nature biomedical engineering

PEER-REVIEW INFORMATION

**Generation of T-cell-receptor-negative CD8αβ-positive CAR T cells from T-cell-derived induced pluripotent stem cells**

Corresponding author: Michel Sadelain

## Editorial note

This document includes relevant written communications between the manuscript's corresponding author and the editor and reviewers of the manuscript during peer review. It includes decision letters relaying any editorial points and peer-review reports, and the authors' replies to these (under 'Rebuttal' headings). The editorial decisions are signed by the manuscript's handling editor, yet the editorial team and ultimately the journal's Chief Editor share responsibility for all decisions.

Any relevant documents attached to the decision letters are referred to as **Appendix #**, and can be found appended to this document. Any information deemed confidential has been redacted or removed. Earlier versions of the manuscript are not published, yet the originally submitted version may be available as a preprint. Because of editorial edits and changes during peer review, the published title of the paper and the title mentioned in below correspondence may differ.

## Correspondence

Fri 21 Jan 2022
**Decision on Article nBME-21-2890**

Dear Dr Sadelain,

Thank you again for submitting to *Nature Biomedical Engineering* your manuscript, "Timed and calibrated CAR expression drives CD8αβ T cell development from T cell-derived induced pluripotent stem cells". The manuscript has been seen by three experts, whose reports you will find at the end of this message.

You will see that the reviewers appreciate the quality of the evidence and the execution and scope of the work, and that they provide a number of useful suggestions, in addition to raising technical questions and concerns, that I hope will help you to improve the work. Also, please do make sure that the reporting of the methodology in the revised manuscript is exhaustive, so as to facilitate the replicability of the work and the adoption of the strategy for the generation of CD8αβ CAR T cells. Also, please provide the specific *p*-values (rather than ranges).

When you are ready to resubmit your manuscript, please upload the revised files, a point-by-point rebuttal to the comments from all reviewers, the reporting summary, and a cover letter that explains the main improvements included in the revision and responds to any points highlighted in this decision.

Please follow the following recommendations:

* Clearly highlight any amendments to the text and figures to help the reviewers and editors find and understand the changes (yet keep in mind that excessive marking can hinder readability).

* If you and your co-authors disagree with a criticism, provide the arguments to the reviewer (optionally, indicate the relevant points in the cover letter).

* If a criticism or suggestion is not addressed, please indicate so in the rebuttal to the reviewer comments and explain the reason(s).

* Consider including responses to any criticisms raised by more than one reviewer at the beginning of the rebuttal, in a section addressed to all reviewers.

* The rebuttal should include the reviewer comments in point-by-point format (please note that we provide all reviewers will the reports as they appear at the end of this message).

* Provide the rebuttal to the reviewer comments and the cover letter as separate files.

We hope that you will be able to resubmit the manuscript within 15 weeks from the receipt of this message. If this is the case, you will be protected against potential scooping. Otherwise, we will be happy to consider a revised manuscript as long as the significance of the work is not compromised by work published elsewhere or accepted for publication at *Nature Biomedical Engineering*.

We hope that you will find the referee reports helpful when revising the work, which we look forward to receive. Please do not hesitate to contact me should you have any questions.

Best wishes,

Pep

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Pep Pàmies
Chief Editor, *Nature Biomedical Engineering*

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Reviewer #1 (Report for the authors (Required)):

General comments / summary:
This manuscript by van der Stegen and coworkers describes that timed calibrated CAR expression drives CD8ab T cell development from iPSCs. Indeed, the iPSC-mediated differentiation to functional T cells is challenging, especially to CD8ab T cells. Readily available and effective CAR T cells could potentially address a broad spectrum of diseases, incl. solid tumors, infectious diseases, autoimmunity, which is why this article of broader relevance. This manuscript is generally well written and addresses a clearly relevant topic with methodological/technological advances.

Major points:
- Fig. 1b: Can the authors comment on the different time points used to compare H1 ES cells and iPSC?
- Is the described differentiation problem and the pre-rearranged TCR locus primarily a problem in iPSC generated from T cells or would they predict this to be a problem in iPSCs generated from other cell sources? The comparison to ES cells is not ideal in this context.
- The iPSC differentiation protocol should be more detailed, so that others can more easily follow this, e.g. sources of cytokines, aMEM, serum, concentrations of cytokines, critical steps, etc. Which FGF was used? Which method was used for CD34 enrichment?
- Also, a bit more info could be given on used vector systems and expression cassettes, e.g. Genbank links to genes/isoforms, as well as donor DNA for HDR.
- Have the authors performed experiments with primary leukemia patient cells or just with the NALM6 cell line? If so, these data should be added to strengthen the paper.

Minor points:
- The work from Sauer, van den Brink could be added that CD3zeta signaling impacts T cell progenitor differentiation, in this case with a shift to NK cell development.
- As an outlook, further gene editing strategies could be mentioned, incl. multiplexing, e.g. for CD52 and others.
- M&M / pluripotency assessment: "lines were assessed for pluripotency by analysis of phenotypic analysis

of ...markers..." --> these marker are surface markers on iPSC but no pluripotency tests. Thus, this should be reworded, e.g. iPSC surface marker assessment.
- For all PCRs, primer sequences should be provided. For all CRISPR-Cas9 modifications, sgRNAs sequences as well.
- Which markers were used to fully define a CLP?
- Fig. 1a: What is shown in the picture labeled with D0? These do not look like prototypical iPSC.

Reviewer #2 (Report for the authors (Required)):

Van der Stegen, Sadelain, and colleagues demonstrate the process of re-differentiating iPSC into CAR-expressing iT cells. They report constitutive expression of a CD19 CAR diverts T-cell differentiation away from the DP stage and directs them instead towards the gamma-delta lineage, which is consistent with the current knowledge of mechanisms regulating T-cell differentiation in the thymus. This process is mitigated by reducing tonic CAR signaling and by placing the CAR construct under the control of the TRAC promoter to achieve a TCR-like pattern of CAR expression. Following positive selection with CD19-presenting cells, which is augmented by concurrent 4-1BB costimulation, functional CD19 CAR iT cells are formed. These iT cells largely resemble normal abCD8+ T-cells and mediate anti-tumor activity in a mouse xenograft model of a CD19+ leukemia.

The study is performed at the highest technical level and address the critical issue of optimal cell source as a platform for engineered cell therapy. Addressing the following points would help further enhance the impact of this report.

1. The authors posit “pre-rearrangement” of endogenous TCR genes and tonic CD19 CAR signaling in T-iPSC interfere with DP lineage commitment and skew T-cell differentiation towards the gamma-delta lineage. Disrupting TRAC expression produces only a mild increase in the frequency of DP cells (Fig. 1C) suggesting tonic CAR signaling plays a major role in gdT lineage determination. These results are in agreement with early studies by Paul Love and his lab (cited by the authors in discussion) that suggested high basal TCR signaling at the DN stage dictates gdT-cell commitment whereas low TCR signaling permits abT-cell differentiation. This raises the question whether the observed “gamma-delta-ness” of CD19 CAR iT-cells is due to high spontaneous signaling of that particular CAR construct at the DN stage? Would the authors observe the same with a 1XX CAR or an FMC63 based CAR (or any other CAR with minimal tonic signaling) that is ubiquitously expressed outside the TRAC locus?
In other words, would simply replacing the full-length CD19 CAR with a non-tonically signaling CAR construct under the UbC promoter help reduce the observed gamma delta T-cell commitment.

2. The authors demonstrate transcriptional similarity of the resulting iT cells to normal CD8 ab T-cells but also highlight important phenotypic and functional differences between the two subsets. CAR iT cells resemble activated effector cells, with minimal expression of CCR7, CD62L, reduced CD27 and CD5, and increased CD45RO, whereas the majority of normal abT-cells that exit thymus have a naïve phenotype with inverse expression of these markers. The phenotypic differences are supported by the decreased functionality of iT cells in vivo (Fig 6) where the curative activity is achieved with a 40-fold higher T-cell dose than is needed for PBMC-derived TRAC-1XX CD19 CAR T-cells (reported by the same group in Feucht et al, 2019). The effector-like iT cells produce robust yet short-lived activity in mice, even with IL2/IL15 supplementation. Such limited activity of iT cells per se does not constitute a major problem in the context of allogeneic cell therapies (and may even be
desirable, in order to avoid long-term on-target toxicities) but still underscores an important difference between iT cells and donor-derived abT-cells. How to the authors explain these disparities and can they offer any strategies to increase “naiveness” of CAR iT cells?

3. While the lack of TCR/CAR signaling at the DP stage leads to “death by attrition”, excessive signaling can also result in apoptosis mimicking negative selection of autoreactive thymocytes. The authors use CD19-expressing 3T3 cells to produce positive selection of mature CD19 CAR iT cells which, depending on the level of CD19 expression, may induce strong CAR signaling. Did the authors evaluate the impact of a milder stimulation of DP cells (eg by modulating CD19 expression level on 3T3 or by titrating recombinant plate-bound CD19) on cell yield and the phenotype of final iT cells? Stimulation with 3T3-CD19 results substantially reduces the frequency of CD62L+ CD27+ T-cells (Ext Fig 6 vs Ext Fig 8) possibly contributing to their terminal differentiation discussed in query #2.

Minor questions
1. How would one distinguish the failure of CD19 CAR TiPS to commit to the DP stage from the global apoptosis of DP cells due to tonic CAR signaling-induced negative selection?
2. The authors mentioned that mice receiving peripheral blood-derived TRAC-1XX-CD8 T cells succumbed to GvHD-like symptoms. How do T-cells lacking TCR induce xenogeneic GvHD and is this a consequence of exogenous cytokine supplementation?
3. These studies are aimed at optimizing differentiation of T-iPSC into “conventional” T-cells via the DP route away from the gdT lineage. Why are gamma-delta-like CAR iT cells inferior to alpha-beta-like CAR iT?

Reviewer #3 (Report for the authors (Required)):

• Key results:
This study describes the technological achievement that consists in generating bona fide CAR T cells from iPSCs. The success of CAR T cells in the clinic in so far untreatable leukemias and lymphomas seems to reside in properties intrinsic to T-cells: the capacity to kill target cells in an antigen-specific manner but also to proliferate in massive amounts in the organism. Complete clinical responses have associated with several logs of proliferations of CAR T cells in the patient.

So far, the attractiveness of iPS as a source of CAR-expressing therapeutic cells has been hampered by the inability to drive their differentiation into cells that recapitulate all the properties of mature T cells.
In this study, the authors perform a step by step dissection of what it takes to push T-iPSC along the path of T cell differentiation.

They use the previous teachings of thymocyte differentiation studies to understand why CAR expression impairs T cell differentiation using first DP cell formation as an indicator of commitment to αβT cell lineage then address the impact of CAR T cell signaling to drive maturation into SP T cells.

Key findings are:
- Unregulated CAR expression disorganizes the fine interplay between notch pathway and TCR signaling that drives αβ vs γδ lineage commitment.
- Strength or rather quality of CAR signaling is a key factor in this context.
- Antigen-specific stimulation is necessary to form SP T cells but they appear immature
- 41BB costimulation allows differentiation into mature CD8ab SP T cells able to proliferate and provide sustained in vivo antitumor activity

• Originality and significance:

I think that the findings presented here are of general interest, not just people involved in cell therapy.

• Data & methodology:
In general, the data and methodology used are of high quality. There are nevertheless serious gaps.

Methodology is not sufficiently to enable reproducing the results: all cytokine concentrations are missing. Also during T lymphoid differentiation, it should be mentioned at what day TPO and IL3 are no longer added to the medium (.806.)

Flow cytometry: In many cases where side by side comparison are made and quantitative /qualitative differences are observed and interpretated, the flow cytometry axis are different between samples suggesting that the examples shown don’t come from the same data sets or questioning whether the gates were set appropriately. (see CD4 axes in Fig1d as one of many examples). In general, there is no mention on how gates were set, no mention is made of the use of unstained, isotype or “fluorescence-minus-one” controls.

• Data interpretation:
101. When comparing wt TiPS to H1 ES cells, the authors illustrate their inability to produce DP cells by showing D35 data in Fig1b whereas for H1 cells, they mention that DP cells only start appearing at D39, also showing D50. The same days should be shown for wt TiPS.

Ext Fig 2a PCR primer position should be illustrated

Why has DTX1 induction (ext fig 4b) not been performed on the same cells, TiPSCs?

133-16: a model is proposed from the indluence of mDLL1 on H1/Trac-/-TiPS vs that of human notch ligands. Nevertheless, fig1c and 1d suggest that WT-TiPS and TRAC-/--TiPS treated with mDLL1 are much more similar to each other (5 v 10% DP) than they are to WT-TiPS treated with human DLL1 (0% DP). Is the 5 to 10% difference relevant?

138. In what sense is CAR signal “stronger” than a TCR signal? During lymphoid differentiation, constitutive CAR expression would trigger an earlier signal than an endogenous rearranged TCR driven by TCR alpha and beta genetic control elements but stronger is difficult to understand without measuring downstream phosphorylation in the TCR cascade.

144. Given that there 68% of cells appear DP at day 35, I imagine they come from WT-TiPS treated with hDLL4-OP9 but it should be mentioned in the text and the legend.
More generally, the type of OP9 cells used for differentiation is missing from the entire second half of the manuscript.

217-219 I wouldn’t call these phenotypic change as ‘characteristics of cell activation’ as CD69 CD25, CD45 isoforms (at least) are also transiently expressed at different stages of T cell selection in the thymus.

238. It’s surprising to interpret Fig 4k as “3T3-CD19-41BBL matured cells exhibited improved persistence upon repeated antigen exposure”. Rather 3T3-CD19 matured cells failed to expand and survive upon antigenic stimulation.

• Suggested improvements:

160-163.
To demonstrate TRAC-encoded 1928z still interfered enough to prevent DP commitment, the authors should show the kinetics of DP induction in WT-TiPS earlier than D35 and correlate that to TRAC encoded TCR expression.

180. It seems the formulation used by the authors doesn’t fairly reflect the data. ITAM phosphorylation is exclusively detected in cells with the highest CAR expression and given that the level of CAR expression is different between retrovirally expressed CAR and TRAC-1XX, the data is difficult to interpret. ITAM1 phosphorylation should be compared between TRAC-1928z and TRAC-1XX (ITAM3 is not relevant as phosphorylatable tyrosines are mutated to F in this construct).

Figure 5a. A vast majority of the cells appear CD45RA-CD62L-, a phenotype typically associated with effector memory properties in peripheral T cells. The authors should comment. Staining should be performed in parallel on primary human abT cells, NK cells and gamma delta T cells for comparison.

Also Figure 5b legend should mention that clustering is performed on transcriptomics data

Additional information should be added including if the D42 cells used in figure 5 and 6 were exposed to 3T3-CD19 cells or 3T3-CD19-41BBL cells.

The authors claim that TRAC-1XX-iT cells achieve full tumor control in vivo, as well as CAR-expressing peripheral T cells. Nevertheless , the experimental conditions used are far from their stress test model. In the original Heucht et al paper, the stress test model is performed using 5x10^5 Nalm6 cells vs 1 to 4x10^5 CAR T cells. Here, much fewer Nalm6 cells are used (1x10^5 cells) and more CAR T cells are used (2x10^6 in figure 4 and 4x10^6 in figure 6). Also, IL2 and IL15 are injected in mice possibly to sustain survival and growth of human cells. It is therefore not rigorous to claim that the model used provides a quantitative assessment of the potency of TRAC-XX-iT cells.

There are paradoxical data in the manuscript, the authors claim that D42 TRAC-XX-iT cells have downregulated CD62L (12% in Fig 5a) but that should correspond to D0 cells in Fig 6i that show 40% CD62L+.

The authors mention the occurrence of GvHD-like syndrome in mice treated with TRAC-1XX-CD8 T cells. The authors should show the level of αβTCR+ cells in the cells injected into the mice as it is very uncommon to observe GvHD-like symptoms in this type of model.

The study should be performed using cells after depletion of remaining αβTCR+ cells and should be performed more than once as it is impossible to match donors for TRAC-1XX-CD8 T cells and TRAC-1XX-CD8 iT cells.

The authors should justify the use of IL2 and IL15 injections.

•References:
171. A reference should be included to justify that CD1a, CD2, CD62L and CD45RO expression confirm T lineage commitment or include data looking at these data in developing thymocytes at various stages.

• Clarity and context:
The abstract is clear and accessible. The introduction presents the appropriate context for the study.

I think the conclusion is a bit of an overstatement. The authors start the discussion by “We report here the generation of therapeutic CD8αβ CAR iT c ells from TiPS. This is an overstatement as several aspects are missing that would allow the claim that these cells are of therapeutic potential as the word ‘therapeutic’ is often understood as ‘clinic-ready’. No mention is made of CD4 CAR T cells where very few scientists would be willing to develop a therapy solely based on CD8 T cells. The positive impact of CD4 CAR T cells has been reported.

Several properties of their cells that are very different from bone fide T cells are just brushed upon and not discussed: their inability to produce IL2. The in vivo experimental conditions used seem to suggest that these cells are not as potent as traditional CAR Ts.
A valid quantitative in vivo comparison with the current standard CAR Ts should be included.

Although the authors do not claim that this platform is applicable as is to all CAR T cells, for this paper to speak to a large audience, a discussion should be included on the applicability of this work to other CAR targets (potential limitations).

• I feel that analysis of expression of gene in the notch pathway in Fig3 goes beyond my field of expertise and I won’t be able to assess if the authors’ interpretation of the data is valid here. Also, a description of error bars is adequately included in each figure but I’m not qualified to verify which statistical test should be used depending on the type of data.

• Minor points/typos:

Fig1a : please add to the right side that T lymhoid differentiation is performed on OP9(+notch ligand) stroma

Ext fig5a “gated on liver CD45+ cells” -> “gated on live CD45+ cells”

216 “(Figure 4a)” should also mention Ext Fig8a as CD1a downregulation is mentioned.

227-228 should read ’30-fold on average’ rather than ‘up to 30-fold’ to be in line with fig 4g

Shouldn’t Figure 5a legend read “γδTCR-T cell markers (bottom panel)” rather than “γδTCR-T cells (bottom panel)”?

Mon 06 Jun 2022
**Decision on Article nBME-21-2890A**

Dear Dr Sadelain,

Thank you for your patience in waiting for the feedback on your revised manuscript, "Timed and calibrated CAR expression drives CD8αβ T cell development from T cell-derived induced pluripotent stem cells", which has been seen by the original reviewers. In their reports, which you will find at the end of this message, you will see that all reviewers acknowledge the improvements to the work and that Reviewer #3 has a few additional relatively minor technical points that I am sure you will be able to consider. In particular, please do make sure to explicitly discuss the limitations of the approach for timed and calibrated CAR expression, and the barriers to overcome towards the clinical use of the T-cell-derived induced pluripotent stem cells.

As before, when you are ready to resubmit your manuscript, please upload the revised files, a point-by-point rebuttal to the comments from Reviewer #3, and the reporting summary.

I look forward to receive a further revised version of the work. Please do not hesitate to contact me should you have any questions.

Best wishes,

Pep

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Pep Pàmies
Chief Editor, *Nature Biomedical Engineering*

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Reviewer #1 (Report for the authors (Required)):

The authors have adequately addressed my concerns.

Reviewer #2 (Report for the authors (Required)):

The authors have answered all queries. I have no more questions.

Reviewer #3 (Report for the authors (Required)):

I appreciate the authors' efforts to greatly improve the manuscript, taking into account remarks from all 3 reviewers.

In general, the authors have adequately addressed questions/concerns in rebuttal letter. When they did not agree with my remarks, they provided additional references or data to illustrate their point.

The methodology is much better described.

Additional data was provided to strengthen evidence, help data interpretation and support conclusions.

The in vivo cell dose titration experiment is very useful (ext fig 9j/k) but I'm not sure I would agree that "2x106 TRAC-1XX-iT cells provided a similar response to 4x105 CD8 TRAC-1XX T cells". The absence of significant difference does not mean there is no difference (except if p value reaches 0.95). All animals died in the TRAC-XX-iT groups whereas the 4x10^5 CD8 TRAC XX has not reach median survival at day 50. Especially because the authors mention the occurrence of GVHD like symptom in this group and what is represented is

overall survival. Could Progression free survival representation help? Was the experiment followed longer? On the other hand, antitumor activity for 2x106 TRAC-1XX-iT cells is clearly stronger than for 1x105 CD8 TRAC-1XX T cells, so if we're comparing orders of magnitude, we're not far off.

I thank the authors for mentioning in the discussion the applicability to other CARs. Do they think that, as T cell differentiation is intimately linked to fine differences in TCR and CAR signals, the protocol and /or CAR signaling motives might need to be re-optimized for other CARs?

Can the authors comment why TRAC-/- TiPS engineering was performed at the iPS stage whereas for TRAC 1928z TiPS and TRAC 1XX TiPS, engineering was performed on T cells that were subsequently reprogrammed ? At first thought, I would expect this to have an impact, but maybe it should be mentioned. Did they need to use that particular TiPS clone for comparability or is AAV6 transduction inefficient in iPSCs?

Also, are the 'CAR-iT' used in Fig 6 the same as CAR-TiPS-iT described in introduction? It seems their generation should be described in methods.

Minor language detail:
The use of the phrase "the latter succumbed" in line 316 might be incorrect as the mice are the ones which succumbed, whereas in the previous sentence, the cells are mentioned and not the mice treated with such cells.

Thu 09 Jun 2022
**Decision on Article nBME-21-2890B**

Dear Dr Sadelain,

Thank you for the latest version of your manuscript, "CD8αβ CAR T cell development from T cell-derived induced pluripotent stem cells". Having consulted with Reviewer #3 (whose brief comments you will find at the end of this message), I am pleased to write that we shall be happy to publish the manuscript in *Nature Biomedical Engineering*.

We will be performing detailed checks on your paper and will send you a checklist detailing our editorial and formatting requirements in due course.

Please do not hesitate to contact me if you have any questions.

Best wishes,

Pep

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Pep Pàmies
Chief Editor, *Nature Biomedical Engineering*

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Reviewer #3 (Report for the authors (Required)):

The authors have answered all my questions and adequately updated the manuscript. I have no further comment.

**Rebuttal 1**

**Point-by-point responses to the Reviewers' comments:**

We would like to thank the Reviewers for their time to review the work and the helpful commentary to further improve the manuscript. Please find below our point-by-point responses to all comments raised by the Reviewers.

**Reviewer #1**

*General comments / summary:*
*This manuscript by van der Stegen and coworkers describes that timed calibrated CAR expression drives CD8ab T cell development from iPSCs. Indeed, the iPSC-mediated differentiation to functional T cells is challenging, especially to CD8ab T cells. Readily available and effective CAR T cells could potentially address a broad spectrum of diseases, incl. solid tumors, infectious diseases, autoimmunity, which is why this article of broader relevance. This manuscript is generally well written and addresses a clearly relevant topic with methodological/technological advances.*

We appreciate the overall positive assessment

*Major points:*
*- Fig. 1b: Can the authors comment on the different time points used to compare H1 ES cells and iPSC?*

We have replaced the data originally presented in Fig. 1b with a complete time course analysis (shown in new Extended Data Fig. 1b), representing multiple time points throughout the differentiation of H1 and WT-TiPS on OP9-mDLL1. An informative time point is highlighted in new Fig. 1b.

*- Is the described differentiation problem and the pre-rearranged TCR locus primarily a problem in iPSC generated from T cells or would they predict this to be a problem in iPSCs generated from other cell sources? The comparison to ES cells is not ideal in this context.*

To address this concern, we added to new Fig. 1b the differentiation of fibroblast-derived iPSCs (FiPS) on OP9-mDLL1. Similar to H1, FiPS show increased expression of early T lineage commitment markers ($CD7^+CD5^+$) compared to TiPS, and also generate a greater $CD4^+$ population (both CD4 ISP and $CD4^+CD8\alpha\beta^+$ DP populations) compared to the TiPS. Along with the addition of the FiPS differentiation in the new Fig. 1b, expression of pluripotency markers on FiPS has been added to new Extended Data Fig. 1a, and the differentiation time-course to the new Extended Data Fig. 1c. We have adjusted the text in the Results section to include the additional comparison:

> Line 99: "The human embryonic stem (ES) cell line H1 and fibroblast-derived iPS (FiPS) cells consistently yielded a DP population, typically arising by day 35 and followed by the appearance of CD3+ cells by day 40 (Figure 1b, Extended Data Figure 1a-c)."

*- The iPSC differentiation protocol should be more detailed, so that others can more easily follow this, e.g. sources of cytokines, aMEM, serum, concentrations of cytokines, critical steps, etc. Which FGF was used? Which method was used for CD34 enrichment?*

Additional details, including the items highlighted by the Reviewer, have been added to the Methods section.

*- Also, a bit more info could be given on used vector systems and expression cassettes, e.g. Genbank links to genes/isoforms, as well as donor DNA for HDR.*

Additional details on the generation of the OP9-lines and the development of the TRAC-KO TiPS, have been provided in the Methods section, as requested. References providing further information on TRAC targeting of the 1928z and 1928z-1XX constructs are supplied in the text (Eyquem et al., Nature 2017, Feucht et al., Nature Medicine 2019).

*- Have the authors performed experiments with primary leukemia patient cells or just with the NALM6 cell line? If so, these data should be added to strengthen the paper.*

We would like to thank the Reviewer for this suggestion. We have performed a cytotoxicity assay against patient-derived primary chronic lymphoblastic leukemia and provide the result in new Fig. 4p, showing effective lysis of primary CLL cells by TRAC-1XX-iT cells. The text has been adjusted to reflect this additional functional assessment (line 256).

*Minor points:*
*- The work from Sauer, van den Brink could be added that CD3zeta signaling impacts T cell progenitor differentiation, in this case with a shift to NK cell development.*

We thank the Reviewer for this suggestion. We have included a reference to this publication in the Results section (line 139; referring to CAR-derived signalling interfering with gene expression required for T lineage differentiation) and another in the discussion (line 327; referring to constitutive CAR expression skewing differentiation towards the NK cell lineage).

*As an outlook, further gene editing strategies could be mentioned, incl. multiplexing, e.g. for CD52 and others.*

We thank the Reviewer for this suggestion. We have added a paragraph on the potential of the TiPS platform to facilitate the generation of therapeutic T cells with multiple gene editing strategies combined to the Discussion:

> Line 398: “Genotype selection, including detection of off-target editing events, renders the TiPS platform particularly suitable for multiplexed gene editing strategies, such as combining *TRAC* locus editing with the ablation of CD52[54], CD70[57], or PD1[55].”

*- M&M / pluripotency assessment: "lines were assessed for pluripotency by analysis of phenotypic analysis of ...markers..." --> these marker are surface markers on iPSC but no pluripotency tests. Thus, this should be reworded, e.g. iPSC surface marker assessment.*

We thank the Reviewer for catching this oversight. The text has been corrected (line 946-948).

*- For all PCRs, primer sequences should be provided. For all CRISPR-Cas9 modifications, sgRNAs sequences as well.*

For the TRAC-1928z and TRAC-1XX CRISPR modification and PCR validations, details can be found in the original publications describing the targeting strategy and construct respectively. The references have been provided in the Methods section. For the generation of TRAC-KO TiPS, the additional detail has been provided in the Methods section. For the ddPCR assays, the primer sequences are not provided by the manufacturer (Bio-Rad), but we provided the Assay ID in the Methods section.

*- Which markers were used to fully define a CLP?*

We have clarified the text to refer to lymphoid commitment based on the appearance of CD45 and CD7 by D20. The term CLP has been removed from the Methods.

*- Fig. 1a: What is shown in the picture labeled with D0? These do not look like prototypical iPSC.*

Fig. 1a indeed showed recently replated WT-TiPS on D0 to initiate the iT differentiation through the monolayer-based iCD34 protocol. We have replaced it in new Fig. 1a with a representative image showing the WT-TiPS in the basic iPSC culture-conditions prior to plating for differentiation.

**Reviewer #2**

*Van der Stegen, Sadelain, and colleagues demonstrate the process of re-differentiating iPSC into CAR-expressing iT cells. They report constitutive expression of a CD19 CAR diverts T-cell differentiation away from the DP stage and directs them instead towards the gamma-delta lineage, which is consistent with the current knowledge of mechanisms regulating T-cell differentiation in the thymus. This process is mitigated by reducing tonic CAR signaling and by placing the CAR construct under the control of the TRAC promoter to achieve a TCR-like pattern of CAR expression. Following positive selection with CD19-presenting cells, which is augmented by concurrent 4-1BB costimulation, functional CD19 CAR iT cells are formed. These iT cells largely resemble normal abCD8+ T-cells and mediate anti-tumor activity in a mouse xenograft model of a CD19+ leukemia.*

*The study is performed at the highest technical level and address the critical issue of optimal cell source as a platform for engineered cell therapy. Addressing the following points would help further enhance the impact of this report.*

We thank the Reviewer for an overall positive assessment.

*1. The authors posit "pre-rearrangement" of endogenous TCR genes and tonic CD19 CAR signaling in T-iPSC interfere with DP lineage commitment and skew T-cell differentiation towards the gamma-delta lineage. Disrupting TRAC expression produces only a mild increase in the frequency of DP cells (Fig. 1C) suggesting tonic CAR signaling plays a major role in gdT lineage determination. These results are in agreement with early studies by Paul Love and his lab (cited by the authors in discussion) that suggested high basal TCR signaling at the DN stage dictates gdT-cell commitment whereas low TCR signaling permits abT-cell differentiation. This raises the question whether the observed "gamma-delta-ness" of CD19 CAR iT-cells is due to high spontaneous signaling of that particular CAR construct at the DN stage? Would the authors observe the same with a 1XX CAR or an FMC63 based CAR (or any other CAR with minimal tonic signaling) that is ubiquitously expressed outside the TRAC locus? In other words, would simply replacing the full-length CD19 CAR with a non-tonically signaling CAR construct under the UbC promoter help reduce the observed gamma delta T-cell commitment.*

The inhibitory effect of early CAR (or TCR) expression on αβTCR-T lineage commitment has been previously observed in several other publications and settings: for example, expression of a Ubc-GPC3-CAR in iPSC resulted in the adoption of a more innate/NK-like phenotype (Ueda et al., Cancer Science 2020); CARs targeting CEA or CD19 introduced into cord-blood derived CD34 cells resulted in reduced T lineage commitment (Maluski et al., J Clin Invest 2019; van Caeneghem et al., Blood (2015)126(23):3097). Disruption of T lineage development is not unique to transgenic CAR expression, as it has also been observed with transgenic TCR expression in both human (Ueda et al., Cancer Science 2020) and murine (Egawa et al., PLoS One 2008) T cell development. Given that TCRs show significantly lower tonic signals than CARs (Ramello et al., Sci Signal 2019) and that even TCR expression can disrupt T lineage development, we postulate that the constitutive expression of a minimally, tonically signalling

CAR would still disrupt T cell differentiation. A recent publication that described the development of CD8αβ SP CAR T cells from TiPSC (Wang et al., Cell Stem Cell 2022) mentioned that CD8αβ SP T cell development had been facilitated by unexpected epigenetic silencing of the transgene (driven by EF1α) during T lymphoid differentiation (albeit without reporting levels of CAR expression or the formation of a DP population prior to CD8αβ SP T cell induction), which is in accord with our data.

*2. The authors demonstrate transcriptional similarity of the resulting iT cells to normal CD8 ab T-cells but also highlight important phenotypic and functional differences between the two subsets. CAR iT cells resemble activated effector cells, with minimal expression of CCR7, CD62L, reduced CD27 and CD5, and increased CD45RO, whereas the majority of normal abT-cells that exit thymus have a naïve phenotype with inverse expression of these markers. The phenotypic differences are supported by the decreased functionality of iT cells in vivo (Fig 6) where the curative activity is achieved with a 40-fold higher T-cell dose than is needed for PBMC-derived TRAC-1XX CD19 CAR T-cells (reported by the same group in Feucht et al, 2019). The effector-like iT cells produce robust yet short-lived activity in mice, even with IL2/IL15 supplementation. Such limited activity of iT cells per se does not constitute a major problem in the context of allogeneic cell therapies (and may even be desirable, in order to avoid long-term on-target toxicities) but still underscores an important difference between iT cells and donor-derived abT-cells. How to the authors explain these disparities and can they offer any strategies to increase "naiveness" of CAR iT cells?*

We agree with the Reviewer that the effector-like phenotype of emerging *TRAC*-1XX-iT cells is distinct from that of recent thymic emigrants, and that this phenotype may be associated with reduced in vivo efficacy compared to peripheral blood T cells. The more effector like phenotype is not unique to our CAR-matured iT cells. Low levels of CCR7 and lack of CD45RA expression have been widely observed in *in vitro* differentiated T cells (Nishimura et al., Cell Stem Cell 2013, Ito et al., Comm Biol 2021, Iriguchi et al., Nat Comm 2021 and Wang et al., Nature Biomed Eng 2021), utilizing different differentiation and maturation protocols (TCR or CAR-based, different feeder cells, different agonistic antibodies). None of these processes has yet yielded T cells with the same phenotype as generated in T cells maturing intra-thymically. We have added the following text to our Discussion:

> Line 365: "This effector-like phenotype is commonly observed following extrathymic differentiation of T cells, irrespective of CAR expression or maturation protocol[49,50]."

*3. While the lack of TCR/CAR signaling at the DP stage leads to "death by attrition", excessive signaling can also result in apoptosis mimicking negative selection of autoreactive thymocytes. The authors use CD19-expressing 3T3 cells to produce positive selection of mature CD19 CAR iT cells which, depending on the level of CD19 expression, may induce strong CAR signaling. Did the authors evaluate the impact of a milder stimulation of DP cells (eg by modulating CD19 expression level on 3T3 or by titrating recombinant plate-bound CD19) on cell yield and the phenotype of final iT cells? Stimulation with 3T3-CD19 results substantially reduces the*

*frequency of CD62L+ CD27+ T-cells (Ext Fig 6 vs Ext Fig 8) possibly contributing to their terminal differentiation discussed in query #2.*

We thank the Reviewer for this suggestion. To address the issue whether the level of CD19 expression on the 3T3-CD19-41BBL induced too-strong a signal, leading to the effector-like phenotype of the *TRAC*-1XX-iT cells, we performed the suggested experiment wherein the maturation of the *TRAC*-1XX-iT cells on 3T3-CD19-41BBL is compared to maturation using titrated, plate-bound recombinant CD19-Fc fusion protein (new Extended Data Fig. 7d). The stimulation of D35 *TRAC*-1XX-iT cells on different doses of recombinant CD19 revealed that cell yields, as well as the yield of CD8$\alpha\beta$ SP cells, were positively correlated with increasing CD19. Although iT cell yields could be increased on high CD19-Fc doses (50$\mu$g/mL or 25$\mu$g/mL), there were fewer CD8$\alpha\beta$ SP cells than obtained with 3T3-CD19-41BBL induced maturation. CD62L and CD27 expression were consistently lower in the CD19-Fc-based maturation, suggesting that the reduction of CD62L$^+$CD27$^+$ cells on D35 (original Extended Data Fig. 6) to D42 (original Extended Data Fig. 8) is not due to excessive CD19 activation from the CD19$^+$ 3T3.

> Line 241: "To determine whether CD19 levels may influence acquisition of an effector-like phenotype of the TRAC-1XX-iT cells, we matured iT cells on titrated levels of recombinant CD19 (Extended Data Figure 7d). Increasing CD19 positively affected the expansion and CD8$\alpha\beta$ SP iT cell content, but did not reduce the effector-like phenotype.

*Minor questions*

*1. How would one distinguish the failure of CD19 CAR TiPS to commit to the DP stage from the global apoptosis of DP cells due to tonic CAR signaling-induced negative selection?*

To determine whether apoptosis occurs at a higher rate in DP cells differentiating from CAR-TiPS, we measured apoptosis levels by flow cytometry (new Extended Data Fig. 3h) in WT-TiPS and CAR-TiPS during differentiation on OP9-DLL4. From day 27-35, when the DP population is established in the WT-TiPS, both WT-TiPS and CAR-TiPS were harvested daily, and the percentage of apoptotic cells (live, Annexin-V$^+$ cells) was determined in each population. Levels of apoptosis were consistently low in WT-TiPS and CAR-TiPS, and no increased levels of apoptosis were detected in the DP population.
We added the following section to the results to describe these observations:

> Line 144: To assess whether premature CAR signalling induces apoptosis in emerging DP cells, we measured apoptotic cells at the different developmental stages (DN, CD4 induced single positive (ISP), DP, CD8$\alpha\beta$ SP) in WT-TiPS and CAR-TiPS from D27 – D35, when the induction of the DP population occurs in WT-TiPS (Extended Data Figure 3h). Levels of apoptosis were uniformly low (<5%) in both WT-TiPS and CAR-TiPS, and similar in all different developmental stages, suggesting that the lack of DP establishment from CAR-TiPS is not due to global apoptosis of the DP population.

*2. The authors mentioned that mice receiving peripheral blood-derived TRAC-1XX-CD8 T cells*

*succumbed to GvHD-like symptoms. How do T-cells lacking TCR induce xenogeneic GvHD and is this a consequence of exogenous cytokine supplementation?*

We observed GvHD-like symptoms in mice infused with CD8 *TRAC*-1XX T cells from day 80 post-T cell infusion. IL-2 and IL-15 were administered to all treatment groups for the first 21 days post-T cell infusion. Contrary to the *TRAC*-1XX-iT cells, the peripheral-blood derived CD8 *TRAC*-1XX T cells are not a 100% pure TCR-KO population. As was shown in Extended Data Fig. 10a, the CD8 *TRAC*-1XX T cell population still contained a 4.72% population of $TCR^+$ T cells. As described in Qasim et al. STM 2017, a small residual population of $TCR^+$ T cells can elicit GvHD in human patients. We thus hypothesize that the observed GvHD-like symptoms were caused by the residual $TCR^+$ population present in the CD8 *TRAC*-1XX T cell population. To better clarify the possible cause for the GvHD like symptoms, we adjusted the Results section to highlight the presence of $TCR^+$ cells in CD8 *TRAC*-1XX T cells, along with a reference to Qasim et al. STM 2017:

> Line 316: "Notably, the latter succumbed while presenting GvHD-like symptoms including weight loss, diarrhoea and loss of fur, likely caused by the remaining small population of $TCR^+$ cells[11], which are not present in *TRAC*-1XX-iT cells (Extended Data Figure 10a, d)."

*3. These studies are aimed at optimizing differentiation of T-iPSC into "conventional" T-cells via the DP route away from the gdT lineage. Why are gamma-delta-like CAR iT cells inferior to alpha-beta-like CAR iT?*

To address this question, we added a functional comparison of CAR-iT cells to *TRAC*-1XX-iT cells in Fig. 6a-d and Extended Data Fig. 9). While the cytolytic potential is similar between the groups in an initial CTL assay (new Fig. 6a), CAR-iT cells have reduced Granzyme B and CD107a compared to *TRAC*-1XX-iT and CD8 *TRAC*-1XX T cells (New Fig. 6b), and fail to control tumour growth in an in vitro tumour rechallenge assay (New Fig. 6c). CAR-iT produce less cytokines (IFNy, TNFa, new Fig. 6d) than *TRAC*-1XX-iT cells. When comparing the CAR-iT and *TRAC*-1XX-iT populations in vivo (new extended data Fig. 9e-g), *TRAC*-1XX-iT cells have superior anti-tumour control compared to the CAR-iT, which could be due to the higher number of persisting cells in the *TRAC*-1XX-iT cells in the bone marrow, blood and spleen (new Extended Data Fig. 9h, i).

> Line 285: "To determine whether the αβTCR-T lineage commitment of *TRAC*-1XX-iT cells enhanced their functional capabilities, we compared their *in vitro* and *in vivo* functions to CAR-iT cells and healthy-donor PBMC-derived $CD8^+$ *TRAC*-1XX αβTCR-T cells […]

**Reviewer #1 (Report for the authors (Required)):**

The authors have adequately addressed my concerns.

*We thank the Reviewer for the positive assessment of our response and revisions.*

**Reviewer #2 (Report for the authors (Required)):**

The authors have answered all queries. I have no more questions.

*We thank the Reviewer for the positive assessment of our response and revisions.*

**Reviewer #3 (Report for the authors (Required)):**

I appreciate the authors' efforts to greatly improve the manuscript, taking into account remarks from all 3 reviewers.

In general, the authors have adequately addressed questions/concerns in rebuttal letter. When they did not agree with my remarks, they provided additional references or data to illustrate their point.

The methodology is much better described.

Additional data was provided to strengthen evidence, help data interpretation and support conclusions.

*We thank the reviewer for the positive assessment of our response and revisions and agree that it has further strengthened the manuscript.*

The in vivo cell dose titration experiment is very useful (ext fig 9j/k) but I'm not sure I would agree that "2x10^6 TRAC-1XX-iT cells provided a similar response to 4x10^5 CD8 TRAC-1XX T cells". The absence of significant difference does not mean there is no difference (except if p value reaches 0.95). All animals died in the TRAC-XX-iT groups whereas the 4x10^5 CD8 TRAC XX has not reach median survival at day 50. Especially because the authors mention the occurrence of GVHD like symptom in this group and what is represented is overall survival. Could Progression free survival representation help? Was the experiment followed longer? On the other hand, antitumor activity for 2x106 TRAC-1XX-iT cells is clearly stronger than for 1x105 CD8 TRAC-1XX T cells, so if we're comparing orders of magnitude, we're not far off.

*The text now states: '2x10$^6$ TRAC-1XX-iT cells provided a survival response that was not significantly different from 4x10$^5$ healthy-donor derived CD8 TRAC-1XX T cells' (lines 304 – 306). Additionally, we have specified in the text (lines 316-317) that GvHD-like symptoms were only observed in mice infused with 4x10$^6$ CD8 TRAC-1XX T cells (with 4.72% TCR$^+$ cells, 80+ days post T cell injection, Extended Data Figure 10a, d). No GvHD was observed in the animals treated*

*with $4x10^5$ CD8 TRAC-1XX T cells (which only retains 1.36% $TCR^+$ cells, Extended Data Figure 9a, j, k); these mice died from tumor progression. We adjusted the graph title and corresponding figure 6 legend to indicate 'Tumour free survival'.*
*The experiment shown in Figure 6 was performed in response to the reviewer's requests, we have extended the survival graph to include the most recent timepoint (D80).*

I thank the authors for mentioning in the discussion the applicability to other CARs. Do they think that, as T cell differentiation is intimately linked to fine differences in TCR and CAR signals, the protocol and /or CAR signaling motives might need to be re-optimized for other CARs?

*Our studies reveal the principle that the timing and strength of TCR/CAR signalling affect critical junctions of T cell development, and that DP development is feasible in the absence of TCR expression through TRAC-controlled CAR expression. Different CAR designs, which may differ in their level of expression or signalling, would nonetheless need to be assessed as we described here.*
*We have added the following to the discussion: 'Nonetheless, CAR designs that provide greater signalling strength or interact with ligands expressed during T cell development, may require the same careful analysis as described here to avert interference with DP development.' (lines 405-408).*

Can the authors comment why TRAC-/- TiPS engineering was performed at the iPS stage whereas for TRAC 1928z TiPS and TRAC 1XX TiPS, engineering was performed on T cells that were subsequently reprogrammed? At first thought, I would expect this to have an impact, but maybe it should be mentioned. Did they need to use that particular TiPS clone for comparability or is AAV6 transduction inefficient in iPSCs?

*While we initially performed experiments by engineering TiPS lines (Figure 1), we eventually recognized that reprogramming engineered T cells is more efficient. All subsequent experiments were performed in that manner, which is clearly indicated in the methods.*

Also, are the 'CAR-iT' used in Fig 6 the same as CAR-TiPS-iT described in introduction? It seems their generation should be described in methods.

*The CAR-iT in Figure 6 are the same as the CAR-TiPS-iT described in the introduction.*
*The generation of the CAR-TiPS (lines 900 - 902), as well as the lymphoid differentiation were already included in the methods (lines 988 - 1003). Stimulation and expansion of iT cells (lines 1003 – 1008) now specifies that it was used for TRAC-1XX-iT and CAR-iT. Additionally, we have adjusted the inconsistency in nomenclature and replaced 'CAR-TiPS-iT' in the introduction with 'CAR-iT'.*

Minor language detail:
The use of the phrase "the latter succumbed" in line 316 might be incorrect as the mice are the

ones which succumbed, whereas in the previous sentence, the cells are mentioned and not the mice treated with such cells.

*We thank the reviewer for alerting us to the grammatical inconsistency. We have corrected the sentence which now reads:*
*'Notably, mice infused with 4x10$^6$ CD8 TRAC-1XX T cells succumbed while presenting GvHD-like symptoms' (lines 316 – 318).*

**Reviewer #3**

*• Key results:*
*This study describes the technological achievement that consists in generating bona fide CAR T cells from iPSCs. The success of CAR T cells in the clinic in so far untreatable leukemias and lymphomas seems to reside in properties intrinsic to T-cells: the capacity to kill target cells in an antigen-specific manner but also to proliferate in massive amounts in the organism. Complete clinical responses have associated with several logs of proliferations of CAR T cells in the patient.*

*So far, the attractiveness of iPS as a source of CAR-expressing therapeutic cells has been hampered by the inability to drive their differentiation into cells that recapitulate all the properties of mature T cells. In this study, the authors perform a step by step dissection of what it takes to push T-iPSC along the path of T cell differentiation.*

*They use the previous teachings of thymocyte differentiation studies to understand why CAR expression impairs T cell differentiation using first DP cell formation as an indicator of commitment to αβT cell lineage then address the impact of CAR T cell signaling to drive maturation into SP T cells.*

*Key findings are:*
*- Unregulated CAR expression disorganizes the fine interplay between notch pathway and TCR signaling that drives αβ vs γδ lineage commitment.*
*- Strength or rather quality of CAR signaling is a key factor in this context.*
*- Antigen-specific stimulation is necessary to form SP T cells but they appear immature*
*- 41BB costimulation allows differentiation into mature CD8ab SP T cells able to proliferate and provide sustained in vivo antitumor activity*

*• Originality and significance:*

*I think that the findings presented here are of general interest, not just people involved in cell therapy.*

We thank the Reviewer for this positive assessment.

*• Data & methodology:*
*In general, the data and methodology used are of high quality. There are nevertheless serious gaps.*

*Methodology is not sufficiently to enable reproducing the results: all cytokine concentrations are missing. Also during T lymphoid differentiation, it should be mentioned at what day TPO and IL3 are no longer added to the medium (.806.)*

The Methods section has been extended to include the requested details.

*Flow cytometry: In many cases where side by side comparison are made and quantitative /qualitative differences are observed and interpretated, the flow cytometry axis are different between samples suggesting that the examples shown don't come from the same data sets or questioning whether the gates were set appropriately. (see CD4 axes in Fig1d as one of many examples). In general, there is no mention on how gates were set, no mention is made of the use of unstained, isotype or "fluorescence-minus-one" controls.*

To better clarify how flow cytometry gating was performed, we have added the following details to the methods section:

> Line 1076: "Flow cytometer voltages were calibrated with Ultra Rainbow Calibration Kit (SpheroTech, URCP-38-2K) prior to every acquisition. […] Negative and positive gates were set based on (un)stained PBMC and TiPS controls (Supplementary Figure 1).

Additionally, we have extended Supplementary Fig. 1 to not only include the gating strategy up to $CD45^+$ $CD7^+$, but also examples of the stained and unstained PBMC, WT-TiPS and CAR-TiPS for gating reference.

• *Data interpretation:*
*101. When comparing wt TiPS to H1 ES cells, the authors illustrate their inability to produce DP cells by showing D35 data in Fig1b whereas for H1 cells, they mention that DP cells only start appearing at D39, also showing D50. The same days should be shown for wt TiPS.*

We thank the Reviewer for this suggestion. As mentioned above in response to Reviewer 1, we have replaced the previous experiment shown in Fig. 1b for a replicate experiment including H1, FiPS and WT-TiPS, showing the same timepoints for all three lines. Cell counts start to decline rapidly for WT-TiPS post day 35, therefore the end-point for the comparison between the groups was set at the last timepoint where quality phenotype data could be obtained for all groups, D40. New Fig. 1b shows the phenotypes of the new differentiations of H1, FiPS and WT-TiPS at D40, the expression of the pluripotency markers on all three lines is shown in the new Extended Data Fig. 1a, and the phenotype time course in the new Extended Data Fig. 1b-d. We have adjusted the text accordingly as detailed above in the answer to question 1 of Reviewer 1.

*Ext Fig 2a PCR primer position should be illustrated.*

The PCR primer positions have been added to Extended Data Fig. 2a.

*Why has DTX1 induction (ext fig 4b) not been performed on the same cells, TiPSCs?*

The DTX1 induction was initially performed on peripheral-blood derived T cells to validate the OP9 lines prior to application in the TiPS differentiations. We agree with the Reviewer that a validation on WT-TiPS is more informative and have replaced the PBMC-based experiment in

the original Extended Data Fig. 4b with an experiment using WT-TiPS (new Extended Data Fig. 3c).

*133-16: a model is proposed from the indluence of mDLL1 on H1/Trac-/-TiPS vs that of human notch ligands. Nevertheless, fig1c and 1d suggest that WT-TiPS and TRAC-/--TiPS treated with mDLL1 are much more similar to each other (5 v 10% DP) than they are to WT-TiPS treated with human DLL1 (0% DP). Is the 5 to 10% difference relevant?*

We agree with the Reviewer that in the original Fig. 1c, the DP population generated in the TRAC-KO versus the WT-TiPS on OP9-mDLL1 was not very striking, and that D35 data did not best represent the difference we consistently observe. We now show D40 phenotype profiles in the Fig. 1c to better illustrate the effect that the TRAC-KO has on the T cell differentiation (the full time course is in new Extended Data Fig. 2d).

*138. In what sense is CAR signal "stronger" than a TCR signal? During lymphoid differentiation, constitutive CAR expression would trigger an earlier signal than an endogenous rearranged TCR driven by TCR alpha and beta genetic control elements but stronger is difficult to understand without measuring downstream phosphorylation in the TCR cascade.*

We thank the Reviewer for this question. The "stronger" tonic signalling strength of CARs relative to TCRs has been previously described (eg, Ramello et al., Sci Signal 2019). To confirm that CAR-TiPS are exposed to stronger signalling than WT-TiPS, we quantified phosphorylated ERK1/2 in WT-TiPS vs CAR-TiPS (D35, differentiated on OP9-DLL4), using the BD cytometric bead array (new Extended Data Fig. 3f), showing that ERK1/2 phosphorylation in the absence of antigen exposure is higher in CAR-TiPS compared to WT-TiPS. Additional to the new pERK1/2 data, Fig. 3 shows the downstream effects of CAR expression on the expression of TCR and Notch signalling responsive genes. We have adjusted the text to include a reference to the new Extended Data Fig. 3f as well as referenced previous publications on the strength of CAR signalling (Ramello et al) and the effect of CAR expression on lymphoid differentiation in mouse cells (Maluski., et al J Clin Invest 2019):

> Line 140: "we found that TiPS that constitutively expressed the 1928z CAR (CAR-TiPS, Extended Data Figure 3e), had increased levels of ERK1/2 phosphorylation (Extended Data Figure 3f)"

*144. Given that there 68% of cells appear DP at day 35, I imagine they come from WT-TiPS treated with hDLL4-OP9 but it should be mentioned in the text and the legend.*
*More generally, the type of OP9 cells used for differentiation is missing from the entire second half of the manuscript.*

The Reviewer is correct that these cells were differentiated on OP9-DLL4. We have added language to specify this in the figure legend.

*217-219 I wouldn't call these phenotypic change as 'characteristics of cell activation' as CD69*

*CD25, CD45 isoforms (at least) are also transiently expressed at different stages of T cell selection in the thymus.*

The Reviewer is correct that CD69, CD25 and CD45 isoforms are transiently expressed at different stages of T cell selection. Because the described phenotypes occurred after CAR-antigen engagement and are associated with a loss of CD4 and CD1a, maturation of the iT cells has occurred, and hence our reference to "activation". We have adjusted the text to better describe that iT cell maturation has occurred, and the phenotypes are interpreted as such:

> Line 225: "The matured SP D42 TRAC-1XX-iT cells displayed a phenotype resembling activated T cells, including the upregulation of CD25, CD69, CD56 and transition from CD45RO to CD45RA (Extended Data Figure 7a)."

*238. It's surprising to interpret Fig 4k as "3T3-CD19-41BBL matured cells exhibited improved persistence upon repeated antigen exposure". Rather 3T3-CD19 matured cells failed to expand and survive upon antigenic stimulation.*

We have adjusted the sentence to reflect that the improvement is over 3T3-CD19 matured cells specifically:

> Line 248: "However, whereas 3T3-CD19 matured cells failed to expand upon repeated exposure to antigen, 3T3-CD19-41BBL maturation improved their expansion and survival (Figure 4k)."

• *Suggested improvements:*

*160-163.*
*To demonstrate TRAC-encoded 1928z still interfered enough to prevent DP commitment, the authors should show the kinetics of DP induction in WT-TiPS earlier than D35 and correlate that to TRAC encoded TCR expression.*

We thank the Reviewer for this suggestion. To better demonstrate the effect the *TRAC*-CARs have, we added the kinetics of differentiation of the WT-TiPS, *TRAC*-1928z-TiPS and *TRAC*-1XX-TiPS to the new Extended Data Fig. 5a. These data show the phenotype of the differentiating cells at the same timepoints as the TCR and CAR expression in Fig. 2a. As Extended Data Fig. 5a shows, the $CD4^+CD8\alpha\beta^+$ DP stage starts to appear between D24 and D27 in WT-TiPS, the same time window during which CAR expression is induced in the *TRAC*-controlled expression (Fig. 2a). The *TRAC*-1928z-iT cells do not show this induction of DP cells but proceed to the $CD8\alpha\alpha$ phenotype. We have adjusted the text to include reference to the added differentiation phenotype data:

> Line 166: The differentiation of *TRAC*-1928z-TiPS towards early T cell lineage commitment improved, as reflected in a greater $CD7^+CD5^+$ population (Figure 2b) compared to CAR-

TiPS (Extended Data Figure 3g). DP induction, however, was still not enhanced (Figure 2b, c; Extended Data Figure 5a).

*180. It seems the formulation used by the authors doesn't fairly reflect the data. ITAM phosphorylation is exclusively detected in cells with the highest CAR expression and given that the level of CAR expression is different between retrovirally expressed CAR and TRAC-1XX, the data is difficult to interpret. ITAM1 phosphorylation should be compared between TRAC-1928z and TRAC-1XX (ITAM3 is not relevant as phosphorylatable tyrosines are mutated to F in this construct).*

We previously showed that CAR expression is higher from retroviral transduced T cell compared to TRAC T cells accounting for the lesser tonic signalling (Eyquem et al., Nature, 2017). Following the Reviewer's comment, we have included *TRAC*-1928z in our assessment of tonic ITAM1 and ITAM3 phosphorylation (new Extended Data Fig. 6). We show that *TRAC*-1928z shows less tonic phosphorylation of both ITAMs compared to the retrovirally expressed 1928z CAR. The phosphorylation of ITAM3 is further reduced and eliminated, as expected, from the point-mutation in the 1XX CAR. We have clarified the text as follows:

Line 179: "*TRAC*-encoded 1928z showed reduced phosphorylation of ITAM1 and ITAM3, while the latter was abolished in *TRAC*-1XX T cells (Extended Data Figure 6a-c)."

*Figure 5a. A vast majority of the cells appear CD45RA-CD62L-, a phenotype typically associated with effector memory properties in peripheral T cells. The authors should comment. Staining should be performed in parallel on primary human abT cells, NK cells and gamma delta T cells for comparison.*

We agree with the Reviewer that the phenotype described in Fig. 5a resembles that of effector memory cells in peripheral T cells and had addressed this in the discussion with the following text: "However, these iT cells do not have a classical naïve phenotype, as they maintain CD5 and CD7 expression but not homogeneously express CD45RA, CD62L and CCR7, a would be expected in naïve T cells and recent thymic emigrants. They rather express CD45RO, CD28, CD25 and CD56, hallmarks of recently activated T cells (Extended Data Fig. 9b)."

In addition to the old Extended Data Fig. 9b, we have added a reference to Fig. 5a in the Discussion and extended the Discussion to elaborate on the more effector-like phenotype of the cells, as also suggested in question 2 from Reviewer 2. Finally, we included a phenotype comparison of the *TRAC*-1XX-iT cells to peripheral blood derived CD8$\alpha\beta$ $\alpha\beta$TCR-T cells, CD4 $\alpha\beta$TCR-T cells $\gamma\delta$TCR-T cells and NK cells in the new Extended Data Fig. 8a.

*Also Figure 5b legend should mention that clustering is performed on transcriptomics data*

The Fig. 5b legend has been updated to clarify that clustering is performed on the transcriptomics data.

*Additional information should be added including if the D42 cells used in figure 5 and 6 were exposed to 3T3-CD19 cells or 3T3-CD19-41BBL cells.*

Figure legends for Fig. 5 and 6 have been extended to include the mention of 3T3-CD19-41BBL used as the maturation step.

*The authors claim that TRAC-1XX-iT cells achieve full tumor control in vivo, as well as CAR-expressing peripheral T cells. Nevertheless, the experimental conditions used are far from their stress test model. In the original Heucht et al paper, the stress test model is performed using 5x10^5 Nalm6 cells vs 1 to 4x10^5 CAR T cells. Here, much fewer Nalm6 cells are used (1x10^5 cells) and more CAR T cells are used (2x10^6 in figure 4 and 4x10^6 in figure 6). Also, IL2 and IL15 are injected in mice possibly to sustain survival and growth of human cells. It is therefore not rigorous to claim that the model used provides a quantitative assessment of the potency of TRAC-XX-iT cells.*

As shown in Fig. 6f, *TRAC*-1XX-iT cells achieve full tumor control in vivo with a single dose of $4x10^6$ iT cells. We did not claim that this function is equivalent to CAR expressing peripheral T cells from a healthy donor, nor that these studies were a "stress test". We only stated that the cells achieved 'comparable tumour control in a systemic *in vivo* leukaemia model' to CD8 *TRAC*-1XX cells (original lines 35 and 280-283). The Reviewer is correct that the stress test conditions used in this manuscript are not the same as in the Feucht et al. or other previous studies. As we described in Zhao et al., where we first introduced the concept of the 'stress test', T cell doses are purposefully lowered to levels where the CAR therapy begins to fail in a given tumour model. In Fig. 6f and Extended Data fig. 9f, it is illustrated that at $4x10^6$, the TRAC-1XX-iT cells are able to achieve full tumour control in the respective model. At $2x10^6$ the CAR T cells start to fail, therefore setting 'stress test' conditions for those TRAC-1XX-iT cells.

*There are paradoxical data in the manuscript, the authors claim that D42 TRAC-XX-iT cells have downregulated CD62L (12% in Fig 5a) but that should correspond to D0 cells in Fig 6i that show 40% CD62L+.*

Numerous differentiations have been performed to generate the data presented in this manuscript. The differentiations performed for the RNA sequencing analysis (the phenotypes shown in Fig. 5a), are separate from the differentiations performed for the in vivo functional analysis (presented as D0 in Fig. 6i). The difference seen in CD62L expression is the result of biological variability between differentiations. To illustrate the biological variability of the phenotype of the D42 cells, we have included the phenotypes of the differentiations used for RNA sequencing in the new Extended Data Fig. 8b.

*The authors mention the occurrence of GvHD-like syndrome in mice treated with TRAC-1XX-CD8 T cells. The authors should show the level of αβTCR+ cells in the cells injected into the mice as it is very uncommon to observe GvHD-like symptoms in this type of model.*

The level of αβTCR+ T cells (4.72%) in the CD8 *TRAC*-1XX population was shown in Extended Data Fig. 10a. As we mention in response to question 2 from Reviewer 2, it has been previously reported that a small residual population of $TCR^+$ T cells can elicit GvHD (Qasim et al., STM 2017).

*The study should be performed using cells after depletion of remaining αβTCR+ cells and should be performed more than once as it is impossible to match donors for TRAC-1XX-CD8 T cells and TRAC-1XX-CD8 iT cells.*

To correct for the GvHD-like symptoms, we have shown the tumour-free survival in Fig. 6g (old Fig. 6f). Since animals with GvHD-like symptoms did not show a difference in tumour control (Fig. 6f-g), anti tumour efficacy was not affected. We agree with the Reviewer that is it challenging to match donor for CD8 *TRAC*-1XX T cells and *TRAC*-1XX-iT cells. We have thus performed one more in vivo experiment (new Extended Data Fig. 9j-k), in which *TRAC*-1XX-iT cells are compared against different doses of CD8 *TRAC*-1XX T cells (please see the further below for details on the experiment), from a different donor.

*The authors should justify the use of IL2 and IL15 injections.*

The use of exogenous cytokines is common practice in the in vivo assessment of iPSC-derived T cells (Themeli et al., Nat Biotech 2013; Ando et al., Stem Cell Reports 2015; Saito et al., Cancer Research 2016; Maeda et al., Cancer Research 2016; Kawamoto et al., Progress In Hematology 2018; Kashima et al., iScience 2020; Ito et al., Communications Biology 2021; Harada et al., Molecular Therapy 2021; Wang et al., CSC 2022) and no different in the present study.

•*References:*
*171. A reference should be included to justify that CD1a, CD2, CD62L and CD45RO expression confirm T lineage commitment or include data looking at these data in developing thymocyte at various stages.*

We agree with the Reviewer that the phrasing of the sentence is unclear regarding the interpretation of the mentioned phenotype markers. We have rephrased the sentence to:

> Line 185: "By day 35, these DP cells express CD1a, CD2 and CD45RO, consistent with the phenotype of human DP thymocytes[29-31] (Extended Data Figure 5b)."

• *Clarity and context:*
*The abstract is clear and accessible. The introduction presents the appropriate context for the study.*

*I think the conclusion is a bit of an overstatement. The authors start the discussion by "We report here the generation of therapeutic CD8αβ CAR iT c ells from TiPS. This is an overstatement as several aspects are missing that would allow the claim that these cells are of therapeutic potential as the word 'therapeutic' is often understood as 'clinic-ready'. No mention*

*is made of CD4 CAR T cells where very few scientists would be willing to develop a therapy solely based on CD8 T cells. The positive impact of CD4 CAR T cells has been reported.*

We disagree with the Reviewer that the term therapeutic implies 'clinic ready'. There are numerous animal studies across many domains that use this term to reflect that a disease model is being treated (not necessarily cured), without clinical implications

*Several properties of their cells that are very different from bone fide T cells are just brushed upon and not discussed: their inability to produce IL2. The in vivo experimental conditions used seem to suggest that these cells are not as potent as traditional CAR Ts.*
*A valid quantitative in vivo comparison with the current standard CAR Ts should be included.*

The disparities in function between the *TRAC*-1XX-iT and CD8 *TRAC*-1XX cells are described in the results section (lines 287-297; 303-318) as well as in the discussion (lines 373-389), including the lack of IL-2 production. They obviously point to the need for further research in the future.

As suggested, we have reiterated in the Discussion the difference in function between TiPS-derived T cells and peripheral blood derived T cells:

> Line 376: "*TRAC*-1XX-iT cells still produced significantly lower levels of cytokines than CD8 *TRAC*-1XX cells, notably lacking IL-2 production (Figure 6d). *TRAC*-1XX-iT cells nonetheless provide substantial anti-tumour activity in a systemic NALM6 model, which CAR-iT cannot achieve (Figure 6g, Extended Data 9g), while requiring a higher dosage than CD8 *TRAC*-1XX T cells (Extended Data Figure 9k)."

To quantify the potential of the *TRAC*-1XX-iT cells more accurately, we have performed an additional *in vivo* study comparing the potency of the *TRAC*-1XX-iT cells to a titration of CD8 TRAC-1XX cells, to determine at what dose level the peripheral-blood derived counterparts of our iT cells start to fail (Extended Data Fig. 9j-k). Based on this assay, we could determine that *TRAC*-1XX-iT cells are about 5 fold less potent than their natural counterparts. We have further elaborated in the Discussion on the difference in function between *TRAC*-1XX-iT cells and the current standard treatment with the following:

> Line 303: "To provide a potency benchmark under these conditions, we administered diminishing doses of CD8 *TRAC*-1XX T cells and found that $2x10^6$ *TRAC*-1XX-iT cells provided a similar response to $4x10^5$ CD8 *TRAC*-1XX T cells (Extended Data Figure 9j, k)."

*Although the authors do not claim that this platform is applicable as is to all CAR T cells, for this paper to speak to a large audience, a discussion should be included on the applicability of this work to other CAR targets (potential limitations).*

We thank the Reviewer for this suggestion. We have extended our discussion to include the wider applicability of TiPS-derived T cells along with the possible multiplexing strategies as suggested by Reviewer 1.

> Line 401: "The use of TiPS-derived T cells is not limited to targeting CD19 as described here and is applicable to other target tumour associated antigens, barring that potential interaction between the CAR with antigen expressed throughout T cell development does not interfere with T lineage commitment, as well as applications beyond cancer immunotherapy[58]."

• *Minor points/typos:*

*Fig1a : please add to the right side that T lymhoid differentiation is performed on OP9(+notch ligand) stroma*

'OP9 + Notch ligand' has been added to the figure.

*Ext fig5a "gated on liver CD45+ cells" -> "gated on live CD45+ cells"*

Typo has been corrected.

*216 "(Figure 4a)" should also mention Ext Fig8a as CD1a downregulation is mentioned.*

A reference to Extended Data Fig. 7a (old Extended Data Fig. 8a) has been added.

*227-228 should read '30-fold on average' rather than 'up to 30-fold' to be in line with fig 4g*

The Reviewer is correct, we have made the adjustment in the text.

*Shouldn't Figure 5a legend read "γδTCR-T cell markers (bottom panel)" rather than "γδTCR-T cells (bottom panel)"?*

The Reviewer is correct. We have made the adjustment in the text.

**Rebuttal 2**

**Reviewer #1 (Report for the authors (Required)):**

The authors have adequately addressed my concerns.

*We thank the Reviewer for the positive assessment of our response and revisions.*

**Reviewer #2 (Report for the authors (Required)):**

The authors have answered all queries. I have no more questions.

*We thank the Reviewer for the positive assessment of our response and revisions.*

**Reviewer #3 (Report for the authors (Required)):**

I appreciate the authors' efforts to greatly improve the manuscript, taking into account remarks from all 3 reviewers.

In general, the authors have adequately addressed questions/concerns in rebuttal letter. When they did not agree with my remarks, they provided additional references or data to illustrate their point.

The methodology is much better described.

Additional data was provided to strengthen evidence, help data interpretation and support conclusions.

*We thank the reviewer for the positive assessment of our response and revisions and agree that it has further strengthened the manuscript.*

The in vivo cell dose titration experiment is very useful (ext fig 9j/k) but I'm not sure I would agree that "2x10^6 TRAC-1XX-iT cells provided a similar response to 4x10^5 CD8 TRAC-1XX T cells". The absence of significant difference does not mean there is no difference (except if p value reaches 0.95). All animals died in the TRAC-XX-iT groups whereas the 4x10^5 CD8 TRAC XX has not reach median survival at day 50. Especially because the authors mention the occurrence of GVHD like symptom in this group and what is represented is overall survival. Could Progression free survival representation help? Was the experiment followed longer? On the other hand, antitumor activity for 2x106 TRAC-1XX-iT cells is clearly stronger than for 1x105 CD8 TRAC-1XX T cells, so if we're comparing orders of magnitude, we're not far off.

*The text now states: '2x10$^6$ TRAC-1XX-iT cells provided a survival response that was not significantly different from 4x10$^5$ healthy-donor derived CD8 TRAC-1XX T cells' (lines 304 – 306). Additionally, we have specified in the text (lines 316-317) that GvHD-like symptoms were only observed in mice infused with 4x10$^6$ CD8 TRAC-1XX T cells (with 4.72% TCR$^+$ cells, 80+ days post T cell injection, Extended Data Figure 10a, d). No GvHD was observed in the animals treated*

*with $4x10^5$ CD8 TRAC-1XX T cells (which only retains 1.36% $TCR^+$ cells, Extended Data Figure 9a, j, k); these mice died from tumor progression. We adjusted the graph title and corresponding figure 6 legend to indicate 'Tumour free survival'.*
*The experiment shown in Figure 6 was performed in response to the reviewer's requests, we have extended the survival graph to include the most recent timepoint (D80).*

I thank the authors for mentioning in the discussion the applicability to other CARs. Do they think that, as T cell differentiation is intimately linked to fine differences in TCR and CAR signals, the protocol and /or CAR signaling motives might need to be re-optimized for other CARs?

*Our studies reveal the principle that the timing and strength of TCR/CAR signalling affect critical junctions of T cell development, and that DP development is feasible in the absence of TCR expression through TRAC-controlled CAR expression. Different CAR designs, which may differ in their level of expression or signalling, would nonetheless need to be assessed as we described here.*
*We have added the following to the discussion: 'Nonetheless, CAR designs that provide greater signalling strength or interact with ligands expressed during T cell development, may require the same careful analysis as described here to avert interference with DP development.' (lines 405-408).*

Can the authors comment why TRAC-/- TiPS engineering was performed at the iPS stage whereas for TRAC 1928z TiPS and TRAC 1XX TiPS, engineering was performed on T cells that were subsequently reprogrammed? At first thought, I would expect this to have an impact, but maybe it should be mentioned. Did they need to use that particular TiPS clone for comparability or is AAV6 transduction inefficient in iPSCs?

*While we initially performed experiments by engineering TiPS lines (Figure 1), we eventually recognized that reprogramming engineered T cells is more efficient. All subsequent experiments were performed in that manner, which is clearly indicated in the methods.*

Also, are the 'CAR-iT' used in Fig 6 the same as CAR-TiPS-iT described in introduction? It seems their generation should be described in methods.

*The CAR-iT in Figure 6 are the same as the CAR-TiPS-iT described in the introduction.*
*The generation of the CAR-TiPS (lines 900 - 902), as well as the lymphoid differentiation were already included in the methods (lines 988 - 1003). Stimulation and expansion of iT cells (lines 1003 – 1008) now specifies that it was used for TRAC-1XX-iT and CAR-iT. Additionally, we have adjusted the inconsistency in nomenclature and replaced 'CAR-TiPS-iT' in the introduction with 'CAR-iT'.*

Minor language detail:
The use of the phrase "the latter succumbed" in line 316 might be incorrect as the mice are the

ones which succumbed, whereas in the previous sentence, the cells are mentioned and not the mice treated with such cells.

*We thank the reviewer for alerting us to the grammatical inconsistency. We have corrected the sentence which now reads:*
*'Notably, mice infused with 4x10$^6$ CD8 TRAC-1XX T cells succumbed while presenting GvHD-like symptoms' (lines 316 – 318).*