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Peer Review File

High efficiency CRISPR knock-in demonstrates that TCF1 is insufficient to reverse T cell exhaustion

Corresponding Author: Dr Ian Parish

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

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
In the study, “High efficiency CRISPR knock-in demonstrates that TCF1 is insufficient to reverse T cell exhaustion,” de Menezes et al. report optimization of a highly efficient CRISPR knock-in editing protocol in murine CD8 T cells and apply this technique to interrogate T cell exhaustion. They specifically ask the question as to whether TCF1 can actively de-differentiate more terminally exhausted T cell subsets back into a stem-like state. The authors highlight that high efficiency homology-directed repair editing in TCR-transgenic CD8 T (P14) cells is achieved with T cell activation and lower template insert size. Additionally, utilizing their protocol and Thy1 gene stop codon, they demonstrate that knock-in editing of GFP in endogenous regulatory elements does not disrupt the endogenous gene expression. The authors applied this technique to specifically target CX3CR1 Tex-int CD8 T cell populations and conditionally over-express TCF1. Interestingly, constitutive but not conditional over-expression of TCF1 lead to an increase in Tpex numbers. In summary, the authors provide an exciting tool that is certain to be a valuable resource. However, the majority of this work is about optimization of a technique using one biological observation.

Major Comments
1. Most of the main figures (Fig. 1-3) describe optimization of conditions to yield high editing efficiency, but these figures could be moved to supplemental to allow for additional characterization on the TCF1 knock-in studies.

2. The authors suggest that conditional overexpression of TCF1 in CXC3CR1 expressing Tex-int cells is insufficient to reprogram Tex-int into Tpex by phenotype. What is the transcriptional and epigenomic landscape of these conditional knock-in cells?

Reviewer #2

(Remarks to the Author)
De Menezes et al. aim to explore the role of the T cell-specific nuclear factor TCF1 in murine models of T cell exhaustion. They utilize the well-established LCMV infection model to investigate the functional importance of TCF1 in precursors of exhausted T cells, known as Tpex. TCF1 expression is lost upon Tpex differentiation. The authors use CRISPR-Cas9 to engineer transgenic CD8+ T cells in this model. However, much of the manuscript focuses on optimizing efficient CRISPR editing, which is not novel.
By targeting the CX3CR1 locus—shown by the authors to be more highly expressed in differentiated Tpex cells—they re-express a form of TCF1 but fail to rescue Tpex development. This result contrasts with previous reports that demonstrated increased Tpex numbers upon ectopic TCF1 expression. The authors conclude that TCF1 cannot revert T cell exhaustion via de-differentiation into Tpex.
1. While the conclusions appear valid based on the reported experiments, it is unclear whether this general conclusion is correct. Importantly, TCF1 expression from the CX3CR1 locus is at least fivefold lower than in native Tpex cells (see Fig 5d of the manuscript), suggesting that TCF1 levels may be insufficient to drive de-differentiation. A more precise quantification of TCF1 protein, such as by Western blot, would be helpful—this also relates to my second point.
2. TCF1 is a highly complex gene/protein with at least eight alternative splice variants and multiple promoters, which have been shown to exert distinct functional effects in certain contexts, most notably in the thymus. Which TCF1 cDNA variant is being used—p33, p45, or p56? This could significantly influence the results, although re-expression experiments in TCF1-/-

CD8 T cells have suggested some splice variant-independent functions.
3. TCF1-mediated stemness is associated with canonical Wnt signaling, and reporter mice indicate active Wnt signaling in these cells. Have the authors considered expressing Wnt pathway components or a TCF1-β-catenin fusion protein to potentially compensate for possibly reduced Wnt signaling under exhaustion conditions?
4. Are there any negative side effects of CRISPR editing at these loci, such as translocations or large deletions? Have the authors considered using a non-integrating lentivirus instead of AAV6, which would allow for the introduction of a much larger DNA template?

Reviewer #3

(Remarks to the Author)
This study explores an intriguing topic: reversing T-cell exhaustion and demonstrates proof of concept novel “knock-in” CRISPR editing of T cells. This technology will be incredibly valuable for the field and thus we are very excited by this study. Moreover, the authors use this technology to reverse engineer T cells to study ways of modifying the differentiation of T cells, particularly hyporesponsive, exhausted T cells. Extensive research has examined the causes of T-cell exhaustion, with ongoing efforts to develop therapeutic strategies to counteract this process. Gaining a deeper understanding of the mechanisms that regulate exhaustion could prolong the functional lifespan of T cells, thereby enhancing immune responses against chronic diseases. After a series of experiments demonstrating the abilities and limitations of performing homologous directed repair (HDR), this research focuses on the transcription factor TCF1, the key regulator of Tpex cells, to determine whether its overexpression can reverse T-cell exhaustion by reprogramming cells that are already progressing toward exhaustion.
Previous studies have shown that high TCF1 expression correlates with longer overall survival (OS) in human patients. This study aims to investigate whether TCF1 alone is responsible for this effect or if other factors contribute to its role in promoting longer OS. To investigate this question, the authors employed an innovative CRISPR-based approach to efficiently engineer T-cells with either constitutive or conditional TCF1 over-expression. The study demonstrates that while constitutive TCF1 over-expression increases the Tpex population, conditional TCF1 over-expression in Tex-int cells fails to revert them back to a Tpex-like state. This is a simple, but interesting point, and unexpected since since TCF1 is touted to be a pioneer factor. It would be great to see how FoxoAAA could differ from TCF1 in this regard…or late stage deletion of ID2 or Blimp1.

While CRISPR-Cas9 knock-in strategies have been widely used, the efficiency of the homology-directed repair (HDR) technique remains a challenge. The authors introduce an innovative approach for achieving high-efficiency CRISPR HDR-based gene knock-in in primary murine CD8+ T cells, making it applicable for both in vitro and in vivo studies. The high-efficiency CRISPR knock-in technique described in this study represents a key methodological advancement, enabling precise genetic modifications within murine T-cells for in vivo experimentation. Thus, this study will also be an important proof of technique for the field and it makes a simple, but interesting observation related to CD8 T cell differentiation.
This reviewer only has a few minor concerns, mostly related to the techniques.

1) It is a bit confusing in Fig. 1 why the non-infected T cells (GFP-) do not have higher levels of Thy1.2. The whole point of showing HDR is that the GFP insertion will disrupt Thy1 open reading frame, so it is expected that the GFP+ infected cells will have low levels of Thy1.2 staining, but what I can’t understand is why the non-infected cells have the same low levels of Thy1.2. Am I misunderstanding the experimental design, or is there an issue with shedding of CD90 since it is a GPI-linked protein in the uninfected cells? This needs to be clarified and perhaps a different locus should be used altogether if they are getting CD90 shedding.
2) Do they authors know the % of homozygous vs heterozygous HDR in the cells? This should be another parameter they discuss.
3) In Figure 5e and h-k, I am very confused how they are gating on CX3CR1+ cells when they are targeting this locus for HDR. In fig 5B they should show CX3Cr1 by TCF1 to show overall expression of teh two proteins and they could also use KLRG1 in addition to CX3CR1 to identify the CX3Cr1+ cells. And for teh TCF1 they should also analyze that with slamf6 and cxcr5. They need to do more diligence in the analysis of these cells. Also, it would be great if indeed KLRG1 is not affected by TCF1 expression, then they could sort out KLRG1+ TCF1+ and KLRG1+ TCF- cells and compare the two transcriptionally to better examine how TCF1 overexpression affects the T cells. Lastly, they should look at IL-2 and see if this is increased at all by TCF1 OE.

In summary, these findings challenge the notion that TCF1 alone is sufficient to drive de-differentiation and suggest that additional factors may be required for reprogramming exhausted T-cells. The research carries significant clinical implications, particularly in the context of immune checkpoint blockade therapies, where restoring stem-like T-cell populations could improve therapeutic outcomes for patients with chronic infections or cancer.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The reviewers have adequately editorially and experimentally responded to this reviewer's the prior comments. The readership at Nature Communications should find this work valuable.

Reviewer #2

(Remarks to the Author)
The authors have addressed most of the comments raised by me and the other reviewers. However, it is surprising that a Western blot could not be performed, given that SDS–PAGE buffers, loading dyes, and protein extraction buffers are generally harsher in terms of experimental conditions than intracellular flow cytometry and threfore easier to do. If the authors could specifically clarify why a Western blot was not feasible in this case, this would help to resolve the issue. Alternatively, inclusion of actual results from such an experiment would substantially strengthen the manuscript.

Reviewer #3

(Remarks to the Author)
I strongly recommend publication of this revised manuscript. The authors have fully addressed my concerns, particularly the efficiency of HET vs HOMO recombination. This study represents a technical advance in the field that is highly deserving of publication. The work is rigorously done.

Point-by-point response to Reviewer's comments

We thank the reviewers for their constructive feedback on our manuscript. Following the reviewers' recommendations, we have added additional data to address the reviewers' concerns which we believe has improved the quality and novelty of the study. The major changes are:

- We have included multi-ome analysis providing evidence that TCF1 over-expression only causes mild transcriptional and epigenetic changes to the CX3CR1^{+} cell population. These new results provide mechanistic insight into our phenotype.
- We provide evidence that our CRISPR HDR optimized protocol works as efficiently as conventional over-expression systems, such as retroviral over-expression.
- We demonstrate that TCF1 isoforms work similarly to increase Tpex when over-expressed and that β-catenin is not a limiting factor in this context.
- We present an estimated quantification of homozygous vs heterozygous integration of transgenes using our CRISPR HDR protocol.

We have updated the manuscript text to reflect these changes and to accommodate additional reviewer requests (revised text is marked in red). Overall, we strongly feel that these revisions have significantly strengthened the manuscript. We provide a point-by-point response to the reviewer comments below.

Reviewer #1

In the study, "High efficiency CRISPR knock-in demonstrates that TCF1 is insufficient to reverse T cell exhaustion," de Menezes et al. report optimization of a highly efficient CRISPR knock-in editing protocol in murine CD8 T cells and apply this technique to interrogate T cell exhaustion. They specifically ask the question as to whether TCF1 can actively de-differentiate more terminally exhausted T cell subsets back into a stem-like state. The authors highlight that high efficiency homology-directed repair editing in TCR-transgenic CD8 T (P14) cells is achieved with T cell activation and lower template insert size. Additionally, utilizing their protocol and Thy1 gene stop codon, they demonstrate that knock-in editing of GFP in endogenous regulatory elements does not disrupt the endogenous gene expression. The authors applied this technique to specifically target CX3CR1 Tex-int CD8 T cell populations and conditionally over-express TCF1. Interestingly, constitutive but not conditional over-expression of TCF1 lead to an increase in Tpex numbers. In summary, the authors provide an exciting tool that is certain to be a valuable resource. However, the majority of this work is about optimization of a technique using one biological observation.

Major Comments
1. Most of the main figures (Fig. 1-3) describe optimization of conditions to yield high editing efficiency, but these figures could be moved to supplemental to allow for additional characterization on the TCF1 knock-in studies.

We thank the reviewer for the suggestion, but given that protocol development for in vivo mouse studies is a major component of the study that we feel is novel and would be of interest to the field, we would respectfully prefer to keep Figures 1-3 as main

figures. In particular, we show that we can achieve high efficiency CRISPR KI with only minimal in vitro culture that does not affect differentiation in vivo. This has not been shown previously and would be of interest to those who wish to apply this approach to their in vivo T cell studies.

2. The authors suggest that conditional overexpression of TCF1 in CXC3CR1 expressing Tex-int cells is insufficient to reprogram Tex-int into Tpex by phenotype. What is the transcriptional and epigenomic landscape of these conditional knock-in cells?

We agree with the reviewer that this is an important point to resolve. To address this point, we performed whole cell multi-ome analysis (combined scRNA-seq + scATAC-seq) on CRISPR-edited P14 cells at day 20 p.i. and the results are now shown in the new **Figure 6** and **Supplementary Figure 4**. Consistent with our flow cytometry phenotyping in the initial submission, we find that TCF1 over-expression only causes mild epigenetic and transcriptional changes within the CX3CR1$^+$ subset (Figure 6d-g). Nevertheless, there was still a significant enrichment of the Tpex transcriptional signature, and depletion of the CX3CR1$^+$ cell transcriptional signature, within TCF1 over-expressing CX3CR1+ cells (Figure 6f) alongside a slight increase in TCF1 motif accessibility (Figure 6g). Collectively, these data support a model whereby TCF1 over-expression within CX3CR1$^+$ cells can cause mild transcriptional and epigenetic reprogramming of cells towards a Tpex phenotype, but overall these changes are not sufficient to push cells into a bona fide Tpex state. These new data are discussed in lines 294-322, 368-369 and 379-381 of the revised manuscript.

Reviewer #2

De Menezes et al. aim to explore the role of the T cell-specific nuclear factor TCF1 in murine models of T cell exhaustion. They utilize the well-established LCMV infection model to investigate the functional importance of TCF1 in precursors of exhausted T cells, known as Tpex. TCF1 expression is lost upon Tpex differentiation. The authors use CRISPR-Cas9 to engineer transgenic CD8+ T cells in this model. However, much of the manuscript focuses on optimizing efficient CRISPR editing, which is not novel. By targeting the CX3CR1 locus—shown by the authors to be more highly expressed in differentiated Tpex cells—they re-express a form of TCF1 but fail to rescue Tpex development. This result contrasts with previous reports that demonstrated increased Tpex numbers upon ectopic TCF1 expression. The authors conclude that TCF1 cannot revert T cell exhaustion via de-differentiation into Tpex.

We thank the reviewer for their summary and comments about the manuscript. Whilst CRISPR-mediated knockout is now routinely used in the field, there have been very few studies that have reported high efficiency CRISPR-mediated knock-in as used in our study. We would therefore respectfully disagree that the optimization of CRISPR knock-in is not novel, since there is no report to date of high efficiency CRISPR HDR editing in primary murine CD8$^+$ T cells for in vivo studies of T cell differentiation. Prior studies were predominantly in vitro, and the little in vivo work involved extensive cell culture prior to in vivo transfer (e.g. using CAR T cells), which is problematic as long-term in vitro culture is known to disrupt in vivo T cell differentiation. It was thus unknown whether the short-term culture conditions required for the study of in vivo T cell differentiation would support CRISPR HDR. Our optimized protocol demonstrates that

this is achievable and represents the only protocol to date suitable for in vivo T cell biology and differentiation studies. As noted by Reviewer 3 "*The high-efficiency CRISPR knock-in technique described in this study represents a key methodological advancement, enabling precise genetic modifications within murine T-cells for in vivo experimentation.*"

Additionally, it's important to note that our results are consistent with previous studies involving constitutive ectopic TCF1 expression. We find that constitutive TCF1 over-expression by knock-in to the *Thy1* locus does increase Tpex numbers, in accordance with previous studies employing constitutive TCF1 expression (eg. Shan et al, *Cell Mol Immunol* 2020). It was only after conditional TCF1 over-expression in CX3CR1^{+} cells that we failed to observe an increase in Tpex. Neither the cited Shan et al study nor any previous study has conditionally over-expressed TCF1 after Tpex differentiation.

1. While the conclusions appear valid based on the reported experiments, it is unclear whether this general conclusion is correct. Importantly, TCF1 expression from the CX3CR1 locus is at least fivefold lower than in native Tpex cells (see Fig 5d of the manuscript), suggesting that TCF1 levels may be insufficient to drive de-differentiation. A more precise quantification of TCF1 protein, such as by Western blot, would be helpful—this also relates to my second point.

Thank you for the comment, and we apologize for the confusion regarding the levels of TCF1 expressed by each population. We have now updated **Figure 5d** to include TCF1 expression levels in native Tpex cells to enable a direct comparison between Tpex and the CRISPR-edited cells. Notably, there is no significant difference in TCF1 expression between the edited cells in the CX3CR1-TCF1 group and native Tpex cells. Moreover, TCF1 levels in edited cells were higher in the CX3CR1-TCF1 group than the Thy1-TCF1 group. Given that TCF1 expression levels in the Thy1-TCF1 group were sufficient to increase Tpex numbers, these results suggest that insufficient TCF1 expression is not the reason why CX3CR1-TCF1 edited cells fail to de-differentiate. These new data are discussed and better clarified in lines 250 to 253 of the revised manuscript. We have also added a label to Figure 5d to make it clearer that the TCF1 MFI within the graph was measured within edited (ie. TCF1+) cells in the CX3CR1-TCF1 and Thy1-TCF1 groups.

We thank the reviewer for the suggestion to quantify the levels of TCF1 by Western blotting. This experiment requires that we first sort on the edited TCF1^{+} cells otherwise the contaminating unedited cells will prevent accurate assessment of TCF1 over-expression after CRISPR HDR knock-in. We attempted to sort TCF1 expressing cells from the different groups and perform Western blotting to quantify TCF1 protein levels in each condition, but the cell fixation, permeabilization and staining required for sorting disrupted downstream TCF1 detection by Western blotting. This meant that TCF1 quantification by Western blotting was unfortunately not feasible.

2. TCF1 is a highly complex gene/protein with at least eight alternative splice variants and multiple promoters, which have been shown to exert distinct functional effects in certain contexts, most notably in the thymus. Which TCF1 cDNA variant is being used—p33, p45, or p56? This could significantly influence the results, although re-expression experiments in TCF1-/- CD8 T cells have suggested some splice variant-independent functions.

Thank you for raising this important point. The isoform used in our over-expression system is the p45 (full-length) isoform and we have added this information to the main text on line 240.

Prior work has demonstrated that the functional domains of TCF1 for T cells reside within the N-terminal domain (Xing et al, *Nat Immunol* 2016) consistent with prior work showing that p56 and p45 are functionally equivalent in T cells (Van de Wetering et al, *Mol Cell Biol* 1996). For this reason, the $CD8^+$ T cell field has focused solely on p33 and p45 when studying TCF1 function (Gullicksrud et al, *J Immunol* 2017; Chen et al, *Immunity* 2019; Shan et al, *Cell Mol Immunol* 2020), and we have thus also focused on these two isoforms to address this question. To examine the effect of different TCF1 isoforms on Tpex differentiation, we measured the impact of retroviral over-expression of the p33 or p45 isoforms on Tpex frequency (**Supplementary Figure 3f,g** in the revised manuscript). Consistent with previous studies (Chen et al, *Immunity* 2019), both isoforms similarly increased Tpex frequency in comparison to the empty vector control. Thus, at least in the context of Tpex differentiation, p33 and p45 are functionally equivalent. Importantly, the increase in Tpex after retroviral expression was similar to that seen after constitutive TCF1 over-expression by our CRISPR KI strategy (ie. TCF1 KI to the Thy1 locus) (**Supplementary Figure 3f,g**). These new data are discussed in lines 277 to 282 of the revised manuscript.

3. TCF1-mediated stemness is associated with canonical Wnt signaling, and reporter mice indicate active Wnt signaling in these cells. Have the authors considered expressing Wnt pathway components or a TCF1-β-catenin fusion protein to potentially compensate for possibly reduced Wnt signaling under exhaustion conditions?

We thank the reviewer for the suggestion. Since the p33 isoform does not contain the β-catenin binding domain, the results in **Supplementary Figure 3f,g** suggest that β-catenin does not play a role in the Tpex increase seen after TCF1 over-expression. The β-catenin (*Ctnnb1*) coding sequence is 2.4 kb long, meaning that the transgene size required for a TCF1-β-catenin fusion protein (or even β-catenin alone) is too large for CRISPR HDR (see **Figure 2** of the manuscript). Thus, to address if β-catenin was a limiting factor preventing reprogramming, we instead retrovirally over-expressed the stable form of β-catenin (Harada et al, *EMBO J* 1999) together with conditional TCF1 knock-in (CX3CR1-TCF1). While β-catenin over-expression alone increased Tpex proportions, Tpex were not further increased in β-catenin over-expressing CX3CR1-TCF1 cells (**Supplementary Figure 3h,i** in the revised manuscript). These results suggest that insufficient β-catenin does not explain why conditional TCF1 over-expression fails to de-differentiate $CX3CR1^+$ cells into Tpex. The increase in Tpex seen after β-catenin over-expression alone could be due to the actions of β-catenin on endogenous TCF1 or LEF1, but it should be noted that β-catenin has TCF1-independent functions (Valenta et al, *EMBO J* 2012). Note that the inability of β-catenin over-expression to synergise with TCF-1 over-expression to increase Tpex is consistent with prior findings (Shan et al, *Cell Mol Immunol* 2020). These new data are discussed in lines 282 to 289 of the revised manuscript.

4. Are there any negative side effects of CRISPR editing at these loci, such as translocations or large deletions? Have the authors considered using a non-integrating

lentivirus instead of AAV6, which would allow for the introduction of a much larger DNA template?

We thank the reviewer for the question. We wish to clarify that the main purpose of the AAV6 and CRISPR-HDR approach was to enable us to insert transgenes under the control of promoters such as CX3CR1 that only become active once the $CD8^+$ T cells begin to differentiate. This enables us to assess the ability of TCF1 overexpression to reverse exhaustion in cells that have already undergone differentiation. Other strategies such as non-integrating lentivirus are only suitable for constitutive expression and so would not enable us to address this research question. Although chromosomal translocations/large deletions have been reported in CRISPR/Cas9 edited T cells, these are rare and in the majority of cases these cells are not viable and so do not have a functional impact. Importantly, CRISPR/Cas9 edited cells have successfully and safely been used in patients (Stadtmauer et al, *Science* 2020). We agree that a careful examination of such events is needed for any CRISPR-edited T cell product that is designed for clinical use. However, given their low frequency, such off-target effects are unlikely to impact our current research question.

We nevertheless acknowledge that one concern with our editing approach may be that it is unable to drive as much transgene expression as retroviral approaches. To further compare our CRISPR KI strategy to more conventional over-expression systems used for mouse T cells, we constitutively over-expressed TCF1 using CRISPR HDR or retrovirus and observed higher TCF1 expression levels in edited cells by CRISPR knock-in. We have now included this data in the manuscript as **Figure 5h**. Furthermore, as noted in point 2 above, in **Supplementary Figure 3f,g** we show that Tpex expansion is comparable between CRISPR edited and retrovirally over-expressing cells. These results suggest that our optimized CRISPR KI protocol works as efficiently as retroviral over-expression systems. These new data are discussed in lines 265 to 276 of the revised manuscript.

Reviewer #3

This study explores an intriguing topic: reversing T-cell exhaustion and demonstrates proof of concept novel "knock-in" CRISPR editing of T cells. This technology will be incredibly valuable for the field and thus we are very excited by this study. Moreover, the authors use this technology to reverse engineer T cells to study ways of modifying the differentiation of T cells, particularly hyporesponsive, exhausted T cells. Extensive research has examined the causes of T-cell exhaustion, with ongoing efforts to develop therapeutic strategies to counteract this process. Gaining a deeper understanding of the mechanisms that regulate exhaustion could prolong the functional lifespan of T cells, thereby enhancing immune responses against chronic diseases. After a series of experiments demonstrating the abilities and limitations of performing homologous directed repair (HDR), this research focuses on the transcription factor TCF1, the key regulator of Tpex cells, to determine whether its overexpression can reverse T-cell exhaustion by reprogramming cells that are already progressing toward exhaustion.

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OS. To investigate this question, the authors employed an innovative CRISPR-based approach to efficiently engineer T-cells with either constitutive or conditional TCF1 over-expression. The study demonstrates that while constitutive TCF1 over-expression increases the Tpex population, conditional TCF1 over-expression in Tex-int cells fails to revert them back to a Tpex-like state. This is a simple, but interesting point, and unexpected since since TCF1 is touted to be a pioneer factor. It would be great to see how FoxoAAA could differ from TCF1 in this regard…or late stage deletion of ID2 or Blimp1.

While CRISPR-Cas9 knock-in strategies have been widely used, the efficiency of the homology-directed repair (HDR) technique remains a challenge. The authors introduce an innovative approach for achieving high-efficiency CRISPR HDR-based gene knock-in in primary murine CD8+ T cells, making it applicable for both in vitro and in vivo studies. The high-efficiency CRISPR knock-in technique described in this study represents a key methodological advancement, enabling precise genetic modifications within murine T-cells for in vivo experimentation. Thus, this study will also be an important proof of technique for the field and it makes a simple, but interesting observation related to CD8 T cell differentiation.

This reviewer only has a few minor concerns, mostly related to the techniques.

We thank the reviewer for the kind words and comprehensive summary. We agree that FoxoAAA is an interesting target, and in line with this idea we are currently conducting large-scale screening for factors that can reprogram cells into Tpex as part of a larger follow-up study. Late-stage deletion of effector-associated transcription factors is also an interesting idea to investigate, although not something that would be achievable using our current CRISPR HDR approach.

1) It is a bit confusing in Fig. 1 why the non-infected T cells (GFP-) do not have higher levels of Thy1.2. The whole point of showing HDR is that the GFP insertion will disrupt Thy1 open reading frame, so it is expected that the GFP+ infected cells will have low levels of Thy1.2 staining, but what I can't understand is why the non-infected cells have the same low levels of Thy1.2. Am I misunderstanding the experimental design, or is there an issue with shedding of CD90 since it is a GPI-linked protein in the uninfected cells? This needs to be clarified and perhaps a different locus should be used altogether if they are getting CD90 shedding.

We apologise for the confusion and thank the reviewer for the comment. While the HDR-mediated insertion of GFP will indeed disrupt Thy1 expression, this is not 100% efficient and a certain percentage of the cells will be edited through the NHEJ pathway, resulting in disruption of the *Thy1* open reading frame by indels without insertion of GFP. In other words, the Thy1.2^{-}GFP^{-} population represents cells where Thy1.2 was successfully knocked out but where GFP was not successfully knocked-in. This is now clarified in lines 130 to 132 of the manuscript.

2) Do they authors know the % of homozygous vs heterozygous HDR in the cells? This should be another parameter they discuss.

We thank the reviewer for raising this important question. To address this question, we performed a CRISPR KI experiment where we added in two different DNA templates

encoding different transgenes (GFP or mCherry) but targeting the same locus (*Cx3cr1*) (**Figure 4i,j** in the revised manuscript). In this experimental setup, cells that are double positive for both GFP and mCherry after CRISPR KI must have undergone heterozygous editing of the *Cx3cr1* locus. The percentage of double-positive cells thus enables an estimate of heterozygous integration frequencies, as previously shown by others (Roth et al, *Nature* 2018). On average we detected ~20% double positive cells, although the frequencies were highly variable, reaching as high as 50%. These data demonstrate that heterozygous editing does variably occur using our protocol. These new data are discussed in lines 225 to 232 of the revised manuscript.

3) In Figure 5e and h-k, I am very confused how they are gating on CX3CR1+ cells when they are targeting this locus for HDR.

We thank the reviewer for the comment and apologize for the confusion. As shown in **Figure 4**, we engineer the CX3CR1-targeting HDR templates (CX3CR1-GFP and CX3CR1-TCF1) to be inserted immediately after the *Cx3cr1* coding sequence separated by a P2A linker, which results in maintenance of CX3CR1 protein expression alongside GFP expression after CRISPR KI. We have added further text to clarify this point in line 222.

In fig 5B they should show CX3Cr1 by TCF1 to show overall expression of teh two proteins and they could also use KLRG1 in addition to CX3CR1 to identify the CX3Cr1+ cells. And for teh TCF1 they should also analyze that with slamf6 and cxcr5. They need to do more diligence in the analysis of these cells. Also, it would be great if indeed KLRG1 is not affected by TCF1 expression, then they could sort out KLRG1+ TCF1+ and KLRG1+ TCF- cells and compare the two transcriptionally to better examine how TCF1 overexpression affects the T cells.

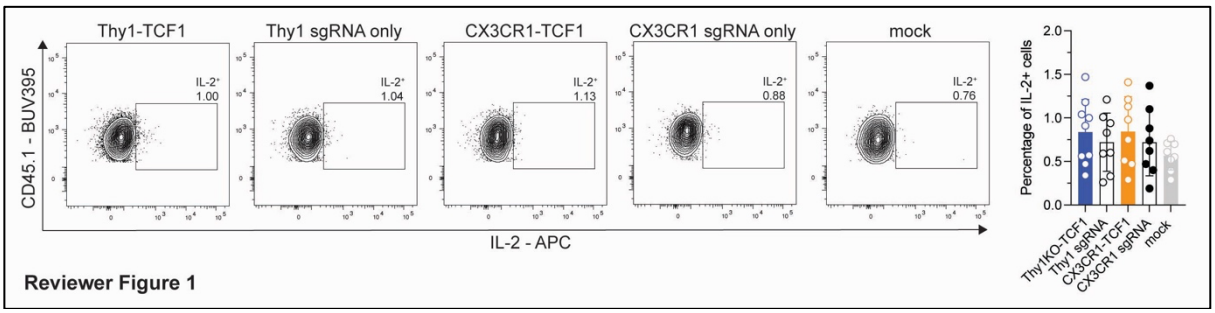
We thank the reviewer for the suggestions and agree that it is important to ensure that we are correctly gating on the CX3CR1$^+$ population. Unfortunately, though, unlike in acute infection, KLRG1 is largely not expressed within the exhausted CX3CR1$^+$ cell population at day 20 p.i. during chronic LCMV as demonstrated in multiple previous studies (Chen et al, *Immunity* 2019; Beltra et al, *Immunity* 2020; Daniel et al, *Nat Immunol* 2022). For this reason, KLRG1 is not a viable alternative marker for identifying effector exhausted cell populations.

We agree that transcriptional analysis is needed to better define the effect of TCF1 over-expression on CX3CR1$^+$ cells. To address this concern, we have now included single-cell multi-ome analysis of control, Thy1-TCF1 and CX3CR1-TCF1 P14 cells (**Figure 6** and **Supplementary Figure 4**). This single cell approach allowed us to more precisely isolate the effector exhausted cell cluster for further analysis, and we find that TCF1 expression only minimally impacts the CX3CR1$^+$ cell phenotype in agreement with our flow cytometry findings in the original submission. Please see our response to Reviewer 1's similar query for a more detailed description of the data.

Lastly, they should look at IL-2 and see if this is increased at all by TCF1 OE.

We agree that investigating if TCF1 over-expression increases IL-2 production is a valuable point. IL-2 production is typically barely detectable in exhausted T cells (Wherry et al, *J Virol* 2003; Wherry et al, *Immunity* 2007) and in **Reviewer Figure 1**

we show that TCF1 over-expression was not able to rescue IL-2 production in exhausted P14 cells at day 20 p.i.



Reviewer Figure 1

In summary, these findings challenge the notion that TCF1 alone is sufficient to drive de-differentiation and suggest that additional factors may be required for reprogramming exhausted T cells. The research carries significant clinical implications, particularly in the context of immune checkpoint blockade therapies, where restoring stem-like T cell populations could improve therapeutic outcomes for patients with chronic infections or cancer.

Point-by-point response to Reviewer's comments

We thank the reviewers for their positive feedback on our revised manuscript. We provide a point-by-point response to the reviewer comments below to address the remaining point raised by Reviewer #2.

Reviewer #1

The reviewers have adequately editorially and experimentally responded to this reviewer's the prior comments. The readership at Nature Communications should find this work valuable.

We thank the reviewer for the kind words.

Reviewer #2

The authors have addressed most of the comments raised by me and the other reviewers. However, it is surprising that a Western blot could not be performed, given that SDS–PAGE buffers, loading dyes, and protein extraction buffers are generally harsher in terms of experimental conditions than intracellular flow cytometry and threfore easier to do. If the authors could specifically clarify why a Western blot was not feasible in this case, this would help to resolve the issue. Alternatively, inclusion of actual results from such an experiment would substantially strengthen the manuscript.

We apologise for the confusion as the reviewer appears to have misunderstood our response to this query. The reviewer is correct that Western blotting is normally simple to perform on primary mouse T cells. However, in order to assess levels of TCF1 expression within CRISPR HDR edited T cells, we must first isolate the edited cells from the bulk T cell population as only ~40% of cells were productively edited in our TCF1 knock-in experiments. If we were to conduct Western blotting on the edited cells without first enriching for the cells that had been edited to knock-in TCF1, then the large number of contaminating TCF1- unedited cells would make it impossible to accurately assess TCF1 over-expression levels within the edited population.

As there was no surface marker to isolate edited cells, the only way that we could isolate these cells is by intracellular flow cytometric staining for TCF1 followed by FACS sorting of TCF1+ cells. We discovered that the fixation and permeabilization steps required for this sorting procedure disrupted protein migration on an SDS gel even after boiling in loading buffer, likely because of the formaldehyde cross-links introduced by the flow cytometry fixation step. For this reason, we could not accurately identify TCF1 by Western blotting, and thus could not conduct the requested experiment.

Given that this query was due to a misunderstanding of our rebuttal response, we have not altered the manuscript text or added more data. We would respectively argue that the original query around TCF1 expression levels in edited cells was already comprehensively quantitatively addressed by flow cytometry in Figure 5d of the revised manuscript in a manner that is arguably more quantitative/accurate than can be achieved by Western blotting.

Reviewer #3

I strongly recommend publication of this revised manuscript. The authors have fully addressed my concerns, particularly the efficiency of HET vs HOMO recombination. This study represents a technical advance in the field that is highly deserving of publication. The work is rigorously done.

We thank the reviewer for the strong endorsement of our manuscript.