examining the resubmission, I find that all my concerns about the original manuscript have been adequately addressed. Hence, I now recommend the revised manuscript for publication in EMBO Reports.

Referee #2:

After revision the manuscript is greatly improved and addresses all my prior concerns. I would recommend it for publication in its current form.

Referee #3:

The authors have strengthened the manuscript by providing additional evidence that the effects of MLLT6 knockout on PD-L1 expression are on target and reproducible in different cancer cell contexts. In addition, the new data highlight the role of MLLT6 as a broad transcriptional regulator and a more balanced discussion of the potential positive and negative effects of interferon and MLLT6 on the anti-tumour immune response is presented.

However, I do have two continuing concerns:

1. As requested, the authors have provided data showing the effect of MLLT6 knockout on the expression of the target antigens EpCAM, EGFR and HER2 for the bispecific antibodies used in the T-cell co-culture experiments in figure 3. This is important as it has been established that, as expected, level of cell surface expression of the target antigen determines the sensitivity of cancer cells to CD8 T-cell killing induced by bispecific T-cell engagers (BITEs). In appendix figure S5 the authors show that MLLT6 knockout substantially increases cell surface expression of EpCAM and EGFR. This is problematic as most of the experiments in figure 3 use the EpCAM-CD3 BITE and therefore the increased sensitivity of the MLLT6 knockout cells to the EpCAM-CD3 BITE could be entirely due to the increased expression of EpCAM itself. This differing level of EpCAM expression also makes the comparison with the effects of PD-L1 knockout on sensitivity to BITE induced T-cell killing very difficult to interpret (figure 3D). For HER2, there does not seem to be a substantial difference in expression following MLLT6 KO, however, there is currently only one panel that uses the anti-HER2 bi-specific antibody. As such, unless the authors can provide evidence that MLLT6 knockout affects sensitivity to T-cell killing independent of its effects on target antigen expression for the BITE, there is almost no evidence in the paper to suggest that 'MLLT6 depletion alleviates suppression of CD8+ cytotoxic T cell-mediated cytolysis' or that the effects of MLLT6 on expression of interferon inducible genes has any functional consequence with respect to sensitivity to T-cell cytolysis.

2. The authors have not addressed my previous request to clearly explain the limitations of their CRISPR screen. Many groups have performed and published similar positive selection CRISPR screens using serial enrichment strategies. With effective sort enrichment, this should not result in loss of almost all the sgRNAs targeting the apparent top 'hits' in the screen. As requested previously, the authors should state in the results section that only a single sgRNA targeting STAT1 (line 100) was enriched in the IFN screen and similarly explicitly state that only one of seven sgRNA targeting MLLT6 was enriched. The statement that has been added (lines 104-105) is meaningless: "Interestingly, we observed enrichment of multiple gRNAs targeting positive control genes such as PD-L1 or eGFP but no redundancy for any other gene." The failure to enrich more than a single sgRNA for many of the candidate hits has nothing to do with redundancy and instead reflects a substantial loss of representation of the CRISPR sgRNA library. This is a major limitation of the screen and should be clearly described.

Response to referees:

Referee #1:
In the revised manuscript, the authors have included additional data, edited the manuscript to address key issues and provided explanation for questions indicated in my initial review. Upon examining the resubmission, I find that all my concerns about the original manuscript have been adequately addressed. Hence, I now recommend the revised manuscript for publication in EMBO Reports.

Referee #2:
After revision the manuscript is greatly improved and addresses all my prior concerns. I would recommend it for publication in its current form.

Referee #3:
The authors have strengthened the manuscript by providing additional evidence that the effects of MLLT6 knockout on PD-L1 expression are on target and reproducible in different cancer cell contexts. In addition, the new data highlight the role of MLLT6 as a broad transcriptional regulator and a more balanced discussion of the potential positive and negative effects of interferon and MLLT6 on the anti-tumour immune response is presented.

However, I do have two continuing concerns:
1. As requested, the authors have provided data showing the effect of MLLT6 knockout on the expression of the target antigens EpCAM, EGFR and HER2 for the bispecific antibodies used in the T-cell co-culture experiments in figure 3. This is important as it has been established that, as expected, level of cell surface expression of the target antigen determines the sensitivity of cancer cells to CD8 T-cell killing induced by bispecific T-cell engagers (BITEs). In appendix figure S5 the authors show that MLLT6 knockout substantially increases cell surface expression of EpCAM and EGFR. This is problematic as most of the experiments in figure 3 use the EpCAM-CD3 BITE and therefore the increased sensitivity of the MLLT6 knockout cells to the EpCAM-CD3 BITE could be entirely due to the increased expression of EpCAM itself. This differing level of EpCAM expression also makes the comparison with the effects of PD-L1 knockout on sensitivity to BITE induced T-cell killing very difficult to interpret (figure 3D). For HER2, there does not seem to be a substantial difference in expression following MLLT6 KO, however, there is currently only one panel that uses the anti-HER2 bi-specific antibody. As such, unless the authors can provide evidence that MLLT6 knockout affects sensitivity to T-cell killing independent of its effects on target antigen expression for the BITE, there is almost no evidence in the paper to suggest that 'MLLT6

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depletion alleviates suppression of CD8+ cytotoxic T cell-mediated cytolysis' or that the effects of MLLT6 on expression of interferon inducible genes has any functional consequence with respect to sensitivity to T-cell cytolysis.

Reply:
We thank the referee for bringing this up. Indeed, *MLLT6* knockout leads to increased expression of the genes EpCAM and EGFR but leaves the expression of HER-2 unaltered (Appendix Fig S5). Although the referee raises a valid point, it appears unlikely that the MLLT6 effects on T cells are entirely due to enhanced expression of the genes EpCAM or EGFR. The bi-specific antibody targeting HER-2 shows a comparable performance to EpCAM or EGFR antibodies although HER-2 expression is not increased by MLLT6 knockout (Fig 3). Furthermore, *MLLT6* knockout downregulates the tumor immune repressor genes *PD-L1* and *IDO1* (Fig 4) and affects the expression of multiple immune related genes (Dataset 4) by impairing interferon-γ signal transduction (Fig 4, Dataset 4). Our conclusions are corroborated by the observations that *MLLT6* is required for oncogenic growth in mice (Beronja et al., 2013) and that the increased expression of *MLLT6* negatively correlates with prognosis in gastric cancer (Szasz et al., 2016). We believe this data to justify that *MLLT6* is of high relevance for the immunological editing conveyed by tumor cells far beyond the increased expression of EpCAM and EGFR. Additionally, increased expression of these important tumor antigens is of high relevance in itself as strategies targeting both genes are in clinical trials. Combining anti-EpCAM or EGFR antibodies with MLLT6 inhibition may be a valid strategy for enhancing respective therapies.

2. The authors have not addressed my previous request to clearly explain the limitations of their CRISPR screen. Many groups have performed and published similar positive selection CRISPR screens using serial enrichment strategies. With effective sort enrichment, this should not result in loss of almost all the sgRNAs targeting the apparent top 'hits' in the screen. As requested previously, the authors should state in the results section that only a single sgRNA targeting STAT1 (line 100) was enriched in the IFN screen and similarly explicitly state that only one of seven sgRNA targeting MLLT6 was enriched. The statement that has been added (lines 104-105) is meaningless: "Interestingly, we observed enrichment of multiple gRNAs targeting positive control genes such as PD-L1 or eGFP but no redundancy for any other gene." The failure to enrich more than a single sgRNA for many of the candidate hits has nothing to do with redundancy and instead reflects a substantial loss of representation of the CRISPR sgRNA library. This is a major limitation of the screen and should be clearly described.

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Reply:
We thank the referee for pointing this out. We now included a statement in line 103 that explicitly states that the screen failed enriching more than a single sgRNA for any of the hit genes including *STAT1* and *MLLT6.* Furthermore, we removed the misleading term “redundancy”.

2nd Revision - Editorial Decision 22nd Sep 2020

Dr. Mirko Theis
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Fetscherstraße 74/PF 64
Dresden, Saxony 01307
Germany

Dear Dr. Theis,

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

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Thank you again for your contribution to EMBO reports and congratulations on a successful publication. Please consider us again in the future for your most exciting work.

Yours sincerely,

Achim Breiling
Editor
EMBO Reports

Referee #3

The authors have adequately addressed my concerns. It would be helpful to readers to describe the increased cell surface EpCAM and EGFR expression following MLLT6 knockout in the text where appendix fig S5 is referenced (line 187/188) to facilitate evaluation of the potential contributory effects of increased target antigen expression on the results in figure 3.

****

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Corresponding Author Name: Mirko Theis
Journal Submitted to: EMBO Reports
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Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

Please fill out these boxes (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	No pre-specified effect size was calculated.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	NA
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No data were excluded.
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	No randomization was done.
For animal studies, include a statement about randomization even if no randomization was used.	NA
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	No blinding was done.
4.b. For animal studies, include a statement about blinding even if no blinding was done	NA
5. For every figure, are statistical tests justified as appropriate?	The type of statistical tests are reported in the respective section of Material and Methods or in the figure legends. Typically, for two group comparison we used a Student's two-tailed t-test and one-way ANOVA was used for comparing multiple groups utilizing Graphpad Prism software (version 6.04).
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	NA
Is there an estimate of variation within each group of data?	We report the SD for averages as given in the figure legends.
Is the variance similar between the groups that are being statistically compared?	NA

C- Reagents

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http://ClinicalTrials.gov
http://www.consort-statement.org
http://www.consort-statement.org/checklists/view/32-consort/66-title

http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tumo

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http://jij.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	All antibodies from commercial sources have been validated by the vendors and their validation data are available on the manufacturer's website. Immunofluorescence: goat anti-eGFP (MPI-CBG, Antibody Facility), mouse anti-α-tubulin (MPI-CBG, Antibody Facility), donkey anti-mouse Alexa594 (ThermoFisherScientific, 13497317), donkey anti-goat FITC (ThermoFisherScientific, 15343856). Flow cytometry: PE Mouse IgG1, κ Isotype Control (BD Pharmingen, 555749), PE Mouse Anti-Human CD274 (BD Pharmingen, 557924). Western Blot: rabbit anti-PD-L1 (E1L3N) XP (Cell Signaling Technology, 13684), goat anti-GAPDH (Acris Antibodies, AP16240PU-N), rabbit anti-IFNGR1 (Cell Signaling Technology, 34808), rabbit anti-IFNGR2, C-term (Gene Tex, GTX81601), rabbit anti-JAK1(6G4) (Cell Signaling Technology, 3344), rabbit anti-IDO1 (Gene Tex, GTX113753), rabbit anti-HLA-DRB1 (N1C3) (Gene Tex, GTX104919), rabbit anti-CD74 (Gene Tex, GTX110477), rabbit anti-GBP5 (Gene Tex, GTX106994), rabbit anti-JAK2 (D2E12, Cell Signaling Technology, 3230) rabbit anti-STAT1 (Cell Signaling Technology, 9172), rabbit anti-Phospho-STAT1 (Tyr701) (D4A7) (Cell Signaling Technology, 7649), IRDye 680LT Donkey anti-Goat IgG (LI-COR Biosciences, 926-68024), IRDye 800 CW Donkey anti-Rabbit IgG (LI-COR Biosciences, 926-32213). T-cells activation:
	APC Mouse anti-Human CD3 (BD Pharmingen, 555342), APC-Cy7 CD8 (BD Pharmingen, 348813), PE-Cy7 Mouse anti-Human CD25 (BD Pharmingen 557741), FITC Mouse anti-Human CD69 (BD Pharmingen, 557049). Bispecific Antibodies: Recombinant anti-EpCAM-CD3 bi-specific T-cell engagers (Creative Biolabs, BITE-L022), anti-Her2-CD3 and anti-EGFR-CD3 were kindly provided by the DKFZ (Heidelberg).
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Source of cell lines was ATCC. Non of the cell lines were authenticated. All cells used were tested negative for mycoplasma contamination (VenorGeM, Minerva Biolabs).

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	NA
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	NA
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	NA

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	Human blood samples were taken from healthy donors under the ethics vote of the the Ethics Committee of the University of Dresden (EK244072018).
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	NA
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	NGS data have been deposited at the NIH Gene Expression Omnibus (GEO) Repository under the number GSE144484.
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	Large scale data such as screening results, sgRNA and nanostring probe sequences and RNA-Seq data are available as supplementary information.
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	NA