

A CD8 $\alpha\beta$ co-receptor modified to contain an intracellular CD28 signaling tail enhances TCR-engineered T cell function independent of solid-tumor-associated co-stimulatory ligands

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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 this study, the authors present a technically elegant and conceptually innovative approach to enhancing the efficacy of TCR-engineered T cells by integrating co-stimulatory signals through a chimeric CD8/CD28 receptor. They provide several in vitro, in vivo, and transcriptional analyses showing evidence for improved function and persistence of modified T cells. However, several important limitations should be addressed to strengthen the translational value of this work:

1. The Introduction section lacks sufficient clarity regarding the well-established limitations of T cells genetically modified with T cell receptors in the treatment of solid tumors. The authors should briefly outline the key mechanisms underlying this limited efficacy, such as tumor antigen heterogeneity, immune suppressive microenvironments, and poor T cell infiltration, and then use this background to clearly articulate their central hypothesis and the rationale for their proposed strategy to overcome these barriers. Providing this context would greatly enhance the coherence and scientific relevance of the study.
2. The presentation of the experimental findings in the Results section lacks coherence and narrative flow. In some places, the transitions between paragraphs are abrupt, and the rationale linking each experimental step is insufficiently developed. As a result, it is difficult for the reader to follow the logical progression of the study or to understand why specific experiments were performed at each stage.
3. The Discussion section largely reiterates the results without offering a focused or integrative analysis of their broader implications. It would benefit from a more critical interpretation of the findings in the context of existing literature, highlighting how this work advances the field. Moreover, key limitations, such as the reliance on a single clinical case, limited T cell persistence, and the use of immunodeficient mouse models, are not adequately addressed. A more balanced discussion acknowledging these weaknesses, along with suggestions for future directions, would strengthen the manuscript considerably.
4. The authors engineered TCR-transduced CD4+ and CD8+ T cells targeting MAGE-A1/HLA-A2 and showed enhanced cytokine production and tumor cell killing in vitro, as well as antitumor activity in vivo. Based on these findings, they treated a patient with MAGE-A1 + NSCLC, demonstrating safety but no clinical efficacy. Although the authors explain the reason for the evaluation of only one case, a single-patient case limits interpretability, and additional cases are needed to draw meaningful conclusions regarding therapeutic potential.
5. Fig. 1D: What is the explanation for the higher proliferative responses observed in the CD4+/CD8+ TCR-MAI-CD8 $\alpha\beta$ group? Does the "coordinated response" contribute to enhanced cytokine expression (what cytokines?), thereby promoting proliferation?
6. Page 7, line 120: Consider replacing "lacks tumor control" with "fails to control tumor growth" for improved clarity and precision.

7. Fig. 2H–J: It is unclear whether the reported scRNA-seq data are representative of samples from multiple patients and whether the findings are reproducible. Have the authors conducted similar analyses using samples from their own patient cohort to validate the findings?

8. Page 7, lines 128–129: The statement “Patient tumors have limited and inconsistent expression of both positive and negative co-stimulatory binders” refers to a well-recognized limitation of CAR T cell therapy in solid tumors, unlike in hematologic malignancies, where co-stimulatory ligands are more consistently present (PMID: 24667962). For clarity, the authors should first provide a brief background on this issue to orient readers before presenting the scRNAseq findings.

9. Importantly, the study lacks translational evidence demonstrating the effectiveness of TCR-engineered T cells incorporating co-stimulatory signaling via a chimeric CD8/CD28 receptor in human patients with solid tumors. Including such data would greatly enhance the clinical relevance of the findings.

Reviewer #2

(Remarks to the Author)

The study investigates strategies to improve HLA-A2-restricted, MAGE-A1-specific TCR-engineered T cell therapy for solid tumors by enhancing CD4+ T cell function through TCR modification and co-stimulatory signaling. The authors found that redirecting CD4+ T cells—engineered to express a tumor-specific TCR and co-expressing the CD8 $\alpha\beta$ co-receptor—is not sufficient to achieve robust anti-tumor responses, likely due to limited co-stimulatory signals within the tumor microenvironment (TME).

To address this, they designed a synthetic construct by tethering the intracellular signaling domain of CD28 to CD8 β . Modifying key signaling motifs within CD28 (LL, YMMN, PRRP, and PYAP) significantly enhanced T cell functionality and in vitro tumor cell killing. These modifications also rendered the engineered T cells more resistant to exhaustion. Furthermore, Zhang et al. demonstrated that the CD8 β /CD28Mut construct enhances the cytotoxic activity of T cells engineered with other tumor-specific TCRs, such as those targeting PRAME and WT1, broadening the potential application of this strategy. The data support the use of engineered CD8 β /CD28 fusion proteins to improve both the durability and efficacy of T cell therapies in the solid tumor setting.

Overall, the study is well-conducted and the conclusions are supported by the data. However, the absence of in vivo evidence showing that T cells expressing the CD8 β /CD28Mut construct provide superior tumor control compared to CD8 β /CD28 wild-type (WT) limits the impact of the findings.

Major Points:

1. The authors should clarify the proportion of engineered CD4+ and CD8+ T cells used in the in vivo experiments.
2. In Figure 4B, the tumor growth curves for mice treated with irrelevant TCR or MAGE-A1 TCR plus CD8 $\alpha\beta$ appear identical to those shown in Figure 2. The authors should either consolidate this data into a single figure or present an independent experiment with a new control group.
3. An in vivo comparison of tumor growth in mice treated with MAGE-A1 TCR-engineered T cells co-expressing CD8 β /CD28WT versus CD8 β /CD28Mut is essential to validate the enhanced efficacy observed in vitro.
4. It would also be important to assess in vivo persistence of the transferred T cells and the overall survival of treated mice.

Reviewer #3

(Remarks to the Author)

- The authors present an interesting manuscript whereby CD8 β is tethered to the costimulatory domain of CD28 and thereby proposed to enhance the activity of TCR-engineered T cells. They claim this results in reduced exhaustion, enhanced tumor infiltration, and improved murine tumor control. Further modifications of a WT or mutated CD28 intracellular domain produced a mutant CD8 β -CD28 construct that conferred superior therapeutic control across tumor models.

Critiques below.

• Abstract

- o The abstract was not clearly written and led to some confusion especially as it relates to describing CD4 T cells. A clearer and more concise description of describing their strategy to engineer CD8 $\alpha\beta$ into CD4 T cells to improve TCR-T cell would be helpful.

• Introduction

- o A point for clarity. The authors state that “modifying the intracellular TCR chain domains decreased TCR function, likely by blocking the initiation of TCR signaling”. However, the publication they cited used a single-chain TCR that wouldn’t engage TCR signaling in the first place as such their rationale seems unfounded.

• Results

o Figure 1

What is the significance of the K278T mutation in MAGE-A1 peptide for selecting TCRMA15 as TCR of interest? How does this relate to the modifications made to the T cell. Is this a higher affinity version? If so, would one want to use a lower affinity version to demonstrate the ability to alter the TCR activation threshold with said modifications?

Figure 1D:

- It is not entirely clear whether the proliferation was determined via incucyte or a separate stimulation. It will be helpful offer a clear description of how they enumerated the CD4 vs CD8 T cells.

- Were the number of CD4 only and CD8 only T cells seeded the same on their own as combined or reduced to half for their 1:1 (or are they doubling the total number of T cells in the combination group)? Could this effect fold-change numbers when determining expansion? One could assume reduced numbers but as written this is point is ambiguous.

- Measuring T cell numbers over 6 days seems too brief. I suggest extending this time to a couple of weeks (at least). How was survival impacted? Please consider using a proliferation dye to visualize the extent of cell division (vs increased numbers due to cell survival).

Figure 1E

- The numbers for the CD4 only and CD8 only treatments vs combined is ambiguous here too.

o Figure 2

Figure 2F:

- PD-1 and TIM-3 aren't sufficient markers to determine differences in exhaustion between single and combined T cell treatment. Suggest looking at transcriptional markers of exhaustion, i.e. TOX.

- The most definitive method to test for exhaustion is to conduct an ex vivo stimulation and assess functional activity.

Are there differences in the frequencies of Tregs in the CD4 only vs combined CD4+CD8 treatments

o Figure 3

Figure E:

- Authors claim enhanced proliferation just by obtaining the T cell counts at the end of all of their stimulations but this method leaves too much room for misinterpretation.

In figure 3B, it looks like there is less p/HLA multimer staining cells than the other CCR groups. Please clarify how you normalize for this because it doesn't look like they stain for the multimer after the stimulation. Does this mean the CD8 $\alpha\beta$ only T cells would have similar cytokine expression if TCR expression was equal across groups in Figure 3C. The data shown actually look like the trend in IFN γ + cells correlates pretty well with TCR expression.

o Figure 4

Figure 4A, please provide a rationale as to T cells are injected 6 days earlier here than they do for the rest of their experiments? At this time point, the tumors do not look like they would be at a palpable size.

Figure 4H—again, consider expanding the markers you use to characterize exhaustion. At this point the tumors are so small that leads one to wonder if the reduced TIM3 phenotype they're seeing is because the cells don't have anything to interact with anymore yet. The authors mention differences in tumor burden in the results section for figure 5 as a reason for why the CD8 $\alpha\beta$ groups have more proliferation associated genes than the CD8/CD28. Repeat some of these studies but euthanizing mice when the tumors are varying sizes might shed some light on this matter.

Authors claim improved infiltration but only show that there are more cells in the tumor. The increased numbers could be due to increased proliferation and/or Survival but not necessarily infiltration.

Figure 4G captions claim that the heightened CD4 to CD8 ratio is indicative of CD4 proliferation. This could be the result of many other mechanisms. CD8 death, for one, in addition to the other concerns I've mentioned about proliferation.

o Figure 5

They mention that the CD8 T cells from the CD8/28 group has more stem-like gene expression, but that is only in reference to the CD8 $\alpha\beta$ combo control and not the CD8 $\alpha\beta$ control. Not sure if that is a fair comparison

If exhaustion is being averted then it is unclear why a cluster of exhausted T cells from this sequencing data does not show up.

o Figure 6

The lack of in vivo antitumor activity using the TCR-PRAME-CD8/28 mut is critical to include but missing from these data sets.

o Figure 7

The manuscript would have benefited from a strong ending (Figures 6 and 7). The lack of data showing that the mutant had any beneficial effects in vivo and characterized it with an in vitro repeat stim/sequencing assay. They show upregulation in proliferation and exhaustion related genes but follow this with flow data that only looks at PD1 and TIM3 again for exhaustion, which also had very little differences between the mutant and wildtype cd8/cd28 groups. Furthermore they saw no differences in cytokine expression after restimulation. Did not show any functional proof of differences in proliferation again.

In figures 6 and 7 they did not show any signaling differences for why their mutant might be affecting the T cells differently than their wildtype version of the CD8/CD28. They mention in their discussion possible signaling molecules that are involved but didn't look at a single one.

Finally, please citing other relevant work demonstrating the use of CD4 or CD8a molecules modified to express costimulatory domains.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The authors have satisfactorily addressed all of my concerns, and the revised manuscript has been substantially improved.

Reviewer #2

(Remarks to the Author)

The authors have thoroughly addressed the reviewer's comments, and the revised manuscript demonstrates substantial

improvement. I consider it suitable for publication in Nature Communications

Reviewer #3

(Remarks to the Author)

The author's have more than adequately addressed each of my critiques. The additional studies outlined in their response, both experimentally and within the body of the manuscript, make for a clearer and more complete report.

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Point by point answers to reviewers:

Reviewer #1:

1-1. The Introduction section lacks sufficient clarity regarding the well-established limitations of T cells genetically modified with T cell receptors in the treatment of solid tumors. The authors should briefly outline the key mechanisms underlying this limited efficacy, such as tumor antigen heterogeneity, immune suppressive microenvironments, and poor T cell infiltration, and then use this background to clearly articulate their central hypothesis and the rationale for their proposed strategy to overcome these barriers. Providing this context would greatly enhance the coherence and scientific relevance of the study.

We appreciate the reviewer's suggestion and have edited the introduction accordingly.

Page 3, line 57 now reads:

"Multiple barriers have been shown to contribute to this lack of efficacy: In addition to tumor heterogeneity limiting antigenic encounter and the presence of immunosuppressive populations the solid tumor microenvironment (TME) is typically rich in inhibitory cytokines, suppressive checkpoint ligands and the general lack of positive co-stimulatory signals necessary for full T cell activation. Together, these obstacles underscore the imminent need to improve T cell trafficking and function within the solid tumor TME."

Page 4, line 79 now reads:

"Using a vector backbone including a TCR that recognizes the MAGE A1 cancer testes antigen ($T_{\text{TCR-MA1}}$) with co-expression of the $CD8\alpha\beta$ co-receptor, we sought to provide co-stimulation within the TCR-associated complex for robust T cell activation independent of the presence/absence of TME-derived co-stimulatory ligands while also directing $CD4^+$ T cells to the tumor"

1-2. The presentation of the experimental findings in the Results section lacks coherence and narrative flow. In some places, the transitions between paragraphs are abrupt, and the rationale linking each experimental step is insufficiently developed. As a result, it is difficult for the reader to follow the logical progression of the study or to understand why specific experiments were performed at each stage.

We have extensively revised the manuscript to clarify the rationales leading to each successive experimental step so that the logical progression is built on the previous finding. For example:

Page 5, line 106 now reads:

"To determine the impact of $CD4^+$ and $CD8^+$ $T_{\text{TCR-MA1-CD8}\alpha\beta}$ both alone and combined, in vitro survival of the MAGE-A1 and HLA-A*0201-expressing ME275 melanoma cell line was assessed in a real-time killing assay where T cells were repeatedly challenged with tumor"

Page 6, line 116-117 now reads:

"To assess the proliferative capacity, $CD4^+$ and $CD8^+$ $T_{\text{TCR-MA1-CD8}\alpha\beta}$ were exposed once to ME275 and their divisions assessed 7 days later (Extended Fig. 3B-G)."

Page 6, line 128 now reads:

"To examine this in vivo, NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ (NSG) mice were engrafted with 1×10^6 ME275 cells (Fig. 1E)."

Page 8, line 180 now reads:

"To achieve ligand-independent signaling, we tethered positive intracellular costimulatory domains to the requisite $CD8\alpha\beta$ co-receptor chains, based on findings that TCR chain modifications

impaired function (Extended Fig. 6A-C) and prior evidence that CD8 α chain modifications can transmit activation signals.”

Page 9, line 200 now reads:

“To standardize TCR expression, constructs were first titrated in CD4⁺ T cells, selected for their lack of endogenous CD8 $\alpha\beta$ expression thus forcing the use of CCRs for class I-restricted responses, ensuring comparable transduction efficiency and transgene expression (Fig. 3B, first 3 panels to the left).”

Page 12, line 276 now reads:

“Based on studies demonstrating that modifications CARs co-stimulatory motifs can enhance function we generated T_{TCR-MA1-CD8/28} constructs with distinct CD28 intracellular domain (ICD) motif mutation combinations.”

1-3. The Discussion section largely reiterates the results without offering a focused or integrative analysis of their broader implications. It would benefit from a more critical interpretation of the findings in the context of existing literature, highlighting how this work advances the field. Moreover, key limitations, such as the reliance on a single clinical case, limited T cell persistence, and the use of immunodeficient mouse models, are not adequately addressed. A more balanced discussion acknowledging these weaknesses, along with suggestions for future directions, would strengthen the manuscript considerably.

We appreciate the reviewer’s suggestions and have revised the Discussion to acknowledge the use of immunodeficient mouse models. Regarding the single patient reported, we have clarified that the rationale to undertake the task of increasing efficacy does not rely on the patient case, rather the patient case adds a dimension, but the lack of efficacy was also evident in mouse models.

We have now included a discussion regarding that limitation and revised other parts of discussion to address broader implications.

The revised Discussion, pages 15&16, starting on lines 347 and 385 now reads:

“CD4⁺ T cells can be transduced with a Class I TCR by co-expressing the CD8 $\alpha\beta$ co-receptor thus enhancing the persistence and function of engineered CD8⁺ and CD4⁺ T cells that recognize the same tumor target when class II expression is often limited. However, both our experience in one patient-case - although hampered by limited interpretability - and stringent murine models, point to the insufficiency of this intervention alone in consistently controlling solid tumors.”

“This study relies on immunodeficient NSG models, which lack critical components of the human immune system and do not fully recapitulate the complexity of the TME. Furthermore, insufficient number of patients received T_{TCR-MA1-CD8 $\alpha\beta$} which impedes drawing meaningful conclusions of the effect of co-engaging CD4⁺ T cells towards the same Class I target. While human studies further assessing whether the T_{TCR-MA1-CD8/CD28} and T_{TCR-MA1-CD8/CD28mut} mediate similar effects in patients are being implemented, humanized mouse models may provide a reflective platform, as they replicate key constraints of the solid tumor microenvironment such as stromal barriers, immunosuppressive cells and cytokines that are likely to prevent infiltration and negatively impact the efficacy of transferred T cells.”

1-4. The authors engineered TCR-transduced CD4⁺ and CD8⁺ T cells targeting MAGE-A1/HLA-A2 and showed enhanced cytokine production and tumor cell killing in vitro, as well as antitumor activity in vivo. Based on these findings, they treated a patient with MAGE-A1 + NSCLC, demonstrating safety but no clinical efficacy. Although the authors explain the reason for the evaluation of only one case, a single-

patient case limits interpretability, and additional cases are needed to draw meaningful conclusions regarding therapeutic potential.

We appreciate the reviewer's comment regarding the limitations of a single-patient clinical case. The trial was ended after 1 patient received cells due to sponsorship withdrawal as a result of a change in corporate strategy. We are acutely aware of the limitations to drawing firm conclusions about either safety and have therefore revised our claims to clarify these points to the reader. See response to point 1-3 above regarding the changes made to the Discussion.

Additionally, we have modified the text in the Results section.

Results Section Title change. Page 7, line 137 now reads:

"CD4⁺ and CD8⁺ T_{TCR-MAI-CD8αβ} are safely infused in one patient, require potency enhancement in stringent murine models"

In the Results section, Page 7, line 147 now reads:

"Given the limitation of a single patient, this result cannot be conclusive but suggests additional measures may be needed to improve efficacy."

1-5. Fig. 1D: What is the explanation for the higher proliferative responses observed in the CD4⁺/CD8⁺ T_{TCR-MAI-CD8αβ} group? Does the "coordinated response" contribute to enhanced cytokine expression (what cytokines?), thereby promoting proliferation?

Relevant to this point and in line with **Reviewer 3 Comment 5 & 6**'s suggestion, we have updated the manuscript to characterize the increased T cell numbers observed at the conclusion of the live killing assays while intentionally avoiding attribution to either enhanced survival or increased proliferation. To address cytokine expression of CD4 alone, CD8 alone and in combination, we have now referenced our previously published manuscript¹ (Supplementary Figure S15 shown below for this Reviewer's ease): For WT1-specific T cells (also an over-expressed self-antigen), wells in which CD4 alone (T_{TCR37-45} dpCD4⁺ - same design as T_{TCR-MAI-CD8αβ} where CD4 T cells co-express CD8αβ) were exposed to supra-physiological levels of cognate peptide (10 µg/ml) had overall increased cytokine concentrations including IL-2 and cytokines associated with helper function compared to wells in which CD8 alone were exposed to peptide (eCD8⁺ - same as CD8 T_{TCR-MAI-CD8αβ} in this manuscript).

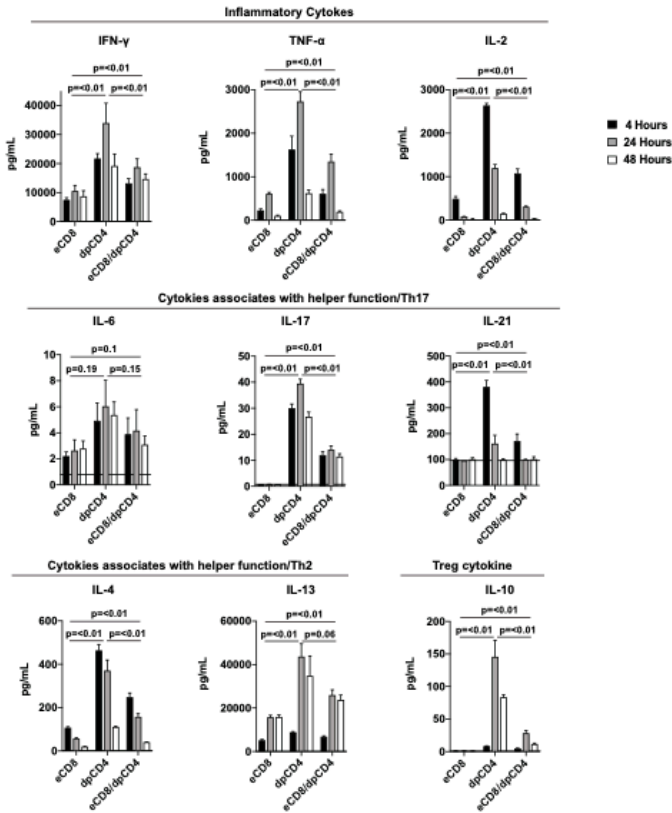
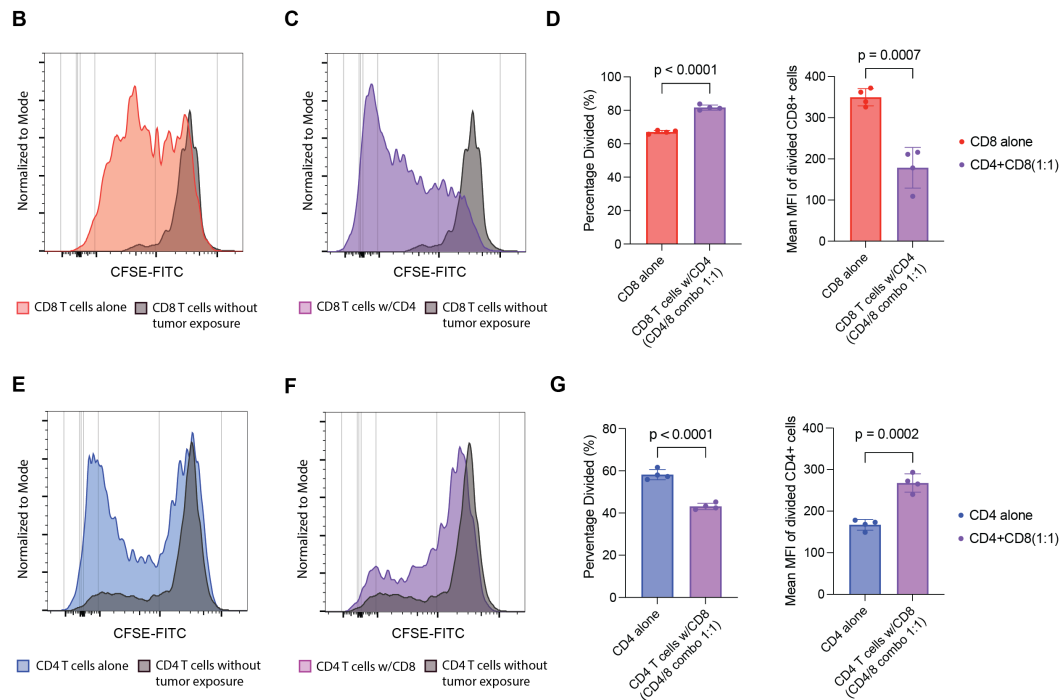


Fig. S15. Cytokine profiles of peptide stimulated eCD8⁺, dpCD4⁺ and eCD8⁺/dpCD4⁺ TCR37-45 T cells. Cytokine production by TCR37-45 T cells was measured after 4, 24, and 48 hours of stimulation with T2 cells exogenously loaded with 10μg peptide. Cytokines are found at significantly higher concentrations in cultures of dpCD4⁺ cells compared to eCD8⁺ cells and the mixture of eCD8⁺/dpCD4⁺. The mixture of eCD8⁺/dpCD4⁺ produced significantly more cytokine than eCD8⁺ cells alone. Data are presented as mean+ standard deviation (SD). Differences between groups were calculated using a student t test.

For this manuscript and the experiment shown in **Fig. 1D**, to address cell proliferation for CD4 alone, CD8 alone or each subset in combination with the other, we have now included a new **Extended Fig. 3** shown below.

The revised Result Section, Page 6, line 116 now reads:

“To assess the proliferative capacity, CD4⁺ and CD8⁺ T_{TCR-MA1-CD8αβ} were exposed once to ME275 and their divisions assessed 7 days later (Extended Fig. 3B-G). CD8⁺ T_{TCR-MA1-CD8αβ} divided more in the presence of CD4⁺ T_{TCR-MA1-CD8αβ} (Extended Fig. 3B-D), while CD4⁺ T_{TCR-MA1-CD8αβ} divided less when combined with CD8⁺ T_{TCR-MA1-CD8αβ} (Extended Fig. 3E-G). Despite the increased ability of TCR-transgenic CD4⁺ T cells co-transduced with CD8αβ to secrete helper cytokines when exposed to supraphysiological levels of peptide¹ and proliferate after tumor exposure (Extended Fig. 3E), they showed reduced tumor killing upon repeated challenge (Fig. 1C). In contrast, CD8⁺ T cells divided less after single tumor exposure but were more effective at repeated killing (Extended Fig. 3B, 3E, Fig. 1C). Together, CD4⁺ and CD8⁺ T cells sustained tumor control (Extended Fig. 3C), likely through CD4⁺-derived cytokine support that enhances CD8⁺ T cells proliferation and cytotoxicity. The reduced proliferation of CD4⁺ T cells in co-culture (Extended Fig. 3F), may reflect rapid tumor clearance by CD8⁺ T cells and/or sequestration of CD4⁺ T cell-derived cytokines.”



Extended Figure 3: CD8⁺ *T_{CR-MA1-CD8αβ}* cell enhanced proliferation with presence of CD4⁺ *T_{CR-MA1-CD8αβ}*
(A) Growth kinetics for the ME275 cell line in a live tumor visualization assay (Incucyte S3) in the absence (black line) or presence of CD8⁺ *T_{CR-MA1-CD8αβ}* (red line) or CD4⁺ + CD8⁺ *T_{CR-MA1-CD8αβ}* (purple line). The E:T ratio was 2:1, and arrows indicate the addition of tumor cells to the culture. The mean and standard deviation (SD) of triplicate wells is shown. (n=3 wells/group). **(B-G)** Co-culture of ME275 with CD4⁺ *T_{CR-MA1-CD8αβ}* (blue), CD8⁺ *T_{CR-MA1-CD8αβ}* (red), or CD4⁺ + CD8⁺ *T_{CR-MA1-CD8αβ}* (purple) at E:T ratio of 5:1 for 7 days. **(B-C)** Histogram representing cell division between CD8⁺ *T_{CR-MA1-CD8αβ}* alone without tumor stimulation (gray), CD8⁺ *T_{CR-MA1-CD8αβ}* alone (red) or CD8⁺ *T_{CR-MA1-CD8αβ}* with CD4⁺ *T_{CR-MA1-CD8αβ}* (purple) with tumor stimulation identified by CFSE proliferation dye. **(D)** Percentage divided (left panel) and mean MFI of divided cells (right) of CD8⁺ *T_{CR-MA1-CD8αβ}* alone (red) compared with CD8⁺ *T_{CR-MA1-CD8αβ}* (purple) from CD4⁺/CD8⁺ *T_{CR-MA1-CD8αβ}* combination group. The mean and standard deviation (SD) of duplicated wells from two experiment is shown (n=4). p value determined by unpaired two-tailed t-test. (n=4 wells) **(E-F)** Histogram representing cell division between CD4⁺ *T_{CR-MA1-CD8αβ}* alone without tumor stimulation (gray), CD4⁺ *T_{CR-MA1-CD8αβ}* alone (red) or CD4⁺ *T_{CR-MA1-CD8αβ}* with CD8⁺ *T_{CR-MA1-CD8αβ}* (purple) with tumor stimulation identified by CFSE proliferation dye. (n=4 wells/group). **(G)** Percentage divided (left panel) and mean MFI of divided cells (right) of CD4⁺ *T_{CR-MA1-CD8αβ}* alone (red) compared with CD4⁺ *T_{CR-MA1-CD8αβ}* (purple) from CD4⁺/CD8⁺ *T_{CR-MA1-CD8αβ}* combination group. The mean and standard deviation (SD) of duplicated wells from two experiment is shown. p value determined by unpaired two-tailed t-test. (n=4 wells/group).

Lahman, M. C. *et al.* Targeting an alternate Wilms' tumor antigen 1 peptide bypasses immunoproteasome dependency. *Sci Transl Med* **14**, eabg8070 (2022).
<https://doi.org/10.1126/scitranslmed.abg8070>

1-6. Page 7, line 120: Consider replacing “lacks tumor control” with “fails to control tumor growth” for

improved clarity and precision.

We have revised the manuscript and replaced “lacks tumor control” with “fails to control tumor growth” throughout.

Page 7, line 120 now reads:

*“Indeed, mice infused with half doses of CD4⁺ plus CD8⁺ T_{TCR-MAI-CD8αβ} (5 x 10⁶ vs. 10⁷ total cells) (**Fig. 2D**) failed to control tumor growth (**Fig. 2E**).”*

1-7. Fig. 2H–J: It is unclear whether the reported scRNA-seq data are representative of samples from multiple patients and whether the findings are reproducible. Have the authors conducted similar analyses using samples from their own patient cohort to validate the findings?

We investigated the tumor heterogeneity expression of the costimulatory ligands by analyzing publicly available single-cell RNA-seq datasets taken from patients with NSCLC (n= 42 patients) and acral or cutaneous melanoma (n=8 patients). We do not have additional patient samples from our own cohort to perform additional scRNA-seq analysis. We have now clarified the number of patients from which the scRNAseq analysis was performed.

Page 8, line 166 now reads:

“To examine the expression of both positive and negative co-stimulatory ligands across human TMEs, in silico analysis of previous published single-cell RNA sequencing (scRNA-seq) datasets from NSCLC (n=42 patients) and melanoma patients (n=8 patients)^{2,3} was performed.”

1-8. Page 7, lines 128–129: The statement “Patient tumors have limited and inconsistent expression of both positive and negative co-stimulatory binders” refers to a well-recognized limitation of CAR T cell therapy in solid tumors, unlike in hematologic malignancies, where co-stimulatory ligands are more consistently present (PMID: 24667962). For clarity, the authors should first provide a brief background on this issue to orient readers before presenting the scRNAseq findings.

We appreciate the reviewer for this helpful suggestion and have integrated a brief background well as the suggested reference.

Page-9, line 162 now reads:

*“T cells can receive co-stimulation either naturally through ligands in the TME or synthetically via immunomodulatory fusion proteins (IFPs) that couple inhibitory receptor ectodomains to costimulatory endodomains⁴. The CD28 co-stimulatory molecule was naturally expressed on T_{TCR-MAI-CD8αβ} infused in a patient (**Fig. 2A**) and in our in vivo murine models⁵ (**Extended Fig. 5A**), indicating their capacity to respond to positive co-stimulation. Unlike in hematologic malignancies, costimulatory ligands are less consistently present in solid tumors⁶. ”*

Kakarla, S. & Gottschalk, S. CAR T cells for solid tumors: armed and ready to go? *Cancer J* **20**, 151-155 (2014). <https://doi.org/10.1097/PPO.000000000000032>

1-9. Importantly, the study lacks translational evidence demonstrating the effectiveness of TCR-engineered T cells incorporating co-stimulatory signaling via a chimeric CD8/CD28 receptor in human patients with solid tumors. Including such data would greatly enhance the clinical relevance of the

findings.

Incorporating the chimeric CD8/CD28 coreceptor in future clinical trials is the planification stages (typically from the planification stages to completion of a clinical trial with associated immune monitoring at our institution can take 3-5 years) of our group and outside of the scope of this manuscript. We have now addressed this point in the Discussion.

Page 16, line 388 now reads:

“While human studies further assessing whether the $T_{TCR-MA1-CD8/CD28}$ and $T_{TCR-MA1-CD8/CD28mut}$ mediate similar effects in patients are being implemented, humanized mouse models may provide a reflective platform, as they replicate key constraints of the solid tumor microenvironment such as stromal barriers, immunosuppressive cells and cytokines that are likely to prevent infiltration and negatively impact the efficacy of transferred T cells”

Reviewer #2:

2-1. The authors should clarify the proportion of engineered CD4⁺ and CD8⁺ T cells used in the *in vivo* experiments.

In all experiments equal total T cell numbers were infused across groups. We have clarified the proportion of engineered CD4⁺ and CD8⁺ T cells used in the *in vivo* experiments updating the relevant figures and figure legends (updated **Figures 1E, 1F, 2D**) and explicitly stating “equal total number of T cell across groups” on the Figures and the proportions in the text. Updated text, figures and figure legends for **Figures 1E, F and 2D** shown below:

Pages 6, Line 128 now reads:

*“To examine this *in vivo*, NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ (NSG) mice were engrafted with 1×10^6 ME275 cells (**Fig. 1E**). CD4⁺, CD8⁺, or combined CD4⁺/CD8⁺ $T_{TCR-MA1-CD8\alpha\beta}$ (1:1 ratio, 1×10^7 total) mediated tumor control,...”*

Pages 6, Line 132 now reads:

*“In this model, CD4⁺ or CD8⁺ $T_{TCR-MA1-CD8\alpha\beta}$ alone slowed but did not prevent tumor growth while the CD4⁺/CD8⁺ combination (1:1 ratio, 1×10^7 total) had significantly reduced tumor volumes compared to controls (**Fig. 1F and G**),...”*

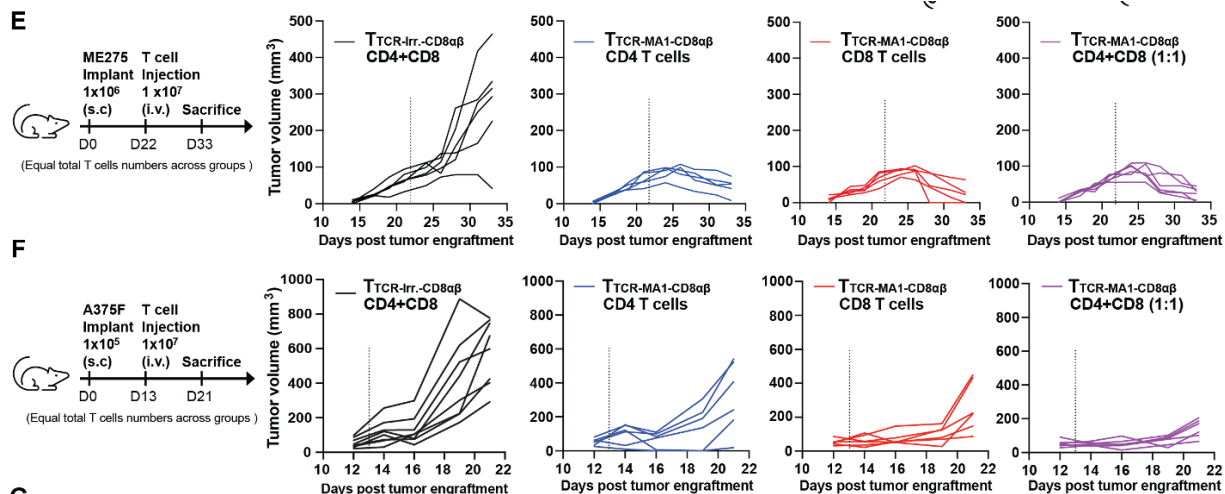


Figure 1 (E) Schematic (left) and representative growth kinetics (right) of 1×10^6 ME275 tumors engrafted subcutaneously into NSG mice with subsequent transfer of 1×10^7 equal numbers of total irrelevant T cells ($T_{Irr-CD8\alpha\beta}$), $CD4^+$ $T_{TCR-MA1-CD8\alpha\beta}$ alone, $CD8^+$ $T_{TCR-MA1-CD8\alpha\beta}$ alone, or a 1:1 ratio of combined $CD4^+$ and $CD8^+$ $T_{TCR-MA1-CD8\alpha\beta}$, 22 days after engraftment. The tumors were allowed to reach $\sim 60\text{mm}^3$ in size before T cell infusion ($n=3$ mice/group, 2 tumors/mouse). **(F)** Schematic (left) and representative growth kinetics (right) of 1×10^5 A375F tumors engrafted into NSG mice with subsequent transfer of 1×10^7 equal numbers of total T cells ($T_{Irr-CD8\alpha\beta}$, $CD4^+$ $T_{TCR-MA1-CD8\alpha\beta}$ alone, $CD8^+$ $T_{TCR-MA1-CD8\alpha\beta}$ alone, or a 1:1 ratio of combined $CD4^+$ and $CD8^+$ $T_{TCR-MA1-CD8\alpha\beta}$) 12 days after engraftment. The tumors were allowed to reach $\sim 60\text{mm}^3$ in size before T cell infusion ($n=3-4$ mice/group, 2 tumors/mouse).

Page 7, line 149 now reads:

“Indeed, mice infused with half doses of $CD4^+$ plus $CD8^+$ $T_{TCR-MA1-CD8\alpha\beta}$ (5×10^6 vs. 1×10^7 total cells) (**Fig. 2D**) failed to control tumor growth (**Fig. 2E**).”

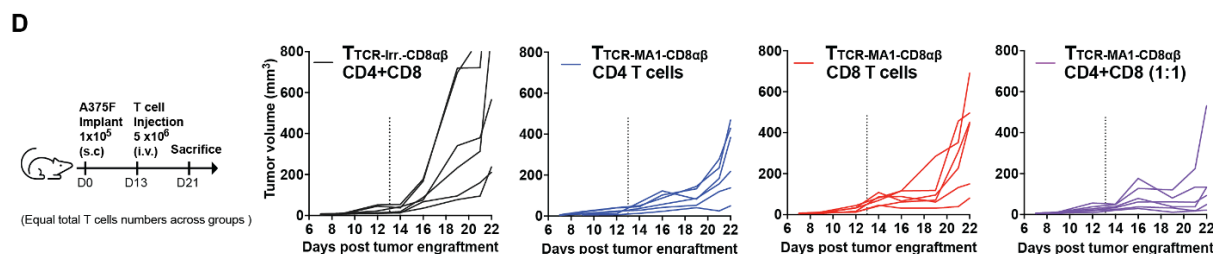


Figure 2 (D) Schematic (left) and growth kinetics (right) of 1×10^5 A375F tumors engrafted into NSG mice with subsequent transfer of 5×10^6 total T cells ($T_{Irr-CD8\alpha\beta}$, $CD4^+$ $T_{TCR-MA1-CD8\alpha\beta}$ alone, $CD8^+$ $T_{TCR-MA1-CD8\alpha\beta}$ alone, or a 1:1 ratio of combined $CD4^+$ and $CD8^+$ $T_{TCR-MA1-CD8\alpha\beta}$), 12 days after engraftment. ($n=3$ mice/group, 2 tumors/mouse, data shown are representative of three independent experiments.)

2-2. In Figure 4B, the tumor growth curves for mice treated with irrelevant TCR or MAGE-A1 TCR plus CD8αβ appear identical to those shown in Figure 2. The authors should either consolidate this data into a single figure or present an independent experiment with a new control group.

We appreciate the reviewer for pointing this out. To address the concern, we have updated **Figure 2D** to present data from a new, independent experiment. This ensures that the tumor growth curves shown in

Figures 2 and Figure 4 represent separate experimental replicates. We have updated the figure legends to state as below.

D

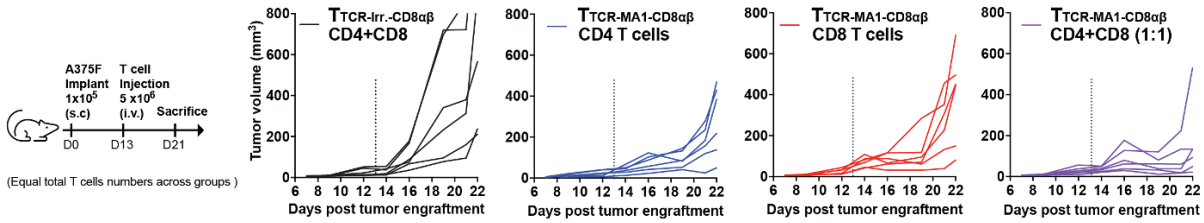


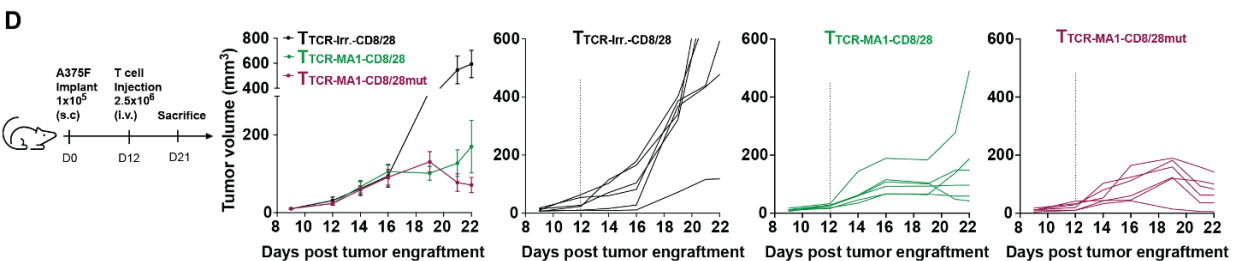
Figure 2 (D) Schematic (left) and growth kinetics (right) of 1×10^5 A375F tumors engrafted into NSG mice with subsequent transfer of 5×10^6 total T cells ($T_{Irr.-CD8\alpha\beta}$, $CD4^+$ $T_{TCR-MA1-CD8\alpha\beta}$ alone, $CD8^+$ $T_{TCR-MA1-CD8\alpha\beta}$ alone, or a 1:1 ratio of combined $CD4^+$ and $CD8^+$ $T_{TCR-MA1-CD8\alpha\beta}$), 12 days after engraftment. ($n=3$ mice/group, 2 tumors/mouse, data shown are representative of three independent experiments.)

2-3. An *in vivo* comparison of tumor growth in mice treated with MAGE-A1 TCR-engineered T cells co-expressing $CD8\beta/CD28^{WT}$ versus $CD8\beta/CD28^{Mut}$ is essential to validate the enhanced efficacy observed *in vitro*.

We agree with the review's comment. To validate the enhanced efficacy observed *in vitro*, we conducted a new *in vivo* comparison of $CD8\beta/CD28^{WT}$ versus $CD8\beta/CD28^{Mut}$ using the A375F tumor model. When administering a reduced number of T cells (2.5×10^6 instead of 5×10^6 as shown in Fig. 6C) 12 days post tumor engraftment, this approach effectively revealed differences in antitumor activity between MAGE-A1 TCR-engineered T cells co-expressing $CD8\beta/CD28^{WT}$ versus $CD8\beta/CD28^{mut}$. These results are now included in the revised manuscript (new Fig. 6D).

The Results Section, Page 13, line 291 now reads:

"However, when the total infused dose was halved (2.5×10^6 T cells), $T_{TCR-MA1-CD8/28^{mut}}$ achieved more robust tumor burden reduction relative to controls (Fig. 6D-E)."



E

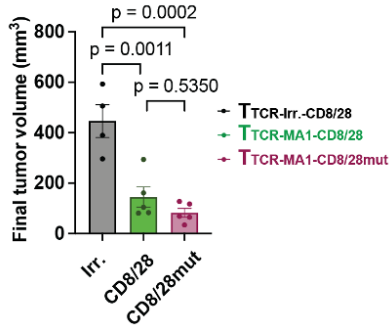


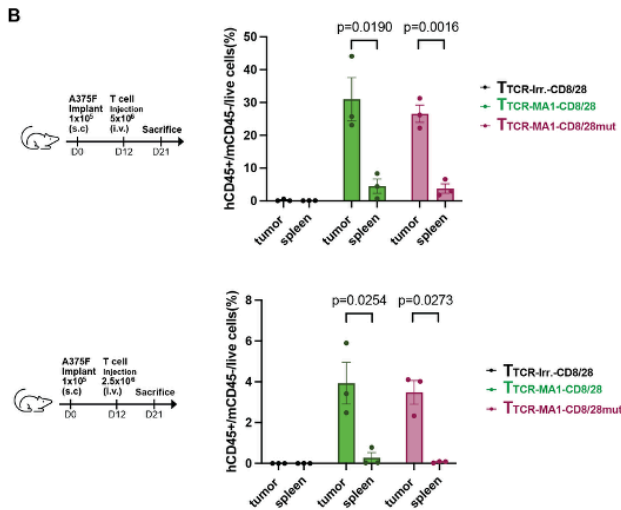
Figure 6 (D) NSG mice were engrafted with 1×10^5 A375F and subsequently infused with total 2.5×10^6 $CD4^+$ and $CD8^+$ T cells ($T_{TCR-Irr.-CD8/28}$, $T_{TCR-MA1-CD8/28}$, and $T_{TCR-MA1-CD8/28mut}$) at a ratio of 1:1 on day 12 post engraftment. (n=3mice/group, 2 tumors/mouse, data shown are representative of two independent experiments.). **(E)** A375F tumor volume assessed at sacrifice 9 days after T cell infusions for each indicated condition same as **(D)**. p value determined by one-way ANOVA with Tukey's multiple comparison test. (n=4-5 mice/group, data shown are representative of two independent experiments).

2-4. It would also be important to assess *in vivo* persistence of the transferred T cells and the overall survival of treated mice.

We thank the reviewer for this suggestion. To assess *in vivo* persistence we have now added a new **Extended Data Figure 11B** which shows T cell percentage obtained from the single cell suspension of spleens and tumors at the time of sacrifice from the experiment shown in **Figure 6C/6D**.

The revised manuscript Page 13, line 294 now reads:

“Cells preferentially localized to tumors while remaining detectable in concurrent spleens (Extended Fig. 11B).



Extended Fig. 11 (B) Schematic (left) and T cell counts in spleens and tumors (right) of A375F tumors engrafted NSG mice with subsequent transfer of 5×10^6 or 2.5×10^6 equal numbers of T cells ($T_{TCR-Irr.-CD8/28}$, $T_{TCR-MA1-CD8/28}$, $T_{TCR-MA1-CD8/28mut}$ 1:1 ratio of combined $CD4^+$ and $CD8^+$) 12 days after implantation. Spleens and tumors were collected at 8 days after T cell infusion and T cells were identified in flow cytometry

analysis. The mean and standard error of mean (SEM), p values determined with unpaired two-tailed t -test. ($n=3$ mice per group).

To address survival of the treated we added a new **Figure 6G** showing a murine model with the SK-MEL37 melanoma tumor cell line, which also addresses reviewer #2 Comment 3 regarding CD8 β /CD28WT versus CD8 β /CD28Mut *in vivo*. In this model, CD8 $\alpha\beta$ /CD28Mut presented superior tumor control compared to CD8 $\alpha\beta$ /CD28WT *in vivo* 60 days after T cell transfer (new **Figure 6G**):

The revised manuscript Page 13, line 296 now reads:

“ $T_{\text{TCR-MA1-CD8/28mut}}$ also suppressed growth of the cell death-resistant HLA-A2⁺ MAGE-A1⁺ SK-MEL-37 melanoma cell line *in vitro* (Extended Fig. 2, Fig. 6F),⁷ and mediated superior tumor reduction *in vivo* at 60 days compared to $T_{\text{TCR-MA1-CD8/28}}$ (Fig 6G). Thus, $T_{\text{TCR-MA1-CD8/28mut}}$ conferred modest but consistent improvements in both short- (10 days) and long-term (60 days) tumor control, potentially due to an increased proliferative capacity.”

G

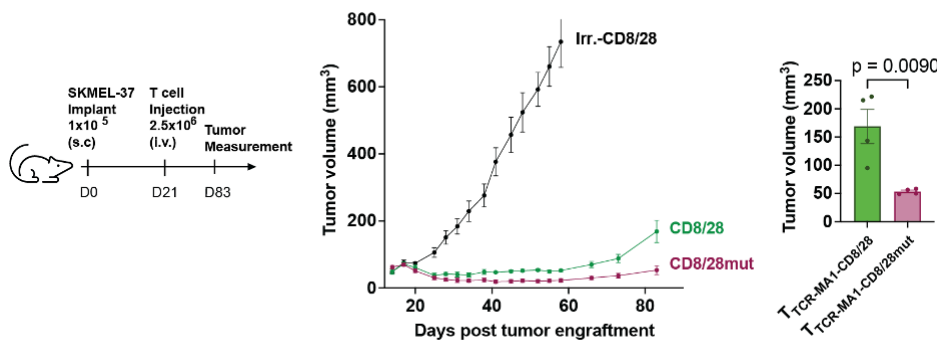


Figure 6 (G) Non-humanized MISTRG mice were engrafted with 1×10^6 SK-MEL-37 (left) and subsequently infused with total 2×10^7 CD4⁺ and CD8⁺ T cells ($T_{\text{TCR-Irr-CD8/28}}$, $T_{\text{TCR-MA1-CD8/CD28}}$, and $T_{\text{TCR-MA1-CD8/CD28mut}}$) at a ratio of 1:1 on day 21 post engraftment ($n=4$ mice, 2 tumors per mouse). SK-MEL-37 tumor volume (right) assessed at sacrifice 62 days after T cell infusions for each indicated condition. p value determined by two-tailed unpaired t -test. ($n=4$ mice/group, 2 tumors/mouse averaged).

Reviewer #3:

3-1. Abstract: The abstract was not clearly written and led to some confusion especially as it relates to describing CD4 T cells. A clearer and more concise description of describing their strategy to engineer CD8 $\alpha\beta$ into CD4 T cells to improve TCR-T cell would be helpful.

We appreciate the reviewer’s suggestion and have re-written the abstract for conciseness and to clarify the CD4 strategy.

The revised abstract (Page 2, lines 35-50) now reads:

“Adoptive transfer of T cells engineered with tumor-specific T cell receptors (TCR-T) has shown limited efficacy in solid tumors, hindered by insufficient persistence, tumor trafficking, and dependence on tumor-associated co-stimulatory ligands. In one clinical case and NSG murine models, infusion of CD4⁺ and CD8⁺ T cells co-expressing a class I MAGE-A1-specific TCR and CD8 $\alpha\beta$ failed to control tumor

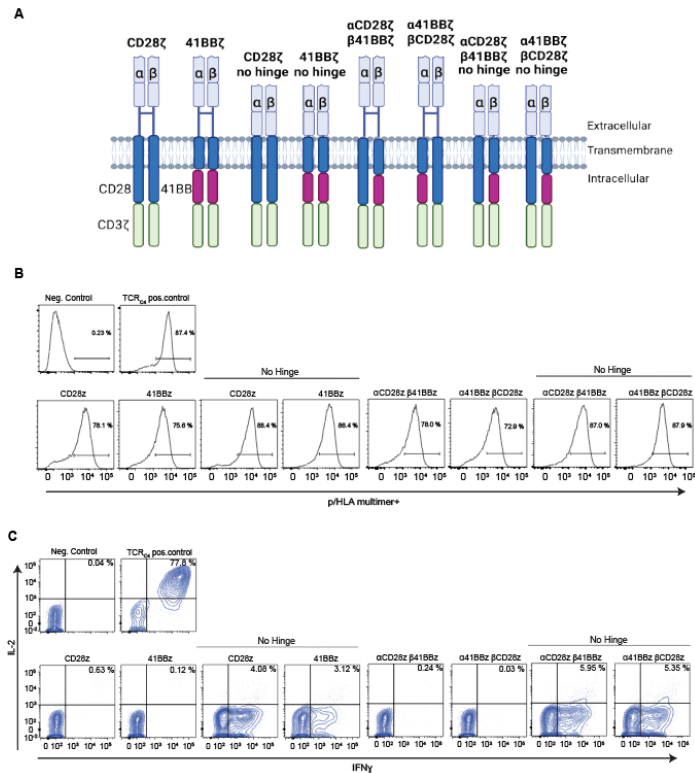
progression. To enhance function downstream of TCR signaling, we explored the adaptability of intracellular TCR components to synthetic modification. By leveraging the obligate co-expression of CD8 $\alpha\beta$ required for CD4⁺ T cells engagement, the CD8 β chain provided a tractable site for engineering without loss of function. In vitro screening of CD4⁺ T cells identified CD28 intracellular tail as the most effective co-stimulatory signal. The synthetic chimeric co-receptor (CD8/CD28) enhanced cytokine production, persistence, and tumor control in immunodeficient murine models with preservation of stem-like transcriptional programs. Rational modification of CD28 cytoplasmic motifs further augmented in vivo tumor control associated with increased intratumoral T cell proliferation and reduced expression of exhaustion-associated genes. These benefits extended in vitro to PRAME- and WT1-specific TCRs, suggesting generalizability. Our findings establish a framework for integrating synthetic co-stimulation directly into the TCR complex independent of co-stimulatory ligand expression.”

3-2. Introduction: A point for clarity. The authors state that “modifying the intracellular TCR chain domains decreased TCR function, likely by blocking the initiation of TCR signaling”. However, the publication they cited used a single-chain TCR that wouldn’t engage TCR signaling in the first place as such their rationale seems unfounded.

We thank the reviewer for this insightful comment. We have removed the reference and the related statement in the introduction and have now supplemented the manuscript with new experimental data (new **Extended Fig. 6A-C**) showing that in the case of a TCR specific a similarly over-expressed self-antigen (WT1), modification of the intracellular TCR signaling domain directly impairs TCR function. These findings are now included in the revised Results section.

The Results section (Page 8, lines 180-183) now reads:

*“To achieve ligand-independent signaling, we tethered positive intracellular costimulatory domains to the requisite CD8 $\alpha\beta$ co-receptor chains, based on findings that TCR chain modifications impaired function (**Extended Fig. 6A-C**) and prior evidence that CD8 α chain modifications can transmit activation signal.”*



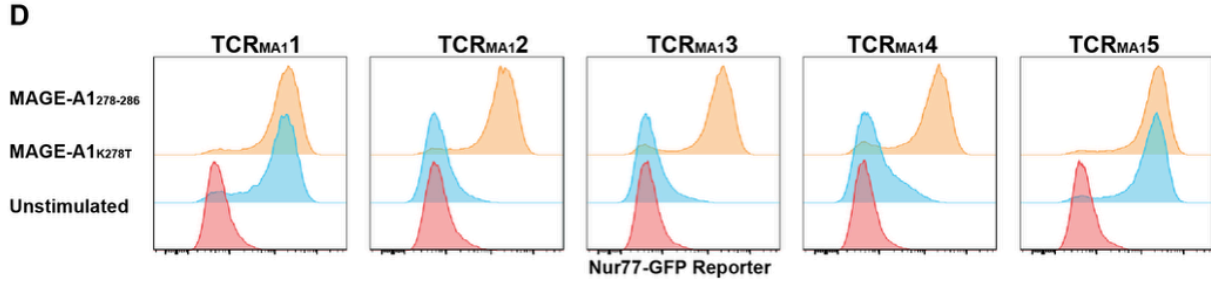
Extended Data Fig. 6. TCR signaling dampened upon TCR intracellular modification. (A) Schematic of construct design; (B) Chimeric TCRs are expressed on the cell surface identified by p/HLA multimer. CD8⁺ T cells were transduced with the modified TCR_{MA15} constructs and p/HLA multimer binding was analyzed by flow cytometry. (C) TCR transduced CD8⁺ T cells were stimulated with cognate peptide at 1 μM and IL-2 and IFN γ levels were analyzed by flow cytometry.

3-3. Figure 1: What is the significance of the K278T mutation in MAGE-A1 peptide for selecting TCR_{MA15} as TCR of interest? How does this relate to the modifications made to the T cell. Is this a higher affinity version? If so, would one want to use a lower affinity version to demonstrate the ability to alter the TCR activation threshold with said modifications?

We appreciate the reviewer's request for clarification regarding the significance of the K278T mutation in the MAGE-A1 peptide and the rationale for selecting TCR_{MA15}. First, we noted that the legend to **Extended Figure 1D** was erroneous and led to believe that TCR modifications were made (not the case in this figure) rather than the TCR recognizing a variant of the MAGE peptide. This is now corrected in the manuscript and shown below.

The revised manuscript Page 5, line 95 now reads:

"TCR_{MA15} had a similar functional avidity towards the wild-type peptide compared to TCR_{MA12}, 3 and 4 (range 48.1-85.8 nM) (**Extended Fig. 1C**), and also recognized the previously described K278T mutated MAGE-A1₂₇₈₋₂₈₆ peptide (**Extended Fig. 1D**). Based on these characteristics, TCR_{MA15} was selected for future studies."



“Extended Data Fig. 1. (D) Recognition of wildtype MAGE-A1₂₇₈₋₈₆ peptide and MAGE-A1₂₇₈₋₈₆ K278T mutant peptide [loaded on TAP-deficient T2 B lymphoblastoid cell lines (T2-BLCL)] by the TCR-deficient Nur-77 reporter Jurkat cells transduced TCR_{MA1}1-5. Histograms represent GFP expression downstream of Nur77-GFP triggered when the TCR is engaged”.

A new section was added in the Methods: Page 18 line 438 now reads:

“Selection of the MA1-specific TCR for clinical translation

“While other somatic mutations exist within the MAGE-A1 gene, only the K278T missense mutation falls within the targeted epitope (MAGE-A1₍₂₇₈₋₂₈₆₎). The K278T mutation has been identified in publicly available datasets and is including in the breast cancer cell line HCC1008⁸. Of the 5 isolated TCRs and to choose one for clinical translation, we proceeded by first excluding the least avid TCR_{MA1}1 (mean EC50 of 134 nM). The remaining 4 TCRs had very similar functional avidity (ranging from 48.1-85.8 nM). TCR_{MA1}5 was chosen based on its ability to recognize the wild-type and mutated peptide, thus broadening the targeted epitope to include the K278T mutation. All 5 TCRs (TCR_{MA1}1-5) were isolated from healthy HLA-A2+ donors. To avoid safety concerns, none were modified or affinity enhanced.”

3-4. Figure 1D: It is not entirely clear whether the proliferation was determined via incucyte or a separate stimulation. It will be helpful offer a clear description of how they enumerated the CD4 vs CD8 T cells.

CD4⁺ and CD8⁺ T cell counts shown in **Figure 1D** were analyzed from the live tumor killing assay shown in **Figure 1C** at the 144-hour timepoint. Cell counts were determined by counting beads via flow cytometry. The Results and Methods were revised to clarify this point.

The Results Section Page 5, line 109 now reads:

“Although CD8⁺ T_{TCR-MA1-CD8αβ} remained superior at repeated tumor killing compared to CD4⁺ T_{TCR-MA1-CD8αβ}, the 1:1 combination of CD4⁺ and CD8⁺ T_{TCR-MA1-CD8αβ} (with each subset contributing half of the total cell number) significantly outperformed an equal total number of CD8⁺-only or CD4⁺-only T_{TCR-MA1-CD8αβ} at 144 hours and 192 hours (Fig. 1C, right panel). At 144 hours, CD4⁺ and CD8⁺ T_{TCR-MA1-CD8αβ} remaining in wells within the combination group were ~30x more abundant whereas CD4⁺ and CD8⁺ T_{TCR-MA1-CD8αβ} alone were ~3x more abundant (Fig. 1D).”

The Methods Section Page 21, line 509 now reads:

“Analysis of T cell counts in Real-time killing assays: At indicated timepoints remaining T cells in the wells were counted via flow cytometry with count bright counting beads (Invitrogen).”

3-5. Figure 1D: Were only T cells seeded the same on their own as combined or reduced to half for their

1:1 (or are they doubling the total number of T cells in the combination group)? Could this affect fold-change numbers when determining expansion? One could assume reduced numbers but as written this is point is ambiguous.

We thank the reviewer for this clarification request which was also a concern for **Reviewer 2**. We confirm that throughout the manuscript for each experiment where the combination of CD8 and CD4 is compared to each subset alone, the total cell remains the same. We have revised the relevant figures to avoid any ambiguity. Additionally, the measure of CD4 and CD8 fold change in alone or combined conditions compared to input is now clarified in **Figure 1D**.

The Results Section Page 5, line 109 now reads:

“Although CD8⁺ T_{TCR-MA1-CD8αβ} remained superior at repeated tumor killing compared to CD4⁺ T_{TCR-MA1-CD8αβ}, the 1:1 combination of CD4⁺ and CD8⁺ T_{TCR-MA1-CD8αβ} (with each subset contributing half of the total cell number) significantly outperformed an equal total number of CD8⁺-only or CD4⁺-only T_{TCR-MA1-CD8αβ} at 144 hours and 192 hours (Fig. 1C, right panel). At 144 hours, CD4⁺ and CD8⁺ T_{TCR-MA1-CD8αβ} within the combination group were ~30x more abundant whereas CD4⁺ and CD8⁺ T_{TCR-MA1-CD8αβ} alone were ~3x more abundant (Fig. 1D).”

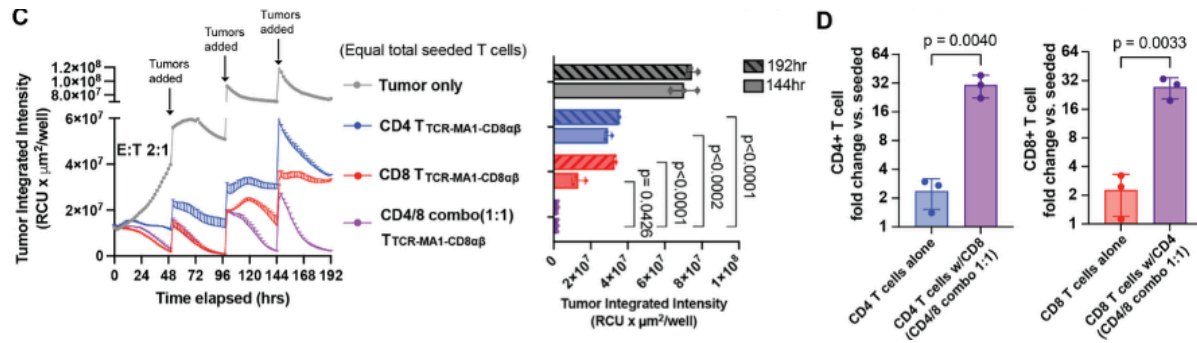


Figure 1 (C) Growth kinetics (left panel) for the ME275 cell line in a live tumor visualization assay (Incucyte S3) in the absence (gray line) or presence of CD4⁺ T_{TCR-MA1-CD8αβ} (blue line), CD8⁺ T_{TCR-MA1-CD8αβ} (red line), or CD4⁺ + CD8⁺ T_{TCR-MA1-CD8αβ} (purple line). The E:T ratio was 2:1, and arrows indicate the addition of tumor cells to the culture. Final tumor integrated intensity (right panel) for the same experiment at 144 and 192 hours. The mean and standard deviation (SD) of triplicate wells is shown. Data were analyzed using a one-way ANOVA with Tukey's multiple comparison test. (n=3 wells/group). (D) Counts of survived T cells were measured at 144 hours post tumor simulation from Incucyte S3 live tumor assay (C). Change in absolute numbers of CD4⁺ T_{TCR-MA1-CD8αβ} (left panel) and CD8⁺ T_{TCR-MA1-CD8αβ} (right panel) in either alone (CD4 - blue bars, CD8 - red bars) or combined (purple bars) conditions compared to seeded cells. The mean ± SD from triplicated wells is shown (n=3 wells/group). p value determined by unpaired two-tailed t-test.

3-6. Figure 1D Measuring T cell numbers over 6 days seems too brief. I suggest extending this time to a couple of weeks (at least). How was survival impacted? Please consider using a proliferation dye to visualize the extent of cell division (vs increased numbers due to cell survival).

We have modified/added 3 additional figures. 1) The measurements for intensity of tumor for the live killing assay shown in **Figure 1C, left panel** was extended from 144 hours (6 days) to 192 hours (~8 days) and statistical analysis for both the 144 and 192 hours timepoints added to **Figure 1C, right panel**. 2) We added a new **Extended Figure 3A** with the combination group continuing to show tumor control at 13 days. 3) We added a new **Extended Figure 3B** showing proliferation (using a proliferation dye) of

CD8⁺ alone, CD4⁺ alone and each subset combined (see response to **Reviewer 1, Comment 5** for specifics).

The revised Results Section Page 5, line 110 now reads:

“.....the 1:1 combination of CD4⁺ and CD8⁺ T_{TCR-MA1-CD8αβ} (with each subset contributing half of the total cell number) significantly outperformed an equal total number of CD8⁺-only or CD4⁺-only T_{TCR-MA1-CD8αβ} at 144 hours and 192 hours (Fig. 1C, right panel). At 144 hours, CD4⁺ and CD8⁺ T_{TCR-MA1-CD8αβ} within the combination group were ~30x more abundant whereas CD4⁺ and CD8⁺ T_{TCR-MA1-CD8αβ} alone were ~3x more abundant (Fig. 1D). CD4⁺ and CD8⁺ T_{TCR-MA1-CD8αβ} combination group continued to control tumor for 13 days (Extended Fig. 3A).”

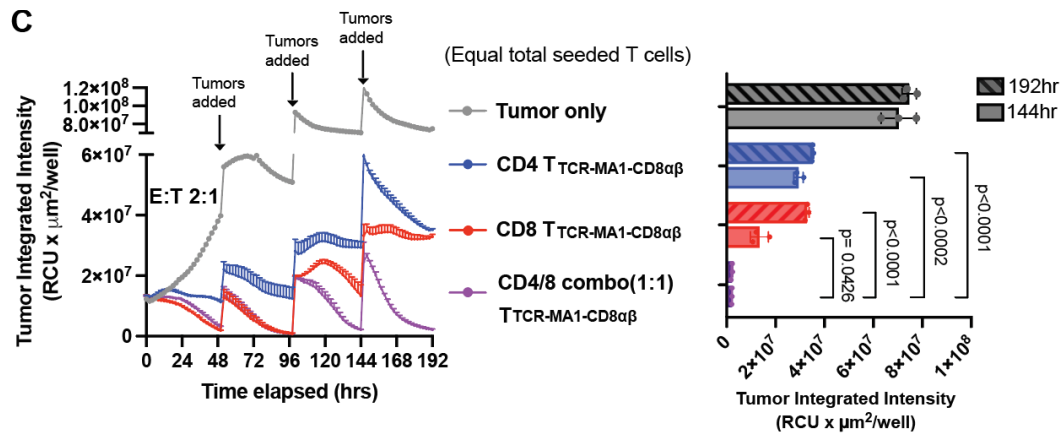
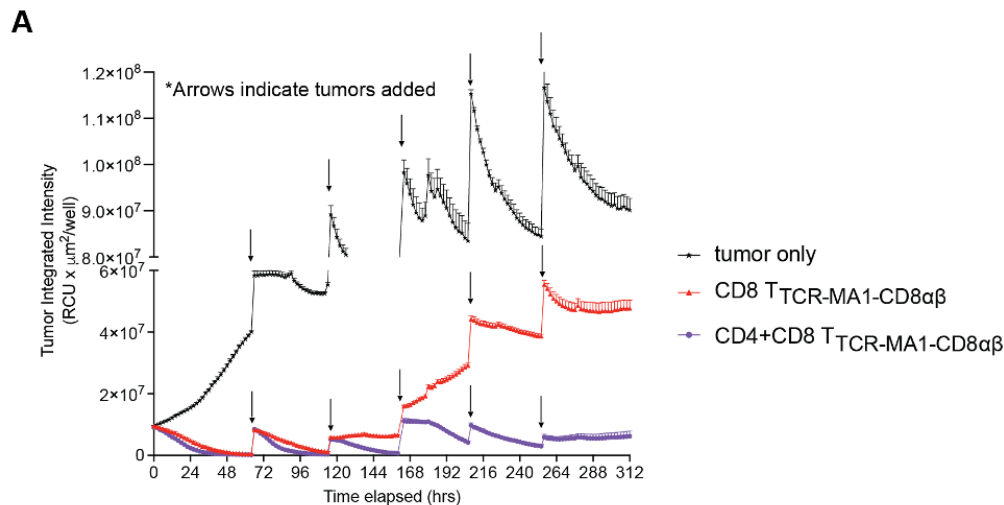


Figure 1. (C) Growth kinetics (left panel) for the ME275 cell line in a live tumor visualization assay (Incucyte S3) in the absence (gray line) or presence of CD4⁺ T_{TCR-MA1-CD8αβ} (blue line), CD8⁺ T_{TCR-MA1-CD8αβ} (red line), or CD4⁺ + CD8⁺ T_{TCR-MA1-CD8αβ} (purple line). The E:T ratio was 2:1, and arrows indicate the addition of tumor cells to the culture. Final tumor integrated intensity (right panel) for the same experiment at 144 and 192 hours. The mean and standard deviation (SD) of triplicate wells is shown. Data were analyzed using a one-way ANOVA with Tukey's multiple comparison test. (n=3 wells/group).



Extended Fig. 3. (A) Growth kinetics for the ME275 cell line in a live tumor visualization assay for 312 hours(13 days) (Incucyte S3) in the absence (black line) or presence of equal total numbers of seeded CD8⁺

$T_{TCR-MA1-CD8\alpha\beta}$ (red line) or $CD4^+ CD8^+ T_{TCR-MA1-CD8\alpha\beta}$ (purple line). The E:T ratio was 2:1, and arrows indicate the addition of tumor cells to the culture. The mean and standard deviation (SD) of triplicate wells is shown. (n=3 wells/group).

3-7. Figure 1E: The numbers for the CD4 only and CD8 only treatments vs combined is ambiguous here too.

We thank the reviewer for pointing this out and have clarified the total cell infused into mice throughout the manuscript. Please see answer to **Reviewer 2 Comment 1**.

3-8. Figure 2F:

- PD-1 and TIM-3 aren't sufficient markers to determine differences in exhaustion between single and combined T cell treatment. Suggest looking at transcriptional markers of exhaustion, i.e. TOX.
- The most definitive method to test for exhaustion is to conduct an ex vivo stimulation and assess functional activity.

We appreciate the reviewer's suggestion regarding a more comprehensive assessment of T cell exhaustion. **1)** To provide a more nuanced view of the expression of markers associated with an exhausted phenotype, we have added the expression of CD39 and 2B4 alongside PD-1 and TIM-3 in the new **Figures 2F and Extended Fig. 4B**. **2)** Intratumoral $T_{TCR-MA1-CD8\alpha\beta}$ from $CD4^+$ alone, $CD8^+$ alone and $CD4^+ & CD8^+$ combined groups were further ex vivo stimulated with peptide and their function assessed. The results are shown in a new **Figure 2G**. Of note, similar revisions were performed in new **Figure 4 G and H** addressed in Reviewer 3 Comment 13.

The revised Results Section Page 7, line 149 now reads:

"Indeed, mice infused with half doses of $CD4^+$ plus $CD8^+ T_{TCR-MA1-CD8\alpha\beta}$ (5×10^6 vs. 1×10^7 total cells) (Fig. 2D) failed to control tumor growth (Fig. 2E). In the combined infusion group, only intratumoral $CD4^+$ exhibited reduced expression of markers exhaustion-associated markers (Tim3, PD-1, CD39, and 2B4), compared to either subset infused alone (Extended Fig. 4B, Fig. 2F), yet all $T_{TCR-MA1-CD8\alpha\beta}$ subsets - either alone or combined - produced <10% median IFN γ and TNF α upon cognate peptide exposure (Fig. 2G).

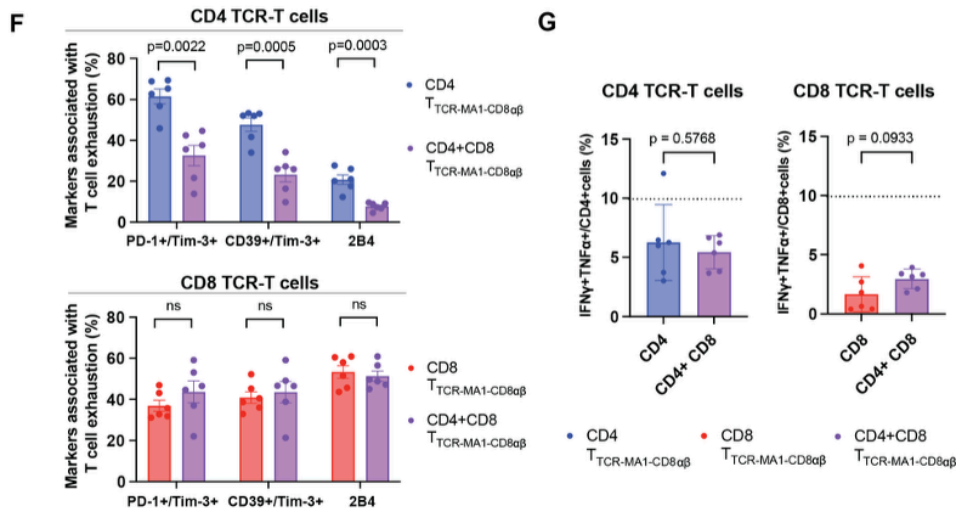
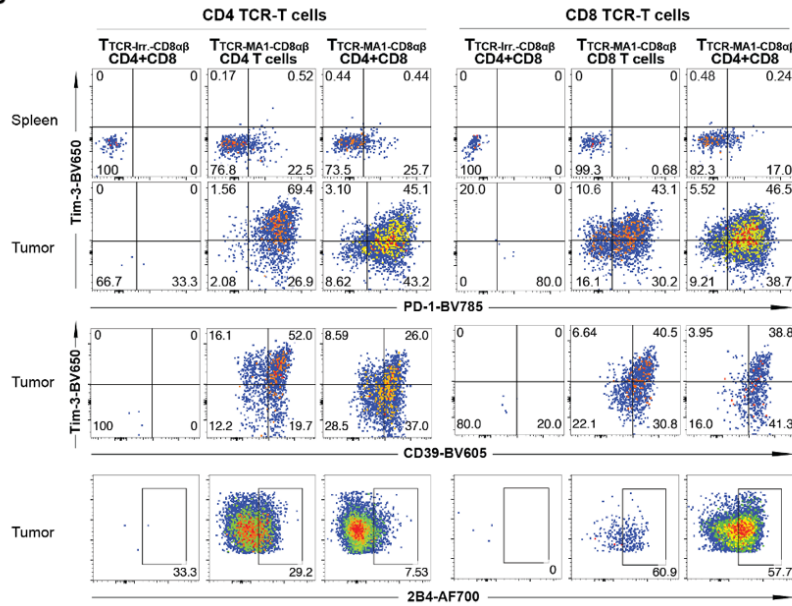


Figure 2. (F) Quantification of $Tim3^+/PD-1^+$, $Tim3^+/CD39^+$ and $2B4^+$ binding across the experimental groups from (D) in $CD4^+$ (top panel) and $CD8^+$ (bottom panel) $T_{TCR-MA1-CD8\alpha\beta}$ for the indicated conditions. Graph represents mean $\pm$ SD. p values determined by unpaired two-tailed t-test. (n=3 mice, 6 tumors/group, 2 tumors/mouse). **(G)** Quantification of $IFN\gamma^+ / TNF\alpha^+$ binding after $1\mu M$ cognate peptide stimulation for 18 hours ex vivo across the same experimental groups from (D) in $CD4^+$ (left) and $CD8^+$ (right) $T_{TCR-MA1-CD8\alpha\beta}$ for the indicated conditions. Single cell suspensions were obtained at sacrifice, 6 days after T cell infusion. Graph represents mean $\pm$ SD. p values determined by unpaired two-tailed t-test. (n=3 mice/group, 2 tumors/mouse).

B



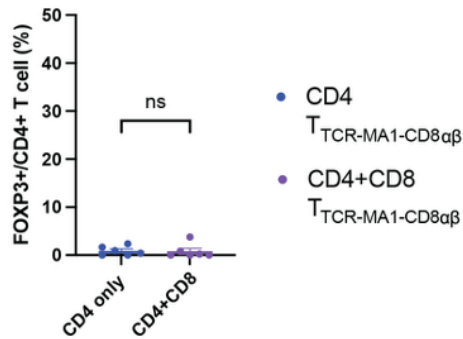
Extended Data Fig.4 (B) Co-expression of PD-1 (x-axis) and Tim-3 (y-axis) in $CD4^+$ (left 3 panels) and $CD8^+$ (right 3 panels) T cells identified in spleen (top panels) or tumor (second row panels) for the indicated conditions. Co-expression of CD39 $^+$ (x-axis) and Tim-3 $^+$ (y-axis) (third row panels) and expression of $2B4^+$ (fourth row panels) in $CD4^+$ $T_{TCR-MA1-CD8\alpha\beta}$ (left 3 panels) and $CD8^+$ $T_{TCR-MA1-CD8\alpha\beta}$ (right 3 panels). Single cell suspensions were obtained at sacrifice, 8 days after T cell infusion from (Fig. 2D).

3-9. Figure 2: Are there differences in the frequencies of Tregs in the $CD4$ only vs combined $CD4+CD8$ treatments

We have now included this analysis in **Extended Fig. 4C**. Using immunohistochemistry on formalin-fixed, paraffin-embedded tumors from the in vivo experiment shown in **Fig. 2D**, we assessed FOXP3 expression in $CD4^+$ $T_{TCR-MA1-CD8\alpha\beta}$ cells. Although studies have found infused CAR T cells become Tregs^{9,10}, we found no evidence infused $CD4$ T cells became Tregs (**Extended Fig. 4C**).

The revised the manuscript page 7, line 154 now reads:

“There were no Treg populations (FOXP3 $^+$) found in $CD4^+$ $T_{TCR-MA1-CD8\alpha\beta}$ alone and $CD4^+$ / $CD8^+$ combined group (**Extended Fig 4C**).”



Extended Figure 4 (C) Immunohistochemistry analysis of FOXP3 intranuclear expression in $CD4^+$ $T_{TCR-MA1-CD8\alpha\beta}$ between the $CD4^+$ alone and the $CD4^+$ + $CD8^+$ combination (1:1 ratio) groups from (Fig. 2D). The mean and $\pm$ SD of 6 tumors is shown ($n=6$). p value determined by unpaired two-tailed t -test. ($n=3$ mice, 2 tumors/mouse).

3-10: Figure 3E: Authors claim enhanced proliferation just by obtaining the T cell counts at the end of all of their stimulations, but this method leaves too much room for misinterpretation.

Relevant to this point and in line with **Reviewer 1 Comment 5's** suggestion and **Reviewer 3 Comment 6**, we have updated language throughout the manuscript to only characterize the increased T cell numbers compared to input observed at the conclusion of the live killing assays while intentionally avoiding attribution to either enhanced survival or increased proliferation.

The Results section Page 9, line 208 now reads:

“Among these, the CD8/CD28 co-receptor (CD8/28) most effectively restrained ME275 tumor growth in repeated killing assays (Fig. 3E, left and middle) and yielded greater live total remaining T cells in wells at 384 hours (16 days) (Fig. 3E, right).”

3-11: Figure 3B: it looks like there is less p/HLA multimer staining cells than the other CCR groups. Please clarify how you normalize for this because it doesn't look like they stain for the multimer after the stimulation. Does this mean the CD8 $\alpha\beta$ only T cells would have similar cytokine expression if TCR expression was equal across groups in Figure 3C. The data shown actually look like the trend in $IFN\gamma^+$ cells correlates pretty well with TCR expression.

We thank the reviewer for the opportunity to clarify this point. T cells were transduced using titrated lentiviral constructs to ensure similar transduction efficiency and vector copy numbers. After transduction and to standardize TCR expression across groups, transduced T cells were further FACS sorted on p/HLA+ cells and expanded using a rapid expansion protocol (REP). We have now revised **Figure 3B** (shown below) to show the MFI of p/HLA multimer binding across groups after FACS sorting and expansion.

The revised Results Section Page 9 199 now reads:

“To standardize TCR expression, constructs were first titrated in $CD4^+$ T cells, selected for their lack of endogenous $CD8\alpha\beta$ expression thus forcing the use of CCRs for class I-restricted responses, ensuring comparable transduction efficiency and transgene expression (Fig. 3B, first 3 panels to the left). Transduced T cells were then sorted on similar p/HLA+ binding and expanded (Fig. 3B, right panel).”

B

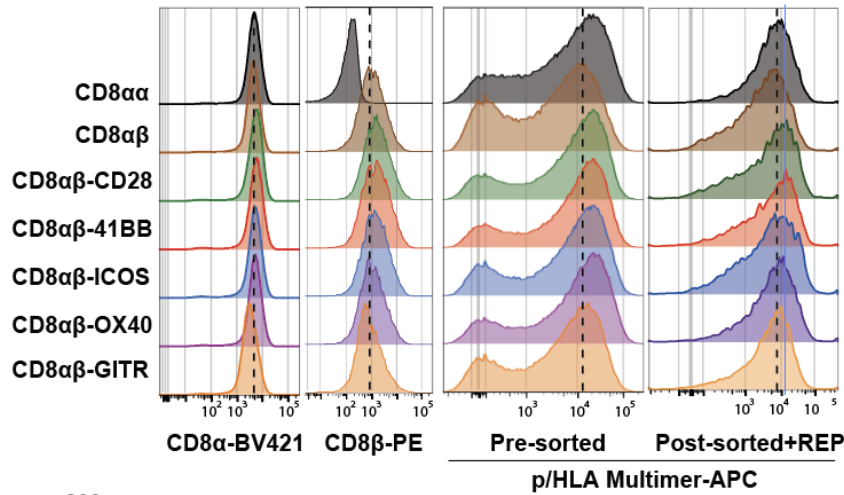


Figure 3 (B) Histogram of CD8α (left), CD8β (middle) and MAGE-A1 p/HLA multimer (right) binding of CD4⁺ transduced to express $T_{TCR-MA1}$ with the indicated CCRs after transduction for equal transduction copy numbers and post-REP (rapid expansion protocol) p/HLA multimer binding.

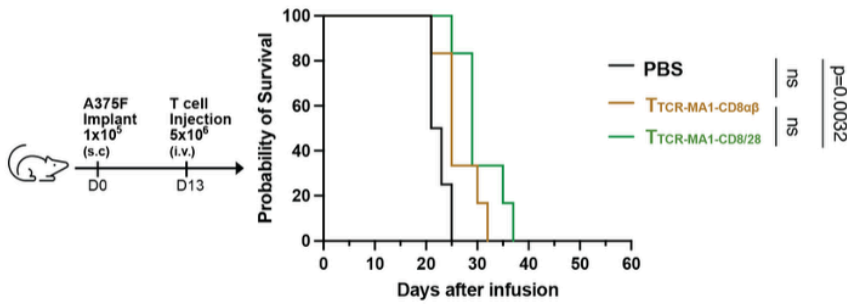
3-12. Figure 4A: please provide a rationale as to T cells are injected 6 days earlier here than they do for the rest of their experiments? At this time point, the tumors do not look like they would be at a palpable size.

The experiment in now revised **Figure 4A** (injection of T cells at day 12 or 13) allowed the assessment of the interaction between tumor and T cells but only produced a modest prolongation of murine overall survival (new **Extended Figure 8D**). However, when the T cell injections were administered 6 days after inoculation (**Figure 4I**, previously **Figure 4A**, with tumor sizes at inoculation now shown in the new **Extended Figure 8E**, the differences between CD8αβ and CD8/28 were evident.

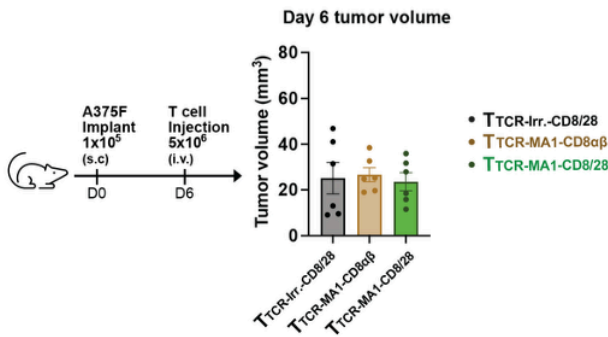
The revised Results Section Page 10, lines 229 now reads:

*“While the survival of all mice infused with $T_{TCR-MA1-CD8/28}$ on day 13 after A375F engraftment was only modestly prolonged compared to controls ($T_{TCR-IT-CD8/28}$) (**Extended Fig. 8D**), when administered on day 6 into mice with measurable tumors (**Extended Fig. 8E**), $T_{TCR-MA1-CD8/28}$ significantly extended survival compared with $T_{TCR-MA1-CD8αβ}$ (**Fig. 4I**).”*

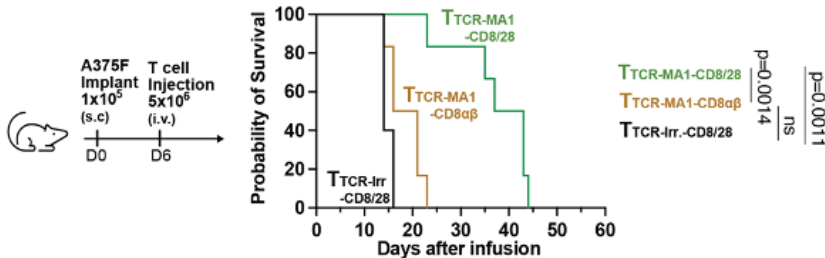
C



D



I



Kaplan-Meier test for analysis of survival applied. p values determined by a log-rank test. (n=6 mice/group).

3-13. Figure 4H: point 1 - again, consider expanding the markers you use to characterize exhaustion.

Similar to the changes made to **Figure 2D**, we expanded the characterization of T cells in **Figures 4G** and **Extended Figure 8B** to include the co-expression of additional markers associated with exhaustion (CD39 with TIM-3, and PD-1 with TIGIT) and added a new **Figure 4H** assessing the function of intratumoral CD4⁺ and CD8⁺ T_{TCR-MA1-CD8/28} compared to T_{TCR-MA1-CD8αβ}.

The revised Results section Page 10, Line 222 now reads:

“T_{TCR-MA1-CD8/28} cells showed reduced expression of surface markers associated with exhaustion (PD1⁺/Tim3⁺, CD39⁺/Tim3⁺, and PD1⁺/TIGIT⁺) (Fig. 4G, Extended Fig. 8C) and produced significantly more cytokines upon cognate peptide exposure ex vivo (Fig. 4H).”

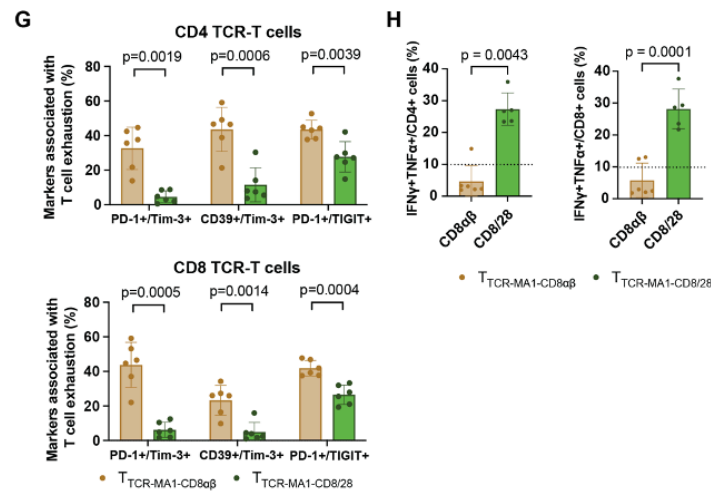
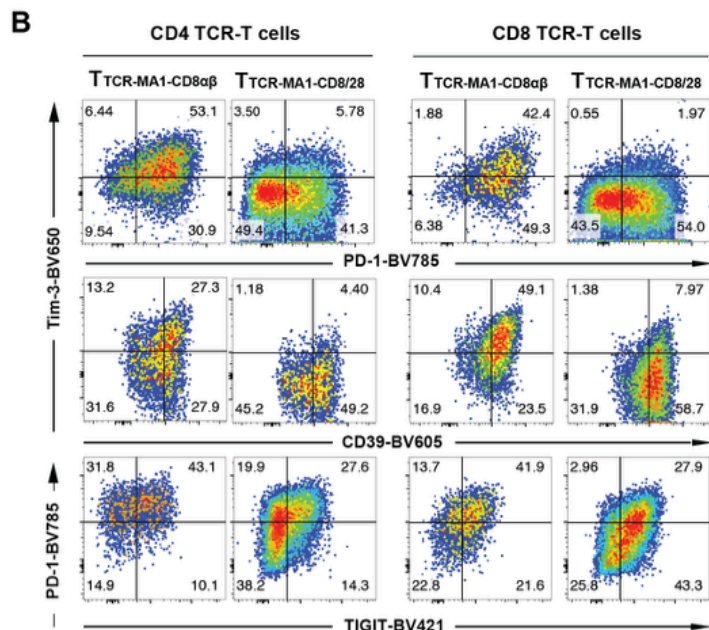


Figure 4 (G) Bar graph showing PD-1⁺/Tim3⁺, CD39⁺/Tim3⁺, and PD-1⁺/TIGIT⁺ frequency of CD4⁺ and CD8⁺ TILs between T_{TCR-MA1-CD8αβ} and T_{TCR-MA1-CD8/28}. Single cell suspensions were obtained at sacrifice, 8 days after T cell infusion from (same experiment design as A). p value determined by unpaired two-tailed t-test. (n=3 mice, 6 tumors/group, 2 tumors/mouse).

(H) Quantification of IFNγ⁺/TNFα⁺ populations after 1μM cognate peptide stimulation for 18 hours ex vivo across the same experimental groups from (A) in CD4⁺ (left panel) and CD8⁺ (right panel) T_{TCR-MA1-CD8αβ} for the indicated conditions. Single cell suspensions were obtained at sacrifice, 6 days after T cell infusion. The mean ± SD of 6 tumors per group is shown. Unpaired two-tailed t-test. (n=3 mice, 5-6 tumors/group, 2 tumors/mouse).



Extended Data Fig. 8 (B) Representative flow plot showing co-expression of PD-1 (x-axis) and Tim-3 (y-axis) (top row panels), co-expression of CD39 (x-axis) and Tim-3 (y-axis) (middle row panels), and co-expression of TIGIT (x-axis) and PD-1 (y-axis) (bottom row panels) in CD4⁺ (left 2 panels) and CD8⁺ (right 2 panels) TILs identified in tumor between $T_{TCR-MA1-CD8\alpha\beta}$ and $T_{TCR-MA1-CD8/CD28}$. Single cell suspensions were obtained at sacrifice, 8 days after T cell infusion from (Fig. 4A).

3-13. Figure 4H: point 2: At this point the tumors are so small that leads one to wonder if the reduced TIM3 phenotype they're seeing is because the cells don't have anything to interact with anymore yet. The authors mention differences in tumor burden in the results section for figure 5 as a reason for why the CD8 $\alpha\beta$ groups have more proliferation associated genes than the CD8/CD28. Repeat some of these studies but euthanizing mice when the tumors are varying sizes might shed some light on this matter.

To address this question, we have updated / added the following figures.

- 1) **Figure 4A:** Additional data points added showing an average tumor volume of 37mm³ for the CD8/28 group at the time of mice euthanasia.
- 2) New **Figure 4G** (also see answer to **Reviewer 3 Comment 13 part 1**): T cell in the CD8/28 group had reduced expression of exhaustion-associated surface markers.
- 3) New **Figure 4H** (also see answer to **Reviewer 3 Comment 13 part 1**): T cells in the CD8/28 group produced more cytokines ex vivo compared to the CD8 $\alpha\beta$ group.

Page 10, line 217 now reads:

"Mice in the $T_{TCR-MA1-CD8/28}$ group markedly reduced tumor size 9 days post-transfer (average tumor volume at euthanasia 37mm³) (Fig. 4A). Tumors from $T_{TCR-MA1-CD8/28}$ -treated mice contained significantly more CD4⁺ and CD8⁺ T cells per mm² (Fig. 4B, quantified in Extended Fig. 8B), with higher T cell-to-tumor ratios overall (Fig. 4C), and within CD4⁺ (Fig. 4D) and CD8⁺ (Fig. 4E) T cell subsets. The CD4⁺:CD8⁺ ratio in this group approached 1 (mean 0.872) versus 0.381 in the $T_{TCR-MA1-CD8\alpha\beta}$ group where CD8⁺ T cells predominated (Fig. 4F). $T_{TCR-MA1-CD8/28}$ cells showed reduced expression of surface markers associated with exhaustion (PD1⁺/Tim3⁺, CD39⁺/Tim3⁺, and PD1⁺/TIGIT⁺) (Fig. 4G, Extended Fig. 8C) and produced significantly more cytokines upon cognate peptide exposure ex vivo (Fig. 4H). Collectively

this data show that $T_{TCR-MA1-CD8/28}$ were detected at higher frequency in tumors, potentially due to either preferential tumor accumulation or an earlier burst of intra-tumoral expansion. Notably, the remaining cells displayed fewer exhaustion-associated features, and exhibited enhanced function compared to $T_{TCR-MA1-CD8\alpha\beta}$.

To link the findings in Figures 4 and 5, the last paragraph associated with Figure 5 was updated.

Page 12, line 269 now reads:

“Taken together these observations suggest that combining $CD8^+$ and $CD4^+$ T cells enhances proliferation of both subsets, while CD28 co-stimulation promotes cytotoxic signatures, consistent with their increased ability to produce cytokines (Fig. 4H) and preserves early memory programs, consistent with their reduced expression of exhaustion-associated surface markers (Fig. 4G).

3-14: Figure 4: Authors claim improved infiltration but only show that there are more cells in the tumor. The increased numbers could be due to increased proliferation and/or Survival but not necessarily infiltration.

To avoid over- or misinterpretation, we have revised the manuscript and now refer to these findings more accurately as an increase in “The number of $CD4^+$ and $CD8^+$ per mm^2 of tumor” rather than “improved infiltration.”

The revised manuscript Page 10, lines 219 now reads:

“Tumors from $T_{TCR-MA1-CD8/28}$ -treated mice contained significantly more $CD4^+$ and $CD8^+$ T cells per mm^2 (Fig. 4B, quantified in Extended Fig. 8B), with higher T cell-to-tumor ratios overall (Fig. 4C), and within $CD4^+$ (Fig. 4D) and $CD8^+$ (Fig. 4E) T cell subsets.”

3-15. Figure 4G: captions claim that the heightened CD4 to CD8 ratio is indicative of CD4 proliferation. This could be the result of many other mechanisms. CD8 death, for one, in addition to the other concerns I’ve mentioned about proliferation.X

We have removed language referencing proliferation or speculation on the mechanisms leading to increased T cell numbers/ mm^2 to avoid over-interpretation. The text in reference to **Figure 4G (now Figure 4F)** and the corresponding Figure Legend have been revised.

The Results Section Page 10, line 221 now reads:

“The $CD4^+:CD8^+$ ratio in this group approached 1 (mean 0.872) versus 0.381 in the $T_{TCR-MA1-CD8\alpha\beta}$ group where $CD8^+$ T cells predominated (Fig. 4F).”

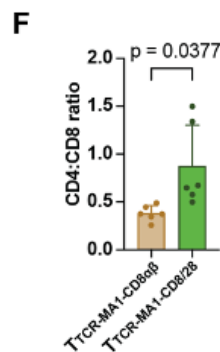


Figure legend 4F now reads: *CD4⁺ and CD8⁺ T cell ratio (F), p value determined by Welch's unpaired two-tailed t-test. Analysis performed using HALO Link software on IHC slides. (n=3 mice, 6 tumors/group, 2 tumors/mouse).*

3-16: Figure 5: They mention that the CD8 T cells from the CD8/28 group has more stem-like gene expression, but that is only in reference to the CD8αβ combo control and not the CD8αβ control. Not sure if that is a fair comparison

We appreciate the comment. In our scRNA-seq data, CD8/28 T cells display an intermediate phenotype: they up-regulate cytotoxic programs while retaining features consistent with early memory relative to the CD8αβ combo control. This suggests that adding CD28 co-stimulation can enhance cytotoxicity yet preserve elements of an early memory program. We have now clarified the text by showing first pairwise comparisons between the different CD8⁺ T cell groups (**Fig. 5D and F**) with corresponding tumor volumes (**Fig. 5E and G**) and then the comparison regarding the 3 groups (**Extended data Fig. 9C**). We note that CD4⁺ T cells in the combo conditions likely sustain proliferation and provide help, whereas the single CD8αβ control, which lacks CD4⁺ cells, is less proliferative and may therefore maintain some early memory features, which could explain why the contrast between CD8/28 and pure CD8αβ is not significant. These mechanistic interpretations are hypotheses and will require further study – this is now clearly stated in the text.

The Results Section Page 11 line 255 now reads:

*“Relative to CD8⁺-only cells, CD8⁺ T cells in the combo CD8αβ group showed enriched cytotoxic and proliferative but reduced stem-like signatures (**Fig. 5D**), aligning with their failure to achieve tumor volume reduction (**Fig. 5E**). In contrast, CD8⁺ T cells in the combo CD8/28 group showed enriched cytotoxic and stem-like but reduced proliferative signatures (**Fig. 5F**), correlating with tumor volume reduction (**Fig. 5G**). Although speculative, the lower proliferative signature may reflect diminished antigen availability after tumor shrinkage, favoring the acquisition of self-renewing programs. Comparisons across groups suggest that CD8⁺-only and CD8⁺ combo CD8/28 proliferate and differentiate less than CD8⁺ combo CD8αβ cells, thereby maintaining stem-like features. For CD8⁺-only, this may reflect absence of CD4⁺ help (less proliferative stimulation), whereas for CD8⁺ combo CD8/28 this may be linked to tumor burden reduction (**Extended Fig. 9C**). Cell proportions (**Extended Fig. 9D**) and absolute numbers (**Extended Fig. 9E**) further support this model: CD8⁺ in the combo CD8/28 group showed a predominance of cytotoxic and stem-like rather than proliferative signatures (tumor burden reduction), whereas the CD8⁺ in the combo CD8αβ group displayed cytotoxicity and proliferative rather than stem-like signatures (no tumor burden reduction compared to control).*

*Taken together these observations suggest that combining CD8⁺ and CD4⁺ T cells enhances proliferation of both subsets, while CD28 co-stimulation promotes cytotoxic signatures, consistent with their increased ability to produce cytokines (**Fig. 4H**) and preserves early memory programs, consistent with their reduced expression of exhaustion-associated surface markers (**Fig. 4G**).”*

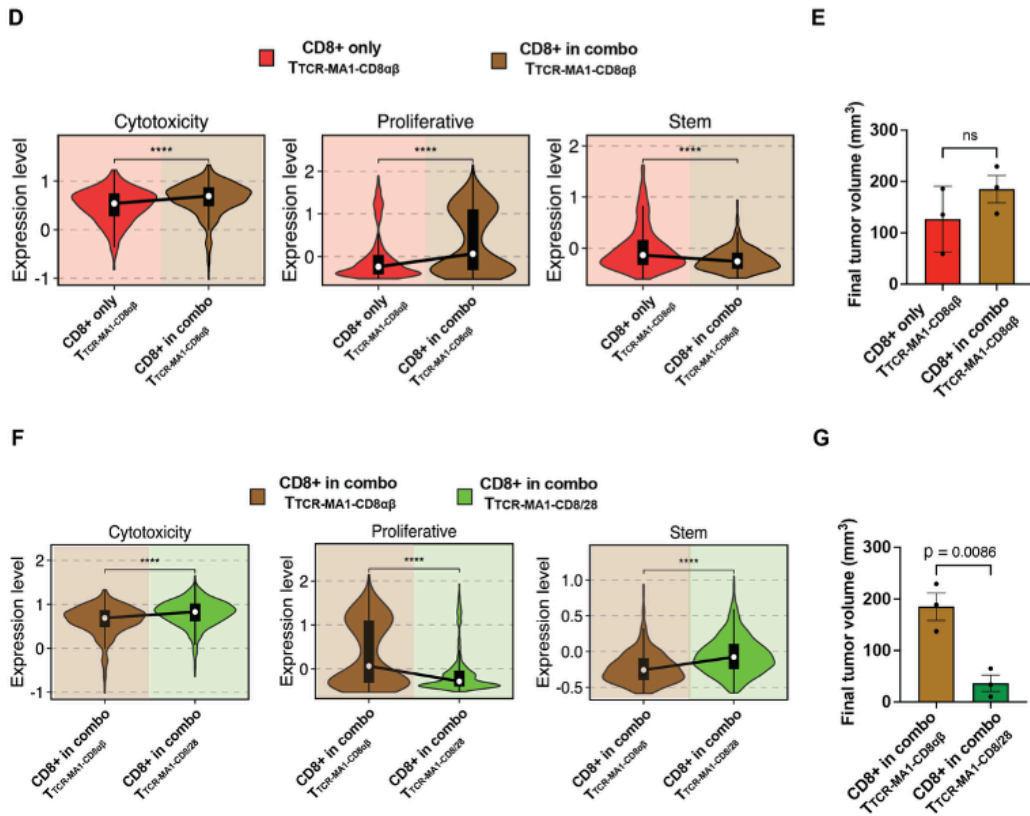


Figure 5. (D) Violin plots displaying the expression levels (y-axis) of stem-like (TCF7, SELL, IL7R, LTB, LEF1, NELL2), proliferation (MKI67, TOP2A, PCNA), and cytotoxicity (KLRG1, KLRD1, NKG7, GZMB, GNLY, PRF1, GZMA, GZMM, CSTA, CSTW) markers across CD8⁺ T-cell transcriptional states between CD8⁺-alone and CD8⁺ in TCR-MA1-CD8αβ combo (x-axis). Statistical significance was assessed using the Wilcoxon rank sum test. Asterisks indicate the following thresholds of significance: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

(E) Tumor burden measurements for NSG mice engrafted with 1×10^5 cells A375F and subsequently infused with total 5×10^6 T cells of CD4⁺ and CD8⁺ (1:1 ratio combo) TCR-Irr.-CD8αβ, CD8⁺ only TCR-MA1-CD8αβ, CD4⁺ and CD8⁺ (1:1 ratio combo) TCR-MA1-CD8αβ 12 days post engraftment. (n=3 mice, 2 tumors/group averaged). p value determined by unpaired two-tailed t-test. **(F)** Violin plots displaying the expression levels (y-axis) of stem-like (TCF7, SELL, IL7R, LTB, LEF1, NELL2), proliferation (MKI67, TOP2A, PCNA), and cytotoxicity (KLRG1, KLRD1, NKG7, GZMB, GNLY, PRF1, GZMA, GZMM, CSTA, CSTW) markers across CD8⁺ T-cell transcriptional states between TCR-MA1-CD8αβ and TCR-MA1-CD8/28 (x-axis). Statistical significance was assessed using the Wilcoxon rank sum test. Asterisks indicate the following thresholds of significance: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

(G) Tumor burden measurements for NSG mice engrafted with 1×10^5 cells A375F and subsequently infused with total 5×10^6 T cells of CD4⁺ and CD8⁺ (1:1 ratio combo) TCR-MA1-CD8αβ, and CD4⁺ and CD8⁺ (1:1 ratio combo) TCR-MA1-CD8/28 and CD4⁺ and CD8⁺ (1:1 ratio combo) TCR-Irr.-CD8/28 12 days post engraftment. (n=3 mice, 2 tumors/group averaged). p value determined by unpaired two-tailed t-test.

3-17 Figure 6: If exhaustion is being averted then it is unclear why a cluster of exhausted T cells from this sequencing data does not show up.

In scRNA-seq data, it is often challenging to clearly define exhausted T cells and to distinguish them from activated or cytotoxic T cells, a difficulty noted by others in the field. In our cytotoxic cluster, we observed expression of some exhaustion-associated markers (e.g., TIGIT, DUSP2); however, a distinct exhausted T-cell cluster was not clearly separated from the cytotoxic population in our dataset.

We have modified the manuscript to acknowledge this point and added relevant references. Page 11, line 252 now reads:

“A distinct “exhausted” cluster was not identified, consistent with prior studies showing that exhaustion markers can also be expressed in activated T cells, complicating their distinction in scRNA-seq data-sets¹¹⁻¹³.”

Gros, A. *et al.* PD-1 identifies the patient-specific CD8⁺ tumor-reactive repertoire infiltrating human tumors. *Journal of Clinical Investigation* **124**, 2246-2259 (2014).
<https://doi.org/10.1172/JCI73639>

Inozume, T. *et al.* Selection of CD8⁺PD-1⁺ lymphocytes in fresh human melanomas enriches for tumor-reactive T cells. *J Immunother* **33**, 956-964 (2010).
<https://doi.org/10.1097/CJI.0b013e3181fad2b0>

Mietz, J. *et al.* Human effector CD8(+) T cells with an activated and exhausted-like phenotype control tumour growth in vivo in a humanized tumour model. *EBioMedicine* **106**, 105240 (2024).
<https://doi.org/10.1016/j.ebiom.2024.105240>

3-18: Figure 6: The lack of *in vivo* antitumor activity using the TCR-PRAME-CD8/28 mut is critical to include but missing from these data sets.

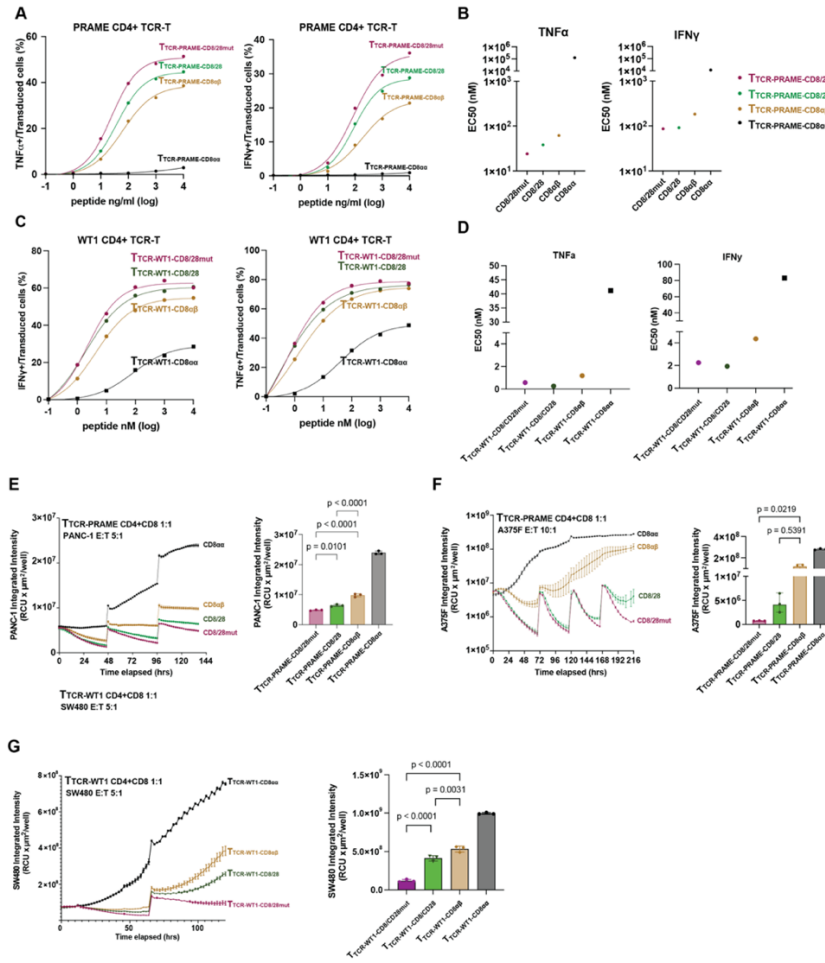
For this manuscript, we prioritized maintaining the focus on the *in vivo* validation of the TCR-MAGE CD8/28 mut and have included two additional *in vivo* murine models detailed in the responses to **Reviewer 2, Comments 3 and 4**. To show the potential applicability of this approach to other TCRs, we have added the effect of TCR-PRAME CD8/28 mut *in vitro* targeting 2 different PRAME-expressing tumors as well as TCR-WT1 CD8/28mut and moved these to **Extended Data Figure 12 (shown below)**. We have also clarified the title and the text of the paragraph to reflect this point.

The Results Section Page 12, line 274 (title) now reads:

“CD8-CD28 CCR tail mutations enhance anti-tumor activity in MAGE tumor models”

The Results Section Page 13, line 301 now reads:

“To test generalizability, we applied the CD28-mut strategy to PRAME- and WT1-specific TCRs (Extended Fig. 12). $T_{\text{TCR-PRAME-CD8/28mut}}$ displayed slightly improved functional avidity (EC_{50} IFN γ 86.78 vs 91.79; TNF α 24.2 vs 38.37nM) compared with $T_{\text{TCR-PRAME-CD8/28}}$ (Extended Fig. 12A & B), while $T_{\text{TCR-WT1-CD8/28mut}}$ showed similar functional avidity to $T_{\text{TCR-WT1-CD8/28}}$ (EC_{50} IFN γ 2.25 vs 0.57; TNF α 1.92 vs 0.26 nM) (Extended Fig. 12C & D). However, both $T_{\text{TCR-PRAME-CD8/28mut}}$ and $T_{\text{TCR-WT1-CD8/28mut}}$ achieved superior tumor control after repeated tumor challenge *in vitro* against HLA A2⁺ PRAME⁺ PANC1 (pancreatic ductal adenocarcinoma), HLA-A2⁺ PRAME⁺ A375F and HLA-A2⁺ WT1⁺ SW480 (colon adenocarcinoma) lines compared with their non-mutated counterparts (Extended Fig. 12E-G). These findings indicate that CD28 tail mutations may enhance efficacy beyond TCR_{MAL}, across to other TCR therapies.”



Extended Data Fig. 12: CD8/CD28 CCR integrated into WT1, and PRAME TCR

(A) Dose response curves showing IFN γ and TNF α levels in CD4⁺ T_{TCR-PRAME-CD8aa}, T_{TCR-PRAME-CD8 $\alpha\beta$} , T_{TCR-PRAME-CD8/CD28}, and T_{TCR-PRAME-CD8/CD28mut} cells incubated with decreasing concentrations of cognate peptide overnight. (B) Mean EC₅₀ of IFN γ and TNF α cytokine expression for CD4⁺ T_{TCR-PRAME-CD8aa}, T_{TCR-PRAME-CD8 $\alpha\beta$} , T_{TCR-PRAME-CD8/CD28}, and T_{TCR-PRAME-CD8/CD28mut} cells from previous dose response curve (A).

(C) Dose response curve showing IFN γ and TNF α levels in CD4⁺ T_{TCR-WT1-CD8aa}, T_{TCR-WT1-CD8 $\alpha\beta$} , T_{TCR-WT1-CD8/CD28}, and T_{TCR-WT1-CD8/CD28mut} cells incubated with decreasing concentrations of cognate peptide overnight. (D) Mean EC₅₀ of IFN γ and TNF α cytokine expression for CD4⁺ T_{TCR-WT1-CD8aa}, T_{TCR-WT1-CD8 $\alpha\beta$} , T_{TCR-WT1-CD8/CD28}, and T_{TCR-WT1-CD8/CD28mut}.

(E) (left) Growth kinetics of the PANC1 cell line in the presence of CD4⁺ with the indicated CCR constructs (T_{TCR-PRAME-CD8aa}, T_{TCR-PRAME-CD8 $\alpha\beta$} , T_{TCR-PRAME-CD8/CD28}, and T_{TCR-PRAME-CD8/CD28mut}) at a ratio of E:T 5:1. (right) Final tumor integrated intensity of Incucyte killing assay was analyzed. *p* values were determined with one-way ANOVA with Tukey's multiple comparison test. (*n*=3 wells/group).

(F) (left) Growth kinetics of the A375F cell line in the presence of CD4⁺ with the indicated CCR constructs (T_{TCR-PRAME-CD8aa}, T_{TCR-PRAME-CD8 $\alpha\beta$} , T_{TCR-PRAME-CD8/CD28}, and T_{TCR-PRAME-CD8/CD28mut}) at a ratio of E:T 10:1. (right) Final tumor integrated intensity of Incucyte killing assay was analyzed. *p* values were determined by one-way ANOVA with Tukey's multiple comparison test. (*n*=3 wells/group).

(G) (left) Growth kinetics of SW480 cell line in the presence of CD4⁺ with the indicated CCR constructs (T_{TCR-WT1-CD8aa}, T_{TCR-WT1-CD8 $\alpha\beta$} , T_{TCR-WT1-CD8/CD28}, and T_{TCR-WT1-CD8/CD28mut}) at a ratio of E:T 5:1. Final tumor

integrated intensity of Incucyte killing assay was analyzed. *p* values were determined by one-way ANOVA with Tukey's multiple comparison test. (*n*=3 wells/group).

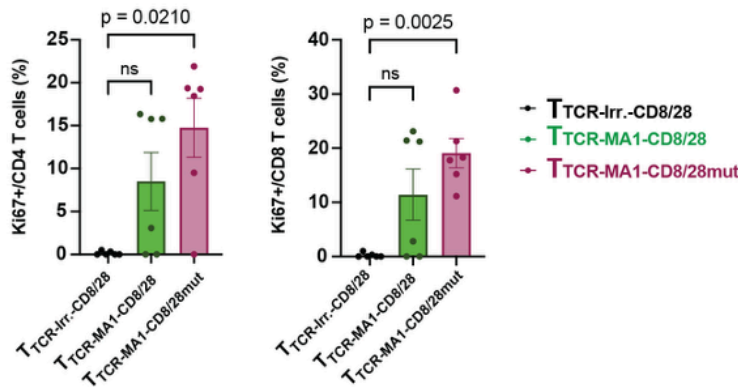
3-19: Figure 7: The manuscript would have benefited from a strong ending (Figures 6 and 7). The lack of data showing that the mutant had any beneficial effects *in vivo* and characterized it with an *in vitro* repeat stim/sequencing assay. They show upregulation in proliferation and exhaustion related genes but follow this with flow data that only looks at PD1 and TIM3 again for exhaustion, which also had very little differences between the mutant and wildtype cd8/cd28 groups. Furthermore, they saw no differences in cytokine expression after restimulation. Did not show any functional proof of differences in proliferation again.

We agree with the reviewer's comments, and we have included two additional *in vivo* murine models detailed in the responses to **Reviewer 2, Comments 3 and 4** regarding the effects on CD8/CD28mut compared to CD8/28wt. We have also included a new **Extended Figure 11C** showing Ki-67 expression on CD4⁺ and CD8⁺ T_{TCR-MA1-CD8αβ} vs T_{TCR-MA1-CD8/28} from *in vivo* tumor IHC analysis.

The result Section Page 13, line 294 now reads:

"CD4⁺ and CD8⁺ T_{TCR-MA1-CD8/28mut} had higher expression of the Ki67 proliferation marker than T_{TCR-MA1-CD8/28} by IHC (Extended Fig. 11C)."

C



Extended Figure 11(C) Immunohistochemistry analysis of Ki-67 expression in CD4⁺ or CD8⁺ T cells between T_{TCR-Irr-CD8/28}, T_{TCR-MA1-CD8/28} and T_{TCR-MA1-CD8/28mut} groups from (Fig. 6D). *p* value determined with Brown-Forsythe and Welch ANOVA test with Dunnett's multiple comparison test. (*n*=3 mice/group, 2 tumors per mouse).

3-20: – point 1: In figures 6 and 7 they did not show any signaling differences for why their mutant might be affecting the T cells differently than their wildtype version of the CD8/CD28. They mention in their discussion possible signaling molecules that are involved but didn't look at a single one.

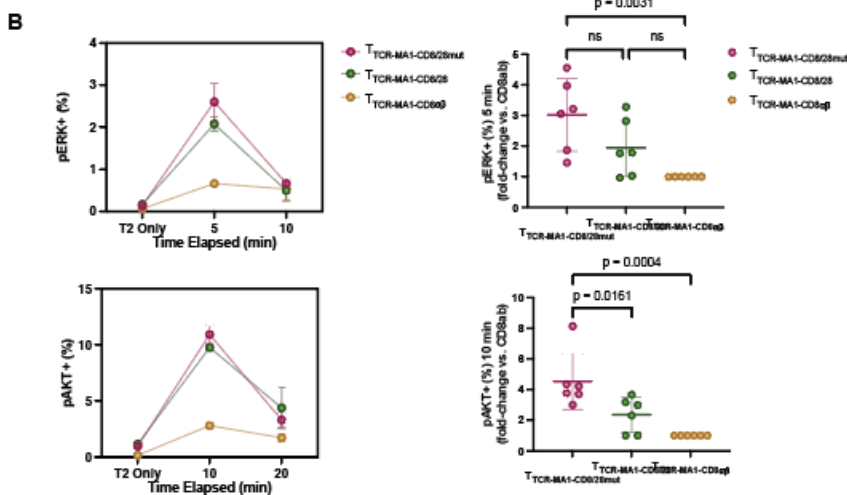
We have now included a new figure (**Extended Fig. 10B**) that examines signaling patterns between the wildtype and mutant CD8/CD28 constructs. Specifically, we analyzed phosphorylation of both AKT (p-AKT) and ERK

(p-ERK) as key downstream effectors of CD28-mediated costimulatory signaling and TCR-driven activation¹⁴. This approach allows for distinguishing the signaling pathways downstream of the CD28 domain in the canonical TCR signaling pathway.

The Results Section Page 14, lines 317 now reads:

“Exposure to cognate peptide-loaded TAP-deficient T2 B lymphoblastoid cell lines (T2-BLCL) revealed higher phosphorylated downstream TCR and CD28 signaling molecules (p)-ERK and (p)-AKT in $T_{TCR-MA1-CD8/CD28mut}$ than $T_{TCR-MA1-CD8/CD28}$ at 5 minutes, with both exceeding $T_{TCR-MA1-CD8\alpha\beta}$ (Extended Fig. 13A), consistent with more pronounced early CD28-mediated costimulatory signaling and TCR-driven signaling¹⁴.”

Adachi, K. & Davis, M. M. T-cell receptor ligation induces distinct signaling pathways in naive vs. antigen-experienced T cells. *Proc Natl Acad Sci U S A* **108**, 1549-1554 (2011). <https://doi.org/10.1073/pnas.1017340108>



Extended Data Fig. 13 (A) Intracellular expression of (p)-ERK(top) and (p)-AKT(bottom) across $CD4^+$ T cells constructs ($T_{TCR-MA1-CD8\alpha\beta}$, $T_{TCR-MA1-CD8/CD28}$, and $T_{TCR-MA1-CD8/CD28mut}$) stimulated with 1uM of cognate peptide at three different time point. Time course (left) and fold change (right) compared to $CD8\alpha\beta$. The mean and standard deviation (SD) of triplicate wells from 2 independent experiment is shown. p value determined by one-way ANOVA with Tukey's multiple comparison test. n= 6 wells/group, from 2 separate experiments).

3-20: – point 2: Finally, please citing other relevant work demonstrating the use of CD4 or CD8a molecules modified to express costimulatory domains.

We have now referenced three relevant studies that inspired our chimeric co-receptor (CCR) design: Modification of the CD8 α intracellular domain to incorporate MyD88 resulting in enhanced CD8 T-cell function and anti-tumor activity *in vivo*, and CD8 α homodimers fused with either CD3 ζ or CD28 intracellular domain shown to transmit activation signals upon crosslinking.

The Result Section Page 8, line 179 now reads:

*“To achieve ligand-independent signaling, we tethered positive intracellular costimulatory domains to the requisite CD8 $\alpha\beta$ co-receptor chains, based on findings that TCR chain modifications impaired function (**Extended Fig. 6A-C**) and prior evidence that CD8 α chain modifications can transmit activation signals¹⁵⁻¹⁷.*