nature portfolio

Peer Review File

Iron-mediated ferroptosis impairs CAR-T cell function and antitumor efficacy

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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)
The manuscript by Kong and colleagues describes the role of ferroptosis in the persistence of CAR T cells and the impact of this process on overall therapeutic efficacy. The role of ferroptosis in cancer immunotherapy has recently gained much attention. This is the first description of this process in relation to CAR T cell therapy and so has the potential to provide important insights to the field. The major strength is this novelty. The studies are well conducted although some conclusions are, in my opinion, slightly overstated and could be rephrased. The authors demonstrate that that inhibition of ferroptosis can enhance CAR T cell efficacy and although these results are clear and well defined, the improvement in efficacy is moderate. However, this work sets the scene for further interrogation of this pathway in future works. The manuscript could also benefit from greater mechanistic insight into the phenotypes observed in CAR T that are engineered to be resistant to ferroptosis through ASCL4 knockout.

Major comments
1) In Figure 1h-m the authors present a number of biomarkers of iron metabolism in the expansion, peak time and diminution phase of CAR T cell response. It would significantly strengthen the conclusions of this work if these biomarkers could be correlated with clinical outcomes observed in these patients. Is this data available?

2) The persistence of CAR T cells in CLL patients is correlated with the proportion of CD45RO-CD27+ subset (https://www.nature.com/articles/s41591-018-0010-1), whilst ither studies have described the importance of CD45RA+CD62L+ TSCM like CAR T cells. Under the culture conditions used by the authors it appears that the cells are 100% CD45RO+ so it is difficult to interpret how inhibition of ferroptosis is affecting the phenotype of CAR T cells within this paradigm. The authors should repeat these experiments under conditions where CD45RA+CD62L+ cells are observed in order to determine what impact ferroptosis inhibition has on the capability of the cells to persist. The authors should also increase the numbers of markers being looked at here e.g. CD27, TCF1. It is also unclear from the analyses whether the authors have gated on CD8+ CAR T cells or if this includes all T Cells. It would be useful information to know if CD8+ or CD4+ T cells were more affected.

3) The phenotypes observed with the Fer-1 pretreatment are impressive, but the mechanism is unclear. It seems unlikely that treatment with Fer-1 can make the CAR T cells resistant to ferroptosis at later timepoints since this reagent is described to have a short half life of only a few hours and the CAR T cells will be proliferating and dividing in response to antigen in vivo. What seems more likely is that Fer-1 is improving the qualities of the CAR T cell product e.g. through enriching memory like CAR T cells but this has not come through in the analyses presented so far – see point number 2. It would be useful to provide more insight into the impact of Fer-1 on CAR T cell phenotype e.g. through bulk or single cell RNA-sequencing.

4) With regards to the patient data presented in Figure 3a, the authors present a longitudinal analyses of serum iron concentrations in patients undergoing CAR T cell therapy. Given that chemotherapy is known to impact on iron levels and can cause anemia, to what extent are these changes merely reflective of the preconditioning regimen rather than relating to the CAR T cell therapy specifically? As per comment #1 it would enhance the impact of this data if these iron levels could be correlated to outcome or CAR T expansion kinetics.

5) Why does Extended Fata Figure 3h stop at 96 hours. There is only one timepoint where the conditions are different from each other- this would be more convincing if later timepoints were shown.

6) Can the authors comment on why (in Extended Data Figure 4) Fer-1 only partially reduces changes in lipid ROS?

7) The authors claim that iron treated CAR T cells exhibit shrunken mitochondria (Extended Data Fig4i). Please quantify these images.

8) In studies with the low iron diet (Figure 3) can the authors please provide BLI images beyond day 28. In particular, it is of interest to clarify why in the survival curves 60% of mice with low iron diet are presented as long term survivors, yet in the BLI images and quantification in panel o all mice appear to have progressive disease at the last time point measured and therefore one would assume all mice ultimately relapse.

9) On Line 373 the authors state that ASCL4 KO reversed the iron induced upregulation of COX2. This does not appear to be the case with the data in Figure5g. Perhaps the authors mean partially reversed. This should be quantified.

10) The data with ASCL4 KO is a strength of the manuscript but should be further developed. The data in the GD2 model are not currently convincing although it looks like there could be a phenotype there. In the BLI images only 3/8 of the control CAR T treated mice appear to have tumors making it difficult to conclude the ASLC4 KO CAR T are more effective. The data also stops at day 20. It would be impactful if these experiments could be repeated under conditions where the control CAR T are less effective (bigger tumors or fewer CAR T cells) and present longer follow up data.

11) Following on from point 10, the significance of the study would also be elevated if a much more comprehensive analyses of CAR T cell phenotype could be provided. Currently this is just a limited analysis of CAR T cells in the bone marrow of the NALM6 tumor bearing mice. Ideally the authors would assess both the spleens and tumors of tumor bearing mice and analyze memory status (CD45RA, TCF7, CD62L), effector function and metabolic fitness.

Minor comments

1) Figure 1D- Please provide statistical analyses for these e.g. FDR.

2) Line 123 refers to oxidative phosphorylation, fatty acid metabolism and ROS regulation as ferroptosis-related pathways. As acknowledged by the authors later n the manuscript, these pathways are associated for a range of cellular processes so it is inaccurate to characterize them in this way. I think it would be better to see that GSVA revealed enrichment of pathway x, y and z, which is of interest as these pathways have been shown to be associated / upregulated in the context of ferroptosis.

3) Line 131- I do not think it is possible from the analyses presented to claim that ferroptosis is the “predominant mechanism of cell loss”. Suggest this statement is softened.

4) Line 166 states that Fer-1 increased the proportion of memory cells but none of the figure panels cited show this data.

5) Line 204. The authors state that their data suggest that chronic tonic signaling sensitizes CAR T cells to ferroptosis but no data showing tonic signaling of the CAR being used is shown. The data could equally reflect that more differentiated cells (as will naturally increase in proportion with extended culture time) are more sensitive to ferroptosis. There are ways to test the impact of tonic signaling e.g. dasatinib treatment if the authors wish to corroborate this claim. Otherwise, these statement should be softened.

Reviewer #2

(Remarks to the Author)
In this manuscript, Kong and colleagues characterize the mechanisms underlying CAR-T cell dysfunction, and reveal that CAR-T cells undergo ferroptosis during their dimunition phase due to elevated serum iron levels. Mechanistically, serum iron promotes mitochondrial ROS, while in parallel enhancing ACSL4 activation. Importantly, knockout of ACSL4 in CAR-T cells significantly improves antitumor efficacy. Overall, the manuscript uncovers a novel mechanism supporting a vulnerability of CAR-T cells towards ferroptosis with potential implications for improving CAR-T cell therapy. Addressing the comments below will benefit the manuscript and bolster the authors’ claims.

Major Comments:

To substantiate the claim that ferroptosis is responsible for the loss of CAR-T cells in vivo, the authors should use systemic delivery of ferroptosis inhibitors in the animal studies (for example use of ferrostatin-1, liproxstatin-1, or vitamin E).

While the authors provide a convincing phenotype with CART cells +/- pre-treatment with Fer-1 in the experiments in Fig. 2b, quantification of the number of live CAR-T cells (using live/dead stains) within these animals would bolster the claim that these CAR-T cells are indeed undergoing ferroptosis. Similar data should be shown for experiments related to Fig. 3L. Additional live/dead staining should be undertaken in in vitro experiments.

To rule out any potential confounding effects from cancer cells, the authors should repeat the experiment in Fig. 3e with CAR-T cells in tumor free mice with or without systemic administration of a ferroptosis inhibitor in vivo.

Given the importance of iron in augmenting CAR-T cell dysfunction and the utility of iron chelators in the clinic, can the authors provide the data showing that “neither iron chelation, nor knockdown of iron-related genes, effectively inhibited iron-induced ferroptosis in CAR-T cells” as mentioned in the discussion.

Could the authors provide IHC and/or IF quantification of the numbers of CAR-T cells infiltrating in the 143B tumors in Fig. 6e.

Lipidomics should be performed on CAR-T cells during expansion, peak, and diminution phase.

Minor Comments:

Inclusion of images of in vitro FC treated CART cells to verify a ferroptotic morphology.

The authors claim that CAR-T cells enter a ferroptosis susceptible state upon FC treatment in part due to upregulation of ACSL4. Given the role of ACSL4 in lipid remodeling, lipidomics should be performed on WT and ACSL4 KO CAR-T cells +/- FC.

The authors claim that CAR-T cells become especially vulnerable to ferroptosis due to increased serum iron levels compared to endogenous T-cells (Fig. 1l-m). Could the authors provide any explanation for this observation?

The authors demonstrate a delay in CAR-T cell loss in humans, this suggests that systemic iron alone is not sufficient to induce acute ferroptosis in CAR-T cells upon infusion. Could the authors provide a scientific rationale for this observation?

Can the authors comment on why serum iron levels are altered in ALL and MM patients at defined time points during CAR-T cell treatment?

Could the authors offer an explanation for the discrepancy between Fer-1 and Lip-1 in the phenotypes observed in extended data figure 2j?

Please include the entire uncropped portion of 4HNE blot in fig 1f and g.

It is unclear how the timepoints to assess tumor formation following CAR-T injection as shown in Figure 2a were chosen. Do they correlate with CAR-T cell levels in human blood as shown in Figure 1a (exhibit distinct expansion, peak, dimunition phases)?

Formatting:

Please adjust the colors of points in extended data figure 1b.

Please add the y-axis label to extended data figure 1i.

Please describe in the figure legend what timepoints are being used to calculate the p-values in Figure 2c.

Please add an axis to extended data figure 4c.

Reviewer #3

(Remarks to the Author)
Kong et al. investigated how iron metabolism may underly CAR-T cell dysfunction and diminished long-term persistence in patients through multi-omics analyses of clinical samples. They found that CAR-T cells undergo ferroptosis during their diminution phase due to elevated serum iron levels, with excessive intracellular iron accumulation impairing CAR-T cell functions both in vivo and in vitro. Mechanistically, they demonstrated that iron promotes ferroptosis by inducing mitochondrial reactive oxygen species accumulation and directly enhancing ACSL4 phosphorylation and enzymatic activity, while showing that pharmacological ferroptosis inhibition o ACSL4 ablation in CAR-T cells significantly improved antitumor efficacy and CAR-T persistence in multiple murine models.

Overall Assessment
This is a highly impressive and data-rich manuscript that is well-presented and well-written. The authors comprehensively explored ferroptosis in CAR-T cells using multiple orthogonal methods including patient samples, in vitro studies, and in vivo models. The study provides convincing data that iron modulation can affect CAR-T persistence and efficacy, though the magnitude of the effect may not be very high according to their data.

I note that CAR-T cells necessarily undergo a diminution phase (as T cells do after any immune response; otherwise exponential T cell growth would occur), and inhibiting this is not necessarily a goal per se. Overall, I believe this is an excellent manuscript deserving of publication and of sufficient scientific and technical merit to interest readers.

Specific Comments for the Authors
Figure 1
• Figure 1g: Where is GPX4? The bands look similar between peak vs diminution compared to the actin band.
• Figure 1h-k: Please label the y-axes so readers know what they're looking at. It's unclear what flow cytometry analysis is being performed here or in panels l-m.
Figure 2
• Figure 2b: The scale keeps changing in the images, making it appear that there is no disease at late timepoints when there presumably is. Please just show the data consistently.
• Figure 2c: Why only show the quantification until day 24?
• Figure 2d: What did the mice die from?
• Figure 2f-i: Why only 3 mice per group? Was this a small cohort or did you narrow down the mice? Why use 5 per group for cytokines (Figure 2j-q) when you had 10?

Methodological Clarification
Line 167-168: It doesn't look like you used the Giavridis et al. model referenced in citation 36 here. Is that indeed the case, or are these the same mice as above? The statement reads: "Importantly, safety evaluations using established cytokine release syndrome (CRS) models36 revealed that Fer-1 treatment did not exacerbate systemic inflammation or induce cytokine storms."
Figure 3
Figure 3l-p and Extended Figure 4: I note that mouse survival and tumor control here are much worse than previously shown in Figure 2. Indeed, the CAR-T cells in the standard and low iron groups essentially had no survival benefit over the no-CAR condition. Do the authors have an explanation here? The low-iron group here looks similar to the previous normal iron condition in Figure 2. Was the experiment set up differently, or is this due to donor variability or another explanation?

Suggestions for Strengthening the Study
Ideally, I would like to see an immunocompetent model, as the interplay with the full immune system would be fascinating (i.e., mouse CAR-T cells and tumor). This would strengthen the data, though there is already a substantial amount of data presented and this by no means essential.

Minor Point
I note a recent publication on GPX4 (10.1007/s00262-025-04133-w) as a master regulator, which would be interesting to comment on in the discussion.

Conclusion
This manuscript represents a significant contribution to our understanding of CAR-T cell biology and provides mechanistic insights into ferroptosis-mediated dysfunction. The comprehensive approach and robust methodology make this work suitable for publication in Nat Cancer.

Reviewer #4

(Remarks to the Author)
Kong et al identified the critical roles of environmental iron and ferroptosis in CAR-T cell dysfunction and provides insights for enhancing CAR-T therapeutic outcome.

This is novel and high-level interesting story, with potential translational impact.
Some concerns or issues to be considered:
1. Fig 2. Fer-1 is here used as ferroptosis inhibitor (in ex vivo setting maybe OK), however much better ferroptosis inhibitors for in vivo use are around such as Lip-1 or UAMC-3203. Were they also considered or tested. Can these further improve survival of mice (Fig 2d)?
2. Work performed related to MoA, seems a bit biased to involvement of mitochondria and related ROS. Fig 4c illustrates that MoA is more complex than involvement of mito's. Iron is stored in lysosomes, which can release labile iron pool. More extended study on role of lysosomes and labile iron pool could strengthen paper. Fig 3c also highlight some other potential interesting pathways contributing to sensitivity such as estrogen, WNT signaling… maybe worth looking into?
3. What is effect of ACSL-4 KO on CAR-T functionality?
4. Authors favor gen therapy over pharmacological targeting with ferroptosis inhibitors. Not sure if this is correct. Maybe more balanced view on pro and contra's of both approaches should be considered in discussion.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors have added significant amounts of new data that significantly strengthens the manuscript. In particular the new in vivo data with ASCL4 KO is highly compelling.

I only have a few minor points that require clarification.

1) The authors provide new data showing that ferroptosis inhibitors significantly elevate CD27 and CD62L expression. However I am confused that the authors show data showing that these cells are almost 100% CD45RO+ (e.g. Ex Data 4d) and now also ~100% CD45RA+ (e.g. Ex Data 4f). Distinct CD45RA and CD45RO populations should be discernible and it is the CD45RA+CD45RO+ population that is generally considered more prognostic. For example, see Figure 4b of Fraietta et al. Nature Med 2018.

2) The new data with ferroptosis inhibition in vivo (revised Fig 2a-g) are well received and to my reading suggests that the pre-treatment of CAR T cells with these inhibitors is relatively more important than inhibition in vivo given the strength of the respective phenotypes. I suggest the results text is modified to reflect this point.

Reviewer #2

(Remarks to the Author)
The authors have adequately addressed our prior comments, and the manuscript has improved significantly. It is well written, the findings are timely and of clear potential clinical importance, and I anticipate this work will stimulate further research on the interplay between ferroptosis, iron metabolism, and cancer immunology. I therefore support acceptance of the manuscript in its current form with minor copyediting (see notes below).

Re: Reporting Summary and the revised statistical/methodological reporting.
Overall, the statistical approaches appear appropriate for the study design. The remaining issues are limited to clarity and consistency of reporting which I assume will be addressed during copyediting:
1. Replicate reporting / definition of n: In several figure panels, n is provided but not described in a way that clearly distinguishes biological replicates/independent experiments from technical replicates. I recommend adding a brief descriptor (e.g., "independent biological replicates, "independent experiments," etc.) in the figure legends or Methods.

2. Minor statistical reporting/legend inconsistencies: A small number of figure-legend statements describing statistical tests appear inconsistent or unclear and should be corrected for precision. In Figure 2 legend, the assignments "two-way ANOVA (d,f)" and "log-rank test (e,g)" should instead be "(c,f)" and "(d,g)," respectively. In addition, in Fig. 4e, the legend/text indicates "unpaired two-tailed t test (e)," but this comparison should be analyzed via ANOVA rather than a t-test. This may simply be a typographical inconsistency, as panel (e) appears to be included in the ANOVA statement elsewhere; however, it should be corrected to avoid confusion.

Reviewer #3

(Remarks to the Author)
I'm happy that the authors have addressed my points and the manuscript is much improved. I would still have liked to see the immunocompetent model - it should be straightforward to make mouse CAR-T cells. these don't expand in vitro but you just make more at the start.

Reviewer #4

(Remarks to the Author)
Congrats, impressive revision of manuscript.

RE: Manuscript ID: NATCANCER-A19808

Title: Identification and targeting of iron-mediated ferroptosis in CAR-T cells

Reviewer's comments：

Reviewer #1:

The manuscript by Kong and colleagues describes the role of ferroptosis in the persistence of CAR T cells and the impact of this process on overall therapeutic efficacy. The role of ferroptosis in cancer immunotherapy has recently gained much attention. This is the first description of this process in relation to CAR T cell therapy and so has the potential to provide important insights to the field. The major strength is this novelty. The studies are well conducted although some conclusions are, in my opinion, slightly overstated and could be rephrased. The authors demonstrate that that inhibition of ferroptosis can enhance CAR T cell efficacy and although these results are clear and well defined, the improvement in efficacy is moderate. However, this work sets the scene for further interrogation of this pathway in future works. The manuscript could also benefit from greater mechanistic insight into the phenotypes observed in CAR T that are engineered to be resistant to ferroptosis through ASCL4 knockout.

Summary： We thank the reviewer for the supportive assessment of the novelty and overall quality of this study. In response to the reviewer's suggestions, we have revised the manuscript to temper the wording of several conclusions so that they more accurately reflect the magnitude of the observed therapeutic effects.

To further strengthen the mechanistic insight into ferroptosis-resistant CAR-T cells, we have additionally incorporated lipidomics analyses of ACSL4-knockout CAR-T cells. These data provide complementary metabolic evidence supporting a role for ACSL4 in regulating lipid peroxidation susceptibility in CAR-T cells, and help explain the functional phenotypes observed following ACSL4 ablation. The relevant results have been added to the revised manuscript.

Below, we provide point-by-point responses to the reviewer's specific comments.

Major Comments:

Q1. In Figure 1h-m the authors present a number of biomarkers of iron metabolism in the expansion, peak time and diminution phase of CAR T cell response. It would significantly strengthen the conclusions of this work if these biomarkers could be correlated with clinical outcomes observed in these patients. Is this data available?

Response:

We thank the reviewer for this insightful suggestion regarding the clinical relevance of iron metabolism-related biomarkers.

Regarding the markers shown in Fig. 1h-m (e.g., lipid ROS and related parameters), it is important to note that these samples were collected longitudinally at different phases following CAR-T cell infusion. During this period, patients are exposed to multiple concurrent and dynamic influences, including lymphodepleting chemotherapy (as the reviewer also pointed out in comment #4), cytokine release syndrome (CRS), and the release of intracellular contents resulting from extensive CAR-T and tumor cell death. These confounding factors substantially affect systemic iron homeostasis and ferroptosis-related readouts, thereby limiting the interpretability and robustness of direct correlations between serum iron/ferroptosis biomarkers and clinical outcomes within this longitudinal setting.

To directly address the reviewer's concern and strengthen the clinical relevance of our findings, we performed additional analyses in an independent cohort of 10 CAR-T-treated patients (5 complete responders [CR] and 5 non-responders [NR]). In this cohort, serum protein levels of ferritin light chain (FTL) and ferritin heavy chain (FTH1) were measured prior to CAR-T infusion. Notably, patients with higher serum ferritin levels exhibited inferior clinical responses compared with those achieving CR (**revised Fig. 1l and m**). Furthermore, we assessed baseline serum iron levels prior to CAR-T cell infusion and found that patients with elevated serum iron concentrations tended to have poorer therapeutic outcomes (**revised Fig. 3c**). These results provide independent clinical evidence supporting a negative association between systemic iron burden and CAR-T cell efficacy. Corresponding results and descriptions have been incorporated into the revised manuscript (**revised manuscript lines154-157 and 214-219**).

Q2. The persistence of CAR T cells in CLL patients is correlated with the proportion of CD45RO-CD27+ subset (https://www.nature.com/articles/s41591-018-0010-1), whilst ither studies have described the importance of CD45RA+CD62L+ TSCM like CAR T cells. Under the culture conditions used by the authors it appears that the cells are 100% CD45RO+ so it is difficult to interpret how inhibition of ferroptosis is affecting the phenotype of CAR T cells within this paradigm. The authors should repeat these experiments under conditions where CD45RA+CD62L+ cells are observed in order to determine what impact ferroptosis inhibition has on the capability of the cells to persist. The authors should also increase the numbers of markers being looked at here e.g. CD27, TCF1. It is also unclear from the analyses whether the authors have gated on CD8+ CAR T cells or if this includes all T Cells. It would be useful information to know if CD8+ or CD4+ T cells were more affected.

Response:
We thank the reviewer for these helpful and constructive suggestions. As recommended, we expanded our phenotypic analyses to include a CD45RA-CD27 panel in addition to the original CD45RO-CD62L markers. Consistent with our previous observations, iron

loading induced a pronounced reduction in CD27 expression, whereas ferroptosis inhibitors significantly elevated CD27 expression, and CD45RA expression remained uniformly high across conditions. (**revised Extended Data Fig. 2j-l, and Extended data Fig. 4f-h**).

Notably, following adoptive transfer in vivo, we detected the emergence of a CD45RA-negative CAR-T cell population (**revised Fig. 2i**). This observation is consistent with clinical reports in CLL patients (PMID: 29713085) and suggests that phenotypic diversification of CAR-T cells is shaped by distinct in vivo environmental cues that are not fully recapitulated during ex vivo expansion.

Finally, all phenotypic analyses were performed on gated CAR^+ T cells derived from bulk $CD3^+$ T cell populations, which therefore comprised both $CD4^+$ and $CD8^+$ subsets. Under these conditions, iron loading and ferroptosis inhibition significantly altered T-cell differentiation states as well as the proportion of $CD8^+$ cells (**revised Extended Data Fig. 2j, l and Extended Data Fig. 4g, h**). Consistent with the scRNA-seq results (**Extended Data Fig. 5f**), these findings indicate that iron loading or ferroptosis inhibition markedly influences the relative abundance of $CD8^+$ CAR-T cells in the experimental settings examined.

Q3. The phenotypes observed with the Fer-1 pretreatment are impressive, but the mechanism is unclear. It seems unlikely that treatment with Fer-1 can make the CAR-T cells resistant to ferroptosis at later timepoints since this reagent is described to have a short half life of only a few hours and the CAR T cells will be proliferating and dividing in response to antigen in vivo. What seems more likely is that Fer-1 is improving the qualities of the CAR T cell product e.g. through enriching memory like CAR-T cells but this has not come through in the analyses presented so far – see point number 2. It would be useful to provide more insight into the impact of Fer-1 on CAR T cell phenotype e.g. through bulk or single cell RNA-sequencing.

Response:
We thank the reviewer for these insightful comments regarding the durability and mechanism of Fer-1-mediated effects on CAR-T cells. We agree that, given the short reported half-life of Fer-1, it is unlikely that transient pretreatment alone would confer long-term resistance to ferroptosis in vivo, particularly in the context of antigen-driven CAR-T cell proliferation and expansion.

In response to this concern, and in line with suggestions from multiple reviewers, we have substantially expanded our in vivo and ex vivo analyses (**revised manuscript lines 163-202**). First, we included additional ferroptosis inhibitors (Lip-1 and UAMC) during ex vivo CAR-T cell culture and evaluated their effects in tumor-bearing mouse models (**revised Extended Data Fig. 2n-q**). Second, we implemented a continuous in vivo dosing regimen to directly assess whether sustained ferroptosis inhibition enhances CAR-T cell efficacy, thereby minimizing the confounding effects of short-

term drug instability (**revised Fig. 2a-g**). Across these complementary approaches, inhibition of ferroptosis consistently enhanced CAR-T cell-mediated tumor control and prolonged survival, supporting a functional role for ferroptosis suppression in promoting CAR-T cell efficacy in vivo.

In addition, as suggested by the reviewer, we further expanded our phenotypic analyses using a CD45RA-CD27 panel to assess the impact of Fer-1 on memory-associated CAR-T cell populations both ex vivo and in vivo (**revised Extended Data Fig. 2j-l and Fig. 2i, p, q**). These results demonstrate that Fer-1 significantly promotes stemness-associated features of CAR-T cells in both settings, while concomitantly reducing the expression of exhaustion markers (**revised Fig. 2k-m**).

Moreover, single-cell RNA-sequencing analysis revealed that Fer-1 treatment effectively reversed iron-induced oxidative stress in CAR-T cells, as evidenced by the downregulation of ROS-related and DNA repair-associated pathways (**revised Fig. 4c**). Fer-1 has been reported to function as a ROS inhibitor(PMID: 31571665), and excessive ROS accumulation is known to impair CAR-T cell functionality (PMID: 38171332). Consistently, in the CAR-T manufacturing setting, Fer-1 acted as an oxidative stress-mitigating agent that preserved mitochondrial activity (**revised Extended Data Fig. 3p-r**), thereby enhancing CAR-T cell fitness. In contrast, in post-infusion in vivo models, Fer-1 primarily functioned as a ferroptosis inhibitor, counteracting iron-induced ferroptotic toxicity, as demonstrated by the sustained suppression of lipid ROS in vivo (**revised Fig. 2h**), ultimately preserving CAR-T cell survival.

Q4. With regards to the patient data presented in Figure 3a, the authors present a longitudinal analyses of serum iron concentrations in patients undergoing CAR T cell therapy. Given that chemotherapy is known to impact on iron levels and can cause anemia, to what extent are these changes merely reflective of the preconditioning regimen rather than relating to the CAR T cell therapy specifically? As per comment #1 it would enhance the impact of this data if these iron levels could be correlated to outcome or CAR T expansion kinetics.

Response:
We thank the reviewer for this important comment regarding the interpretation of longitudinal serum iron measurements during CAR-T cell therapy.

Accordingly, consistent with our response to **Comment #1**, to strengthen the clinical relevance of the iron-related analyses while minimizing confounding effects from preconditioning chemotherapy, we additionally examined baseline serum iron levels measured prior to lymphodepletion and CAR-T cell infusion. Although baseline serum iron showed only a non-significant trend toward higher levels in non-responders compared with complete responders (**revised Fig. 3c**), this trend was concordant with the significantly elevated serum levels of FTL and FTH1 proteins observed in non-

responders (**revised Fig. 1l and m**). Together, these findings suggest that pre-existing alterations in systemic iron homeostasis, rather than acute treatment-related fluctuations, may be associated with inferior clinical responses to CAR-T cell therapy.

We acknowledge that, due to cohort size limitations and the confounding impact of lymphodepletion, we were unable to robustly correlate longitudinal serum iron levels with CAR-T outcomes. We have revised the manuscript to clarify this limitation in discussion (**revised manuscript lines 511-529**) and to avoid over-attribution of iron dynamics to CAR-T cell-specific effects.

Q5. Why does Extended Fata Figure 3h stop at 96 hours. There is only one timepoint where the conditions are different from each other- this would be more convincing if later timepoints were shown.

Response:
We thank the reviewer for this helpful suggestion. In the revised manuscript, we have repeated the experiment and extended the expansion curve in **revised Extended Data Fig. 3h** to day 8. With the inclusion of later time points, a clear divergence between conditions becomes evident, demonstrating that prolonged iron exposure leads to progressively impaired CAR-T cell expansion. These updated data strengthen the conclusion that iron loading exerts cumulative inhibitory effects on CAR-T cell proliferation over time.

Q6. Can the authors comment on why (in Extended Data Figure 4) Fer-1 only partially reduces changes in lipid ROS?

Response:
We appreciate the reviewer's careful examination of Extended Data Fig. 4. We apologize for any potential confusion regarding the interpretation of lipid ROS levels following Fer-1 treatment.
As shown in revised **Extended Data Fig. 3m**, ferric citrate (FC) significantly increased lipid ROS levels in CAR-T cells, whereas co-treatment with Fer-1 reduced lipid ROS to levels that were not significantly different from untreated CAR-T cells ($p = 0.8366$). Thus, under the conditions tested, Fer-1 effectively restored iron-induced lipid ROS accumulation to near-baseline levels rather than only partially suppressing it.

Q7. The authors claim that iron treated CAR T cells exhibit shrunken mitochondria (Extended Data Fig4i). Please quantify these images.

Response:
We thank the reviewer for this important suggestion. We have now quantitatively analyzed mitochondrial morphology in CAR-T cells using transmission electron microscopy images. Specifically, mitochondrial area and length (Feret's diameter) were measured across multiple cells per condition in a blinded manner (**revised Extended**

Data Fig. 3q and r).

Q8. In studies with the low iron diet (Figure 3) can the authors please provide BLI images beyond day 28. In particular, it is of interest to clarify why in the survival curves 60% of mice with low iron diet are presented as long-term survivors, yet in the BLI images and quantification in panel all mice appear to have progressive disease at the last time point measured and therefore one would assume all mice ultimately relapse.

Response:
We thank the reviewer for the careful examination of the in vivo bioluminescence imaging (BLI) data in Fig. 3. In response to this comment, we have extended the BLI analysis beyond day 28 and now include imaging data at day 33 in the revised **Fig. 3g-i**. At this time point, four mice in the low-iron diet group remained alive, whereas all mice in the standard-iron and high-iron diet groups had succumbed to disease.

We also apologize for any confusion caused by the presentation of the survival curves in the original Fig. 3p. During figure assembly, the censoring marks (vertical ticks) corresponding to deaths in the low-iron diet group were inadvertently omitted, which may have given the impression that all mice were long-term survivors. The corrected survival plot now includes the missing censoring information, and the statistical analysis was re-performed (**revised Fig. 3i**).

Together, the updated BLI data and corrected survival curves clarify that a subset of mice receiving a low-iron diet exhibit prolonged survival, consistent with delayed disease progression rather than complete absence of relapse.

Q9. On Line 373 the authors state that ASCL4 KO reversed the iron induced upregulation of COX2. This does not appear to be the case with the data in Figure5g. Perhaps the authors mean partially reversed. This should be quantified.

Response:
We thank the reviewer for this careful observation. In the revised version, we have performed densitometric quantification of all immunoblot data and provided the quantitative results in **revised Supplementary Table 4**.

We agree that the original wording describing COX2 regulation was imprecise. Quantitative analysis shows that iron exposure still significantly increased COX2 protein levels in ACSL4-KO CAR-T cells, although the magnitude of induction was lower than that observed in control CAR-T cells. Thus, ACSL4 deletion did not completely reverse iron-induced COX2 upregulation.

Importantly, ACSL4 knockout significantly reduced the basal expression level of COX2 in CAR-T cells, thereby limiting the extent of COX2 induction upon iron treatment. We have revised the text accordingly to reflect that ACSL4 deletion partially

attenuates, rather than fully reverses, iron-induced COX2 upregulation (**revised manuscript lines 429–433**).

Q10. The data with ASCL4 KO is a strength of the manuscript but should be further developed. The data in the GD2 model are not currently convincing although it looks like there could be a phenotype there. In the BLI images only 3/8 of the control CAR T treated mice appear to have tumors making it difficult to conclude the ASLC4 KO CAR T are more effective. The data also stops at day 20. It would be impactful if these experiments could be repeated under conditions where the control CAR T are less effective (bigger tumors or fewer CAR T cells) and present longer follow up data.

Response:
We thank the reviewer for this constructive suggestion and agree that the original solid tumor experiment did not sufficiently challenge the efficacy of control CAR-T cells, thereby limiting the interpretability of the ACSL4-KO phenotype.

In response, we repeated the GD2-expressing 143B solid tumor experiments under more stringent conditions. Specifically, to reduce the therapeutic efficacy of control CAR-T cells, we lowered the infused CAR-T cell dose from 1.3×10^7 to 5×10^6 cells per mouse and extended longitudinal bioluminescence imaging follow-up to 60 days. Under these conditions, all mice receiving control CAR-T cells developed progressive tumor burden, allowing a clearer comparison of therapeutic efficacy.

Notably, under this more stringent setting, Fer-1 treated mice exhibited durable tumor control with 2 of 7 mice remaining tumor-free at day 60, whereas ACSL4-KO CAR-T cells achieved superior efficacy, with 3 of 7 mice demonstrating long-term disease-free survival (**revised Fig. 6e-g**). These results demonstrate that inhibition of ferroptosis—particularly via genetic ablation of ACSL4—confers a robust therapeutic advantage in solid tumor settings when control CAR-T efficacy is limited.

Q11. Following on from point 10, the significance of the study would also be elevated if a much more comprehensive analyses of CAR T cell phenotype could be provided. Currently this is just a limited analysis of CAR T cells in the bone marrow of the NALM6 tumor bearing mice. Ideally the authors would assess both the spleens and tumors of tumor bearing mice and analyze memory status (CD45RA, TCF7, CD62L), effector function and metabolic fitness.

Response:
We thank the reviewer for this important suggestion regarding a more comprehensive in vivo phenotypic characterization of CAR-T cells.

In the revised manuscript, we have expanded our analyses beyond the bone marrow compartment. Specifically, in the ferroptosis inhibitor–treated Nalm6 leukemia model (Fig. 2), we now include phenotypic assessment of CAR-T cells in both bone marrow

and spleen, with a focus on memory-associated markers using a CD45RA–CD27 panel (**revised Fig. 2i, p, q**). These data demonstrate that ferroptosis inhibition preserves a memory-like CAR-T phenotype in multiple lymphoid compartments in vivo.

In addition, in the GD2-expressing 143B solid tumor model, we performed histological and immunofluorescence analyses of residual tumors from non-responding mice. These analyses revealed pronounced CAR-T cell infiltration and localized tumor cell death in both the Fer-1–treated and ACSL4-KO groups (**Fig. 6h-j**), supporting enhanced effector activity within the tumor microenvironment.

Furthermore, although metabolic fitness could not be directly assessed in the mouse models due to the limited number of CAR-T cells recovered after treatment, we addressed this question mechanistically by performing comprehensive lipidomic profiling of ACSL4-KO CAR-T cells under iron stress in vitro. This analysis revealed a marked suppression of polyunsaturated fatty acid (PUFA) incorporation into phosphatidylethanolamines, accompanied by attenuation of ferroptosis-associated lipid remodeling (**revised Fig. 5f-j**). Collectively, these phenotypic, functional, and metabolic data provide further evidence that ferroptosis contributes to CAR-T cell dysfunction under iron stress and that its inhibition preserves CAR-T cell fitness across multiple disease contexts.

Minor comments:

Q1. Figure 1D- Please provide statistical analyses for these e.g. FDR.

Response:
We thank the reviewer for this suggestion. In response, we have now provided the corresponding statistical analyses for Fig. 1d. Specifically, FDR values for all gene sets shown in Fig. 1d are listed in **revised Supplementary Table 2**. The manuscript has been updated accordingly to clarify the statistical significance of these analyses.

Q2. line 123 refers to oxidative phosphorylation, fatty acid metabolism and ROS regulation as ferroptosis-related pathways. As acknowledged by the authors later n the manuscript, these pathways are associated for a range of cellular processes so it is inaccurate to characterize them in this way. I think it would be better to see that GSVA revealed enrichment of pathway x, y and z, which is of interest as these pathways have been shown to be associated / upregulated in the context of ferroptosis.

Response:
We thank the reviewer for this important clarification. We agree that oxidative phosphorylation, fatty acid metabolism, and ROS regulation are not specific to ferroptosis and are involved in a broad range of cellular processes. In the revised manuscript (**lines 130–136**), we have therefore softened our original wording and no longer describe these pathways as ferroptosis-determining pathways. Instead, we now

state that these pathways are consistent with a cellular state predisposed to ferroptotic stress.

Q3. Line 131- I do not think it is possible from the analyses presented to claim that ferroptosis is the "predominant mechanism of cell loss". Suggest this statement is softened.

Response:
We thank the reviewer for this important point. We agree that the sequencing analyses presented here are not sufficient to support the claim that ferroptosis represents the predominant mechanism of cell loss. Instead, we now state that the observed transcriptional changes are consistent with a cellular state predisposed to ferroptotic stress (**lines 133-136**). This revised phrasing more accurately reflects the scope of the data and avoids overinterpretation.

Q4. Line 166 states that Fer-1 increased the proportion of memory cells but none of the figure panels cited show this data.

Response:
We thank the reviewer for pointing out this issue. We agree that the originally cited figure panels did not sufficiently support the statement that Fer-1 increased the proportion of memory CAR-T cells. In response, and as detailed in Major **Comment #2**, we have expanded our phenotypic analyses by including CD45RA and CD27 markers to more comprehensively assess memory-associated CAR-T cell subsets following Fer-1 treatment. These data are now presented in **Extended Data Fig. 2d-l** and demonstrate that Fer-1 promotes memory-like CAR-T cell phenotypes. The corresponding description has been added to the revised manuscript (**lines 163–166**).

Q5. Line 204. The authors state that their data suggest that chronic tonic signaling sensitizes CAR T cells to ferroptosis but no data showing tonic signaling of the CAR being used is shown. The data could equally reflect that more differentiated cells (as will naturally increase in proportion with extended culture time) are more sensitive to ferroptosis. There are ways to test the impact of tonic signaling e.g. dasatinib treatment if the authors wish to corroborate this claim. Otherwise, these statement should be softened.

Response:
We thank the reviewer for this important comment. Accordingly, we have revised the manuscript to soften this statement and removed the attribution to chronic tonic signaling. The revised text now states that late-stage CAR-T cells exhibit increased susceptibility to iron-induced cell death, indicating a preferential sensitivity of more terminally differentiated T cells to ferroptotic stress (**lines 241-244**), consistent with previous reports (PMID: 36882513).

Reviewer #2:

In this manuscript, Kong and colleagues characterize the mechanisms underlying CAR-T cell dysfunction, and reveal that CAR-T cells undergo ferroptosis during their dimunition phase due to elevated serum iron levels. Mechanistically, serum iron promotes mitochondrial ROS, while in parallel enhancing ACSL4 activation. Importantly, knockout of ACSL4 in CAR-T cells significantly improves antitumor efficacy. Overall, the manuscript uncovers a novel mechanism supporting a vulnerability of CAR-T cells towards ferroptosis with potential implications for improving CAR-T cell therapy. Addressing the comments below will benefit the manuscript and bolster the authors' claims.

Summary:
We thank the reviewer for the thorough evaluation of our manuscript and for recognizing the novelty and potential translational significance of identifying ferroptosis as a vulnerability underlying CAR-T cell dysfunction. We particularly appreciate the reviewer's insightful comments regarding the in vivo relevance of ferroptosis inhibition and the need for more detailed characterization of animal phenotypes.

In response, we have expanded our in vivo analyses by incorporating additional ferroptosis inhibitors, extending treatment regimens, and providing more comprehensive phenotypic assessments in tumor-bearing mouse models. These revisions strengthen the mechanistic link between ferroptosis suppression and improved CAR-T cell persistence and antitumor efficacy, and further support the conclusions of the study.
Below, we provide point-by-point responses to the reviewer's specific comments.

Major Comments:

Q1. To substantiate the claim that ferroptosis is responsible for the loss of CAR-T cells in vivo, the authors should use systemic delivery of ferroptosis inhibitors in the animal studies (for example use of ferrostatin-1, liproxstatin-1, or vitamin E).

Response:
We thank the reviewer for this important comment regarding the design of the in vivo studies in Fig. 2. We agree that systemic delivery of ferroptosis inhibitors is necessary to substantiate a causal role of ferroptosis in CAR-T cell loss in vivo.
In response to this suggestion, and in line with comments from multiple reviewers, we have substantially redesigned and expanded the relevant animal experiments. First, we treated CAR-T cells ex vivo with multiple ferroptosis inhibitors, including ferrostatin-1 (Fer-1), liproxstatin-1 (Lip-1), and UAMC-3203 (UAMC), during CAR-T cell

expansion and subsequently evaluated their therapeutic efficacy in tumor-bearing mice (**revised Extended Data Fig. 2n-q**). Second, we implemented a continuous in vivo dosing strategy in which mice receiving equivalent numbers of CAR-T cells were administered Fer-1, Lip-1, or UAMC intraperitoneally every two days for three weeks, allowing us to directly assess the impact of sustained ferroptosis inhibition on CAR-T cell efficacy in vivo (**revised Fig. 2a-g**).
Across both experimental settings, inhibition of ferroptosis—either through ex vivo pretreatment or sustained systemic administration—significantly enhanced CAR-T cell antitumor efficacy. These new data and corresponding descriptions have been incorporated into the revised manuscript (**lines 173–188**).

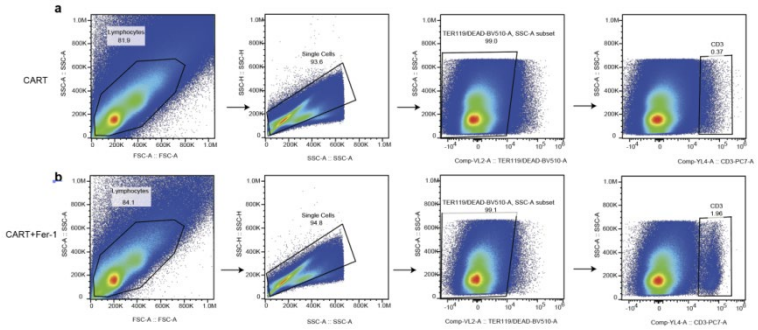
Q2. While the authors provide a convincing phenotype with CART cells +/- pre-treatment with Fer-1 in the experiments in Fig. 2b, quantification of the number of live CAR-T cells (using live/dead stains) within these animals would bolster the claim that these CAR-T cells are indeed undergoing ferroptosis. Similar data should be shown for experiments related to Fig. 3L. Additional live/dead staining should be undertaken in in vitro experiments.

Response:
We thank the reviewer for this constructive suggestion and apologize for not sufficiently detailing the in vivo flow cytometry strategy in the original submission. For all in vivo analyses (cells isolated from mouse bone marrow and spleen), viability staining was systematically performed. As shown in **Response Letter Fig. 1a and b**, lymphocytes and singlets were first gated, followed by exclusion of erythroid cells and dead cells by selecting TER-119$^-$/Zombie Aqua™ Fixable Viability Dye$^-$ double-negative events. Live CAR-T cells were then identified by gating on CD3$^+$ cells prior to downstream analyses.

Importantly, as requested, we quantitatively assessed the frequency of live CAR-T cells in vivo. As shown in **revised Fig. 2j and Fig. 3j**, systemic administration of Fer-1 significantly increased the proportion of viable CAR-T cells. In contrast, a high-iron diet markedly induced ferroptotic death of CAR-T cells compared with control conditions. Together, these data provide direct in vivo evidence that ferroptosis contributes to CAR-T cell loss and that pharmacological inhibition of ferroptosis improves CAR-T cell survival.

In addition, to further substantiate iron-induced CAR-T cell death under controlled conditions, we complemented the in vitro iron-loading experiments with real-time live-cell imaging (**revised Extended Data Fig. 3e and f**), which directly visualizes CAR-T cell death following iron exposure.

Response Letter Figure 1



Response Letter Figure 1 | Gating strategy for identification of live CAR-T cells from mouse tissues

Representative flow cytometry gating strategy used to identify live CAR-T cells isolated from mouse bone marrow and spleen. (**a**) CAR-T group and (**b**) CAR-T + Fer-1 group. Lymphocytes were first gated based on FSC-A and SSC-A, followed by exclusion of doublets using SSC-A versus SSC-H. Dead cells and erythroid cells were excluded by gating on TER-119$^-$ and Zombie Aqua™ Fixable Viability Dye (BV510)$^-$ events. Live CAR-T cells were subsequently identified by CD3$^+$ gating. All downstream phenotypic and quantitative analyses in Fig. 2 and Fig. 3 were performed on these live CD3$^+$ CAR-T cell populations.

Q3. To rule out any potential confounding effects from cancer cells, the authors should repeat the experiment in Fig. 3e with CAR-T cells in tumor free mice with or without systemic administration of a ferroptosis inhibitor in vivo.

Response:

We thank the reviewer for this valuable suggestion. To exclude potential confounding effects from tumor cells, we have now extended the analysis to tumor-free mice. As shown in **revised Fig. 3l–o**, CAR-T cells were transferred into mice without tumor engraftment and exposed to a high-iron systemic environment.

Under these tumor-free conditions, elevated iron levels still resulted in significant intracellular iron accumulation in CAR-T cells and a marked increase in the ferroptosis-associated marker lipid ROS. Importantly, sustained systemic administration of the ferroptosis inhibitor Fer-1 effectively reversed these iron-induced changes, restoring intracellular iron and lipid ROS levels toward baseline. These results demonstrate that high systemic iron directly induces ferroptotic stress in CAR-T cells in vivo, independent of tumor-derived influences, and that pharmacological ferroptosis inhibition can effectively restrain this process.

Q4. Given the importance of iron in augmenting CAR-T cell dysfunction and the utility of iron chelators in the clinic, can the authors provide the data showing that "neither iron chelation, nor knockdown of iron-related genes, effectively inhibited iron-induced ferroptosis in CAR-T cells" as mentioned in the discussion.

Response:

We apologize for the lack of clarity in the original description. In the revised manuscript, we have now provided the relevant experimental evidence supporting this statement. Specifically, in **revised Extended Data Fig. 4n and o**, we performed a systematic screen of 126 ferroptosis-related compounds, which included three clinically used iron chelators—deferoxamine mesylate, deferasirox, and deferiprone. In CAR-T cell killing assays conducted under iron-replete conditions, all three iron chelators exhibited pronounced cytotoxic effects on CAR-T cells rather than rescuing iron-induced dysfunction. These results are highlighted in **Statistical_Source Data Figure 4b** and have been explicitly described in the revised manuscript (**lines 296-299**).

In addition, in **revised Extended Data Fig. 6a-f**, we genetically disrupted multiple key iron transport-related genes, including TFRC, FTH1, DMT1, and MFRN1. Knockdown of each of these genes resulted in a marked impairment of CAR-T cell proliferation and effector function, indicating that a basal level of intracellular iron availability is essential for CAR-T cell survival and activity.

We acknowledge that we did not perform iron re-supplementation experiments following genetic disruption of these iron-related genes, as CAR-T cells with these knockdowns exhibited profound growth arrest and cell death within 3-5 days, precluding further functional analyses. In light of this limitation, we have revised the Discussion to avoid overinterpretation and now more precisely describe the role of iron as a double-edged requirement for CAR-T cell function (**revised manuscript lines 552-554**).

Q5. Could the authors provide IHC and/or IF quantification of the numbers of CAR-T cells infiltrating in the 143B tumors in Fig. 6e.

Response:
We thank the reviewer for this helpful suggestion. In response, we have performed quantitative IHC and immunofluorescence analyses based on CAR-T positive area measurements to assess CAR-T infiltration in GD2-expressing 143B solid tumors.

In the revised manuscript, CAR-T infiltration was quantified as the percentage of CAR-T positive area relative to total tumor area across treatment groups. Using this area-based approach, tumors from Fer-1 treated mice and, more prominently, from ACSL4-KO CAR-T treated mice exhibited significantly increased CAR-T positive infiltration areas, accompanied by a marked reduction in tumor size, compared with tumors from mice receiving control CAR-T cells (**revised Fig. 6h-j**).

These quantitative histological analyses support enhanced CAR-T tumor infiltration following ferroptosis inhibition, with ACSL4 deletion conferring the most robust effect.

Q6. Lipidomics should be performed on CAR-T cells during expansion, peak, and diminution phase.

Response:
We thank the reviewer for this important suggestion. We agree that lipidomic profiling of CAR-T cells across the expansion, peak, and diminution phases in patients would be highly informative. During our initial attempts to obtain in vivo evidence of ferroptosis in patient-derived CAR-T cells, we sought to perform metabolomic analyses on sorted CAR-T cells from different treatment phases. However, during the expansion phase, the number of recoverable CAR-T cells was extremely limited and sufficient only for immunoblotting analyses, falling well below the cell numbers required for reliable lipidomic profiling.

To partially address this limitation, we performed untargeted metabolomic analysis of patient serum across treatment phases, which revealed dynamic changes in the lipid environment associated with the diminution phase (**revised Fig. 3d** and **e**). In addition, in our **revised Extended Fig. 8a**, we performed lipid peroxidation–focused metabolomic analyses on CAR-T cells under iron stress conditions, providing direct evidence of ferroptotic lipid remodeling.

Importantly, in response to the reviewer's suggestion (**Minor Comment #2**), we have now expanded our lipidomic analyses by performing comprehensive lipidomics on control and ACSL4-KO CAR-T cells under iron exposure. These data demonstrate that ACSL4 deletion markedly attenuates iron-induced lipid remodeling, particularly by reducing the incorporation of polyunsaturated fatty acids into phospholipids, thereby limiting the accumulation of ferroptotic lipid substrates (**revised Fig. 5f-j**). Notably, consistent with reduced availability of oxidizable substrates, lipid substrate consumption following iron treatment was substantially lower in ACSL4-KO CAR-T cells than in control CAR-T cells, in agreement with our functional data showing suppression of iron-induced lipid peroxidation.

Together, these complementary analyses provide mechanistic insight into how iron-driven lipid peroxidation contributes to CAR-T cell dysfunction and how ACSL4 deletion confers resistance to ferroptotic stress, despite the technical constraints associated with phase-specific lipidomics in patient-derived CAR-T cells.

Minor Comments:
Q1. Inclusion of images of in vitro FC treated CART cells to verify a ferroptotic morphology.

Response:
We thank the reviewer for this helpful suggestion. In the revised manuscript, we have included representative in vitro images of ferric citrate (FC) treated CAR-T cells to illustrate ferroptosis-associated morphological changes.

Specifically, in **revised Extended Data Fig. 3e and f**, we show the dynamic process

and characteristic morphological features of CAR-T cell death following iron treatment in vitro. In addition, revised **Extended Data Fig. 3p** presents ultrastructural evidence obtained by transmission electron microscopy, revealing mitochondrial shrinkage and cristae disruption, which are hallmark features of ferroptosis. Together, these complementary morphological analyses provide visual and ultrastructural support that iron exposure induces ferroptotic cell death in CAR-T cells.

Q2. The authors claim that CAR-T cells enter a ferroptosis susceptible state upon FC treatment in part due to upregulation of ACSL4. Given the role of ACSL4 in lipid remodeling, lipidomics should be performed on WT and ACSL4 KO CAR-T cells +/- FC.

Response:
We thank the reviewer for this suggestion. As also addressed in Major Comment #6, we have now performed comprehensive lipidomic analyses on wild-type and ACSL4-KO CAR-T cells in the presence or absence of ferric citrate (FC).

These analyses demonstrate that ACSL4 knockout markedly suppresses iron-induced lipid remodeling, particularly by reducing the incorporation of PUFAs into ferroptosis-relevant phospholipids such as phosphatidylethanolamines (PE). As a consequence, ACSL4 deficiency limits the accumulation of oxidizable lipid substrates and effectively prevents iron-driven lipid peroxidation, thereby attenuating ferroptosis in CAR-T cells. Together, these lipidomic data provide direct mechanistic support for the conclusion that ACSL4 upregulation contributes to the ferroptosis-susceptible state induced by iron exposure in CAR-T cells.

Q3. The authors claim that CAR-T cells become especially vulnerable to ferroptosis due to increased serum iron levels compared to endogenous T-cells (Fig. 1l-m). Could the authors provide any explanation for this observation?

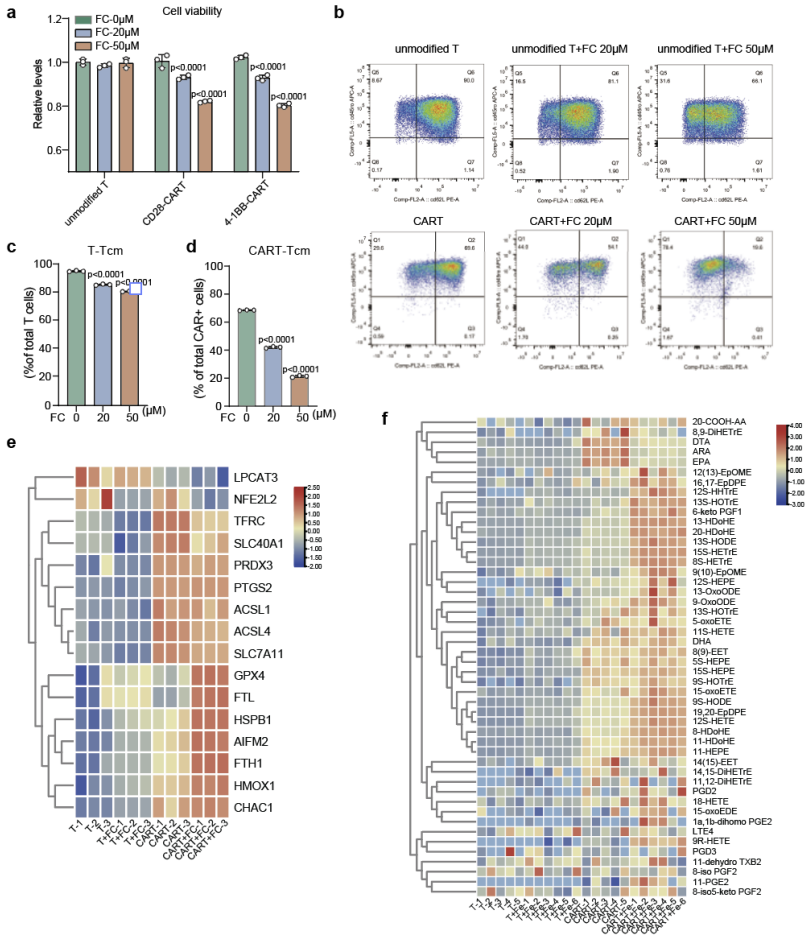
Response:
We thank the reviewer for this insightful question. Indeed, in our in vivo analyses of ferroptosis-associated markers, we observed a clear differential sensitivity to iron-induced ferroptotic stress between endogenous T cells and CAR-T cells. To further examine the robustness of this observation, we directly compared unmodified T cells and CAR-T cells under identical iron exposure conditions. As shown in **Response Letter Fig. 2a-d**, T cell viability and function were relatively preserved at iron concentrations that markedly impaired CAR-T cells.

We further found that CAR-T cells exhibited enhanced expression of iron transport- and ferroptosis-related genes compared with unmodified T cells (**Response Letter Fig. 2e**). Consistent with this transcriptional profile, iron-induced lipid peroxidation was significantly more pronounced in CAR-T cells than in T cells, (**Response Letter Fig. 2f**) providing functional evidence that CAR-T cells are intrinsically more susceptible

to ferroptosis.

We acknowledge that the precise molecular mechanisms underlying this differential sensitivity remain incompletely understood. In particular, how CAR signaling rewires redox homeostasis and iron-handling pathways to predispose CAR-T cells toward an oxidative, ferroptosis-prone state warrants further investigation and will be the subject of future studies.

Response Letter Figure 2



Response Letter Fig. 2 | Differential sensitivity of CAR-T cells and unmodified T cells to iron-induced ferroptotic stress.

(a) Cell viability of unmodified T cells, CD28-CAR-T cells, and 4-1BB-CAR-T cells following exposure to increasing concentrations of ferric citrate (FC; 0, 20, and 50 μM). (**b**) Representative flow cytometry plots showing live/dead staining of unmodified T cells and CAR-T cells treated with FC (20 or 50 μM). (**c and d**) Quantification of central memory T cell (T-Tcm) frequency in unmodified T cells (**c**) and CAR-T cells (**d**) following iron treatment. (**e**) Heatmap showing relative expression of iron metabolism and ferroptosis-associated genes in unmodified T cells and CAR-T cells under control and iron-treated conditions. (**f**) Targeted lipidomic profiling demonstrating enhanced

accumulation of oxidized polyunsaturated fatty acid species in CAR-T cells compared with unmodified T cells following iron exposure.

Q4. The authors demonstrate a delay in CAR-T cell loss in humans, this suggests that systemic iron alone is not sufficient to induce acute ferroptosis in CAR-T cells upon infusion. Could the authors provide a scientific rationale for this observation?

Response:
We agree with the reviewer that the delayed loss of CAR-T cells indicates that systemic iron alone is not sufficient to induce acute ferroptosis immediately upon infusion. Following adoptive transfer, CAR-T cells initially engage tumor antigens and undergo robust antigen-driven expansion and cytotoxic activity, during which dominant proliferative and effector signals likely outweigh iron-associated functional impairment or cell death.

With prolonged in vivo exposure to elevated iron levels, CAR-T cells progressively develop functional impairment, reduced proliferative capacity, and increased susceptibility to ferroptotic stress, ultimately culminating in late-phase ferroptosis that limits long-term persistence. Consistent with this model, our single-cell RNA-sequencing data indicate that exhausted $CD8^+$CAR-T cells are significantly more susceptible to ferroptosis than memory-like CAR-T cells (**revised Extended data Fig. 5f**). Importantly, iron is unlikely to act in isolation in vivo. The enrichment of polyunsaturated fatty acids observed during the late phase of therapy (**revised Fig. 3d and e**) suggests that therapy-associated metabolic remodeling may cooperate with sustained iron exposure to lower the threshold for ferroptosis.

This perspective has been incorporated into the revised manuscript (**lines 219–229**). Further studies will be required to define the precise interplay between iron, metabolic state, and CAR-T cell differentiation in governing ferroptosis susceptibility in vivo.

Q5. Can the authors comment on why serum iron levels are altered in ALL and MM patients at defined time points during CAR-T cell treatment?
Response:

We observed pronounced changes in serum iron levels at distinct stages of CAR-T cell therapy, which we believe reflect the combined effects of CAR-T treatment and systemic iron homeostasis. During the early post-infusion phase, rapid antigen-driven CAR-T cell expansion is expected to increase iron utilization, while peak cytokine release syndrome (CRS), through IL-6–mediated induction of hepcidin, further limits circulating iron availability. In addition, prior lymphodepleting chemotherapy and associated anemia likely contribute to reduced serum iron levels at this stage.

At later stages of therapy, extensive death of target cells (including tumor cells and B cells), as well as CAR-T cells themselves, is expected to release intracellular iron.

Concurrently, resolution of CRS under anti-inflammatory treatment may reduce hepcidin levels, together with systemic feedback mechanisms that mobilize iron from macrophages to restore iron homeostasis, resulting in elevated serum iron levels.

We acknowledge that these mechanisms are difficult to directly interrogate in patients. Therefore, in the revised manuscript, we examined whether baseline serum iron and ferritin levels correlate with clinical outcome and observed higher ferritin and serum iron levels in NR patients (**Fig.1l, m and Fig.3c**), supporting a clinically relevant association between systemic iron status and CAR-T therapeutic efficacy. These considerations have been incorporated into the Discussion (**revised manuscript lines 511-529**).

Q6. Could the authors offer an explanation for the discrepancy between Fer-1 and Lip-1 in the phenotypes observed in extended data figure 2j?

Response:
We thank the reviewer for this insightful question regarding the discrepancy between ferrostatin-1 (Fer-1) and liproxstatin-1 (Lip-1) observed in Extended Data Fig. 2j. Although both compounds are widely used ferroptosis inhibitors and share the ability to suppress iron-dependent lipid peroxidation, they may differ in their biochemical properties, cellular distribution, and functional potency in vivo, which could contribute to divergent phenotypes (**revised Fig. 2a-g**).

Both ferrostatin-1 (Fer-1) and liproxstatin-1 (Lip-1) are radical-trapping antioxidants that inhibit ferroptosis by suppressing lipid peroxidation rather than by directly targeting specific enzymatic components of the pathway (Zilka et al., Chem Rev, 2017; DOI: 10.1021/acs.chemrev.6b00500). However, recent studies suggest that Lip-1 exhibits a more compartment-restricted mode of action, preferentially suppressing iron-mediated lipid oxidation within lysosomal and endolysosomal compartments (Zhang , Nature, 2025; DOI: 10.1038/s41586-025-08974-4). This organelle-biased activity may limit the breadth of Lip-1-mediated ferroptosis suppression, whereas Fer-1, through broader radical-trapping activity across cellular membranes, may provide more effective global protection against iron-induced oxidative toxicity.

In our study, mitochondrial structural disruption and dysfunction emerged as major contributors to CAR-T cell functional impairment and cell death. We therefore speculate that the broader radical-trapping activity of Fer-1 may confer more effective suppression of iron-induced oxidative toxicity across multiple cellular membranes, including mitochondrial membranes, thereby resulting in more pronounced functional rescue in vitro and superior therapeutic efficacy in vivo compared with Lip-1. We have revised the manuscript to clarify this interpretation and emphasize that the observed differences likely reflect context-dependent modes of ferroptosis inhibition rather than fundamentally distinct biological effects (**revised manuscript lines 572-578**).

Q7. Please include the entire uncropped portion of 4HNE blot in fig 1f and g.

Response:
We thank the reviewer for this suggestion. The full, uncropped 4-HNE immunoblots corresponding to Fig. 1f and g are now provided in the **unprocessed blots** section.

Q8. It is unclear how the timepoints to assess tumor formation following CAR-T injection as shown in Figure 2a were chosen. Do they correlate with CAR-T cell levels in human blood as shown in Figure 1a (exhibit distinct expansion, peak, dimunition phases)?

Response:
We thank the reviewer for this thoughtful question. The time points selected for longitudinal tumor imaging in Fig. 2a do not strictly correspond to the expansion, peak, and diminution phases defined in patients (as shown in Fig. 1a). Nevertheless, the imaging schedule was designed to encompass the overall period spanning CAR-T cell expansion through subsequent diminution in vivo. Weekly imaging was chosen as a balanced and practical approach to capture dynamic changes in tumor burden in mice, allowing for consistent longitudinal assessment while minimizing variability and stress associated with more frequent imaging.

In our mouse studies, CAR-T cells were administered at subclinical and deliberately stringent doses to accelerate disease recurrence and thereby sensitively evaluate differences in CAR-T persistence and therapeutic durability between treatment groups. Under these conditions, tumor progression occurs more rapidly than in the clinical setting. Accordingly, the imaging time points in Fig. 2 were selected to capture the onset and rate of tumor relapse, which provides a functional readout of CAR-T efficacy under ferroptotic stress, rather than to model distinct clinical expansion or peak phases.

Formatting:
Q1. Please adjust the colors of points in extended data figure 1b.

Response:
We apologize for the confusion caused by the color presentation in **Extended Data Fig. 1b and c**. In the revised manuscript, we have separated the two panels and adjusted the point colors accordingly to ensure clear visual distinction and accurate interpretation.

Q2. Please add the y-axis label to extended data figure 1i.

Response:
We thank the reviewer for this careful observation. The y-axis label has now been added to **Extended Data Fig. 1i** in the revised manuscript.

Q3. Please describe in the figure legend what timepoints are being used to calculate

the p-values in Figure 2c.

Response:
Thank you for this important point. We apologize for the lack of clarity in the original figure legend.

In the revised version, we have explicitly clarified that the p-values reported in Fig. 2c are derived from a two-way ANOVA assessing the main effect of treatment over time, rather than from comparisons at individual time points.

Specifically, all longitudinal bioluminescence measurements across the indicated time points were included in the model, and the statistical significance reflects the overall difference in tumor burden trajectories between treatment groups, rather than differences at a single selected day.

This statistical approach was applied consistently to all longitudinal in vivo BLI analyses (including **revised Fig. 2c and 2f**), and the corresponding statistical details have now been clearly described in each relevant figure legend.

Q4. Please add an axis to extended data figure 4c.

Response:
Thank you for your careful suggestion. We have now added the requested axis to **extended data Fig. 3m** (previously labeled as 4c in the original version).

Reviewer #3

Kong et al. investigated how iron metabolism may underly CAR-T cell dysfunction and diminished long-term persistence in patients through multi-omics analyses of clinical samples. They found that CAR-T cells undergo ferroptosis during their diminution phase due to elevated serum iron levels, with excessive intracellular iron accumulation impairing CAR-T cell functions both in vivo and in vitro. Mechanistically, they demonstrated that iron promotes ferroptosis by inducing mitochondrial reactive oxygen species accumulation and directly enhancing ACSL4 phosphorylation and enzymatic activity, while showing that pharmacological ferroptosis inhibition o ACSL4 ablation in CAR-T cells significantly improved antitumor efficacy and CAR-T persistence in multiple murine models.

Overall Assessment
This is a highly impressive and data-rich manuscript that is well-presented and well-written. The authors comprehensively explored ferroptosis in CAR-T cells using multiple orthogonal methods including patient samples, in vitro studies, and in vivo models. The study provides convincing data that iron modulation can affect CAR-T persistence and efficacy, though the magnitude of the effect may not be very high according to their data.

I note that CAR-T cells necessarily undergo a diminution phase (as T cells do after any immune response; otherwise exponential T cell growth would occur), and inhibiting this is not necessarily a goal per se. Overall, I believe this is an excellent manuscript deserving of publication and of sufficient scientific and technical merit to interest readers.

Summary:
We sincerely thank the reviewer for their thoughtful and positive feedback. We are pleased to hear that the reviewer found our manuscript both comprehensive and data-rich. We appreciate the recognition of our multi-omics approach to explore ferroptosis in CAR-T cells, as well as the use of orthogonal methods, including clinical samples, in vitro studies, and in vivo models.

We also acknowledge the reviewer's point regarding the natural diminution phase of T cells post-infusion. We agree that the goal is not necessarily to completely inhibit this phase but to explore how ferroptosis, driven by elevated serum iron levels, impedes CAR-T cell function and long-term persistence. Our findings provide valuable insights into how ferroptosis contributes to CAR-T cell dysfunction and highlight potential strategies for enhancing CAR-T therapy, such as ferroptosis inhibition and ACSL4 ablation.

Once again, we greatly appreciate the reviewer's encouraging remarks, and we believe the revisions we have made in response to their comments have further strengthened

the manuscript.

Minor Comments:

Q1. Figure 1g: Where is GPX4? The bands look similar between peak vs diminution compared to the actin band.

Response:
Thank you for pointing this out. We apologize for the omission of GPX4 in the original version of Figure 1g. The GPX4 immunoblot was inadvertently left out during figure assembly and has now been included in the **revised Fig. 1g**.

Regarding the reviewer's observation that several ferroptosis-related protein bands appear comparable between the peak and diminution phases when normalized to actin, this similarity reflects the biological state of CAR-T cells at these two closely related stages. Although peak expansion represents the maximal numerical abundance of CAR-T cells, it also marks the onset of functional decline. Our accompanying data show that intracellular lipid ROS and labile iron levels are already elevated at the peak phase and remain high during the diminution phase (**revised Fig. 1h-k**). Thus, ferroptotic stress is already strongly engaged at peak expansion, resulting in similar expression levels of ferroptosis-associated proteins across these two time points.

Q2. Figure 1h-k: Please label the y-axes so readers know what they're looking at. It's unclear what flow cytometry analysis is being performed here or in panels l-m.

Response:
Thank you for pointing this out. In the revised manuscript, we have clarified the y-axis labels in **Fig. 1h–k**, which are now explicitly indicated as "CAR-T lipid ROS MFI" and "CAR-T labile iron MFI", respectively. These revisions are intended to clearly specify the flow cytometry readouts being presented and to improve interpretability for readers.

Q3. Figure 2b: The scale keeps changing in the images, making it appear that there is no disease at late timepoints when there presumably is. Please just show the data consistently.

Response:
We thank the reviewer for raising this important concern regarding the dynamic range of the bioluminescence images in Fig. 2b. We agree that changes in image scaling can create the impression that disease is absent at later time points, which may be misleading.

In response, and in line with comments from multiple reviewers, we have repeated and substantially expanded the in vivo experiments shown in Fig. 2. These revised experiments include both ferroptosis inhibitor pretreatment of CAR-T cells (**revised**

Fig. 2n-q) and continuous in vivo administration of ferroptosis inhibitors (**revised Fig. 2a-g**). During image visualization, enforcing a single fixed scale across all time points results in near-complete signal saturation at later stages, causing images from all groups to appear uniformly red and obscuring biologically meaningful intergroup differences.

To balance accurate visualization with interpretability, we therefore adopted a consistent scaling strategy across adjacent time points while prioritizing the clear depiction of intergroup differences. Importantly, to avoid underestimation or masking of residual disease at late stages, we extended the imaging period to 49 days in the **revised Fig. 2**, allowing clearer visualization of tumor relapse dynamics. In addition, all conclusions are supported by quantitative total flux analyses, which are independent of image scaling and are consistently analyzed using longitudinal statistical models.

Q4. Figure 2c: Why only show the quantification until day 24?

Response:
Thank you for your insightful comment. In the **revised Fig. 2e**, we have extended the in vivo imaging timeline to Day 49, which allows for a more comprehensive assessment of CAR-T therapy effects and the progression of tumor relapse.

Q5. Figure 2d: What did the mice die from?

Response:
Thank you for raising this important question. We apologize for the confusion caused by the previous scaling issue, which may have obscured the disease burden at the experimental endpoint. In the revised version, we have extended the in vivo imaging timeline and adjusted the scale to clearly show the tumor relapse process. Regarding the cause of death, the mice in the control and inhibitors-treated groups died as a result of tumor progression, as evidenced by the elevated tumor burden observed at later time points.

Q6. Figure 2f-i: Why only 3 mice per group? Was this a small cohort or did you narrow down the mice? Why use 5 per group for cytokines (Figure 2j-q) when you had 10?

Response:

Thank you for your insightful comment regarding the inconsistent mouse sample sizes. In the revised version, we have reanalyzed the flow cytometry data and standardized the mouse numbers to 5 mice per group (**revised Fig. 2h-y**).

The original discrepancy arose because the flow cytometry and cytokine analysis data required euthanasia of mice to collect peripheral blood, bone marrow, and spleen samples, which were different mice from those used in the in vivo imaging experiments. We now realize that this inconsistency could lead to confusion, and we have addressed

this issue by aligning the number of mice across all experiments in Figure 2.

Methodological Clarification

Q1. Line 167-168: It doesn't look like you used the Giavridis et al. model referenced in citation 36 here. Is that indeed the case, or are these the same mice as above? The statement reads: "Importantly, safety evaluations using established cytokine release syndrome (CRS) models36 revealed that Fer-1 treatment did not exacerbate systemic inflammation or induce cytokine storms."

Response:
Thank you for your thoughtful comment. We apologize for the lack of clarity in the initial manuscript. For the cytokine release syndrome (CRS) assessment, we indeed followed the method described by Giavridis et al. (citation 36). Specifically, we induced CRS in immunodeficient mice by administering a high dose of CAR-T cells, as per the referenced protocol.

To avoid any confusion, we have now provided a more detailed description of this method in the revised **Methods section** of the manuscript. This should clarify that the CRS model used is based on the approach described by Giavridis et al. and that it aligns with the referenced citation.

Q2. Figure 3l-p and Extended Figure 4: I note that mouse survival and tumor control here are much worse than previously shown in Figure 2. Indeed, the CAR-T cells in the standard and low iron groups essentially had no survival benefit over the no-CAR condition. Do the authors have an explanation here? The low-iron group here looks similar to the previous normal iron condition in Figure 2. Was the experiment set up differently, or is this due to donor variability or another explanation?

Response:
Thank you for your thoughtful question. We acknowledge that the differences in tumor control and mouse survival between Fig. 3l-p and Extended Fig. 4 compared to Fig. 2 are likely due to several experimental factors.

The primary difference between these experiments lies in the age of the mice used. In the Fig. 3 experiments, the mice had been fed with different iron-containing diets for several weeks and were aged 16-18 weeks before receiving Nalm6 tumor cell injections and subsequent CAR-T treatment. These mice were closer to middle-aged to older adults. In contrast, the mice used in other experiments were 4-6 weeks old, and the tumor and CAR-T cell doses were the same across all experiments. It is likely that the age and size differences influenced tumor progression and CAR-T treatment efficacy.

Additionally, as these experiments were not conducted in the same batch, donor variability in the CAR-T cells could also contribute to the differences observed, despite

our efforts to standardize CAR-T cell expansion times in vitro before injection. We hope this explanation clarifies the discrepancies observed in the tumor control and survival outcomes.

Suggestions for Strengthening the Study

Ideally, I would like to see an immunocompetent model, as the interplay with the full immune system would be fascinating (i.e., mouse CAR-T cells and tumor). This would strengthen the data, though there is already a substantial amount of data presented and this by no means essential.

Response:

We thank the reviewer for this valuable suggestion. We agree that an immunocompetent CAR-T model would provide important insight into the interaction between CAR-T cells and the intact immune system.

In line with published protocols, we attempted to generate mouse CAR-T cells from splenic T cells. However, these cells consistently exhibited profound proliferative impairment during ex vivo expansion. We further tested ACSL4 knockout and pharmacological ferroptosis inhibition with ferrostatin-1 (Fer-1), but neither approach effectively rescued CAR-T expansion. Consequently, we were unable to obtain sufficient numbers of functional mouse CAR-T cells to establish robust in vivo studies in immunocompetent tumor models.

Notably, the key evidence supporting CAR-T ferroptosis in this study is derived from clinical samples of CAR-T treated patients (Fig. 1), which inherently reflect CAR-T behavior in an immunocompetent setting. We therefore believe that these patient-based data already provide physiologically and clinically relevant support for the role of ferroptosis in CAR-T dysfunction.

To further reinforce our conclusions in vivo, we added a model involving sustained administration of ferroptosis inhibitors following infusion of untreated CAR-T cells (**revised Fig. 2a-g**). In this setting, systemic ferroptosis inhibition significantly enhanced CAR-T therapeutic efficacy, supporting the notion that ferroptosis acts as a limiting factor for CAR-T function in vivo.

We fully agree that extending these findings to immunocompetent CAR-T models represents an important future direction. As the reviewer also notes, such experiments are not essential for the central conclusions of the current study and will be pursued in future work as technical feasibility improves.

Minor Point

I note a recent publication on GPX4 (10.1007/s00262-025-04133-w) as a master regulator, which would be interesting to comment on in the discussion.

Response:

We thank the reviewer for this insightful suggestion. In our clinical data, we observed a downregulation of GPX4 expression over time (**revised Fig. 1g**). Its loss reduces T cell resistance to oxidative stress and increases sensitivity to ferroptosis. However, we also noted a recent study published in Cell (PMID: 41494530), which demonstrated that tumor-derived extracellular GPX4 can suppress T cell cytotoxicity through interactions with immune cell receptors. This dual role of GPX4 complicates its potential as a robust candidate for inhibiting ferroptosis in CAR-T cells. We have added this discussion to the revised manuscript (**lines 561–568**).

Reviewer #4

Kong et al identified the critical roles of environmental iron and ferroptosis in CAR-T cell dysfunction and provides insights for enhancing CAR-T therapeutic outcome.

This is novel and high-level interesting story, with potential translational impact.

Summary:
We sincerely thank the reviewer for the thoughtful and encouraging feedback. We greatly appreciate the recognition of the novelty and potential translational impact of our study, particularly in elucidating the critical roles of environmental iron and ferroptosis in CAR-T cell dysfunction. We are grateful for the opportunity to improve our manuscript and have carefully addressed the concerns raised. We believe that the revisions made will strengthen the clarity and rigor of the work, and we look forward to further discussion.

Q1. Fig 2. Fer-1 is here used as ferroptosis inhibitor (in ex vivo setting maybe OK), however much better ferroptosis inhibitors for in vivo use are around such as Lip-1 or UAMC-3203. Were they also considered or tested. Can these further improve survival of mice (Fig 2d)?

Response:
We thank the reviewer for this insightful comment. Based on the feedback from you and other reviewers, we have revisited the in vivo experiments shown in Fig. 2. In **revised extended Fig. 2n-q**, we have added data testing the effects of Lip-1 and UAMC-3203 on ex vivo pretreated CAR-T cells. These ferroptosis inhibitors all extended the antitumor efficacy of CAR-T cells in NSG mice, with Fer-1 showing the most pronounced effect.

To assess the long-term impact of ferroptosis inhibition on CAR-T cell efficacy, we also performed three-week systemic treatments with these inhibitors in vivo. As shown in **revised Fig. 2a-g**, while ferroptosis inhibitors promoted tumor progression in the absence of CAR-T cells, their effects were more pronounced in enhancing CAR-T cell efficacy upon infusion. Notably, Fer-1 significantly prolonged mouse survival, demonstrating the strongest therapeutic benefit.

Regarding the differences in the efficacy of these inhibitors in vivo, we hypothesize that Fer-1 may exert a broader ferroptosis suppression effect compared to Lip-1, which predominantly targets lysosomal iron-dependent lipid peroxidation. We have expanded on this discussion in the revised manuscript (**lines 572-578**).

Q2. Work performed related to MoA, seems a bit biased to involvement of mitochondria and related ROS. Fig 4c illustrates that MoA is more complex than involvement of mito's. Iron is stored in lysosomes, which can release labile iron pool. More extended study

on role of lysosomes and labile iron pool could strengthen paper. Fig 3c also highlight some other potential interesting pathways contributing to sensitivity such as estrogen, WNT signaling… maybe worth looking into?

Response:
We appreciate your insightful comments. As you noted, while we focused on mitochondria and related reactive oxygen species (ROS) in our investigation of ferroptosis, we recognize that the mechanism of action (MoA) is indeed more complex.

Following your suggestion, we further explored the role of lysosomal dysfunction and the labile iron pool in CAR-T cells. We observed compensatory lysosomal expansion after iron exposure, along with an increase in lysosomal pH, indicating functional impairment (**revised extended Fig. 7v and w**). This disruption in lysosomal function inhibits ferritin dissociation, thereby, blocking the normal release of iron from storage. Consequently, newly imported iron accumulates in the cytosol, elevating the labile iron pool. This mechanism is directly corroborated by our FerroOrange probe assays, which show a significant increase in intracellular free iron. These results suggest that lysosomal dysfunction significantly contributes to the toxic effects of iron in CAR-T cells.

In addition, we performed gene knockdown of iron transporters, including Tfrc, Dmt1, and Fth1, to modulate the labile iron pool. However, these interventions did not significantly improve CAR-T fitness and, in fact, led to cell death (**revised extended Fig. 6a-f**). This suggests that modulating intracellular iron levels alone may not be sufficient to counteract the detrimental effects of iron exposure on CAR-T cells.

Notably, while targeting the labile iron pool or mitochondria for rescue did not fully inhibit the toxic effects of iron (**revised extended Fig. 7o-u**), Ferroptosis inhibitors such as Fer-1 effectively suppressed iron-induced ferroptosis and CAR-T cell dysfunction. This supports our hypothesis that targeting the endpoint of ferroptosis—lipid metabolism—may offer an effective strategy for protecting CAR-T cells from iron toxicity. In response to your **comment #3**, we have added further detail on the regulatory role of ACSL4 in CAR-T lipid metabolism in the revised manuscript. Using lipidomics and oxidized lipid metabolism assays, we show that ACSL4 knockout significantly inhibits the incorporation of polyunsaturated fatty acids (PUFAs) into phospholipids, thereby reducing the accumulation of lipid peroxidation substrates and protecting CAR-T cells from iron-induced ferroptosis (**revised Fig. 5f-j and extended Fig. 8a**).

Additionally, we appreciate the reviewer's insightful suggestion regarding the potential involvement of additional signaling pathways, such as WNT, in the regulation of ferroptosis in CAR-T cells. While we agree that investigating these pathways could yield further mechanistic insights, the current study was deliberately focused on elucidating the ACSL4-dependent mechanism underlying iron-induced ferroptosis in

CAR-T cells. Owing to this focused scope, we were unable to comprehensively assess the contribution of these additional pathways in the present work. We plan to address their potential roles in future studies, particularly in the context of iron-induced ferroptosis, to further expand our understanding of the broader regulatory network governing CAR-T cell fate.

In summary, through our studies of lysosomal function and ACSL4-mediated lipid metabolism in CAR-T cells, we have gained deeper insights into the mechanisms by which iron toxicity affects CAR-T cell function. These findings underscore the multifaceted nature of iron toxicity and the potential for targeted interventions to mitigate it.

Q3. What is effect of ACSL-4 KO on CAR-T functionality?

Response:
Thank you for your question regarding the effect of ACSL4 KO on CAR-T functionality. As addressed in **COMMENT#2**, lipidomic and oxidized lipid metabolism profiling revealed that ACSL4 knockout significantly altered lipid remodeling in CAR-T cells. Specifically, it impaired the incorporation of polyunsaturated fatty acids (PUFAs) into phospholipids, reducing the levels of lipid peroxidation substrates associated with ferroptosis and preventing iron-induced toxicity (**revised Fig. 5f-j**).

It is important to note that ACSL4 KO alone did not enhance CAR-T functionality under baseline conditions. We observed no significant differences in memory, exhaustion, or other functional markers between ACSL4 KO and control CAR-T cells. Therefore, ACSL4 KO primarily serves a protective role by modulating lipid metabolism and enabling CAR-T cells to resist the toxic effects of iron, thus restoring CAR-T function in the presence of ferroptotic stress (**revised Fig. 5k-s**).

Q4. Authors favor gene therapy over pharmacological targeting with ferroptosis inhibitors. Not sure if this is correct. Maybe more balanced view on pro and contra's of both approaches should be considered in discussion.

Response:
Thank you for this valuable suggestion. In the revised version, we have added additional in vivo data evaluating the effects of sustained administration of ferroptosis inhibitors (Fer-1, Lip-1, and UAMC). As shown in revised Fig. 2, although these agents effectively suppressed ferroptosis and enhanced CAR-T therapeutic efficacy in vivo (**revised Fig. 2e-g**), they also promoted tumor growth when administered in the absence of CAR-T cells (**revised Fig. 2a-d**). These findings indicate that systemic pharmacological inhibition of ferroptosis requires careful safety evaluation, particularly with respect to potential tumor-protective effects.

Importantly, our data support the efficacy of both strategies to enhance CAR-T

performance: pharmacological inhibition of ferroptosis during CAR-T cell manufacturing and genetic reduction of ferroptosis susceptibility through ACSL4 knockout. Both approaches consistently improved CAR-T persistence and antitumor activity in our experimental systems. Accordingly, in the revised Discussion (**lines 579-588**), we have adopted a more balanced perspective by explicitly discussing the respective advantages and limitations of pharmacological versus genetic strategies for modulating ferroptosis in CAR-T therapy.

RE: Manuscript ID: NATCANCER-A19808

Title: Identification and targeting of iron-mediated ferroptosis in CAR-T cells/Iron-mediated ferroptosis impairs CAR-T cell function and antitumor efficacy

Dear Editors,

We thank the editor and all reviewers for their positive assessment of our revised manuscript. We also apologize for any remaining points that were not sufficiently clarified and for the formatting errors introduced during revision. Below, we provide a point-by-point response to all comments raised by the reviewers, together with our planned revisions.

Reviewer #1：

Q1: The authors provide new data showing that ferroptosis inhibitors significantly elevate CD27 and CD62L expression. However I am confused that the authors show data showing that these cells are almost 100% CD45RO+ (e.g. Ex Data 4d) and now also ~100% CD45RA+ (e.g. Ex Data 4f). Distinct CD45RA and CD45RO populations should be discernible and it is the CD45RA+CD45RO+ population that is generally considered more prognostic. For example, see Figure 4b of Fraietta et al. Nature Med 2018.

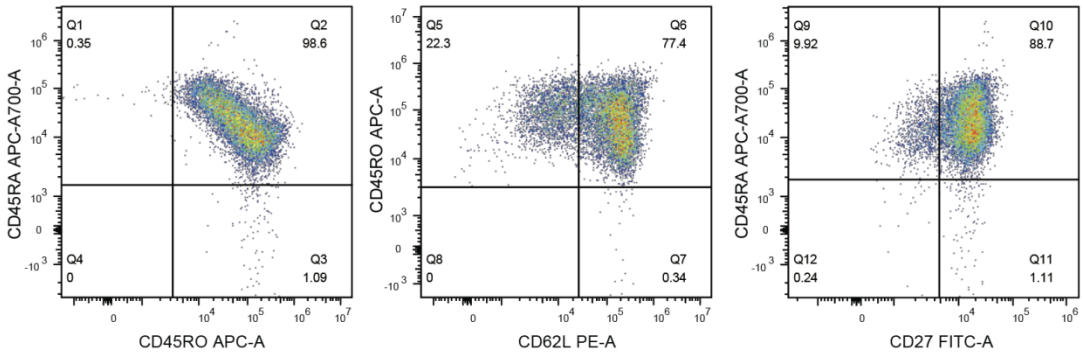
Response：

We thank the reviewer for raising this important point. We agree that CD45RA and CD45RO typically delineate distinct differentiation states in vivo, and that the CD45RA+ CD45RO+ subset is prognostically relevant in clinical CAR-T settings (Fraietta et al., Nat Med 2018; Fig. 4b).

In our IL-2–expanded **in vitro** CAR-T cultures, the dominant CAR+ population reproducibly co-expresses CD45RA and CD45RO. To exclude panel-related artifacts, we re-assessed CD45RA, CD45RO, CD27 and CD62L within the same staining panel (**Response Letter Fig. 1**) using a consistent gating strategy (live lymphocytes → singlets → CAR+). Notably, CD45RA and CD45RO signals display a continuum rather than discrete quadrant-separated populations, consistent with an activation-associated transitional state during ex vivo expansion(Summers et al., 1994 PMID: 8033417, Xu et al., 2014 PMID: 24782509).

Importantly, when CAR-T cells were recovered **in vivo** after adoptive transfer (**revised Fig. 2i**), we observed clearer segregation of CD45RA-defined subsets. This pattern is consistent with Fraietta et al., who profiled CAR-T cells recovered from patients in vivo and reported discernible CD45RA+ CD45RO-defined populations.

Response Letter Fig. 1



Response Letter Figure 1 | Phenotypic characterization of in vitro-expanded CAR-T cells by CD45RA, CD45RO, CD62L, and CD27 staining.

Q2: The new data with ferroptosis inhibition in vivo (revised Fig 2a-g) are well received and to my reading suggests that the pre-treatment of CAR T cells with these inhibitors is relatively more important than inhibition in vivo given the strength of the respective phenotypes. I suggest the results text is modified to reflect this point.

Response：

We thank the reviewer for this helpful suggestion. We have revised the figure organization to better reflect the effects of ferroptosis inhibitors on CAR-T cells. Specifically, we move the CAR-T pre-treatment data with ferroptosis inhibitors (currently shown in **Extended Data Fig. 2o–q**) into the main figures, and relocate the tumor-only cohort with in vivo drug administration (currently shown in **revised Fig. 2b–d**) to the Extended Data. In this way, the key findings supporting both ex vivo pre-treatment and in vivo treatment are clearly presented, and we have revised the Results text accordingly.

Reviewer #2

Q1:Replicate reporting / definition of n: In several figure panels, n is provided but not described in a way that clearly distinguishes biological replicates/independent experiments from technical replicates. I recommend adding a brief descriptor (e.g., "independent biological replicates, "independent experiments," etc.) in the figure legends or Methods.

Response：

We thank the reviewer for this careful suggestion. We will revise all relevant figure legends to explicitly define n and clearly distinguish independent biological replicates/independent experiments from technical replicates (e.g., donors, mice, or independently repeated experiments), to ensure consistency and avoid ambiguity.

Q2:Minor statistical reporting/legend inconsistencies: A small number of figure-legend statements describing statistical tests appear inconsistent or unclear and should be corrected for precision. In Figure 2 legend, the assignments "two-way ANOVA (d,f)" and "log-rank test (e,g)" should instead be "(c,f)" and "(d,g)," respectively. In addition, in Fig. 4e, the legend/text indicates "unpaired two-tailed t test (e)," but this comparison should be analyzed via ANOVA rather than a t-test. This may simply be a typographical inconsistency, as panel (e) appears to be included in the ANOVA statement elsewhere; however, it should be corrected to avoid confusion.

Response：

We apologize for these inconsistencies. They arose from figure reorganization during revision, which led to mismatched panel references in a small number of legends. We have corrected the Figure 2 legend to assign the statistical tests as "two-way ANOVA (c,f)" and "log-rank test (d,g)," as noted by the reviewer. We also revise the Fig. 4e legend/text to ensure the appropriate ANOVA-based analysis is stated consistently (rather than an unpaired two-tailed t test). In addition, we have carefully audited all figure legends across the manuscript to ensure accuracy, clarity, and consistency of statistical reporting and panel labeling.

Reviewer #3

I'm happy that the authors have addressed my points and the manuscript is much improved. I would still have liked to see the immunocompetent model - it should be straightforward to make mouse CAR-T cells. these don't expand in vitro but you just make more at the start.

Response：

We thank the reviewer for this suggestion and fully agree that testing in an immunocompetent setting is an important direction. However, the central focus of our study is a CAR-T cell–intrinsic ferroptosis vulnerability driven by iron overload, supported by extensive mechanistic data in human CAR-T cells, clinical samples, and in vivo efficacy studies using human CAR-T xenograft models. We therefore believe the current dataset is sufficient to support our main conclusions without introducing additional confounding variables inherent to murine CAR-T manufacturing and mouse-specific iron/immunity contexts.

That said, we agree that extending these findings to immunocompetent models will be valuable for

future work. In subsequent studies, we plan to generate murine CAR-T cells (e.g., anti-mouse CD19 CAR-T) and evaluate whether ferroptosis inhibition similarly improves CAR-T persistence and antitumor activity in syngeneic models.

Reviewer #4

Congrats, impressive revision of manuscript.

Response：

We thank the reviewer for the positive and encouraging comments on our revised manuscript.