nature portfolio

Peer Review File

IL7-Receptor–Targeted CAR T-Cell Therapy for T-Cell Acute Lymphoblastic Leukemia

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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 their paper titled “IL7-Receptor–Targeted CAR T-Cell Therapy for T-Cell Acute Lymphoblastic Leukemia” Hocine and coauthors report on the development of IL7Rα 32 (CD127)-targeted chimeric antigen receptor (CAR) T cells with low- and high-affinity single-chain variable fragments. They observed antitumor efficacy of CD127 CAR against T-ALL cells in vitro, in mouse models of T-ALL, and against blasts from patients with T-ALL using the patients’ own T cells transduced with CD127 CAR. They also found that antitumor efficacy was higher with low-affinity CAR T cells compared to high-affinity CAR T cells, albeit with fratricide of CAR T cells. Short-term co-culture with dasatinib facilitated higher low-affinity CAR T-cell yield, improved fitness and preserved functionality. In addition, in vivo dasatinib may serve as an in vivo safety switch to treat potential on-target, off-tumor toxicity.

The article is well written and of potential interest for hematologists and cancer immunologists. This study represents a very extensive and detailed investigation of CD127 as a new target for the treatment of T-ALL using CAR-T. Especially the studies using primary patient material as targets as well as a source for CAR-T production are potentially impactful. However, there are issues with the novelty/innovation of their investigation and there are a number of distinct flaws which need to be addressed to improve the quality of the manuscript.

MAJOR POINTS:

- Dasatinib has extensively been investigated as a reversible on/off switch for CAR T cells and as a factor improving manufacturing of CAR T cells. This does not diminish the relevance of their observations for the treatment of T-ALL using CAR-T but it does reduce the degree of novelty and innovation. They should at least discuss this limitation.

- The same limitation actually applies to the quality of the high-affinity vs low-affinity CAR T cells. This topic has extensively been investigated before.

- The high variability of CD127 expression on T-ALL cell lines is very worrisome and diminishes the value of CD127 as a target. Were the authors able to identify any mechanisms underlying this heterogenous expression?

- Same question for the heterogenous and relatively infrequent expression of CD127 on primary patient material.

- In addition, CD127 expression by normal human T cells is obviously a problem even if, as per the authors, they express a relatively “low” expression of “<5,000 molecules/cell”. There is no guarantee that this will protect them from being targeted even by the low affinity CD127 CAR T cells. Overall, I am not sure I would conclude from the combined data that CD127 is definitely “a highly promising target”.

- The difference in the cytotoxic activity of high-affinity vs. low-affinity CAR-T as shown in Figure 1d seems to be statistically significant, however, the actual absolute difference is minimal. To some extent, the same applies to Figure 1e. The authors should acknowledge that.

- The authors do not demonstrate fratricide per se. Lower CAR T cell numbers could be due to a number of alternative factors such as differential responsiveness to proliferation-promoting cytokines, decreased resistance to exhaustion, etc..

- What did the expression of CD127 look like on freshly harvested T cells as well as on untransduced, LOW and HIGH CAR T cells over the whole course of CAR T cell manufacturing? Does activation of T cells during manufacturing lead to a modification of CD127 expression on the RNA and/or protein levels?

- Again, the difference in tumor burden using high-affinity vs. low-affinity CAR-T as shown in Figure 2c seems to be statistically significant, however, the actual absolute difference is minimal.

- Could the lower numbers of BOTH tumor cells (which are T cells as well in the case of T-ALL) and LOW CAR T cells as shown in Figure 2f be unrelated to fratricide? Could this be related to a similarly decreased responsiveness to a third-party factor such as certain cytokines?

Reviewer #2

(Remarks to the Author)
Dr. Hocine and colleagues report on the development of CD127 (IL7Ra)-targeting CAR T cells for the therapy of T-cell ALL. The authors show differential activity of T cells expressing low- vs high-affinity CAR and document expected CAR-mediated fratricide that can be mitigated by either CD127 gene KO or temporary inhibition of CAR signaling with dasatinib during manufacture. The resulting CAR-T cells mediate potent activity against CD127high T-ALL cell lines and primary blasts, both in vitro and in vivo thus supporting feasibility of targeting IL-7Ra with CAR-T cells in T-cell leukemia.

IL7Ra as an immunotherapy target in T-ALL has been established in prior reports (eg Hixon et al., Leukemia 2019) which should be cited and discussed in this manuscript. Several other questions arise upon reading:

1. CD127 is highly expressed in naive and memory T cells, NK cells, and some B-cells, and is downregulated in Tregs and effector T cells. In addition, CD127 is expressed in lymphoid progenitors in BM (CLP) and thymus (DN stage and SP) suggesting systemic activity of CD127 CAR-T would results in a global lymphoid ablation sparing Tregs and the rare activated/effector T cell. What would be the therapeutic advantage of CAR127 compared to CD7 or CD5 CART that are in the advanced clinical development considering higher expression of those antigens in T-ALL and a slightly more restricted presence on normal tissues compared to CD127? Would this be another bridge-to-transplant strategy despite the need for more "standalone" therapies (lines 87-89)?

2. The authors state "Low-affinity CARs preferentially recognize tumor cells with higher antigen expression while sparing normal cells with lower antigen expression" (lines 497-498). Does this imply the activity against normal circulating cells or activated CAR-T? Coculture assays of CAR127 T cells with freshly isolated, non-activated autologous T and NK cells from peripheral blood would help understand the degree of expected on-target toxicity and characterize the resistant T-cell population, if it arises.

3. CD127 is commonly expressed in ETP and cortical T-ALL but downregulated in more mature TCR+ subsets (Courtois et al., Blood 2023). Considering most experiments are done with CD127high cell lines, where would be the cut-off in %CD127 expression on T-ALL blasts below which this therapy becomes ineffective? This may be difficult to definitively answer without a clinical trial but any experimental data could be helpful for the clinical community to better understand potential impact of this therapy in a broader patient population.

4. The authors conclude that CD127KO does not affect T-cell functon(line 267). However, it is difficult to infer from the presented data considering the established role of IL7R in the long-term maintenance of memory T cells thus requiring prolonged assays in vivo. Either those experiments need to be performed or the authors should temper the conclusion and limit it only to short-term effector function in the models used.

5. Experiments with dasatinib in vivo are in line with prior reports and serve as a supporting data for the authors to envision it as a "safety switch" post-infusion. However, the experiments were performed with a very high dose of dasa (50mg/kg b.i.d.) whereas the FDA-approved dose is much lower (140mg orally QD - so more like 2mg/kg, with different bioavailability and twice less frequent). It is unclear if dasatinib would work at this dose or would require a separate dose-finding study. Further, it would likely work as a short-term break to mitigate acute tox rather than a real "safety switch" to terminate CAR-T activity and limit lymphopenias (line 605-613). Long-term dasatinib administration at doses inhibiting CAR activity would likely also suppress TCR activation and therefore produce severe immune deficiency. The authors should clarify the intent of dasatinib administration post-infusion.

6. In the results and discussion (lines 419-421 and 536-538) the authors interpret the results of RNA-seq where the genes encoding key signaling kinases downstream of IL7R (JAK/STAT/PI3K/mTOR) are not perturbed in CD127KO T cells as this gene deletion has a minimal effect on T-cell function. It is hard to be convinced by these statements when the activity of these signaling pathways are regulated by post-translational modification (phosphorylation, localization) rather than by RNA expression. Further, these pathways would light up in T cells cultured in the presence of IL-2 as indicated in the methods, but would not be active in vivo where resting/memory T-cells rely on IL7R signaling for long-term survival. Therefore, this statement must be tempered or removed.

Reviewer #3

(Remarks to the Author)

Hocine et al present a manuscript describing the development of an IL7R-specific CAR T cell therapy. The authors posit that IL7R, which is expressed on ~70% of T-ALL blasts and 40% of B-ALL blasts, is a good target for cellular therapy of these leukemias. IL7R is also overexpressed on normal T cells but is transiently downregulated upon activation.
They generate 2 different CAR T cell constructs, one with high binding affinity (HIGH-CAR) and one with low binding affinity (LOW-CAR) and determine that the low affinity CAR T cells have more potent antitumor activity in vitro and in vivo. However, they also have increased fratricide, limiting the long-term antitumor activity. To bypass this, the authors propose 3 different strategies including knocking out IL7R (KO CAR T cells), naturally selecting T cells (NS) or using dasatinib to downregulate IL7R.
The manuscript shows thoughtful experiments and robust results, is very thorough and well written. It could be enhanced by addressing the following queries.
-Weight is not an accurate tumor burden monitoring tool as it can happen in the absence of tumor if there is inflammation present. Did the authors evaluate tumor burden in peripheral blood? If so, serial (e.g. weekly or biweekly) bleeds and evaluation of peripheral tumor burden will provide better information.
-In certain tumor models, antigen downregulation happens in the absence of CAR T cell infusion. What is the IL7R expression on mice receiving tumor only or tumor and UTD T cells?
-Please comment on why LOW NS CAR T cell dose was 6x10^6 vs LOW KO dose was 1x10^6? Why was a higher dose needed? Where these cells less effective?

Figures:
-Please include labels for each panel throughout the figures, as sharing labels between panels generates confusion.
-Figure 1. b) Please include transduction expression data of additional replicates, not only representative examples. g) Please comment on E:T used
-Figure 5. Please align duration of follow up between survival curves and bioluminescence curves.
-Figure 5g. Please show data for blood and intestine for LOW WT at relapse if available, so that it can be compared with LOW KO at relapse.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
All my comments were taken into account and the authors responded appropriately. I have no additional concerns.

Reviewer #2

(Remarks to the Author)
The authors have addressed some queries but others remain outstanding.

#2. The rationale provided in the response does not match that in the introduction (lines 77-93) where the authors instead discuss CD127 as a better target than CD5 or CD7 because the latter two are broadly expressed in T cells thus only offering a bridge-to-transplant approach. The current study indicates CD127 targeting would be just as toxic, if not more toxic, than targeting the other two antigens and therefore does not offer a better option like it is positioned in the introduction/rationale. CD127 is highly expressed in the vast majority of normal T cells and other cell types where CD5/CD7 are not present. Further, CD127 expression in T-ALL is much more heterogeneous and commonly downregulated in subtypes most prevalent among patients with r/r disease (cortical T-ALL responds well to standard therapies). Therefore, there is no evidence CD127 is a better or safer target than the other two, as stated in the introduction. The authors have to either convincingly (experimentally) demonstrate the opposite or re-write the rationale of targeting CD127 in the intro, to match experimental results.

#3. It is very hard to be convinced by the coculture results where the % of necrotic cells is the main readout. Robust on-target off-tumor experiments would include quantification of live cell counts before/after coculture, and an expected accumulation of apoptotic cells - neither of these parameters have been quantified. Further, these results contradict authors' earlier data where dasatinib or CD127 KO is required to mitigate normal T-cell killing by CART127. The new supplementary figure has to be completely re-done or removed altogether.

#4. The response is factually incorrect. In the Courtois 2023 study, it was found that 70% of T-ALL express CD127 at various levels, often much lower than the physiologic counterparts. A citation from that paper:
"Conversely, CD127 expression was minimal in ETP-ALL and mature TCRαβ T-ALL, again in contrast with the physiological pattern of normal thymocytes, which normally express the receptor at these differentiation stages. (Figure 1A,E). We found a similar expression (68%; P = not significant) in a retrospective analysis of 100 pediatric T-ALL cases (Figure 1D)."
(https://ashpublications.org/blood/article/142/2/158/495237/IL-7-receptor-expression-is-frequent-in-T-cell)

That study highlighted elevated CD127 expression mainly in cortical T-ALL which, again, has a good prognosis and will rarely be treated with CAR-T. cT-ALL also commonly express CD1a which is a much better target not expressed in normal T

cells (stuides by Pablo Menendez, currently in clinical trial).

Therefore, this erroneous statement must be removed. Further, considering this and prior discrepancies, the authors should critically re-analyze existing literature and re-think the rationale of targeting CD127 with CAR-T.

#6. In the abstract, the authors still state "In addition, in vivo, dasatinib serves as a reversible safety switch to treat potential on-target, off-tumor toxicity or prolonged lymphopenia." I thought we have agreed that this strategy would not be effective in preventing off-tumor toxicity/lymphopenia as it would require prolonged administration which by itself would be toxic and immunosuppressive. In vivo dasatinib can only be applied to halt acute toxicities like CRS/ICANS etc but NOT to prevent unwanted targeting of normal tissues. To achieve this, CAR-T must be effectively eliminated, not temporarily inhibited. Unfortunately, the authors continue to contradict themselves.

#7. Given the agreed-upon limitation in interpreting the results of RNA-seq, are these data needed at all? I am not sure this adds anything of value to the study.

Reviewer #3

(Remarks to the Author)
The authors have addressed the reviewers' queries satisfactorily.

Version 2:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
Thank you for the opportunity to review the revised manuscript and the additional correspondence regarding the remaining concerns raised by Reviewer #2. After carefully evaluating the revised manuscript and rebuttal, I believe the authors have addressed the major scientific concerns sufficiently for publication, although a few wording modifications would still strengthen the manuscript.

Reviewer #2 raises reasonable points regarding the positioning of CD127 as a target antigen in T-ALL. I agree that the original framing may have implied that CD127-directed CAR T cells are inherently safer or superior to CD5/CD7-directed approaches. However, in the revised version, the authors substantially moderated these claims and now position CD127 primarily as a biologically motivated target based on IL7R pathway dependence in selected T-ALL subsets rather than as a universally safer alternative. In my opinion, this clarification adequately resolves the conceptual concern. While additional refinement of wording in the Introduction may still be beneficial, I do not believe this issue represents a fatal flaw in the study.

Regarding the concerns about the on-target/off-tumor coculture experiments, Reviewer #2 is correct that live-cell quantification approaches could provide additional orthogonal support. However, the authors did perform additional apoptosis analyses with appropriate Annexin V methodology and independent donor validation. Although the experimental system is complex and not perfectly optimized for absolute live-cell quantification, I believe the presented data are sufficient to support the manuscript's main conclusions regarding fratricide and relative off-tumor effects. Importantly, the central conclusions of the manuscript are supported by multiple complementary datasets, including repeated in vitro assays, in vivo efficacy studies, knockout experiments, and manufacturing optimization approaches.

I also agree with Reviewer #2 that the wording regarding dexamethasone should remain carefully limited to the available data. The revised wording provided by the authors, clarifying that dasatinib acts as a reversible pharmacologic suppressor of CAR activity rather than a definitive long-term safety solution, appears appropriate. I do not interpret this remaining issue as a major scientific concern.

Overall, I believe the manuscript presents substantial conceptual and translational advances, including:

- identification of CD127 as a biologically relevant target in IL7R-high T-ALL subsets,
- systematic comparison of affinity tuning approaches,
- demonstration of fratricide mitigation strategies,
- and translationally relevant manufacturing optimization approaches.

In my opinion, the remaining concerns primarily relate to framing, interpretation, and preferred experimental design choices rather than deficiencies that invalidate the major conclusions of the work. I therefore believe the manuscript is suitable for publication after final editorial refinement of wording and claims.

Reviewer #2

(Remarks to the Author)
The authors addressed the questions in their response which was a good start but did not completely resolve the issues.
1. The clarification is appreciated but it does not resolve the main concern around positioning of this paper. From the current

introduction:
"Furthermore, because these potential side effects affect regeneration of normal T cells and immune reconstitution, CD5 and CD7 CAR T-cell therapies are considered to be only a bridge to allogeneic hematopoietic stem cell transplant. Therefore, there is a clear unmet need to develop effective and safe targeted cellular immunotherapies that spare normal hematopoietic cells and that can serve as a definitive, standalone treatment for patients with T-ALL. In this study, we investigated low- and high-affinity CD127-targeted CAR T-cell therapy in clinically relevant T-ALL models, including in a T-ALL patient–derived xenograft model, to investigate the potential of CD127–targeted CAR T-cell therapy for the treatment of T-ALL."
This clearly positions the study to demonstrate CD127 CAR-T as a safer alternative to other CAR-T cells and use as a standalone treatment for patients with T-ALL. The rationale for that is not explained in the introduction and the results do not support this positioning, as agreed by the authors in the added paragraph earlier in that section. This is contradictory and needs to be reconciled. There is nothing wrong in exploring CD127 as an alternative target to CD5 or CD7 etc but the authors should not mislead the reader on the positioning of this antigen. As agreed by the authors, current literature indicates its expression is more prevalent on normal lymphocytes and less prevalent in leukaemia compared to CD5 or CD7 thus making it (in theory) an inferior choice. An argument about T-ALL dependency on IL-7R signaling is valid but weak considering its weak/heterogeneous expression in most subtypes outside of the cortical lineage. So exploring CD127 as a target is fine but the rationale has to be rectified based on the supportive evidence to avoid self-contradiction.

2. Quantifying residual live cells is critical and can be easily done by flow cytometry using counting beads. There is no need to mix CAR-T with leukaemia and normal PBMC, those cocultures should be done separately and live CTV+ cells quantified by flow cytometry. Day 0 counts should be taken as a baseline. Relative frequency of apoptotic cells in a 3-way co-culture is not a standard or rigorous way to evaluate cytotoxicity. This experiment supports a central claim of this paper and has to be done right. Luckily, it is not technically hard. The %CAR-negative data is irrelevant since those are effector cells that downregulate CD127 anyway.

4. Dex data is in line with prior publications. However, the authors again mislead the reader in the abstract stating "In addition, in vivo, dasatinib can be used to temporarily and reversibly suppress CAR T-cell activity, and dexamethasone can be used to control CAR T cells.". There is no in vivo data for steroid inhibition to support that claim.

Reviewer #3

(Remarks to the Author)
The submitted manuscript continues to show value as an additional targeting strategy for adult and pediatric T-ALL. The authors have satisfactorily answered all queries.

RESPONSE TO REVIEWERS

Response to Reviewer #1:

The article is well written and of potential interest for hematologists and cancer immunologists. This study represents a very extensive and detailed investigation of CD127 as a new target for the treatment of T-ALL using CAR-T. Especially the studies using primary patient material as targets as well as a source for CAR-T production are potentially impactful. However, there are issues with the novelty/innovation of their investigation and there are a number of distinct flaws which need to be addressed to improve the quality of the manuscript.

Response: Thank you for taking the time to review our manuscript and provide supporting feedback and raise important points to increase the strength of our manuscript.
Please see below specific responses to the questions raised.

Major points:
1. Dasatinib has extensively been investigated as a reversible on/off switch for CAR T cells and as a factor improving manufacturing of CAR T cells. This does not diminish the relevance of their observations for the treatment of T-ALL using CAR-T but it does reduce the degree of novelty and innovation. They should at least discuss this limitation.

Response: Thank you for the comment. We agree that dasatinib has been previously explored both as a reversible on/off switch and as a modulator in CAR T-cell manufacturing. In our study, we leveraged these established properties and demonstrated their combined application in the context of a fratricidal CAR targeting T-ALL. Specifically, we show that dasatinib not only enables reversible control of anti-CD127 CAR activity *in vivo* but can also be integrated into the manufacturing process to improve anti-CD127 CAR T-cell fitness and overall performance. While these individual concepts are not entirely novel, their integration and exploitation in a fratricidal CAR system provides rationale-based translational value and broadens the therapeutic applicability of this approach.

As suggested by the reviewer, the below clarification has been included in the revised Discussion section:

> **Page 29-30, Line 639:** Dasatinib has been reported both as a reversible on/off switch for CAR T cells[1,2] and as a manufacturing modulator that enhances product quality.[3] In this study, we leveraged these complementary properties in the context of a fratricide-prone CAR targeting T-ALL, integrating transient pharmacologic control *in vivo* with an optimized manufacturing approach. Postinfusion dasatinib is envisioned as a short, event-driven intervention to transiently pause CAR signaling; chronic use at CAR-inhibitory levels is not intended, given potential suppression of TCR. Prior studies have documented rapid and reversible inhibition of CAR activity *in vivo* with brief exposure, with full recovery upon drug withdrawal.[1,2]

2. The same limitation actually applies to the quality of the high-affinity vs low-affinity CAR T cells. This topic has extensively been investigated before.

Response: Thank you for the comment. While we agree that the impact of CAR affinity on T-cell performance has been studied, the role of CAR affinity in fratricidal CARs has not been investigated before. In our work, we specifically demonstrate that a low-affinity CAR design confers superior efficacy in T-ALL, a context in which fratricide poses a major challenge. Importantly, we investigated and report the differential efficacy of low- and high-affinity anti-CD127 CAR in the context of dynamic expression of target antigen in T cells and of fratricide. Recognizing the reviewer's comment, we expanded our Discussion to emphasize that, although the concept of affinity modulation is known, its integration with pharmacologic and target-selection strategies in a fratricidal CAR framework represents a meaningful and translationally relevant advance.

> **Page 25, Line 527:** Building on this concept, we applied affinity modulation in the context of T-ALL, where fratricide remains a major challenge. Here, we show that a low-affinity CAR design enhances efficacy by reducing self-recognition due to downregulation of CD127 after activation, and combining this strategy with transient dasatinib modulation provides additive benefits. This integrated approach mitigates off-tumor activity while improving CAR persistence and performance, representing a refined strategy for safer and effective immunotherapy for T-ALL.

3. The high variability of CD127 expression on T-ALL cell lines is very worrisome and diminishes the value of CD127 as a target. Were the authors able to identify any mechanisms underlying this heterogenous expression?

Response: We appreciate this important comment. We recognize the variability of CD127 expression across T-ALL cell lines. However, CD127 expression is dynamic and varies among normal and malignant cells. In addition to hematological malignancies, our ongoing investigations show similar variability in sold tumor cells as well (data not shown). Our attempts to knock down or knock out CD127 in the same cell line (high, moderate, low, or no antigen expression) for investigational purposes were not successful, as CD127 is oncogenic in T-ALL cells and attempts to decrease the expression affected cell viability, proliferation, and accumulation, unlike in normal T cells.

4. Same question for the heterogenous and relatively infrequent expression of CD127 on primary patient material.

Response: We appreciate the reviewer's observation. While we did not investigate the mechanisms underlying CD127 distribution, published reports cited in the manuscript show that elevated CD127 expression is linked to proliferative and survival advantages in T-ALL, supporting its biological and clinical relevance. In our study, the majority of patient samples were collected during remission, and not at diagnosis or relapse, perhaps indicating the variability in CD127 expression.

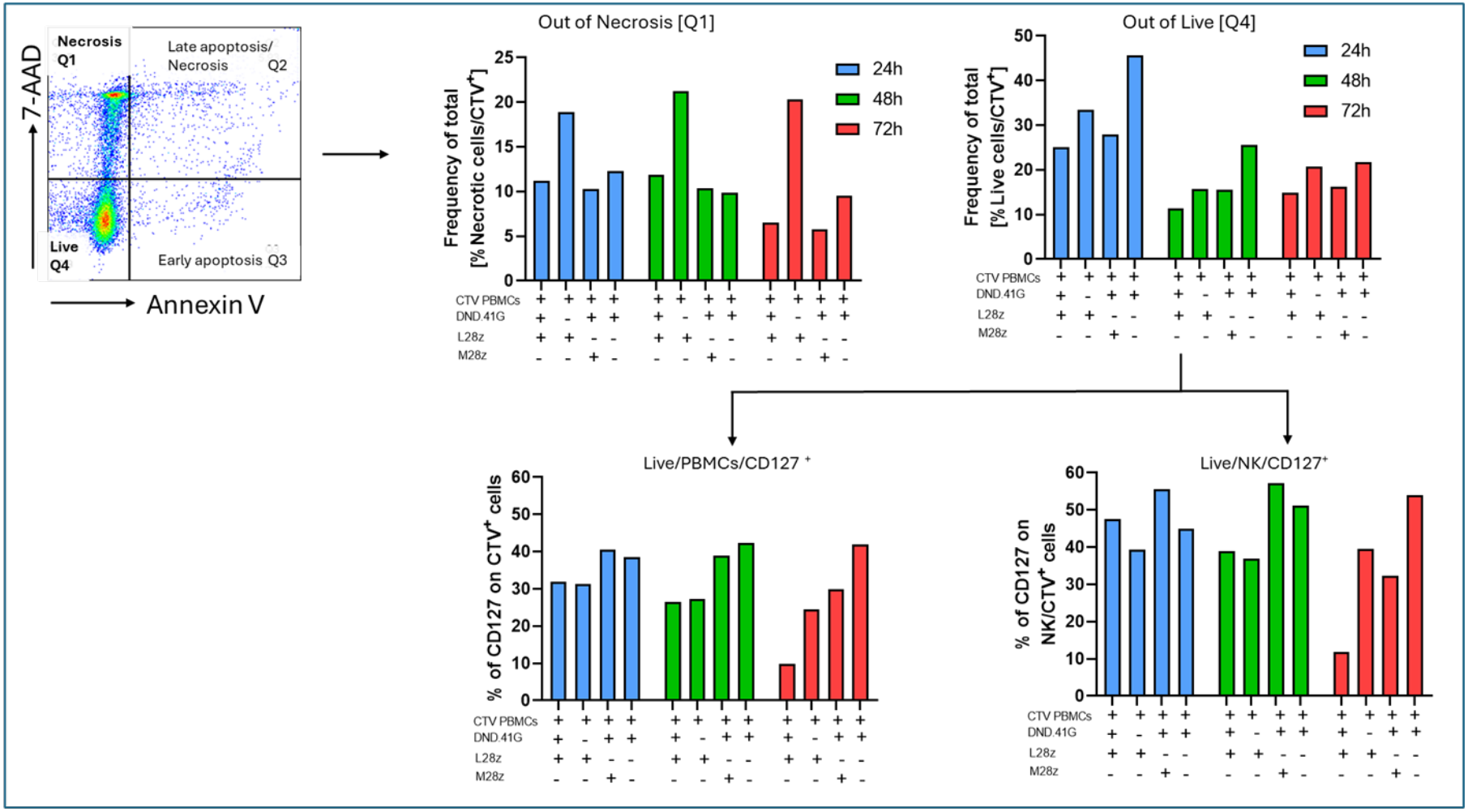
5. In addition, CD127 expression by normal human T cells is obviously a problem even if, as per the authors, they express a relatively "low" expression of "<5,000 molecules/cell". There is no guarantee that this will protect them from being targeted even by the low affinity CD127 CAR T

cells. Overall, I am not sure I would conclude from the combined data that CD127 is definitely "a highly promising target".

Response: We appreciate this insightful comment and agree that CD127 expression on normal T cells represents an important consideration. Notably, CD127 expression in healthy T cells is dynamic, rather than constant, as it is transiently downregulated upon activation, and it remains considerably lower at rest, compared with malignant T-ALL cells such as DND.

Nevertheless, to further address this valid concern, we recently performed additional coculture experiments including PBMCs, CD127-targeted CAR T cells, and DND T-ALL targets. We performed coculture assays using low-affinity CD127-targeted CAR T cells and fresh, nonactivated autologous PBMCs (CTV-labeled) in the presence or absence of T-ALL targets. At 24, 48, and 72 h, in the presence of tumor targets, the proportion/percentage of nonviable CTV-labeled PBMCs was comparable to controls (cocultured with irrelevant CAR or no CAR), indicating minimal bystander cytotoxicity in the presence of tumor cells overexpressing antigen.

In contrast, in the absence of tumor-cell antigen overexpression, we observed cytotoxicity against PBMCs, consistent with antigen density–dependent recognition. Collectively, these observations confirm that CD127-targeted CAR T cells preferentially engage CD127-high targets first. Additionally, within the live-cell compartment, CTV-positive T and NK cells persisted at all time points and retained detectable CD127 expression. These observations, now included as **Supplementary Figure 7d**, support that the low-affinity CD127 CAR preferentially targets malignant cells expressing high antigen density while limiting activity against normal lymphocytes in the presence of tumor antigen.



Supplementary Figure S7d. On-target off-tumor killing of low-affinity CD127 CAR T cells in autologous co-culture assays. Representative flow-cytometry plots showing gating strategy for live (Annexin V^- 7-AAD^-), apoptotic (Annexin V^+ 7-AAD^-), and necrotic (Annexin V^+ 7-AAD^+) CTV-labeled PBMCs after co-culture with CAR T cells. Quantification of necrotic (Q1) and live (Q4) fractions among CTV^+ PBMCs at 24 h (blue), 48 h (green), and 72 h (red). Expression of IL-7R (CD127) on residual live CTV^+ PBMCs and NK-cell subsets across time points and conditions. Autologous cytotoxicity was minimal in the presence of tumor targets (first bar of each time point) and comparable to irrelevant/no-CAR controls, whereas moderate killing emerged in tumor-free conditions (Second bar), consistent with antigen-density–dependent recognition by low-affinity CD127 CAR T cells.

6. The difference in the cytotoxic activity of high-affinity vs. low-affinity CAR-T as shown in Figure 1d seems to be statistically significant, however, the actual absolute difference is minimal. To some extent, the same applies to Figure 1e. The authors should acknowledge that.

Response: Thank you for this valuable observation. We agree that the absolute differences in cytotoxic activity between high- and low-affinity CAR T cells in *Figure 1d–e* are modest, despite being statistically significant. This point is acknowledged in the revised manuscript. Notably, the differences became more evident *in vivo*, where low-affinity CAR T cells achieved superior tumor control and extended survival, highlighting the functional relevance of affinity tuning.

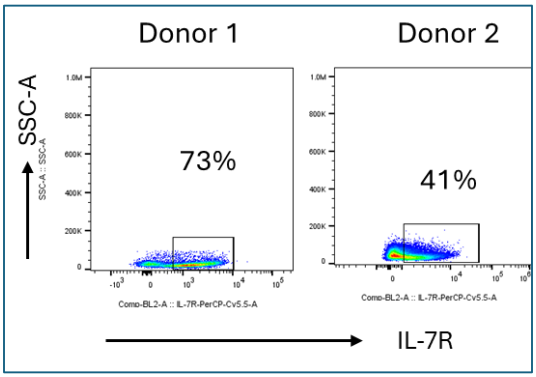
> **Page 24, Line 517**: Although the differences in cytotoxicity between high- and low-affinity CAR T cells were modest *in vitro, in vivo* experiments revealed that low-affinity CAR T cells provided greater tumor control and prolonged survival, confirming that affinity tuning confers a meaningful therapeutic advantage in a disease-relevant setting.

7. The authors do not demonstrate fratricide per se. Lower CAR T cell numbers could be due to a number of alternative factors such as differential responsiveness to proliferation-promoting cytokines, decreased resistance to exhaustion, etc..

Response: We appreciate the reviewer's comment and agree that factors such as cytokine responsiveness or proliferative capacity could contribute to differences in CAR T-cell recovery. However, several observations support that fratricide is the predominant mechanism. In coculture assays using anti-CD127 CARs with CFSE-labeled autologous T cells, we observed a selective reduction of labeled cells, which was not detected when IL7R-knockout (CRISPR 7R KO) T cells were used as targets (Figure 4c). This control strongly suggests that the effect is antigen dependent.

8. What did the expression of CD127 look like on freshly harvested T cells as well as on untransduced, LOW and HIGH CAR T cells over the whole course of CAR T-cell manufacturing? Does activation of T cells during manufacturing lead to a modification of CD127 expression on the RNA and/or protein levels?

Response: We appreciate the reviewer's question. Flow cytometry analysis showed that CD127 protein expression on freshly isolated PBMCs ranged from 40% to 80%, depending on the donor (see representative examples to the right). Untransduced T cells maintained similar expression levels (e.g., Figure 4a). Throughout CAR T-cell manufacturing, CD127 surface expression progressively decreased after activation and transduction, typically remaining below 20% in low-affinity CARs and below 30% in high-affinity CARs (e.g., Figure 1e).



Most importantly, we are happy to report that we are able to successfully manufacture IL7R-targeted low-affinity CAR T cells in our cell manufacturing facility using optimized protocols, described in this manuscript, for our upcoming clinical trial.

9. Again, the difference in tumor burden using high-affinity vs. low-affinity CAR-T as shown in Figure 2c seems to be statistically significant, however, the actual absolute difference is minimal.

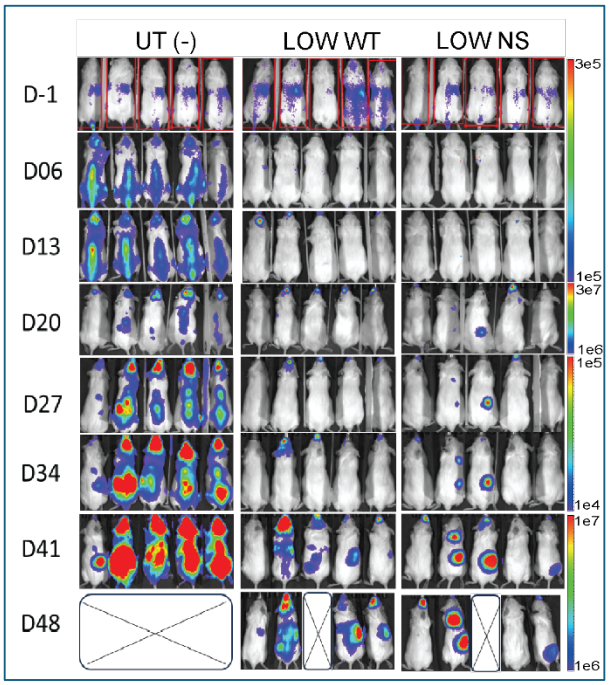
Response: We thank the reviewer for this thoughtful observation. While the absolute differences in tumor burden between high- and low-affinity CAR T cells in Figure 2c are modest, they are more pronounced than those observed *in vitro* and, importantly, translate into a survival benefit of approximately 1 month for the low-affinity CAR–treated group. This outcome was reproducible across multiple donors, with a similar pattern to that shown in Figure 5b.

10. *Could the lower numbers of BOTH tumor cells (which are T cells as well in the case of T-ALL) and LOW CAR T cells as shown in Figure 2f be unrelated to fratricide? Could this be related to a similarly decreased responsiveness to a third-party factor such as certain cytokines?*

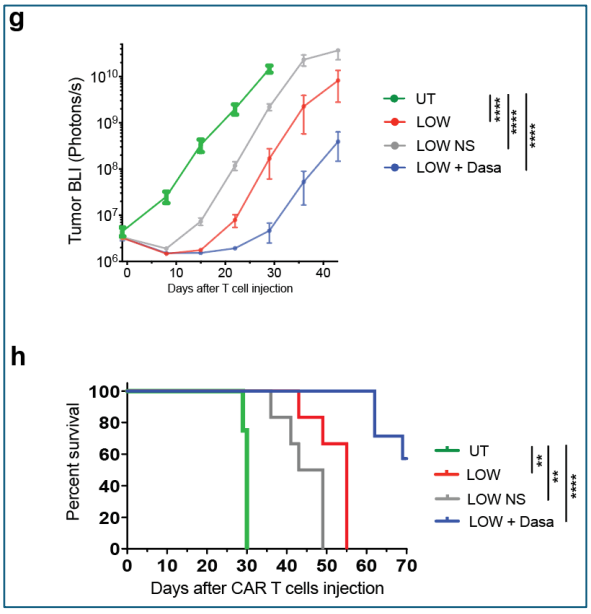
Response: We thank the reviewer for this thoughtful comment. We agree that the reduced numbers of both tumor and LOW CAR T cells observed in Figure 2f could theoretically reflect multiple factors, including differential cytokine responsiveness. However, on the basis of our data, fratricide remains a contributing factor. Following tumor clearance, CAR T cells are exposed to one another, and given that CD127 expression levels increase at this stage, it is plausible that CAR^+ cells recognize and eliminate each other. This interpretation aligns with our prior observations indicating antigen-dependent self-targeting. While we cannot completely exclude other contributing mechanisms, the combined evidence supports that fratricide may be a significant factor underlying these findings.

Additional Updates to Figure Panels

In addition to addressing the reviewers' comments, we made a few minor figure updates:



- Figure 6e: The bioluminescence imaging scale bar was adjusted to match the photon-flux range used during imaging.



- Figure 7 (panels g and h): The *in vivo* experiment was ongoing at the time of the original submission; we have now included the completed survival and bioluminescence imaging follow-up data.

Responses to Reviewer #2:
We are grateful to the reviewer for the time and effort in reviewing our manuscript and providing valuable suggestions to increase the impact of the study.

Dr. Hocine and colleagues report on the development of CD127 (IL7Ra)-targeting CAR T cells for the therapy of T-cell ALL. The authors show differential activity of T cells expressing low- vs high-affinity CAR and document expected CAR-mediated fratricide that can be mitigated by either CD127 gene KO or temporary inhibition of CAR signaling with dasatinib during manufacture. The resulting CAR-T cells mediate potent activity against CD127high T-ALL cell lines and primary blasts, both in vitro and in vivo thus supporting feasibility of targeting IL7Ra with CAR-T cells in T-cell leukemia.

1. IL7Ra as an immunotherapy target in T-ALL has been established in prior reports (e.g. Hixon et al., Leukemia 2019) which should be cited and discussed in this manuscript.

Response: We thank the reviewer for this valuable suggestion and regret this oversight. We have now cited Hixon et al., *Leukemia* 2019, and included a corresponding statement in the revised manuscript to acknowledge prior work establishing IL7Rα as a therapeutic target in T-ALL.

> **Page 5, Line 73:** Building on this biological relevance, IL7Rα (CD127) has been identified as a potential immunotherapy target in T-ALL.[4,5] Its restricted expression pattern and functional importance in leukemic cell survival support its value as a selective target for therapeutic intervention, providing a rationale for exploring IL7R-directed strategies alongside other T-lineage antigens.

2. CD127 is highly expressed in naive and memory T cells, NK cells, and some B-cells, and is downregulated in Tregs and effector T cells. In addition, CD127 is expressed in lymphoid progenitors in BM (CLP) and thymus (DN stage and SP) suggesting systemic activity of CD127 CAR-T would results in a global lymphoid ablation sparing Tregs and the rare activated/effector T cell. What would be the therapeutic advantage of CAR127 compared to CD7 or CD5 CART that are in the advanced clinical development considering higher expression of those antigens in T-ALL and a slightly more restricted presence on normal tissues compared to CD127? Would this be another bridge-to-transplant strategy despite the need for more "standalone" therapies (lines 87-89)?

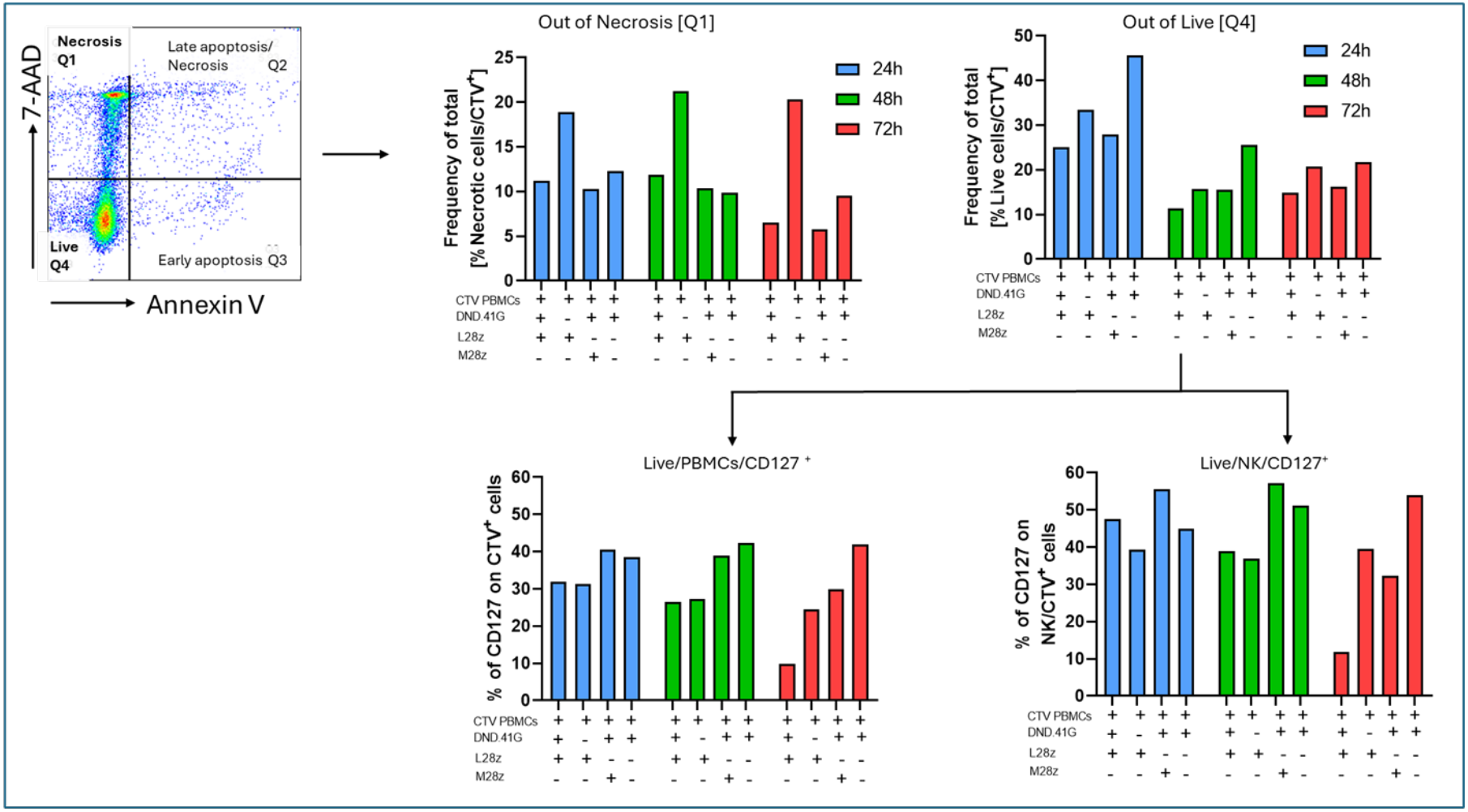
Response: We appreciate the reviewer's insightful question. In comparing antigen targets, it is important to note that CD5 and CD7, although broadly expressed on malignant and normal T cells, are primarily recognized as lineage markers, rather than established oncogenic drivers of T-ALL proliferation or survival.[6,7] By contrast, IL7Rα (CD127) is functionally implicated in T-ALL pathogenesis: activating mutations and overexpression engage key survival pathways (JAK/STAT5, PI3K/AKT) and can contribute to leukemogenesis. Accordingly, our CD127-directed strategy with affinity tuning and pharmacologic control is designed to exploit a disease-dependent vulnerability, rather than simply effect lineage ablation.

3. The authors state "Low-affinity CARs preferentially recognize tumor cells with higher antigen expression while sparing normal cells with lower antigen expression" (lines 497-498). Does this

imply the activity against normal circulating cells or activated CAR-T? Coculture assays of CAR127 T cells with freshly isolated, non-activated autologous T and NK cells from peripheral blood would help understand the degree of expected on-target toxicity and characterize the resistant T-cell population, if it arises.

Response: Thank you for this comment. To address this valid concern, we recently performed additional coculture experiments including PBMCs, CD127-targeted CAR T cells, and DND T-ALL targets. We performed coculture assays using low-affinity CD127-targeted CAR T cells and fresh, nonactivated autologous PBMCs (CTV-labeled) in the presence or absence of T-ALL targets. At 24, 48, and 72 h, in the presence of tumor targets, the proportion/percentage of nonviable CTV-labeled PBMCs was comparable to controls (cocultured with irrelevant CAR or no CAR), indicating minimal bystander cytotoxicity in the presence of tumor cells overexpressing antigen.

In contrast, in the absence of tumor-cell antigen overexpression, we observed cytotoxicity against PBMCs, consistent with antigen density–dependent recognition. Collectively, these observations confirm that CD127-targeted CAR T cells preferentially engage CD127-high targets first. Additionally, within the live-cell compartment, CTV-positive T and NK cells persisted at all time points and retained detectable CD127 expression. These observations, now included as **Supplementary Figure 7d**, support that the low-affinity CD127 CAR preferentially targets malignant cells expressing high antigen density while limiting activity against normal lymphocytes in the presence of tumor antigen.



Supplementary Figure S7d. On-target off-tumor killing of low-affinity CD127 CAR T cells in autologous co-culture assays. Representative flow-cytometry plots showing gating strategy for live (Annexin V⁻ 7-AAD⁻), apoptotic (Annexin V⁺ 7-AAD⁻), and necrotic (Annexin V⁺ 7-AAD⁺) CTV-labeled PBMCs after co-culture with CAR T cells. Quantification of necrotic (Q1) and live (Q4) fractions among CTV⁺ PBMCs at 24 h (blue), 48 h (green), and 72 h (red). Expression of IL-7R (CD127) on residual live CTV⁺ PBMCs and NK-cell subsets across time points and conditions. Autologous cytotoxicity was minimal in the presence of tumor targets (first bar of each time point) and comparable to irrelevant/no-CAR controls, whereas moderate killing emerged in tumor-free conditions (Second bar), consistent with antigen-density–dependent recognition by low-affinity CD127 CAR T cells.

4. CD127 is commonly expressed in ETP and cortical T-ALL but downregulated in more mature TCR+ subsets (Courtois et al., Blood 2023). Considering most experiments are done with CD127high cell lines, where would be the cut-off in %CD127 expression on T-ALL blasts below which this therapy becomes ineffective? This may be difficult to definitively answer without a clinical trial but any experimental data could be helpful for the clinical community to better understand potential impact of this therapy in a broader patient population.

Response: We thank the reviewer for this insightful comment. Indeed, CD127 expression exhibits variability among T-ALL subtypes, with higher levels typically observed in ETP and cortical T-ALL and lower levels in more-mature TCR^+ subsets, as described by Courtois et al., *Blood* 2023. Of note, that study also indicated that, when CD127 is expressed, the IL7R signaling pathway remains functionally active, independent of the presence of IL7R mutations.

In our study, we evaluated two representative T-ALL cell lines (DND-41 and HPB-ALL), both exhibiting high CD127 surface expression, and additionally demonstrated CAR efficacy against a patient-derived T-ALL sample *ex vivo*. In parallel, CD127 expression was assessed in several patient-derived T-ALL samples, revealing heterogeneous expression levels, with some cases exhibiting high IL7R expression (Extended Fig. 3). As the reviewer suggested, while a precise cutoff for therapeutic efficacy would ultimately require clinical validation, our findings suggest that effective CAR activity can be achieved in samples with moderate-to-high CD127 expression and that future inclusion criteria could be guided by antigen expression levels comparable to those observed in our tested samples.

Attesting to the reviewer's comment, we have now included the following in the revised manuscript.

> **Page 25, lines 538:** The CD127 expression cutoff percentage and intensity on T-ALL blasts in patients to be recruited for a trial of anti-CD127 CAR T cells are yet to be determined. However, approximately 70% of patients with T-ALL exhibit significantly elevated expression of CD127 on blast cells, compared with their counterparts with healthy T cells.

5. The authors conclude that CD127KO does not affect T-cell function (line 267). However, it is difficult to infer from the presented data considering the established role of IL7R in the long-term maintenance of memory T cells thus requiring prolonged assays in vivo. Either those experiments need to be performed or the authors should temper the conclusion and limit it only to short-term effector function in the models used.

Response: We thank the reviewer for this valuable observation. We acknowledge that the current data primarily support functional preservation within the experimental settings evaluated, and we have modified the corresponding statement in the manuscript to clarify this interpretation. The text has been revised to ensure that our conclusion regarding CD127KO T-cell function accurately reflects the scope of our data.

> **Page 17, Line 364:** Although CD127 knockout did not impair T-cell activity in our experimental settings, the role of IL7R in long-term T-cell maintenance warrants further

evaluation in extended models to fully define its impact on memory formation and persistence.

6. Experiments with dasatinib in vivo are in line with prior reports and serve as a supporting data for the authors to envision it as a "safety switch" post-infusion. However, the experiments were performed with a very high dose of dasa (50mg/kg b.i.d.) whereas the FDA-approved dose is much lower (140mg orally QD - so more like 2mg/kg, with different bioavailability and twice less frequent). It is unclear if dasatinib would work at this dose or would require a separate dose-finding study. Further, it would likely work as a short-term break to mitigate acute tox rather than a real "safety switch" to terminate CAR-T activity and limit lymphopenias (line 605-613). Long-term dasatinib administration at doses inhibiting CAR activity would likely also suppress TCR activation and therefore produce severe immune deficiency. The authors should clarify the intent of dasatinib administration post-infusion.

Response: We thank the reviewer for raising this important point. Our intent was to ensure full suppression of CAR signaling *in vivo*, consistent with previous reports demonstrating rapid and reversible CAR inhibition with dasatinib in murine models. Notably, Mestermann et al. and Weber et al.[1,2] showed that dasatinib at 10 mg/kg can achieve complete but transient inhibition of CAR activity, with full recovery upon drug withdrawal. These studies collectively indicate that the on/off effect is reversible, supporting the feasibility of modulation even ***at lower, clinically relevant exposures.***

In our study, although we did not test additional *in vivo* doses, the *in vitro* assays using ~100 nM dasatinib are estimated to correspond to low single-digit oral doses in mice (approximately 3–5 mg/kg) on the basis of published pharmacokinetic data.[8,9] This range is within exposures previously shown to transiently inhibit CAR activity *in vivo.*[1,2] These observations are consistent with prior reports demonstrating that dasatinib can reversibly modulate CAR signaling *in vivo.*[1,2] In the revised text, we have clarified that our data support this concept at the *in vitro* level and that the experimental dose is now discussed in the context of physiologically relevant exposures.

> **Page 30, Line 643:** Postinfusion dasatinib is envisioned as a short, event-driven intervention to transiently pause CAR signaling; chronic use at CAR-inhibitory levels is not intended, given potential suppression of TCR. Prior studies have documented rapid and reversible inhibition of CAR activity *in vivo* with brief exposure, with full recovery upon drug withdrawal.[1,2]

7. In the results and discussion (lines 419-421 and 536-538) the authors interpret the results of RNA-seq where the genes encoding key signaling kinases downstream of IL7R (JAK/STAT/PI3K/mTOR) are not perturbed in CD127KO T cells as this gene deletion has a minimal effect on T-cell function. It is hard to be convinced by these statements when the activity of these signaling pathways are regulated by post-translational modification (phosphorylation, localization) rather than by RNA expression. Further, these pathways would light up in T cells cultured in the presence of IL-2 as indicated in the methods, but would not be active in vivo where resting/memory T-cells rely on IL7R signaling for long-term survival. Therefore, this statement must be tempered or removed.

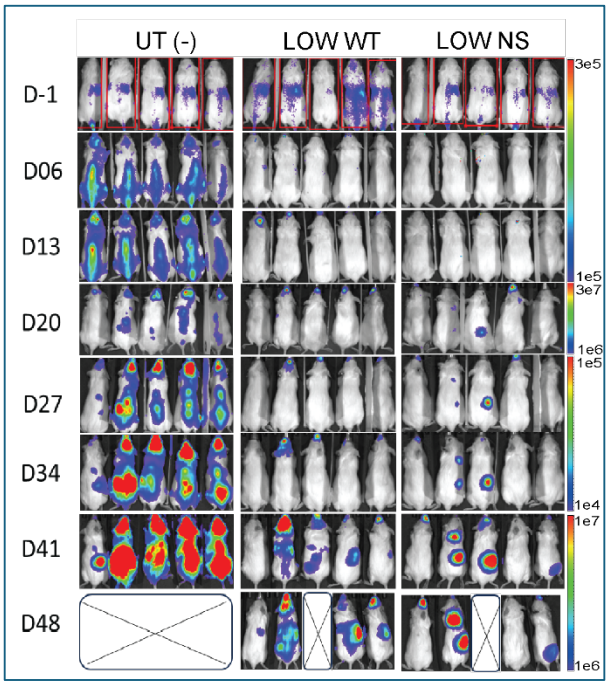
Response: We thank the reviewer for this thoughtful comment and agree that the activity of the JAK/STAT and PI3K/mTOR pathways is primarily governed by posttranslational modifications, rather than by transcript abundance. Moreover, because our assays were conducted in the presence of IL2, downstream signaling through these pathways is likely maintained independently of IL7R engagement, which could mask potential differences observable under resting conditions. To address this, we have tempered our interpretation and revised the corresponding text in the Results and Discussion to clarify that the RNA-seq findings reflect only transcriptional steadiness, without implying preserved functional signaling. We have also noted that further experiments in resting conditions could help confirm the potential effects of IL7R deletion on long-term T-cell survival.

Results (page 20, line 428):
RNA-seq analysis revealed no major transcriptional changes in canonical IL7R-associated genes (JAK/STAT/PI3K/mTOR) after CD127 knockout, suggesting that IL7R deletion did not markedly alter these pathways at the RNA level under the culture conditions used.

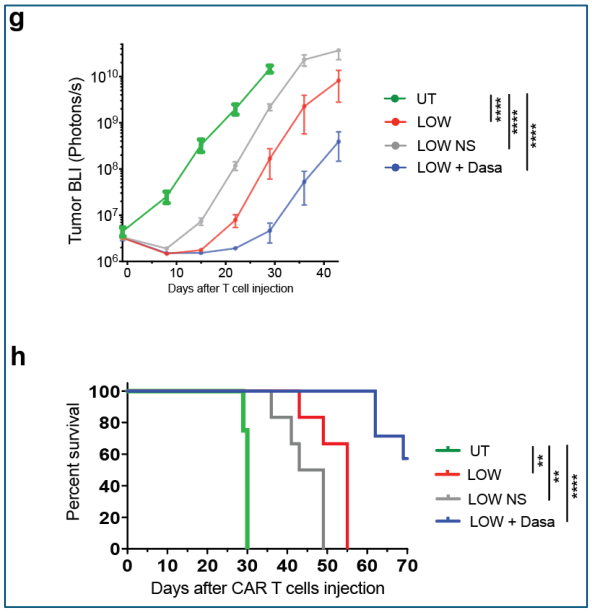
Discussion (page 26, line 561):
As JAK/STAT and PI3K/mTOR signaling are largely regulated posttranslationally and as our assays were performed in the presence of IL2, the RNA-seq profiles likely reflect transcriptional steadiness, rather than pathway activity. Analyses under resting conditions could further delineate the impact of IL7R deletion on downstream signaling and long-term T-cell maintenance.

Additional Updates to Figure Panels

In addition to addressing the reviewers' comments, we made a few minor figure updates:



- Figure 6e: The bioluminescence imaging scale bar was adjusted to match the correct photon-flux range used during imaging.



- Figure 7 (panels g and h): The *in vivo* experiment was ongoing at the time of the original submission; we have now included the completed survival and bioluminescence imaging follow-up data.

Response to Reviewer #3:
Hocine et al present a manuscript describing the development of an IL7R-specific CAR T cell therapy. The authors posit that IL7R, which is expressed on ~70% of T-ALL blasts and 40% of B-ALL blasts, is a good target for cellular therapy of these leukemias. IL7R is also overexpressed on normal T cells but is transiently downregulated upon activation….

The manuscript shows thoughtful experiments and robust results, is very thorough and well written. It could be enhanced by addressing the following queries.

Response: Thank you for taking the time to review our manuscript and provide supporting feedback.

1. Weight is not an accurate tumor burden monitoring tool as it can happen in the absence of tumor if there is inflammation present. Did the authors evaluate tumor burden in peripheral blood? If so, serial (e.g. weekly or biweekly) bleeds and evaluation of peripheral tumor burden will provide better information.

Response: We thank the reviewer for this comment. We agree that body weight *alone* is not a reliable indicator of tumor burden. In our study, serial bioluminescence imaging served as the primary readout of tumor progression. Although serial blood sampling was not performed, we did monitor GFP+ leukemic cells in the peripheral blood and in tissues, specifically when the mice were euthanized; relevant data are included in the Resultssection (Figs. 2f&g, 3h&i, 5g&h, 6g, and Supplemental Data). In addition, increasing bioluminescence imaging signals in untreated and irrelevant CAR controls correlated with higher tumor burden.

2. In certain tumor models, antigen downregulation happens in the absence of CAR T cell infusion. What is the IL7R expression on mice receiving tumor only or tumor and UTD T cells?

Response: We thank the reviewer for this insightful comment. In mice receiving tumor plus untransduced T cells, the proportion of GFP^+ leukemic cells expressing IL7R typically ranged between 60% and 95%, with the highest expression observed in the bone marrow. A representative example of this distribution is shown in Figure 2f.

3. Please comment on why LOW NS CAR T cell dose was 6x10^6 vs LOW KO dose was 1x10^6? Why was a higher dose needed? Where these cells less effective?

Response: We thank the reviewer for this important question. The different doses were designed to evaluate how CAR T-cell number influences the kinetics of tumor control and persistence. The higher dose (6×10^6) led to faster initial tumor clearance, which we hypothesized could increase self-recognition among IL7R-expressing CAR T cells once the leukemic burden was reduced. In contrast, the lower dose (1×10^6) achieved slower tumor elimination but a more delayed relapse due to longer persistence of CAR T cells. Interestingly, higher doses did not provide additional therapeutic benefit, compared with the lower dose, in our experimental setup.

To further explore this observation, we performed new *in vitro* repeat antigen stimulation assays using low-affinity wild-type (WT) and natural selection (NS) CAR T-cell products from 2

different donors. These experiments showed that cultures initiated at lower effector-to-target ratios tended to expand and accumulate more robustly over time than those started at higher effector-to-target ratios.

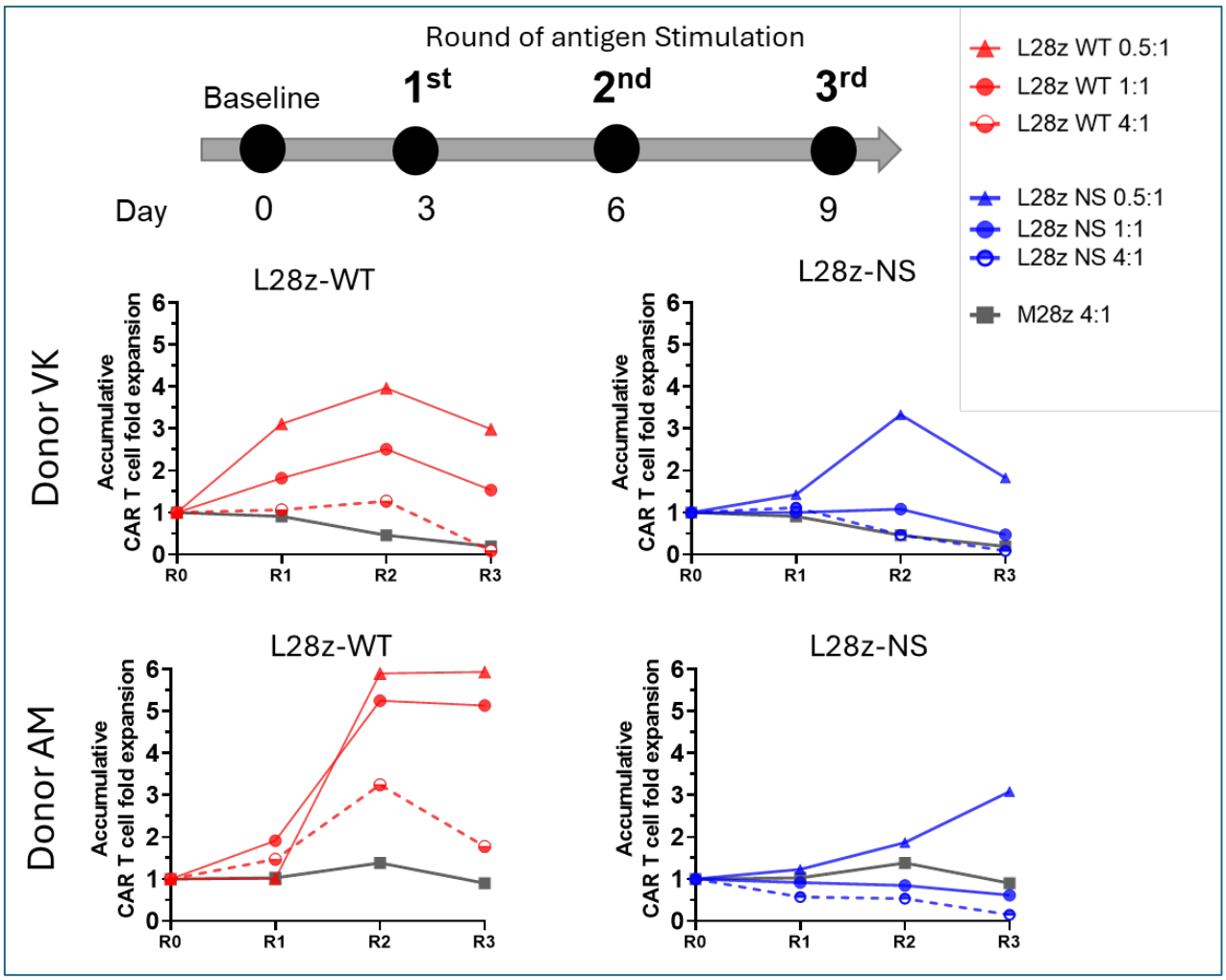


Figure: Repeat antigen stimulation shows dose-dependent CAR T-cell expansion dynamics.

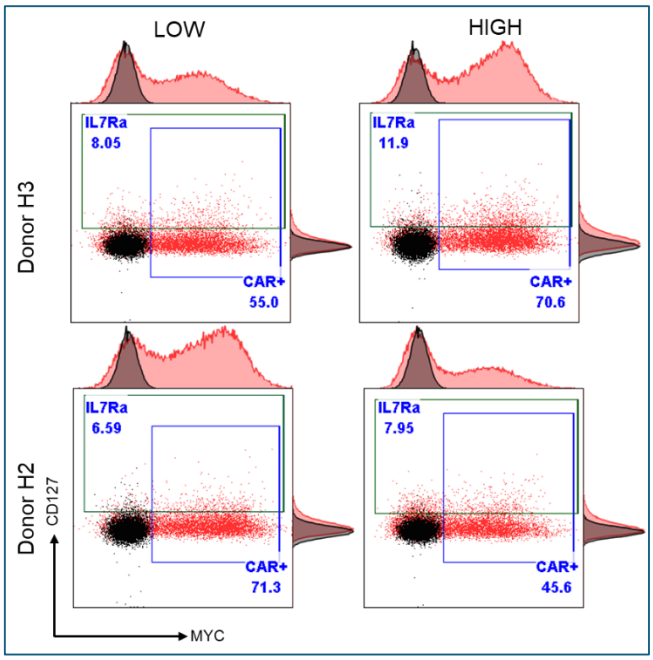
Figures:

4. Please include labels for each panel throughout the figures, as sharing labels between panels generates confusion.

Response: We thank the reviewer for this helpful suggestion. To improve clarity, individual panel labels have been added to Figures 2 and 4. Figure legends have been modified accordingly.

5. Figure 1. b) Please include transduction expression data of additional replicates, not only representative examples. g) Please comment on E:T used

Response: Transduction expression data from additional replicates are now included as Supplementary Figure 1g, and the legend for Fig. 1g has been updated to indicate an effector-to-target ratio of 1:1.



Supplementary Figure 1g

6. Figure 5. Please align duration of follow up between survival curves and bioluminescence curves.

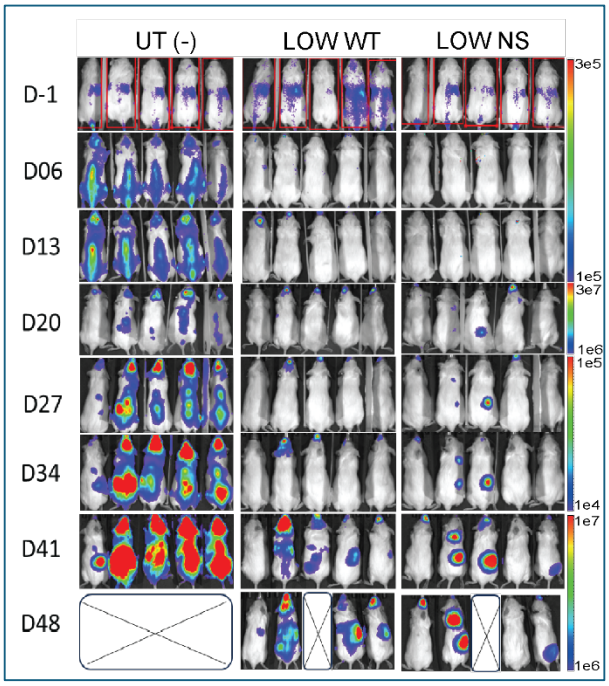
Response: We thank the reviewer for this observation. Once tumor progression is confirmed over multiple time points, the bioluminescence imaging series was concluded earlier than the survival follow-up, to minimize cost and effort, and cumulative stress and anesthesia exposure in mice during extended monitoring was recorded. Survival data were therefore tracked beyond the last imaging time point to capture long-term outcomes.

7. Figure 5g. Please show data for blood and intestine for LOW WT at relapse if available, so that it can be compared with LOW KO at relapse.

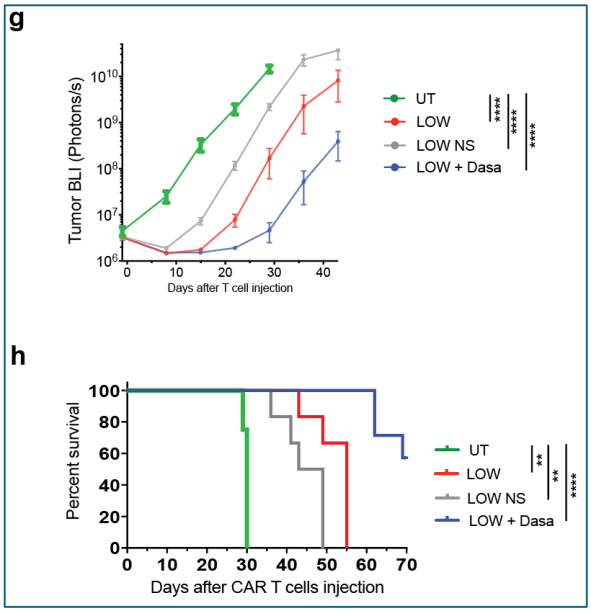
Response: We appreciate this comment. CAR T cells were not detected in blood or intestine at relapse in the LOW WT group, owing to limited sample quality and low cellular recovery at that time point, which precluded reliable detection of rare CAR^+ events.

Additional Updates to Figure Panels

In addition to addressing the reviewers' comments, we made a few minor figure updates:



- Figure 6e: The bioluminescence imaging scale bar was adjusted to match the correct photon-flux range used during imaging.



- Figure 7 (panels g and h): The *in vivo* experiment was ongoing at the time of the original submission; we have now included the completed survival and bioluminescence imaging follow-up data.

References

1 Mestermann, K. *et al.* The tyrosine kinase inhibitor dasatinib acts as a pharmacologic on/off switch for CAR T cells. *Sci Transl Med* **11**, eaau5907, (2019).

2 Weber, E. W. *et al.* Transient rest restores functionality in exhausted CAR-T cells through epigenetic remodeling. *Science* **372**, eaba1786, (2021).

3 Rosselle, L. *et al.* Protocol for production of tonic CAR T cells with dasatinib. *STAR Protoc* **6**, 103529, (2025).

4 Akkapeddi, P. *et al.* A fully human anti-IL-7Ralpha antibody promotes antitumor activity against T-cell acute lymphoblastic leukemia. *Leukemia* **33**, 2155-2168, (2019).

5 Hixon, J. A. *et al.* New anti-IL-7Ralpha monoclonal antibodies show efficacy against T cell acute lymphoblastic leukemia in pre-clinical models. *Leukemia* **34**, 35-49, (2020).

6 Kim, M. Y. *et al.* CD7-deleted hematopoietic stem cells can restore immunity after CAR T cell therapy. *JCI Insight* **6**, e149819, (2021).

7 Schwarz, S. & Linnebacher, M. CD5: from antiquated T cell marker to immunotherapy's new hope. *Signal Transduct Target Ther* **8**, 216, (2023).

8 Luo, F. R. *et al.* Dasatinib (BMS-354825) pharmacokinetics and pharmacodynamic biomarkers in animal models predict optimal clinical exposure. *Clin Cancer Res* **12**, 7180-7186, (2006).

9 Yoshimura, S. *et al.* Preclinical pharmacokinetic and pharmacodynamic evaluation of dasatinib and ponatinib for the treatment of T-cell acute lymphoblastic leukemia. *Leukemia* **37**, 1194-1203, (2023).

RESPONSE TO THE REVIEWERS

Reviewer #1:

All my comments were taken into account and the authors responded appropriately. I have no additional concerns.

Response: Thank you.

Responses to Reviewer #2:
We thank the reviewer for their continued time and thoughtful evaluation of our manuscript. The reviewer's suggestions have significantly strengthened the clarity and overall impact of the study.

1. The rationale provided in the response does not match that in the introduction (lines 77-93) where the authors instead discuss CD127 as a better target than CD5 or CD7 because the latter two are broadly expressed in T cells thus only offering a bridge-to-transplant approach. The current study indicates CD127 targeting would be just as toxic, if not more toxic, than targeting the other two antigens and therefore does not offer a better option like it is positioned in the introduction/rationale. CD127 is highly expressed in the vast majority of normal T cells and other cell types where CD5/CD7 are not present. Further, CD127 expression in T-ALL is much more heterogeneous and commonly downregulated in subtypes most prevalent among patients with r/r disease (cortical T-ALL responds well to standard therapies). Therefore, there is no evidence CD127 is a better or safer target than the other two, as stated in the introduction. The authors have to either convincingly (experimentally) demonstrate the opposite or re-write the rationale of targeting CD127 in the intro, to match experimental results

Response: We thank the reviewer for the comment. We agree that our study does not demonstrate that CD127 is categorically safer or superior to CD5 or CD7 as a CAR T-cell target. Our intention was not to position CD127 as a replacement for these lineage antigens, but rather as a biologically distinct and complementary target based on its functional role in T-ALL biology. While CD5- and CD7-directed CAR T cells eliminate T-lineage cells through lineage targeting and are often considered bridge-to-transplant strategies, CD127 targeting is grounded in pathway dependence in defined T-ALL subsets. Accordingly, our data support the feasibility of CD127-directed therapy in selected contexts, without implying broader safety or superiority.

We have added a new paragraph (**line 75-80**) to clarify the rationale for targeting CD127 and to better align the Introduction with our experimental findings.

> "Its functional importance in leukemic cell survival supports its value as a biologically relevant therapeutic target, providing a rationale for exploring IL7R-directed

strategies alongside other T-lineage antigens. Although CD127 is broadly expressed on normal T cells and its expression in T-ALL can be heterogeneous, its role in IL-7–mediated leukemic cell survival provides a rationale for therapeutic targeting on the basis of pathway dependence, rather than lineage restriction."

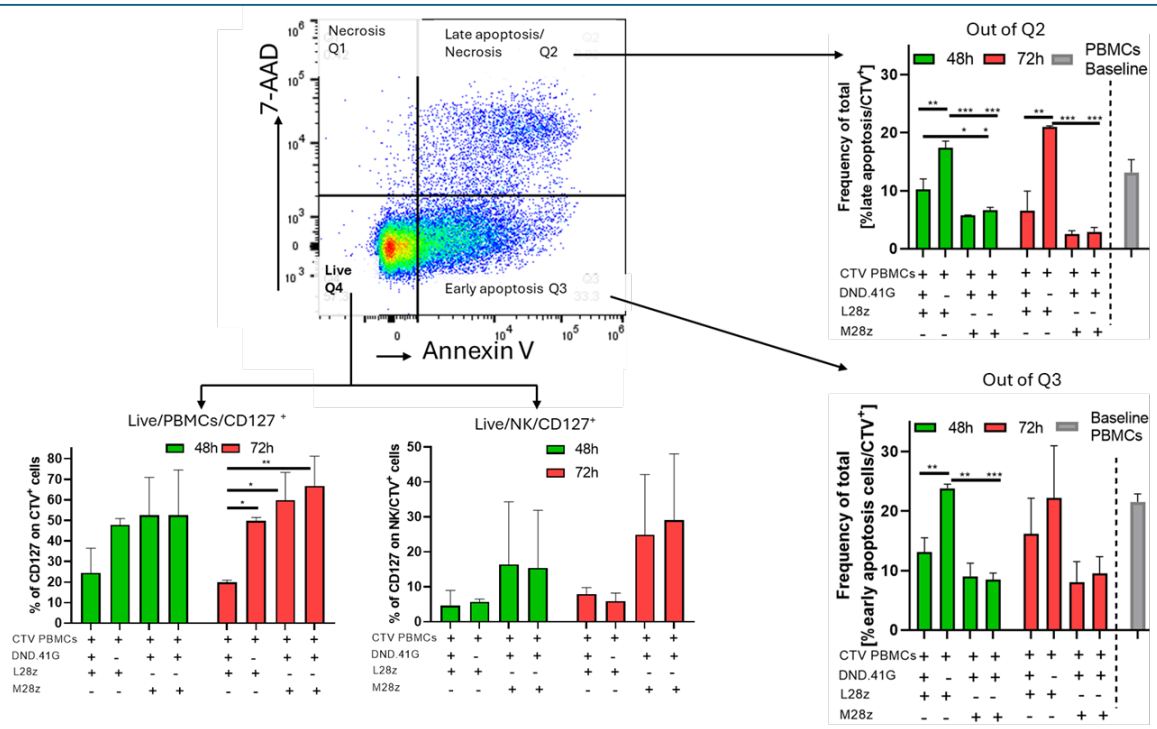
2. It is very hard to be convinced by the coculture results where the % of necrotic cells is the main readout. Robust on-target off-tumor experiments would include quantification of live cell counts before/after coculture, and an expected accumulation of apoptotic cells - neither of these parameters have been quantified. Further, these results contradict authors' earlier data where dasatinib or CD127 KO is required to mitigate normal T-cell killing by CART127. The new supplementary figure has to be completely re-done or removed altogether.

Response: We thank the reviewer for the comment, which prompted us to perform repeat apoptosis experiments. In the initial assay, Annexin V staining was performed using a non-calcium-containing buffer, which limited detection of apoptotic events. We have now repeated the assay using the calcium-dependent buffer using two independent PBMC donors.

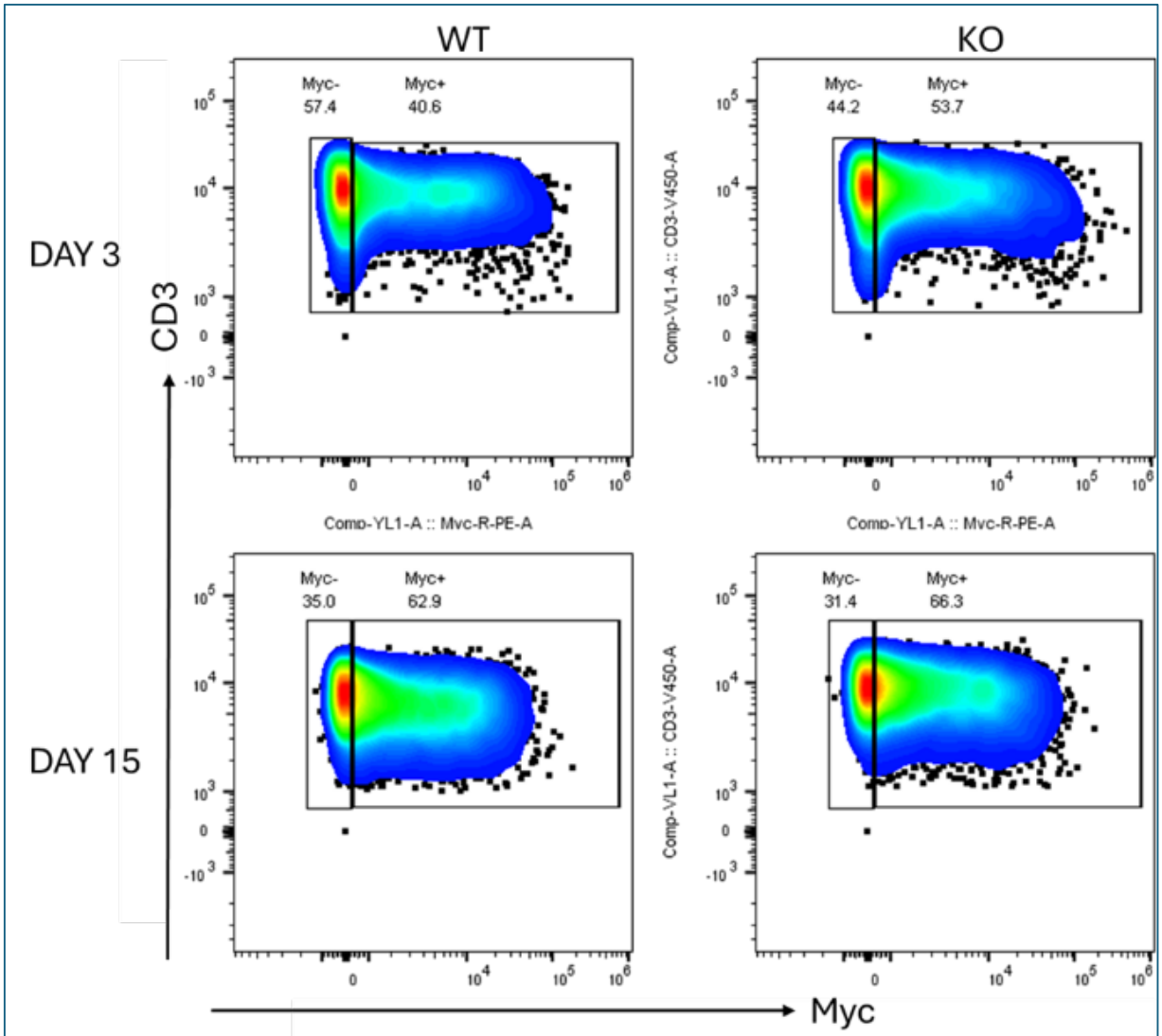
Total cell counts were quantified before and after coculture. We performed flow cytometric quantification of apoptotic and necrotic events within defined PBMC subsets to accurately assess on-target, off-tumor effects (as the assay involves three cellular components [CAR T cells, tumor cells, and PBMCs], absolute post-coculture counts alone are difficult to interpret without phenotypic discrimination of the dying population).

Our results show that the off-tumor PBMC apoptosis in the presence of tumor cells remains low and consistent across apoptotic compartments; results were reproduced with independent donors. PBMC killing becomes evident primarily in the absence of tumor cells and preferentially affects IL7R/CD127^high subsets, rather than causing broad PBMC depletion. The supplementary figure is revised to reflect the apoptosis analysis.

Consistent with these results, even during extended CAR T-cell cultures (~15 days), the frequency of Myc^{-} (CAR^{-}/nontransduced) T cells remains comparable between control and CD127-KO conditions (Response Figure R1).



Supplementary Figure S7d. On-target off-tumor killing of low-affinity CD127 CAR T cells in autologous co-culture assays. Representative flow cytometry plots showing the gating strategy for live (Annexin V^- 7-AAD^-), early apoptotic (Annexin V^+ 7-AAD^-), and late apoptotic/necrotic (Annexin V^+ 7-AAD^+) CTV-labeled PBMCs following co-culture with CAR T cells. Bar graphs show quantification of live and apoptotic fractions among CTV^+ PBMCs at 48 and 72 h. Data represent two independent donors and are presented as mean ± s.d. Expression of IL-7R (CD127) on residual live CTV^+ PBMCs and NK-cell subsets is shown across time points and experimental conditions. Autologous cytotoxicity in the presence of tumor targets was comparable to irrelevant or no-CAR controls. Statistical analysis was performed using one-way ANOVA with multiple comparisons. Significance is indicated as $P < 0.05$ (*), $P < 0.01$ (**), and $P < 0.001$ (***).



Response Figure R1. Comparable persistence of CAR⁻ (Myc⁻) T cells in WT and CD127-KO cultures after extended expansion. Representative flow cytometry density plots showing Myc tag expression (CAR marker) after ~15 days of culture. The frequency of Myc⁻ (CAR⁻/non-transduced) T cells remains comparable between WT and CD127-KO conditions across donors, indicating that CD127 disruption does not result in preferential loss of non-transduced cells during prolonged expansion.

3. The response is factually incorrect. In the Courtois 2023 study, it was found that 70% of T-ALL express CD127 at various levels, often much lower than the physiologic counterparts. A citation from that paper:
"Conversely, CD127 expression was minimal in ETP-ALL and mature TCRαβ T-ALL, again in contrast with the physiological pattern of normal thymocytes, which normally express the

receptor at these differentiation stages. (Figure 1A,E). We found a similar expression (68%; P = not significant) in a retrospective analysis of 100 pediatric T-ALL cases (Figure 1D)." (https://ashpublications.org/blood/article/142/2/158/495237/IL-7-receptor-expression-is-frequent-in-T-cell)

That study highlighted elevated CD127 expression mainly in cortical T-ALL which, again, has a good prognosis and will rarely be treated with CAR-T. cT-ALL also commonly express CD1a which is a much better target not expressed in normal T cells (stuides by Pablo Menendez, currently in clinical trial).

Therefore, this erroneous statement must be removed. Further, considering this and prior discrepancies, the authors should critically re-analyze existing literature and re-think the rationale of targeting CD127 with CAR-T.

Response: We agree with the correction. We acknowledge that CD127 expression in T-ALL is heterogeneous. Our study does not propose CD127 as a universal CAR T-cell target for all patients with T-ALL. Rather, our data demonstrate the feasibility of targeting CD127 in CD127-high T-ALL patients, consistent with the biomarker-driven approach used in other cell therapies. Clinical application would therefore require patient stratification based on CD127 expression levels.

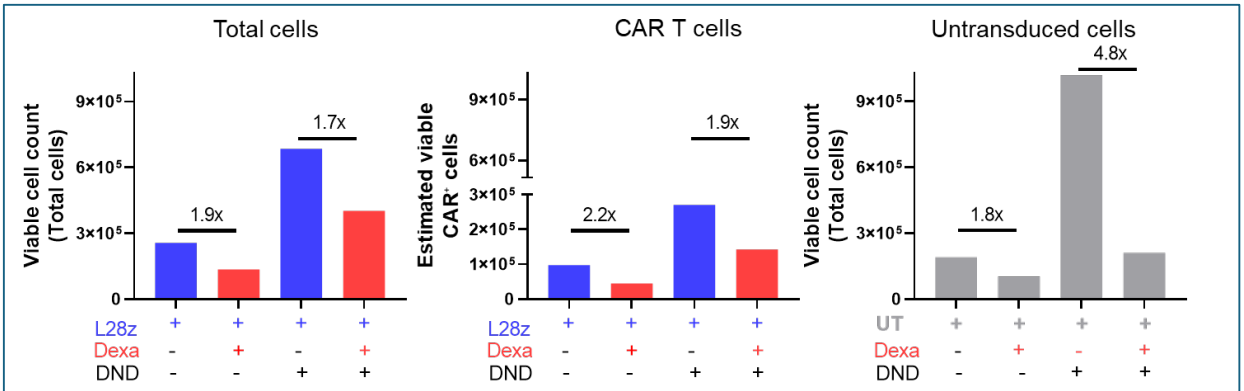
> The paragraph has been modified accordingly (**line 65-66**):
>
> "Approximately 70% of leukemic blasts from patients with T-ALL and 30% to 40% of blasts from patients with B-ALL express IL7R, although expression levels vary across disease subtypes."

4. In the abstract, the authors still state "In addition, in vivo, dasatinib serves as a reversible safety switch to treat potential on-target, off-tumor toxicity or prolonged lymphopenia." I thought we have agreed that this strategy would not be effective in preventing off-tumor toxicity/lymphopenia as it would require prolonged administration which by itself would be toxic and immunosuppressive. In vivo dasatinib can only be applied to halt acute toxicities like CRS/ICANS etc but NOT to prevent unwanted targeting of normal tissues. To achieve this, CAR-T must be effectively eliminated, not temporarily inhibited. Unfortunately, the authors continue to contradict themselves.

Response: We thank the reviewer for raising this point. We agree that prolonged in vivo administration of dasatinib would not be an appropriate long-term strategy to prevent sustained on-target, off-tumor toxicity or lymphopenia and that its clinical utility would be better suited to transient suppression of acute CAR T-cell activity. To avoid ambiguity, we have revised the abstract to clarify that dasatinib functions as a reversible pharmacologic inhibitor to temporarily modulate CAR signaling, rather than as a definitive strategy to prevent prolonged tissue targeting.

We have also modified the corresponding text accordingly in the manuscript (**lines 471, 583-588, and 652**), as well as the title of Figure 7.

In addition, to address concerns regarding toxicity management, we performed new *in vitro* experiments using dexamethasone, an FDA-approved agent routinely used to manage CAR T-cell–associated toxicities in T-ALL patients, to evaluate its effects on CD127 CAR T cells. These studies show that dexamethasone reduces CAR T-cell viability (**Supplementary Fig. 7d)**. Notably, DND41 tumor cells are also sensitive to dexamethasone. In *in vivo* experiments, we similarly observed that dexamethasone affects both CAR T cells and tumor cells, making the interpretation of antitumor effects challenging (data not shown). To better clarify these effects, additional studies incorporating dexamethasone-resistant tumor controls are planned for future IND-enabling work.



Supplementary Figure 7d. Effect of dexamethasone on CAR T-cell expansion. Estimated CAR⁺ cell numbers were calculated from total viable counts adjusted by CAR expression measured by flow cytometry at 72 h. In the absence of tumor targets, dexamethasone reduced CAR⁺ cell numbers (~2.2-fold). In the presence of DND41 cells, a similar reduction was observed (~1.9-fold). Comparable trends were observed in untransduced (UT) T cells cultured under the same conditions.

5. Given the agreed-upon limitation in interpreting the results of RNA-seq, are these data needed at all? I am not sure this adds anything of value to the study.

Response: We agree that RNA-seq should not be interpreted as a functional assessment of IL7R-dependent signaling. However, the dataset was included to evaluate whether CD127 knockout induced widespread or unintended transcriptional changes under the conditions tested. Thus, it provides an unbiased global assessment of transcriptional integrity following gene editing, supporting the specificity of the approach.

We thank the reviewer for helping us to improve the strength of our manuscript. We hope that the additional experiments and revisions address the points raised.

Reviewer #3:

The authors have addressed the reviewers' queries satisfactorily.

Response: Thank you.

REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

1. Thank you for the opportunity to review the revised manuscript and the additional correspondence regarding the remaining concerns raised by Reviewer #2. After carefully evaluating the revised manuscript and rebuttal, I believe the authors have addressed the major scientific concerns sufficiently for publication, although a few wording modifications would still strengthen the manuscript.

Reviewer #2 raises reasonable points regarding the positioning of CD127 as a target antigen in T-ALL. I agree that the original framing may have implied that CD127-directed CAR T cells are inherently safer or superior to CD5/CD7-directed approaches. However, in the revised version, the authors substantially moderated these claims and now position CD127 primarily as a biologically motivated target based on IL7R pathway dependence in selected T-ALL subsets rather than as a universally safer alternative. In my opinion, this clarification adequately resolves the conceptual concern. While additional refinement of wording in the Introduction may still be beneficial, I do not believe this issue represents a fatal flaw in the study.

Response: We thank the reviewer for this positive assessment. We agree that CD127 should be presented as a biologically motivated target in selected T-ALL subsets, rather than as a universally safer alternative to CD5- or CD7-directed approaches.

2. Regarding the concerns about the on-target/off-tumor coculture experiments, Reviewer #2 is correct that live-cell quantification approaches could provide additional orthogonal support. However, the authors did perform additional apoptosis analyses with appropriate Annexin V methodology and independent donor validation. Although the experimental system is complex and not perfectly optimized for absolute live-cell quantification, I believe the presented data are sufficient to support the manuscript's main conclusions regarding fratricide and relative off-tumor effects. Importantly, the central conclusions of the manuscript are supported by multiple complementary datasets, including repeated in vitro assays, in vivo efficacy studies, knockout experiments, and manufacturing optimization approaches.

I also agree with Reviewer #2 that the wording regarding dexamethasone should remain carefully limited to the available data. The revised wording provided by the authors, clarifying that dasatinib acts as a reversible pharmacologic suppressor of CAR activity rather than a definitive long-term safety solution, appears appropriate. I do not interpret this remaining issue as a major scientific concern.

Overall, I believe the manuscript presents substantial conceptual and translational advances, including:

- identification of CD127 as a biologically relevant target in IL7R-high T-ALL subsets,
- systematic comparison of affinity tuning approaches,
- demonstration of fratricide mitigation strategies,
- and translationally relevant manufacturing optimization approaches.

In my opinion, the remaining concerns primarily relate to framing, interpretation, and preferred experimental design choices rather than deficiencies that invalidate the major conclusions of the work. I therefore believe the manuscript is suitable for publication after final editorial refinement of wording and claims.

Response: We thank the reviewer for this assessment. We have carefully reviewed the revised manuscript and confirm that CD127 is presented as a biologically relevant target in selected T-ALL subsets, rather than as a universally safer alternative to CD5- or CD7-directed approaches, that dexamethasone and dasatinib are discussed within the limitations of the available data, and that conclusions regarding off-tumor effects remain proportional to the experimental evidence presented.

Reviewer #2 (Remarks to the Author):

The authors addressed the questions in their response which was a good start but did not completely resolve the issues.

1. The clarification is appreciated but it does not resolve the main concern around positioning of this paper. From the current introduction:
"Furthermore, because these potential side effects affect regeneration of normal T cells and immune reconstitution, CD5 and CD7 CAR T-cell therapies are considered to be only a bridge to allogeneic hematopoietic stem cell transplant. Therefore, there is a clear unmet need to develop effective and safe targeted cellular immunotherapies that spare normal hematopoietic cells and that can serve as a definitive, standalone treatment for patients with T-ALL. In this study, we investigated low- and high-affinity CD127-targeted CAR T-cell therapy in clinically relevant T-ALL models, including in a T-ALL patient–derived xenograft model, to investigate the potential of CD127–targeted CAR T-cell therapy for the treatment of T-ALL."
This clearly positions the study to demonstrate CD127 CAR-T as a safer alternative to other CAR-T cells and use as a standalone treatment for patients with T-ALL. The rationale for that is not explained in the introduction and the results do not support this positioning, as agreed by the authors in the added paragraph earlier in that section. This is contradictory and needs to be reconciled. There is nothing wrong in exploring CD127 as an alternative target to CD5 or CD7 etc but the authors should not mislead the reader on the positioning of this antigen. As agreed by the authors, current literature indicates its expression is more prevalent on normal lymphocytes and less prevalent in leukaemia compared to CD5 or CD7 thus making it (in theory) an inferior choice. An argument about T-ALL dependency on IL-7R signaling is valid but weak considering its weak/heterogeneous expression in most subtypes outside of the cortical lineage. So exploring CD127 as a target is fine but the rationale has to be rectified based on the supportive evidence to avoid self-contradiction.

Response:
To the reviewer: We respect the reviewer's interpretation. However, comparing CD5, CD7 CARs to CD127-targeted CARs is neither the goal nor the intention of our study. We did revise the manuscript extensively to remove any such indication as suggested by the reviewer.

To the editorial board: If the editorial board deems it necessary to address the reviewer's comment, we oblige and have no objection to remove the following sentence:

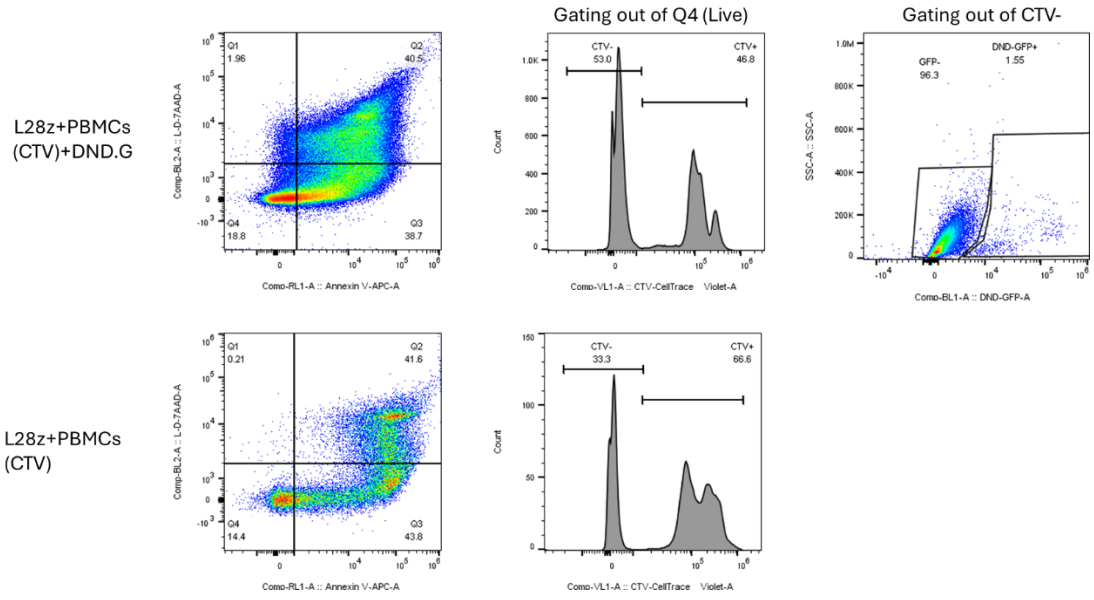
Page 6, top: "Therefore, there is a clear unmet need to develop effective and safe targeted cellular immunotherapies that spare normal hematopoietic cells and that can serve as a definitive, stand-alone treatment for patients with T-ALL."

2. Quantifying residual live cells is critical and can be easily done by flow cytometry using counting beads. There is no need to mix CAR-T with leukaemia and normal PBMC, those cocultures should be done separately and live CTV+ cells quantified by flow cytometry. Day 0 counts should be taken as a baseline. Relative frequency of apoptotic cells in a 3-way co-culture is not a standard or rigorous way to evaluate cytotoxicity. This experiment supports a central claim of this paper and has to be done right. Luckily, it is not technically hard. The %CAR-negative data is irrelevant since those are effector cells that downregulate CD127 anyway.

Response:

In this study, our intent was to model a more clinically relevant setting in which CAR T cells encounter both malignant and normal cells simultaneously. The 3-way co-culture system enables direct comparison of CAR T-cell activity toward leukemia cells and PBMCs under shared conditions.

In addition, we show below the data of an estimation of residual live target cells based on reanalysis of existing flow cytometry data. After 48 hours of co-culture with L28z CAR T cells, we observed a substantially greater reduction in DND41-GFP$^+$ leukemia cells compared to CTV$^+$ PBMCs, supporting preferential depletion of leukemia cells under these conditions.



3. Dex data is in line with prior publications. However, the authors again mislead the reader in the abstract stating "In addition, in vivo, dasatinib can be used to temporarily and reversibly suppress CAR T-cell activity, and dexamethasone can be used to control CAR T cells.". There is

no in vivo data for steroid inhibition to support that claim.

Response:
As mentioned in the previous response to the reviewer, we did observe that dexamethasone control CAR T cells in vivo as well. However, we did not include the data in the manuscript, as repeat experiments are needed with additional controls, which we plan to perform in future studies.

We agree with the reviewer to remove '*and dexamethasone can be used to control CAR T cells'* as we did not include in vivo results in the manuscript.

Reviewer #3 (Remarks to the Author):

The submitted manuscript continues to show value as an additional targeting strategy for adult and pediatric T-ALL. The authors have satisfactorily answered all queries.

Response: We appreciate the reviewer's careful evaluation of the manuscript and are pleased that the responses and revisions have satisfactorily addressed all concerns.