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Peer Review File

IL-4 Drives Exhaustion of CD8+ CART Cells

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REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

Stewart et utilize orthogonal approaches to identify mechanisms that drive CD19-CD28z CAR T cell exhaustion and poor response in patients. They identify IL-4 as a regulator of CAR T cell exhaustion and confirm through function assessment in in vitro and in vivo models. Th1/Th2 skewing of CAR T cells has been studies but very in very limited reports. Additionally, the data from Stewart et al support that the IL-4 driven exhaustion is not associated with classic features of Th2 polarization, a novel finding. Overall, the data appears to be robust with points outlined below and support the conclusions put forth by the authors in the manuscript. Finally, the output of the work is translationally relevant with high potential for testing in human clinical trials.

Comments:

1) The authors are clear in the manuscript that costimulatory domain impacts exhaustion and clearly state the intent to study CD28 costimulated CARS. The also provide validation their models using a 41BB CAR. Thus, performing a subset of the experiments (not necessarily the transcriptomic and epigenomic profiling) such as the IL-4 coculture, IL-4 neutralization experiments would markedly increase understanding of the generalizability of the axis. Even if the IL-4 axis is restricted to CD28 costimulated CARs, this information would be very important in defining the translational path and point to future work to better define the non-Th2 associated aspect of the axis.
2) Inherent donor to donor variability is a well-known feature of CAR T cells and propensity for Th1/Th2 skewing. The figures state biologic replicates (generally 3). I assume these are multiple donors?
3) The authors use irradiated JeKo-1 cells to assess the role for tumor-T cell interaction. It is not clear how repetitive stimulation with irradiated cells would allow for this. A better approach would be to stimulate with a non-cellular method – for example the anti-FMC63 idiotype.

Reviewer #2 (Remarks to the Author):

In this manuscript, the authors Stewart et al, used three independent approaches, including a genome-wide CRISPR screen, RNA and ATAC sequencing, and identified IL-4 as negative regulator of CAR-T persistent function. The authors reported that IL-4 neutralization with antibody improved CAR-T function. While the subject holds some interest, the experiments are not well controlled. Furthermore, the study leaves various critical questions unanswered, raising concerns about the study's overall rigor.

Major comments,

0. Overall, IL4 is a well-established cytokine and the novelty of biology is limited.

1. The authors have left lots of unanswered questions yet are core to their central claim that IL-4 is a negative regulator of CAR-T function. How is IL-4 signaling regulating CAR-T exhaustion? What would be the impact of knocking out IL4 or IL4 receptor in CAR-T cells? Whether if the IL-4 supplement or IL-4 neutralization directly work on CAR-T cells or possibly on suppressing cancer cells instead?

2. As have been mentioned by the author, the exhaustion mechanism of CAR-T exhaustion varies by CAR constructs and disease models, all the experiments are done with CD19-28zeta CAR. They should at try a few other CAR constructs and/or disease models to strengthen their claims that IL-4 is a negative regulator of CAR-T function.

3. Experiments in Fig. 1, and Fig.3 were not well-controlled, CAR-T cells with same length of in-vitro culturing time, but without antigen stimulation should be included as controls for normalization. They showed that D22 CAR-T cells are less functional than D8 CAR-T cells, and claimed that repeated stimulation resulted CAR-T disfunction. It could also be possible that CAR-T cells cultured in vitro for longer time are less functional.

4. The method they used for screen analysis is less developed. In Fig. 2c, the PCA plot is showing PC2 vs PC3, what about PC1? In Fig. 2d, how come the dots, which represent each gene, in the "volcano" plot are not random distributed, and instead seemed to form multiple line shapes? The authors need to be more detailed about how they performed screen analysis, and have plots demonstrating the quality of their screen. Additionally, an important control is missing. CAR-T transfected with either vector or sgRNA targeting non-essential genes should be included as a control.

5. The authors are jumping conclusions without clear evidence. How would the results in Fig. 3H-J support their claim that IL-4 is an upstream regulator instead of a result of CAR-T exhaustion? Additionally, results in Fig.3I and 3J seem contradictory. As Th2 cells are in general IL-4+, how come the IL4 producing in CD4 is the same in Day8 and Day15, while %Th2 in day 15 is lower than day8? The authors should also show represent flow plots.

6. The author failed to indicate which method they used to present their data in all figures. It is not clear whether they are shown as mean (SD), mean (SE), or a mix of different methods. Either way, the top error bar should be of the same size as the bottom error bar. While this was not the case in Fig. 1c and Fig. 6c. And all other bar plots are shown with a mixed of different methods, which again were not specified.

7. The data shown in Fig.6c do not seem to match with the data shown in 6b, take d21 for example, the error bar is expected to relatively bigger than shown in 6c considering 2 out of 5 mice seem to be cancer free shown in 6b.

Minor points:

1. The lower cell number of total CAR-T cells in Fig.1b, does not necessarily mean reduced antigen specific proliferation, their differences in exhaustion, activation induced cell death, could also play a role.

2. Fig. 3a-c did not seem to add much to their overall conclusion.

3. Statistical tests are missing for fig.5c and 5d.

Reviewer #3 (Remarks to the Author):

The group of S. Kenderian and colleagues should be commended for their 3-pronged-approach to identifying new mechanisms that contribute to the exhaustion of CAR-T cells. These include a CRISPR knockout screen, ATAC sequencing of multiply-stimulated CAR-T, and a transcriptomic/ATAC analysis of products from the ZUMA-1 trial. As previous data has pointed to the increased exhaustion of CD19-28 CAR-T as compared to CD19-41BB CAR-T, the present analyses focus on the former. The data are very convincing, well presented and add significantly to our understanding of CART, identifying a novel role of IL-4 in CD8 CART exhaustion.

Importantly, the authors' data highlight a negative IL-4-targeted impact on CD8 CART exhaustion/dysfunction rather than on Th2 CART differentiation. Given the importance of this message (see points below), it would be important to present these data in the abstract (and possibly even in the title). Furthermore, in light of data pointing to the critical nature of a Th2-like CD4CAR population in the long-term responsiveness of patients receiving a CD19-41BBCAR (doi: 10.1126/sciadv.abj2820, a paper that is not cited here), it would be helpful for the authors to discuss the differences, as relates to both CD4 vs CD8 CART and the CAR construct.

It would be helpful if the authors could address the following points:

-The authors' CRISPR data showing the importance of the IL-4 pathway in CART cell dysfunction is impressive. Nevertheless, it is not clear from the experiments presented in figure 2 (or even in figure 1) as to whether the impact of IL-4 in CART exhaustion is biased as a function of CD4 or CD8 CART. As the authors report that the relative CD4/CD8 ratio decreases significantly between day 8 and 15, this is of interest. Is the enhanced representation of IL-4 gRNAs equivalently detected in CD4 and CD8 CART? Indeed, the authors conclude (based on figure 3J), that the negative impact of IL4 on CART is a CD8-specific phenomenon.

-The authors report that there was an "indeterminant enrichment status" of pathways involved in Th differentiation. However, as the CD4/CD8 ratio decreases by 5-10-fold, is it possible that Th2 differentiation in the context of the population of CD4-CART is actually increasing? This should be discussed. Additionally, the percentages of T cells that are CAR+ are not indicated.

-Do the authors have the potential to determine whether the upregulation of IL-4 and CCR3 in non-responders in the ZUMA trial is occurring in CD8 as compared to CD4 CART?

-Related to the question above, is the negative impact of IL-4 on CD19-28CART (Figure 5) associated with changes in CD4/CD8 CART ratios (and conversely, the impact of anti-IL4, Fig S13)? The authors can potentially assess whether IL-4 has the same impact on purified CD4 as compared to CD8 CART.

-While the authors compare the CAR-T products that they generated from healthy donors with products from the ZUMA-1 trial, the authors are clearly aware that the former were generated with a lentiviral vector expressing the CD19-28 CAR downstream of an EF1a promoter while the latter are

expressed from a retroviral vector. While this might not impact the data, the possibility that there could be differences should be discussed. It is also not clear from the methods as to which experiments were performed with retroviral-generated as compared to lentiviral-generated CD19-28CART (some cells are indicated as being produced by KITE (line 113, Supplemental file)). Additionally, since significant differences have been found with CD19 CARs harboring distinct modular domains (i.e. hinge and transmembrane), the precise regions that compose the CD19 CARs used here should be described in the supplemental.

We would like to thank the editor and reviewers for their consideration and time given while reviewing and providing comments on our manuscript. In the document, we provide a point-to-point response to individual questions, comments, and critiques raised by the three reviewers.

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Stewart et utilize orthogonal approaches to identify mechanisms that drive CD19-CD28z CAR T cell exhaustion and poor response in patients. They identify IL-4 as a regulator of CAR T cell exhaustion and confirm through function assessment in in vitro and in vivo models. Th1/Th2 skewing of CAR T cells has been studies but very in very limited reports. Additionally, the data from Stewart et al support that the IL-4 driven exhaustion is not associated with classic features of Th2 polarization, a novel finding. Overall, the data appears to be robust with points outlined below and support the conclusions put forth by the authors in the manuscript. Finally, the output of the work is translationally relevant with high potential for testing in human clinical trials.

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1) The authors are clear in the manuscript that costimulatory domain impacts exhaustion and clearly state the intent to study CD28 costimulated CARS. The also provide validation their models using a 41BB CAR. Thus, performing a subset of the experiments (not necessarily the transcriptomic and epigenomic profiling) such as the IL-4 coculture, IL-4 neutralization experiments would markedly increase understanding of the generalizability of the axis. Even if the IL-4 axis is restricted to CD28 costimulated CARs, this information would be very important in defining the translational path and point to future work to better define the non-Th2 associated aspect of the axis.

Thank you for the suggestion. We fully agree that testing the IL-4 axis in other constructs would strengthen our findings. As such, we have performed additional experiments to test the impact of IL-4 supplementation on three other CAR constructs that are similar to clinically utilized CARTs. We include these data in the revised manuscript and discussed below:

A. **IL-4's Impact on CART19-BBζ cells:** We performed additional experiments where CART cells targeting the CD19 antigen and containing a 4-1BB costimulatory domain (CART19-BBζ) were treated with human recombinant IL-4 (hrIL-4). Similar to our findings with CART19-28ζ cells, CART19-BBζ cells treated with hrIL-4 showed reduced cytotoxicity and a trend for decreased proliferative ability (Revised Supplementary Fig. S14a-b). Furthermore, CART19-BBζ cells chronically stimulated in the presence of hrIL-4 showed increased functional and phenotypical signs of exhaustion as evident by **1)** a trend for decreased proliferative ability (Revised Supplementary Fig. S14c), **2)** decreased degranulation (Revised Supplementary Fig. S14d), **3)** decreased production of IL-2 and IFN-γ (Revised Supplementary Fig. S14e-f), and **4)** increased co-expression of multiple inhibitory receptors (Revised Supplementary Fig. S14g).

B. **IL-4's Impact on BCMA-targeting CART cells with a 4-1BB costimulatory domain:** We then tested the impact of IL-4 on BCMA-CART cells with 4-1BB costimulatory domain, similar to the FDA-approved products[1,2]. Similar to our findings with CART19-28ζ cells, BCMA CART cells chronically stimulated in the presence of hrIL-4 showed enhanced functional and phenotypical signs of exhaustion such as **1)** decreased proliferative ability (Revised Supplementary Fig. S15a), **2)** decreased production of IL-2 and IFN-γ (Revised Supplementary Fig. S15b-c), and **3)** increased co-expression of multiple inhibitory receptors (Revised Supplementary Fig. S15d).

C. **IL-4's Impact on CS1-targeting CART cells with a CD28 costimulatory domain:** Finally, we tested how IL-4 impacts CS1-targeting CART cells containing a CD28 costimulatory domain, similar to the CS1 CART cells currently being evaluating in clinical trials[2,3]. CS1 CART cells were treated with human recombinant IL-4 (hrIL-4). Similar to our findings with CART19-28ζ cells, CS1 CART cells chronically stimulated in the presence of hrIL-4 showed enhanced functional and phenotypical signs of exhaustion such as **1)** decreased proliferative ability (Revised Supplementary Fig. S16a), **2)** decreased degranulation (Revised Supplementary Fig. S16b), and **3)** decreased production of IFN-γ (Revised Supplementary Fig. S16c).

2) Inherent donor to donor variability is a well-known feature of CAR T cells and propensity for Th1/Th2 skewing. The figures state biologic replicates (generally 3). I assume these are multiple donors?

Thank you for the request for clarification. That is correct, the term “biological replicate” is used to describe multiple T-cell donors. We have clarified it further in the statistics section of the supplementary materials (Line 291-292).

3) The authors use irradiated JeKo-1 cells to assess the role for tumor-T cell interaction. It is not clear how repetitive stimulation with irradiated cells would allow for this. A better approach would be to stimulate with a non-cellular method – for example the anti-FMC63 idiotype.

Thank you for this point. We agree that there is additional value in using a completely tumor-free assay to evaluate if IL-4 works directly on CART cells or if it is dependent on the presence of tumor cells. As such, we followed your suggestion and used CD19-conjugated beads to chronically stimulate CART cells in the presence of either hrIL-4 or diluent. In this tumor-free assay, CART19-28ζ cells chronically stimulated in the presence of hrIL-4 showed increased functional and phenotypical signs of exhaustion such as 1) decreased production of IL-2 (Revised Supplementary Fig. S13a), 2) increased co-expression of multiple inhibitory receptors (Revised Supplementary Fig. S13b), and 3) decreased proliferative ability (Revised Supplementary Fig. S13c). This indicates that IL-4 can drive CART cell exhaustion independently of tumor cells.

Reviewer #2 (Remarks to the Author):

In this manuscript, the authors Stewart et al, used three independent approaches, including a genome-wide CRISPR screen, RNA and ATAC sequencing, and identified IL-4 as negative regulator of CAR-T persistent function. The authors reported that IL-4 neutralization with antibody improved CAR-T function. While the subject holds some interest, the experiments are not well controlled. Furthermore, the study leaves various critical questions unanswered, raising concerns about the study's overall rigor.

Major comments,
Overall, IL4 is a well-established cytokine and the novelty of biology is limited.

We appreciate the reviewer’s concerns regarding novelty. While the effect of IL-4 on CD4 T-cells is well established, its effect on CD8 T-cells is not[4,5]. In addition, IL-4 has not previously been linked to the development of CART cell exhaustion. We hope that our new data looking at IL-4’s effect on CD4 and CD8 CART cells independently will help address this comment further (Revised Fig. 5).

1. The authors have left lots of unanswered questions yet are core to their central claim that IL-4 is a negative regulator of CAR-T function.

Thank you for this comment and for the questions. We have addressed each question below.

How is IL-4 signaling regulating CAR-T exhaustion?

To further explore how IL-4 signaling is regulating CART cell exhaustion, we completed experiments to 1) examine the direct effect of IL-4 on CD8 CART cells and 2) determine if IL-4 drives CART cell exhaustion in CART cells with a 4-1BB costimulatory domain.

1. We isolated CD8 CART cells following CART cell production. At baseline, hrIL-4 significantly reduces CD8 CART cytotoxicity (Revised Fig. 5a). Then, following chronic stimulation of CD8 CART cells with JeKo-1 tumor cells in the presence of either hrIL-4 or diluent, CD8 CART cells treated with hrIL-4 show an increased exhausted phenotype as seen by **1)** decreased proliferative ability (Revised Fig. 5b), **2)** decreased production of effector cytokines such as IL-2 and IFN-γ (Revised Fig. 5c-d), **3)** increased co-expression of multiple inhibitory receptors (Revised Fig. 5e), and **4)** increased transcription of EOMES (Revised Fig. 5f). This indicates that IL-4 can directly affect CD8 CART cells to drive CART cell exhaustion.
2. We performed additional experiments where CART cells targeting the CD19 antigen and containing a 4-1BB costimulatory domain (CART19-BBζ) were treated with human recombinant IL-4 (hrIL-4). Similar to our findings with CART19-28ζ cells, CART19-BBζ cells treated with hrIL-4 showed reduced cytotoxicity and a trend for decreased proliferative ability (Revised Supplementary Fig. S14a-b). Furthermore, CART19-BBζ cells chronically stimulated in the presence of hrIL-4 showed enhanced functional and phenotypical signs of exhaustion as evident by **1)** a trend for decreased proliferative ability (Revised Supplementary Fig. S14c), **2)** decreased degranulation (Revised Supplementary Fig.

S14d), **3)** decreased production of IL-2 and IFN-γ (Revised Supplementary Fig. S14e-f), and **4)** increased co-expression of multiple inhibitory receptors (Revised Supplementary Fig. S14g). This indicates that IL-4 can also drive CART cell exhaustion in CARs containing the 4-1BB costimulatory domain.

What would be the impact of knocking out IL4 or IL4 receptor in CAR-T cells?

Since the original submission, we have created IL-4 knockout cells using CRISPR Cas9 with two gRNAs (Revised Supplementary Fig. S17a-b). IL-4 knockout CART cells exhibit increased cytotoxicity (Revised Supplementary Fig. 17c-d). Then, upon chronic stimulation through our *in vitro* model for exhaustion, IL-4 knockout CART cells show reduced signs of CART cell exhaustion such as 1) increased proliferative ability (Revised Supplementary Fig. S17e-f), 2) increased production of effector cytokines such as IL-2 and IFN-γ (Revised Supplementary Fig. 17g-h), and 3) decreased co-expression of inhibitory receptors (Revised Supplementary Fig. S17i).

Whether if the IL-4 supplement or IL-4 neutralization directly work on CAR-T cells or possibly on suppressing cancer cells instead?

To your question as to whether IL-4 supplement suppresses tumor cells, we have shown that hrIL-4 does not affect the growth or survival of JeKo-1 tumor cells (Revised Supplementary Fig. 12a). To further address this question, we performed experiments where CART19-28ζ cells were exposed to either hrIL-4 or diluent while being chronically stimulated with CD19-conjugated beads according to the *in vitro* model for exhaustion. Even in a tumor-free assay, supplementation with hrIL-4 enhanced the exhausted profile of CART19-28ζ cells as seen by **1)** decreased production of IL-2 (Revised Supplementary Fig. S13a), **2)** increased co-expression of multiple inhibitory receptors (Revised Supplementary Fig. S13b), and **3)** decreased proliferative ability (Revised Supplementary Fig. S13c). This indicates that IL-4 can drive CART cell exhaustion independently of tumor cells.

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Thank you for this question, which was also raised by Reviewer #1. We have performed additional experiments to test the impact of IL-4 on other CAR constructs. We utilized three CAR constructs that are similar to the ones used in the clinic, including 41BB-costimulated CAR19, 41BB-costimulated BCMA CAR, and CD28-costimulated CS1 CAR. Our data demonstrate similar induction of exhaustion in all tested CAR constructs following IL-4 exposure.

A. **IL-4's Impact on CART19-BBζ cells:** We performed additional experiments where CART cells targeting the CD19 antigen and containing a 4-1BB costimulatory domain (CART19-BBζ) were treated with human recombinant IL-4 (hrIL-4). Similar to our findings with CART19-28ζ cells, CART19-BBζ cells treated with hrIL-4 showed reduced cytotoxicity and a trend for decreased proliferative ability (Revised Supplementary Fig. S14a-b). Furthermore, CART19-BBζ cells chronically stimulated in the presence of hrIL-4 showed increased functional and phenotypical signs of exhaustion as evident by **1)** a trend for decreased proliferative ability (Revised Supplementary Fig. S14c), **2)** decreased degranulation (Revised Supplementary Fig. S14d), **3)** decreased production of IL-2 and IFN-γ (Revised Supplementary Fig. S14e-f), and **4)** increased co-expression of multiple inhibitory receptors (Revised Supplementary Fig. S14g).

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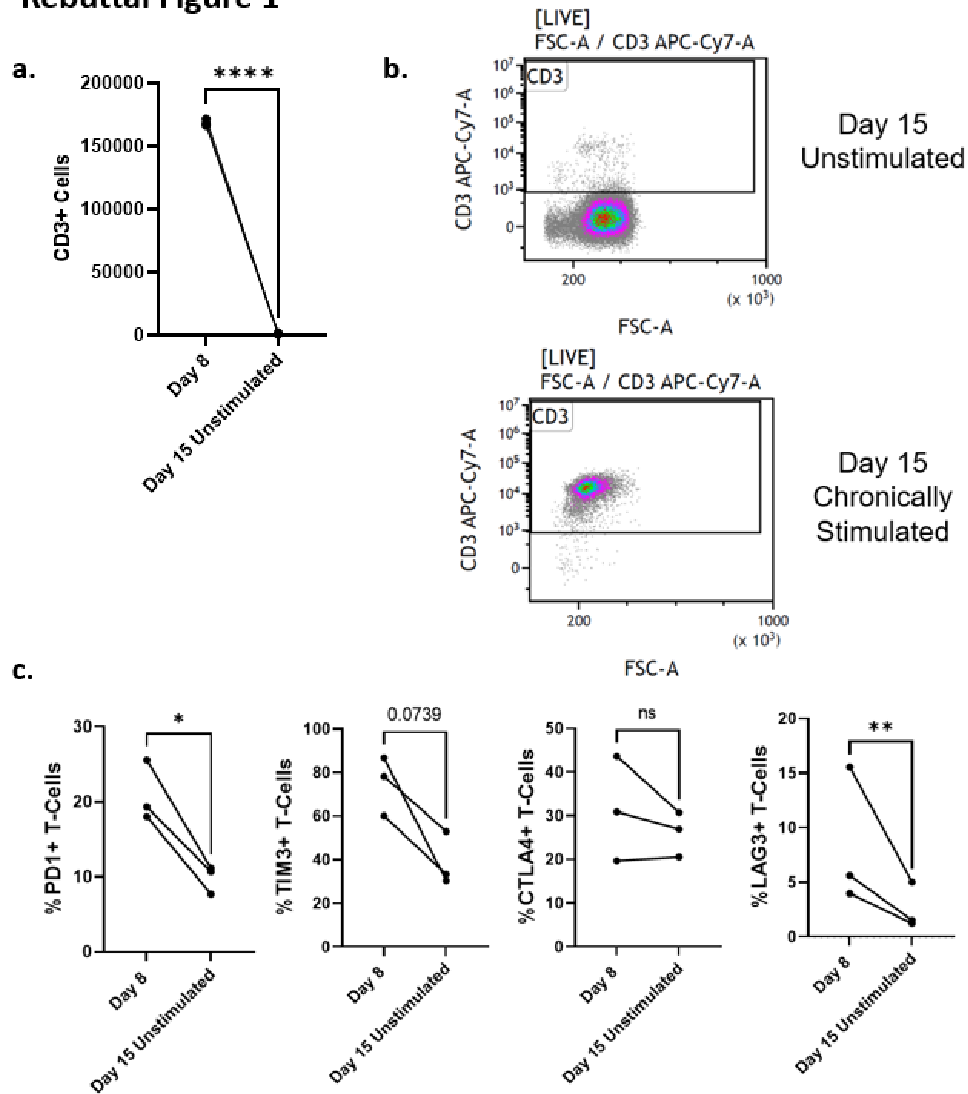
3. Experiments in Fig. 1, and Fig.3 were not well-controlled, CAR-T cells with same length of in-vitro culturing time, but without antigen stimulation should be included as controls for normalization. They showed that D22 CAR-T cells are less functional than D8 CAR-T cells and claimed that repeated stimulation resulted CAR-T disfunction. It could also be possible that CAR-T cells cultured in vitro for longer time are less functional.

Thank you for bringing up this possibility. We considered this factor in our study design. As primary cells, CART cells kept in culture without stimulation will die. We included this data below for your review. This is seen by a lack of proliferative ability and a lack of tumor killing after the CART cells are kept until Day 15 (Rebuttal Fig. 1a-b). However, CART cells kept in culture until Day 15 do not show phenotypical signs of exhaustion. Instead, the percent of cells expressing inhibitory receptors decreases (Rebuttal Fig. 1c). Since unstimulated CART cells are dead or dying by Day 15, we chose not to use this as a control. Instead, we compared chronically stimulated CART cells to freshly produced CART cells.

This model was chosen to mimic the development of exhaustion in patients where CART cells develop a state of exhaustion following chronic stimulation due to a high tumor burden. Further, since we initially began our experiments, other groups have published similar *in vitro* models for exhaustion that compare chronically stimulated CART cells to freshly produced CART cells and reported novel findings using such models[6-9]. Due to the clinical relevance and comparative ability of this experimental set-up to other studies of CART cell exhaustion, we decided to compare chronically stimulated CART cells to freshly produced CART cells in our CRISPR screen and in our RNA and ATAC sequencing experiments.

However, to address this comment further, we completed additional studies to explore if CART cells kept in culture long-term are less functional. To do so, we tried an alternative approach where CART cells were kept in media supplemented with a low-dose (100 IU/mL) of hrIL-2 from Day 8 to Day 22 of the *in vitro* model for exhaustion. This dose of IL-2 is commonly used in CART cell manufacturing protocols[10]. Keeping CART cells until Day 22 in media supplemented with hrIL-2 did not appear to **1)** significantly impair CART proliferative ability (Revised Supplementary Fig. 2a), **2)** increase the coexpression of multiple inhibitory receptors (Revised Supplementary Fig. 2b), or **3)** impair the production of effector cytokines such as IL-2 and TNF-α (Revised Supplementary Fig. 2c-f). This suggests that long-term culture itself does not cause CART cell dysfunction or exhaustion.

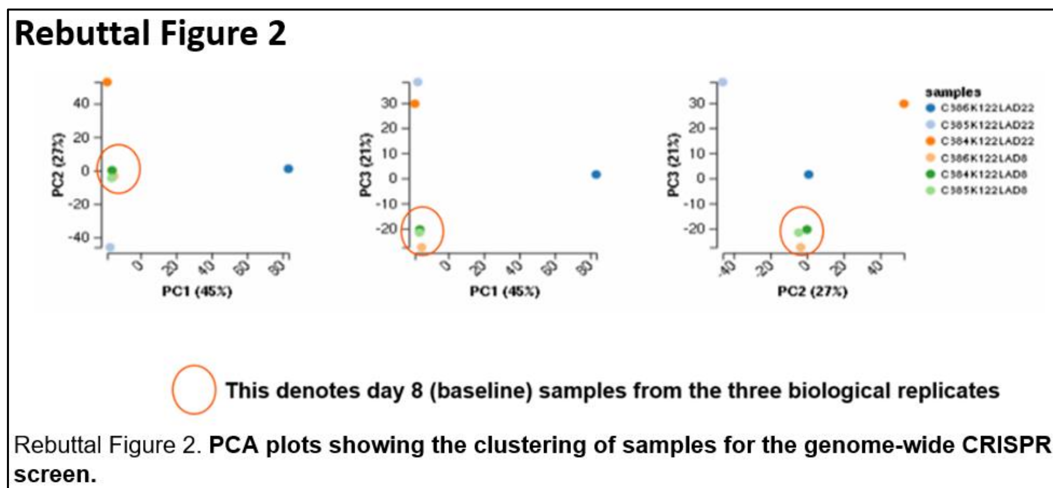


Rebuttal Figure 1. **Long-term co-culture of CART cells without stimulation results in dysfunction. a.** Absolute CD3⁺ cell count after 100,000 CART cells were co-cultured with JeKo-1 cells at 1:1 effector-to-target (E:T) cell ratio for 5-days. Day 15 unstimulated CART cells were kept in T cell media alone until Day 15 (Paired t-test, average of two technical replicates for three biological replicates). **b.** Representative flow-plot of CD3⁺ cells after cells chronically stimulated from Day 8 to Day 15 or cells kept in culture unstimulated from Day 8 to Day 15 were co-cultured with JeKo-1 cells at a 1:1 E:T ratio for 5-days. **c.** The percent of either Day 8 or unstimulated Day 15 CART cells expressing inhibitory receptors as determined by flow cytometric detection of CD3+ cells positive for PD-1, TIM-3, CTLA-4, and LAG-3 (Paired t-test, average of two technical replicated for three biological replicates). (ns: $p>0.05$, *$p<0.05$, **$p<0.01$, ****$p<0.0001$)

4. The method they used for screen analysis is less developed. In Fig. 2c, the PCA plot is showing PC2 vs PC3, what about PC1? In Fig. 2d, how come the dots, which represent each gene, in the "volcano" plot are not random distributed, and instead seemed to form multiple line shapes? The authors need to be more detailed about how they performed screen analysis and have plots demonstrating the quality of their screen. Additionally, an important control is missing. CAR-T transfected with either vector or sgRNA targeting non-essential genes should be included as a control.

We appreciate the request for clarification. The CRISPR screen and analysis methods were followed from validated and published protocols[11,12]. The GeCKO v2 library that we used is commercially available, commonly used, and contains 1,000 non-targeting gRNAs as a control[13]. Analysis was performed using the MAGeCK-VISPR pipeline[12]. This is a comprehensive and widely used pipeline that includes quality control, analysis, and visualization[8,14,15]. Part of the analysis involves inputting the list of non-targeting gRNAs to account for changes in their expression while identifying negatively and positively selected gRNAs. In an effort to further address this concern, we clarified the settings that we chose during MAGeCK-VISPR analysis including the use of maximum likelihood estimation and normalization to the list of 1,000 non-targeting gRNAs included in the library (Supplements Lines 280-283).

In the initial submission, we used MAGeCK-VISPR to assess quality in several ways including: **1)** PCA plots (Revised Fig. 2c), **2)** Gini index calculations (Revised Fig. 2b), **3)** gene ontology enrichment analysis of the negatively selected genes (Revised Supplementary Fig. S6b), and **4)** a literature evaluation of the top positively selected genes. Our discussion of the CRISPR screen quality can be found in Lines 101-116 of the revised manuscript. The PCA plot included in the initial submission was a representative plot. In response to this comment, we included the other PCA plots created by MAGeCK-VISPR in this letter for your review (Rebuttal Fig. 2). Also, in response to this comment, we have included an additional quality control plot that showcases changes in the representation of non-targeting gRNAs (Revised Supplementary Fig. S6a) despite our normalization to the list of non-targeting gRNAs in the final analysis strategy.



Rebuttal Figure 2. **PCA plots showing the clustering of samples for the genome-wide CRISPR screen.**

Finally, in response to concern about the non-random distribution of our volcano plot, we revisited our analysis to address this issue. Our initial volcano plot was graphed with ggplot using the p-value and β-score outputs from MAGeCK-VISPR. While this graph effectively showcased the top hits from the CRISPR screen, it also showed a linear relationship between p-value and β-score. A linear relationship makes sense given that the β-score reflects the extent of selection, and the p-value represents the extent of significance[9]. However, we agree that this is not the most effective way to showcase our results. After looking at other published CRISPR screens, we found that a volcano plot generated with the normalized z-score and the log10(p-value) is the most adequate representation of the data given the heterogeneity of our biological replicates, as some other publications have shown[13]. With this approach, our volcano plot shows the random distribution and is able to effectively showcase the positively and negatively selected genes. As a result, we replaced our initial volcano plot with a plot showing p-value versus z-score (Revised Fig. 2d).

5. The authors are jumping conclusions without clear evidence. How would the results in Fig. 3H-J support their claim that IL-4 is an upstream regulator instead of a result of CAR-T exhaustion?

We appreciate the opportunity to clarify this section. Figures 3h-j were not meant to help identify IL-4 as an upstream regulator. IL-4 was identified as an upstream regulator through ingenuity pathway analysis of the differentially expressed and accessible genes when comparing chronically stimulated to baseline CART cells from healthy donors (Revised Fig. 3g). At the same time, ingenuity pathway analysis also identified an enrichment in the T cell exhaustion pathway and an indeterminant change in the T helper cell pathways (Revised Fig. 3f). Since previous literature has implicated IL-4 in the polarization of CD4 cells towards a Th2 phenotype[4,5], the purpose of Fig. 3h-j was to determine if IL-4's regulatory role was identified due to changes in T helper cell polarization during our *in vitro* model for exhaustion. To clarify the purpose of Fig. 3h-j, we have revised the subheading (Line 163) and paragraphs associated with these figures.

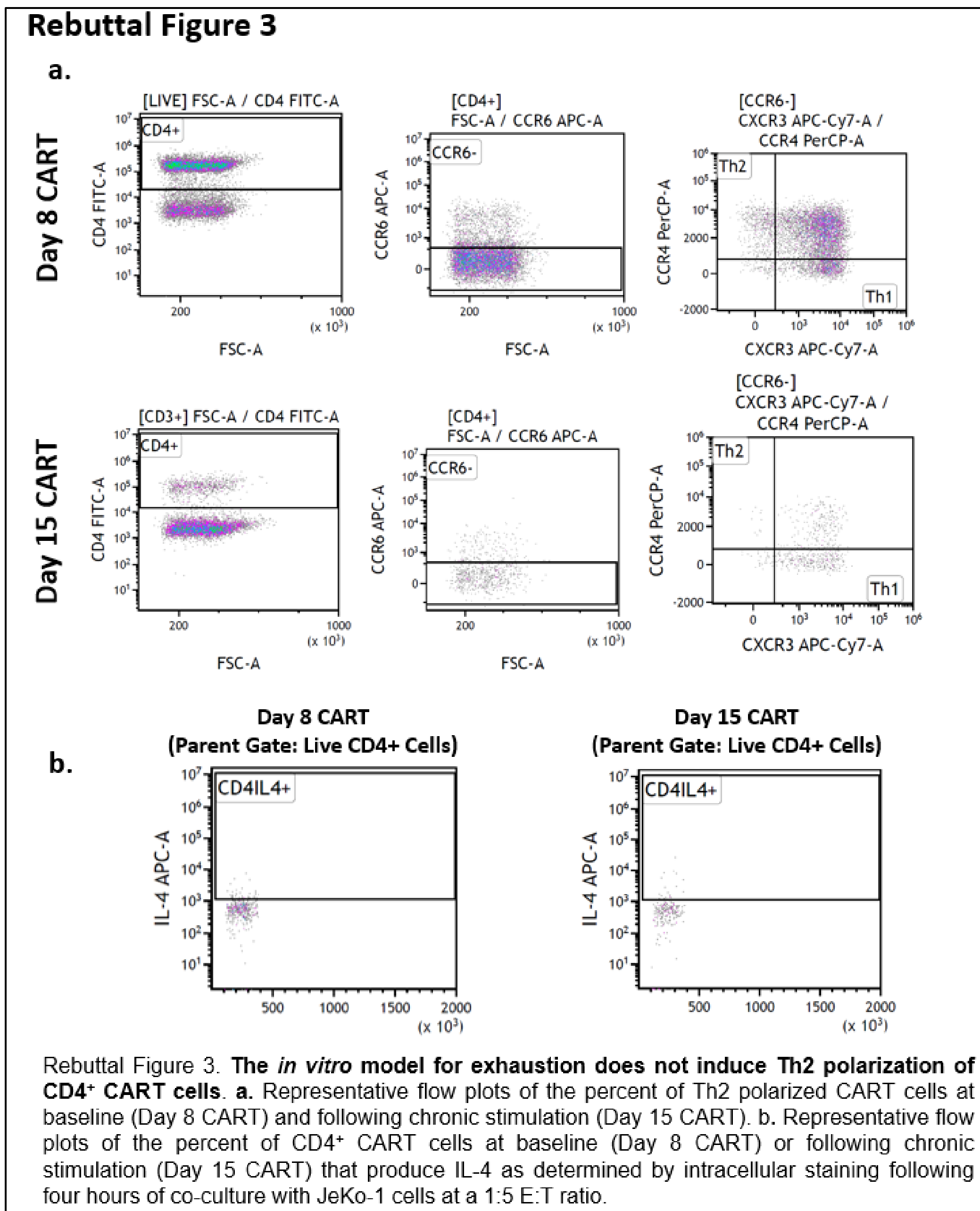
Our additional experiments strongly indicate a regulatory role for IL-4 in the development of exhaustion, as detailed below:

a. In the *in vitro* model for exhaustion, there appears to be a significant loss of CD4 CART cells (Revised Figure 3h). Additionally, there appears to be a small Th2 population that becomes smaller by Day 15 of the assay (Revised Figure 3i). The reduction in both the CD4 and Th2 population indicate that the regulatory role for IL-4 in our *in vitro* model for exhaustion (as identified by ingenuity pathway analysis) would be independent of IL-4's regulatory role in Th2 polarization.
b. This is further supported by evidence that IL-4 production increases in the CD8, but not the CD4 population (Revised Fig. 3j).
c. More definitive evidence of IL-4 driving CART cell exhaustion can be found in Revised Figures 5 and 6 when we perform *in vitro* and *in vivo* validation studies to determine the impact of IL-4 supplement and neutralization on CART cell activity and phenotype. In these figures, we find that IL-4 supplement enhances phenotypical, functional, and transcriptional evidence of exhaustion while IL-4 neutralization does the opposite. Further, in Fig. 5, we see that IL-4 can drive exhaustion in CD8 CART cells independently of CD4 CART cells.

To further clarify, the regulatory role for IL-4 in the development of exhaustion was discussed in detail in the revised manuscript, lines 328-341.

Additionally, results in Fig.3I and 3J seem contradictory. As Th2 cells are in general IL-4+, how come the IL4 producing in CD4 is the same in Day8 and Day15, while %Th2 in day 15 is lower than day8? The authors should also show represent flow plots.

We also appreciate the opportunity to clarify. In this study, we defined Th2 cells as $CD4^+CCR6^-CCR4^+CXCR3^-$ cells by flow cytometry as supported by current literature[16]. Using this gating strategy, we see a reduction in the Th2 population from Day 8 to Day 15 of our *in vitro* model for exhaustion. We included the results in this letter for your review (Rebuttal Fig. 3a). We do not see a significant change in the percent of $CD4^+$ cells producing IL-4 (Rebuttal Fig. 3b). It is possible that $CD4^+$ cells that fall outside our definition of Th2 cells ($CD4^+CCR6^-CCR4^+CXCR3^-$) produce IL-4. For example, Th0 populations have also been associated with the production of IL-4[16].



Rebuttal Figure 3. **The *in vitro* model for exhaustion does not induce Th2 polarization of CD4⁺ CART cells**. **a.** Representative flow plots of the percent of Th2 polarized CART cells at baseline (Day 8 CART) and following chronic stimulation (Day 15 CART). b. Representative flow plots of the percent of CD4⁺ CART cells at baseline (Day 8 CART) or following chronic stimulation (Day 15 CART) that produce IL-4 as determined by intracellular staining following four hours of co-culture with JeKo-1 cells at a 1:5 E:T ratio.

6. The author failed to indicate which method they used to present their data in all figures. It is not clear whether they are shown as mean (SD), mean (SE), or a mix of different methods. Either way, the top error bar should be of the same size as the bottom error bar. While this was not the case in Fig. 1c and Fig. 6c. And all other bar plots are shown with a mixed of different methods, which again were not specified.

We have included clarification for this point in our response to point 7 below.

7. The data shown in Fig.6c do not seem to match with the data shown in 6b, take d21 for example, the error bar is expected to relatively bigger than shown in 6c considering 2 out of 5 mice seem to be cancer free shown in 6b

We appreciate the requests for clarification (#6 and #7). We have included details on how the plots are generated in the methods section (Revised Supplemental Line 288-290), and also detailed in the figure legends of individual figures. For Fig. 1c and Fig. 6c, the original plots showed the mean and standard deviation of tumor flux as graphed in GraphPad Prism. Given the logarithmic y-axis, Prism cannot plot error bars that would go down to a negative number. As such, the original plots only had upward error bars given the presence (Revised Fig. 1c) or absence (Revised Fig. 6c) of tumor in 2 out of 5 mice. To address this comment and avoid any confusion, we have changed the plots to have a linear y-axis. After changing this feature, Prism was able to draw error bars on the top and bottom.

Minor points:
1. The lower cell number of total CAR-T cells in Fig.1b, does not necessarily mean reduced antigen specific proliferation, their differences in exhaustion, activation induced cell death, could also play a role.

Thank you. We agree that there could be several causes for the reduction in antigen specific proliferation. To help characterize the changes induced by the *in vitro* model for exhaustion, we perform a series of test to account for the various cellular fates that could be occurring. For example, we assessed proliferative ability in combination with **1)** effector cytokine production **2)** the expression of multiple inhibitory receptors, **3)** RNA sequencing, and **4)** ATAC sequencing. All of these evaluations together help us confidently conclude that our *in vitro* model for exhaustion pushes CART cells towards an exhausted fate. This was further discussed in the revised manuscript, lined 328 -330.

2. Fig. 3a-c did not seem to add much to their overall conclusion.

Thank you for the opportunity to address this comment and explain the significance of these figures. Since our *in vitro* model for exhaustion is a critical tool in this study, we chose to validate it by evaluating functional, phenotypic, transcriptomic, and epigenetic changes. While Fig. 3a-c may not add directly to the overall conclusions, we believe they are necessary to ensure the rigor of our study and validate the *in vitro* model for exhaustion through a transcriptional analysis, as discussed in Lines 128-138.

3. Statistical tests are missing for fig.5c and 5d.

Thank you. Initially, statistics were not included for these figures because this experiment was only conducted with two biological replicates due to sample availability. Since the original submission, the production of effector cytokines was assessed in three biological replicates and statistical analyses were added (Revised Fig. 5c-d).

Reviewer #3 (Remarks to the Author):

The group of S. Kenderian and colleagues should be commended for their 3-pronged-approach to identifying new mechanisms that contribute to the exhaustion of CAR-T cells. These include a CRISPR knockout screen, ATAC sequencing of multiply-stimulated CAR-T, and a transcriptomic/ATAC analysis of products from the ZUMA-1 trial. As previous data has pointed to the increased exhaustion of CD19-28 CAR-T as compared to CD19-41BB CAR-T, the present analyses focus on the former. The data are very convincing, well presented and add significantly to our understanding of CART, identifying a novel role of IL-4 in CD8 CART exhaustion.

Importantly, the authors' data highlight a negative IL-4-targeted impact on CD8 CART exhaustion/dysfunction rather than on Th2 CART differentiation. Given the importance of this message (see points below), it would be important to present these data in the abstract (and possibly even in the title).

Thank you for your suggestion. We agree that the identification of IL-4's role in driving CD8 CART cells towards exhaustion independently of CD4 CART cells and Th2 differentiation is a critical finding in this study. As such, we have changed the title, highlighted this finding in the abstract, and changed Figure 5 of the manuscript to highlight IL-4's effect on CD8 CART cells.

Furthermore, in light of data pointing to the critical nature of a Th2-like CD4CAR population in the long-term responsiveness of patients receiving a CD19-41BBCAR (doi: 10.1126/sciadv.abj2820, a paper that is not cited here), it would be helpful for the authors to discuss the differences, as relates to both CD4 vs CD8 CART and the CAR construct.

Thank you for the excellent comment and for pointing out the paper. The association of Th2 CD4 cells with long term remissions in this manuscript is consistent with our study's identification for a novel role for IL-4 in CART cell functions that is independent from its role in Th2 polarization. We also expanded our discussion section to address this comment and discuss some of the discrepant data on the impact of Th2 cytokines on CART success (Lines 342-350).

It would be helpful if the authors could address the following points:

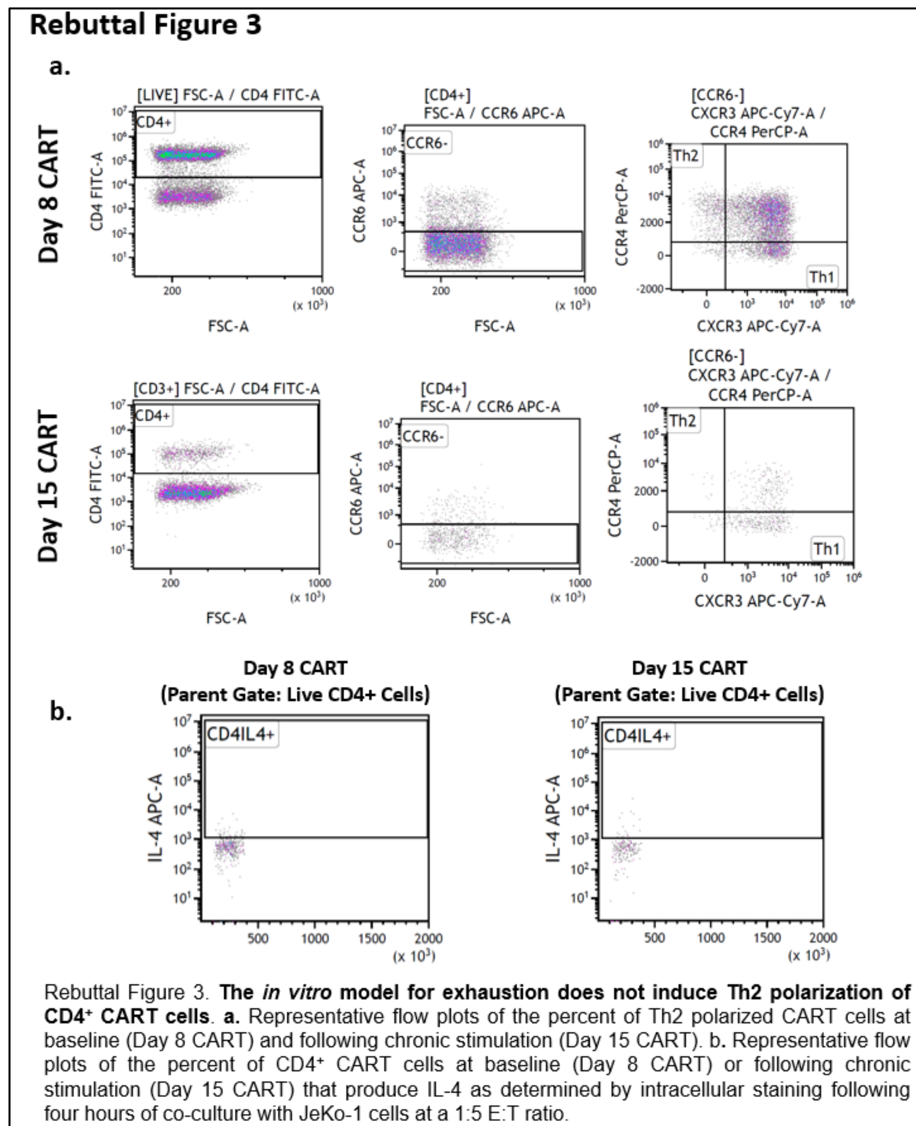
-The authors' CRISPR data showing the importance of the IL-4 pathway in CART cell dysfunction is

impressive. Nevertheless, it is not clear from the experiments presented in figure 2 (or even in figure 1) as to whether the impact of IL-4 in CART exhaustion is biased as a function of CD4 or CD8 CART. As the authors report that the relative CD4/CD8 ratio decreases significantly between day 8 and 15, this is of interest. Is the enhanced representation of IL-4 gRNAs equivalently detected in CD4 and CD8 CART? Indeed, the authors conclude (based on figure 3J), that the negative impact of IL4 on CART is a CD8-specific phenomenon.

Thank you. While we are not able to determine the representation of IL-4 gRNAs in CD4 and CD8 CART cells in the CRISPR screen due to a lack of sample availability, IL-4 knockout in a validation study did not appear to significantly alter the CD4:CD8 ratio across biological replicates (Revised Supplementary Fig. S18). In these validation studies, the CD4:CD8 ratio remained comparable to control gRNA CART cells throughout the *in vitro* model for exhaustion as assessed on Days 8, 15, and 22.

The authors report that there was an “indeterminant enrichment status” of pathways involved in Th differentiation. However, as the CD4/CD8 ratio decreases by 5-10-fold, is it possible that Th2 differentiation in the context of the population of CD4-CART is actually increasing? This should be discussed.

Thank you for this opportunity to clarify our results. As you pointed out, the *in vitro* model for exhaustion does result in a great loss of CD4 CART cells (Revised Fig. 3h). To ensure that this does not skew our results when examining the percentage of Th2 cells at Day 8 and Day 15 of the *in vitro* model for exhaustion, we looked at the percent of CD4⁺ cells that are Th2 polarized (Revised Fig. 3i). To clarify our gating strategy, we have included representative flow plots for your review (Rebuttal Fig. 3, and *below)* and we have clarified this point in the revised manuscript (Line 171-174).



Rebuttal Figure 3. **The *in vitro* model for exhaustion does not induce Th2 polarization of CD4⁺ CART cells**. **a.** Representative flow plots of the percent of Th2 polarized CART cells at baseline (Day 8 CART) and following chronic stimulation (Day 15 CART). b. Representative flow plots of the percent of CD4⁺ CART cells at baseline (Day 8 CART) or following chronic stimulation (Day 15 CART) that produce IL-4 as determined by intracellular staining following four hours of co-culture with JeKo-1 cells at a 1:5 E:T ratio.

Additionally, the percentages of T cells that are CAR+ are not indicated.

Thank you. CD3 antibodies were used to identify CART cells in this study. Importantly, the percent of CAR^+ T cells does not appear to change at the timepoints assessed in the *in vitro* model for exhaustion (Revised Supplementary Fig. S1f). The percent of CAR^+ cells also does not change following IL-4 supplement (Revised Supplementary Fig. S12b) or neutralization (Revised Supplementary Fig. S19e). For the majority of experiments, CAR percentage was greater than 90% on Day 8. This was also discussed in the revised manuscript, lines 66-68.

Do the authors have the potential to determine whether the upregulation of IL-4 and CCR3 in non-responders in the ZUMA trial is occurring in CD8 as compared to CD4 CART?

Thank you for this comment. We agree that it would be interesting to determine the population of cells that is upregulating IL-4 in the pre-infusion CART cell products. Unfortunately, there are no remaining cells or RNA from the products used in this study to evaluate this question further.

Related to the question above, is the negative impact of IL-4 on CD19-28CART (Figure 5) associated with changes in CD4/CD8 CART ratios (and conversely, the impact of anti-IL4, Fig S13)?

The negative impact of IL-4 cannot be entirely attributed to changes in the CD4/CD8 ratios. This is best seen with our new experiments with IL-4 knockout CART cells. IL-4 knockout improved CART cell function and reduced signs of exhaustion throughout long-term culture (Revised Supplementary Fig. S17); however, IL-4 knockout did not significantly alter the CD4:CD8 ratio as compared to CART cells transduced with a control gRNA (Revised Supplementary Fig. S18).

The authors can potentially assess whether IL-4 has the same impact on purified CD4 as compared to CD8 CART.

Thank you for this suggestion. To answer this question, we isolated CD8 CART cells following CART cell production. At baseline, hrIL-4 significantly reduces CD8 CART cytotoxicity (Revised Fig. 5a). Then, following chronic stimulation of CD8 CART cells with JeKo-1 tumor cells in the presence of either hrIL-4 or diluent, CD8 CART cells treated with hrIL-4 show an enhanced exhausted phenotype as seen by 1) decreased proliferative ability (Revised Fig. 5b), 2) decreased production of effector cytokines such as IL-2 and IFN-γ (Revised Fig. 5c-d), 3) increased co-expression of multiple inhibitory receptors (Revised Fig. 5e), and 4) increased transcription of EOMES (Revised Fig. 5f). This indicates that IL-4 can directly affect CD8 CART cells to drive CART cell exhaustion.

Given the importance of this data, we have changed Figure 5 of the manuscript to show how IL-4 supplement affects either bulk ($CD3^+$) or isolated $CD8^+$ CART19-28ζ cells.

While the authors compare the CAR-T products that they generated from healthy donors with products from the ZUMA-1 trial, the authors are clearly aware that the former were generated with a lentiviral vector expressing the CD19-28 CAR downstream of an EF1a promoter while the latter are expressed from a retroviral vector. While this might not impact the data, the possibility that there could be differences should be discussed.

Thank you. This is an important point. It was encouraging for us to identify IL-4 as a regulator of CART cell function using both CART cells generated using a lentiviral vector and CART cells generated with a retroviral vector in independent studies. In response to this comment, we have added a sentence to the discussion (Lines 330-331).

It is also not clear from the methods as to which experiments were performed with retroviral-generated as compared to lentiviral-generated CD19-28CART (some cells are indicated as being produced by KITE (line 113, Supplemental file)).

Thank you for bringing this to our attention, we have clarified this point in the methods section extensively (Supplemental Lines 53 and 99-101). KITE produced all of the cells that we performed RNA and ATAC sequencing on. This includes the healthy donor CART cells and the pre-infusion CART19 cell products from responders and non-responders in the Zuma-1 clinical trial (Fig. 3a-g and Fig. 4). All other experiments, including *in vitro* and *in vivo* validation studies, were completed using healthy donor CART cells generated with lentivirus.

Additionally, since significant differences have been found with CD19 CARs harboring distinct modular domains (i.e. hinge and transmembrane), the precise regions that compose the CD19 CARs used here should be described in the supplemental.

To address this point, we have included a schematic for the lentivirally produced CART cells and included this in the supplementary figures (Supplemental Fig. S21b).

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REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

The authors have adequately responded to my comments including the incorporation of new data addressing my primary concerns

Reviewer #2 (Remarks to the Author):

In this revision, the authors mostly but not sufficiently addressed the concerns I had. While the new data appears to support their previous claim, I still find it hard to be convinced given the lack of in vivo data regarding their key findings.
Major comments are:

1. The data presented in Fig. S17 is crucial for supporting the central claim. However, there are still key data gaps. The authors suggest that CAR-T cells produce IL4 and that IL-4 acts on these cells to suppress their function. In this context, it's essential to know if knocking out the IL-4 receptor on CAR-T cells would yield similar results as knocking out IL-4 itself. Moreover, the assays used to assess IL4-/- CAR-T cells seem too superficial to be persuasive. To establish stronger evidence, comparing the in vivo function of IL4-/- CAR-T vs. IL4+/+ CAR-T and IL4R-/- CAR-T vs. IL4R+/+ CAR-T is necessary, as differences between in vitro and in vivo assays could exist.

2. The claim regarding the distinct roles of IL-4 on CD8 versus CD4 CAR-T cells requires additional evidence. The statements made in Lines 337-341 appear somewhat speculative. To validate this claim, it is essential to conduct separate tests to assess functional disparities between IL4/IL4R-/- and wild-type CAR-T cells within the CD4 and CD8 populations.

Minor comments:
1. Fig. 5c-f, Fig. S13-17 representative flow plots to make their data more convincing.

2. As previously mentioned, in Fig. 5b, Fig. S14b-c, and S17e-f, the lower absolute cell number does not necessarily indicate reduced cell proliferation. Other factors, such as apoptosis and activation-induced cell death, could also influence the overall cell count. To make the claim that IL4 negatively impacts CAR-T proliferation, the authors need to perform CFSE proliferation assay or similar assays.

3. In the rebuttal, the authors mention that all CAR-T cells died by day 22 without stimulation. However, this observation appears unusual, as the tonic signaling introduced by CAR itself typically sustains CAR-T cell proliferation to a certain extent. It's common practice to maintain in vitro CAR-T cultures for over a month without antigen stimulation. I recommend that the authors explore

different CAR-T cell culture protocols to address this discrepancy.

Reviewer #3 (Remarks to the Author):

The authors should be highly commended for the significant numbers of important experiments that they have performed to address the comments of the reviewers. A huge congratulations.

This reviewer does though have one comment that arises based on the revisions:

-The authors perform extensive new experiments with the CD19-BB CAR as well as with the BCMA and CSS1 CAR. In their response to Reviewer 1 and in the revised text (lines 262-267), they conclude that "IL-4 supplementation can drive CART cell exhaustion independent of construct design or tumor models." Nonetheless, the data shown for the CD19-28 CAR (Figure 5) and the new data shown for the CD19-BB CAR (Figure S14) strongly suggest that IL-4 has a less pronounced impact on the CD19-BB CAR as neither CD3+ cell numbers nor IL-2 expression are significantly decreased (Figure S14). As indicated by Reviewer 1, this is of interest as the CD19-28 CAR construct is reported to have a higher propensity for exhaustion. As such, the authors can temper their conclusion to state that "These data strongly suggest that IL-4 supplementation can drive CART cell exhaustion across different constructs and tumor models. Interestingly though, and consistent with data showing a higher level of exhaustion with 28ζ than 28BB constructs (REF), IL-4 appears to have a more dramatic impact on the CART-28ζ construct (Fig. 5) than the CART19-BBζ construct (Fig. S14).

Minor:
-In the new data that the authors performed to knockout IL-4 (congratulations), the percentages of cells expressing IL-2 are extremely low (Fig. S17). The authors may want to just remove these panels to avoid confusion with other data that they present (i.e. second panels in S17g and S17h).

-Would it be helpful to show Fig. S21 (schematics of the different vectors) earlier in the text?

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

The authors have adequately responded to my comments including the incorporation of new data addressing my primary concerns

Thank you for your time spent reviewing this manuscript and for the positive feedback.

Reviewer #2 (Remarks to the Author):

In this revision, the authors mostly but not sufficiently addressed the concerns I had. While the new data appears to support their previous claim, I still find it hard to be convinced given the lack of in vivo data regarding their key findings.

We appreciate your feedback. In response to this comment, we have assessed the activity of IL-4 and IL4R knockdown CART19-28ζ cells in a JeKo-1 xenograft model, as described below.

Major comments are:

1. The data presented in Fig. S17 is crucial for supporting the central claim. However, there are still key data gaps. The authors suggest that CAR-T cells produce IL4 and that IL-4 acts on these cells to suppress their function. In this context, it's essential to know if knocking out the IL-4 receptor on CAR-T cells would yield similar results as knocking out IL-4 itself.

Thank you for the question. To address this comment, we used CRISPR/Cas9 to interrupt the production of the IL-4 receptor (IL4R) in CART19-28ζ cells. We were able to successfully reduce IL4R expression levels (Revised Supplementary Fig. S21a) and test the activity of IL4R knockdown CART19-28ζ cells both *in vitro* and *in vivo* (Revised Supplementary Fig. S21-S23). IL4R knockdown CART19-28ζ cells showed reduced functional and phenotypical signs of exhaustion as compared with control gRNA CART19-28ζ cells following chronic stimulation with JeKo-1 cells in our *in vitro* model for exhaustion. This is demonstrated by 1) increased proliferative ability (Revised Supplementary Fig. S21b), 2) decreased co-expression of multiple inhibitory receptors (Revised Supplementary Fig. S21c), and 3) increased production of effector cytokines such as IL-2 and TNF-α (Revised Supplementary Fig. S21d-e) following IL4R knockdown. This is similar to our previously reported results showing a reduction in the exhausted phenotype of CART cells when IL-4 is knocked down (Revised Supplementary Fig. S18).

Moreover, the assays used to assess IL4-/- CAR-T cells seem too superficial to be persuasive. To establish stronger evidence, comparing the in vivo function of IL4-/- CAR-T vs. IL4+/+ CAR-T and IL4R-/- CAR-T vs. IL4R+/+ CAR-T is necessary, as differences between in vitro and in vivo assays could exist.

Thank you for this comment. To evaluate the activity of IL-4 and IL4R knockdown CART19-28ζ *in vivo*, we utilized a high tumor burden JeKo-1 xenograft model (Revised Supplementary Fig. S23a) similar to the model previously used to test the effect of IL-4 neutralization with a monoclonal antibody (Revised Fig. 6). In this model, both IL-4 and IL4R knockdown CART19-28ζ cells exhibited improved antitumor control as compared with control gRNA CART19-28ζ cells (Revised Supplementary Fig. S23b). Additionally, compared to control gRNA CART19-28ζ cells, IL-4 knockdown CART19-28ζ cells showed a trend towards increased *in vivo* expansion, and IL4R knockdown CART19-28ζ cells showed significantly increased *in vivo* expansion (Revised Supplementary Fig. S23c). This indicates that gene editing of the IL-4 pathway in CART cells can

improve CART cell activity both *in vitro* and *in vivo*. This is consistent with prior literature that suggests interruption of the IL-4 pathway with IL4R switch receptors can improve CART cell activity both *in vitro* and *in vivo*[1,2].

2.The claim regarding the distinct roles of IL-4 on CD8 versus CD4 CAR-T cells requires additional evidence. The statements made in Lines 337-341 appear somewhat speculative. To validate this claim, it is essential to conduct separate tests to assess functional disparities between IL4/IL4R-/- and wild-type CAR-T cells within the CD4 and CD8 populations.

Thank you for this comment. The statement made in the previous Lines 337-341 was meant to highlight a potential role for IL-4 on $CD8^+$ CART cells, that is independent of its effect on $CD4^+$ CART cells, as shown in Revised Fig. 5. This was based on our findings that IL-4 induces an exhausted phenotype in $CD8^+$ CART cells even in the absence of $CD4^+$ T-cells.

As suggested by this reviewer and to further strengthen our findings, we looked specifically at changes in the CD8 population of CART cells when IL-4 or the IL4R is knocked down in CART cells. Supportive of our previous findings that IL-4 supplementation drives $CD8^+$ CART cell exhaustion, knockdown of IL-4 or IL4R reduces the exhausted phenotype of $CD8^+$ CART cells. This is evident by both an increase in the production of effector cytokines and a decrease in the coexpression of multiple inhibitory receptors following chronic stimulation (Revised Supplementary Fig. S20 and Revised Supplementary Fig. S22). This indicates that editing of the IL-4 pathway in CART cells can improve the function and phenotype of the $CD8^+$ population of CART cells. We have modified our previous statement to focus on IL-4's impact on $CD8^+$ CART cells both in the title and abstract and in the results section (Revised Lines 219, 223-224, 280-288, 295-297, and 361-366).

Minor comments:

1. Fig. 5c-f, Fig. S13-17 representative flow plots to make their data more convincing.

Thank you. We have added representative flow plots that correspond to the mentioned figures, where applicable (Revised Supplementary Fig.S13-18).

2. As previously mentioned, in Fig. 5b, Fig. S14b-c, and S17e-f, the lower absolute cell number does not necessarily indicate reduced cell proliferation. Other factors, such as apoptosis and activation-induced cell death, could also influence the overall cell count. To make the claim that IL4 negatively impacts CAR-T proliferation, the authors need to perform CFSE proliferation assay or similar assays.

Thank you for the request to further clarify our findings and previous comment.

To address this comment, we performed experiments to determine if IL-4 directly impacts the proliferative ability of CART cells. As you suggested, we performed CFSE proliferation assays with baseline CART19-28ζ and CART19-BBζ cells supplemented with hrIL-4. We found that IL-4 supplementation reduces the proliferative ability of both CART19-28ζ (Revised Supplementary Fig. S12a) and CART19-BBζ (Revised Supplementary Fig. S15b) cells as evident by a decrease in $CFSE^-$ $CD3^+$ cells. Additionally, IL4R knockdown CART cells are able to proliferate more than control gRNA CART cells following chronic stimulation (Revised Supplementary Fig. S21b) as determined by CFSE staining.

Finally, we have modified our interpretation of findings from Fig. 5b, Fig. S14b-c, and S17e-f to state that our findings suggest changes in CART cell expansion following IL-4 pathway modulation (Revised Lines 60, 65, 236, 255, and 277).

3. In the rebuttal, the authors mention that all CAR-T cells died by day 22 without stimulation. However, this observation appears unusual, as the tonic signaling introduced by CAR itself typically sustains CAR-T cell proliferation to a certain extent. It's common practice to maintain in vitro CAR-T cultures for over a month without antigen stimulation. I recommend that the authors explore different CAR-T cell culture protocols to address this discrepancy.

Thank you for this comment and request for clarifications. While it is true that some CART cells are able to survive in long-term cultures due to antigen-independent tonic signaling, tonic signaling appears to be CAR dependent. For example, in a 2015 paper by Dr. Crystal Mackall's group, they show the presence of tonic signaling in their GD2.28ζ CART cells, but not in their CD19.28ζ cells[3]. In another paper from Dr. Maksim Mamonkin's group in 2017, they showed variation in the occurrence of tonic signaling based on the expression vector used to generate CART cells[4]. The CART cells used in our study did not exhibit antigen-independent signaling, and therefore they were unable to survive until Day 22 of our assay without IL-2 supplementation.

Reviewer #3 (Remarks to the Author):

The authors should be highly commended for the significant numbers of important experiments that they have performed to address the comments of the reviewers. A huge congratulations.

Thank you so much for this comment and your time in review.

This reviewer does though have one comment that arises based on the revisions:

-The authors perform extensive new experiments with the CD19-BB CAR as well as with the BCMA and CSS1 CAR. In their response to Reviewer 1 and in the revised text (lines 262-267), they conclude that "IL-4 supplementation can drive CART cell exhaustion independent of construct design or tumor models." Nonetheless, the data shown for the CD19-28 CAR (Figure 5) and the new data shown for the CD19-BB CAR (Figure S14) strongly suggest that IL-4 has a less pronounced impact on the CD19-BB CAR as neither CD3+ cell numbers nor IL-2 expression are significantly decreased (Figure S14). As indicated by Reviewer 1, this is of interest as the CD19-28 CAR construct is reported to have a higher propensity for exhaustion. As such, the authors can temper their conclusion to state that "These data strongly suggest that IL-4 supplementation can drive CART cell exhaustion across different constructs and tumor models. Interestingly though, and consistent with data showing a higher level of exhaustion with 28ζ than 28BB constructs (REF), IL-4 appears to have a more dramatic impact on the CART-28ζ construct (Fig. 5) than the CART19-BBζ construct (Fig. S14).

Thank you for this feedback. We agree with you. In response to this comment, we have modified our conclusion and have stated that the impact of IL-4 depletion appears to be more profound on CART19-28ζ (Line 362-366).

Minor:
-In the new data that the authors performed to knockout IL-4 (congratulations), the percentages of cells expressing IL-2 are extremely low (Fig. S17). The authors may want to just remove these panels to avoid confusion with other data that they present (i.e. second panels in S17g and S17h).

Thank you for this comment. Based on feedback from you and from reviewer 2, we have added representative flow plots for Supplementary Figure 17 (now Revised Supplementary Fig. S18). We believe these representative flow plots better show changes in the low IL-2 production.

-Would it be helpful to show Fig. S21 (schematics of the different vectors) earlier in the text?

Thank you. As you suggested, we now reference Fig. S21 (now Revised Supplementary Fig. S1) in two parts of the main text (Revised Lines 53 and 263).

1 Zhou, Y. *et al.* Co-expression of IL-4/IL-15-based inverted cytokine receptor in CAR-T cells overcomes IL-4 signaling in immunosuppressive pancreatic tumor microenvironment. *Biomed Pharmacother* **168**, 115740, doi:10.1016/j.biopha.2023.115740 (2023).

2 Wang, Y. *et al.* An IL-4/21 Inverted Cytokine Receptor Improving CAR-T Cell Potency in Immunosuppressive Solid-Tumor Microenvironment. *Front Immunol* **10**, 1691, doi:10.3389/fimmu.2019.01691 (2019).

3 Long, A. H. *et al.* 4-1BB costimulation ameliorates T cell exhaustion induced by tonic signaling of chimeric antigen receptors. **21**, 581-590 (2015).

4 Gomes-Silva, D. *et al.* Tonic 4-1BB Costimulation in Chimeric Antigen Receptors Impedes T Cell Survival and Is Vector-Dependent. *Cell Rep* **21**, 17-26, doi:10.1016/j.celrep.2017.09.015 (2017).

REVIEWERS' COMMENTS

Reviewer #2 (Remarks to the Author):

Prior comments addressed.

Reviewer #3 (Remarks to the Author):

The authors have fully responded to the comments of all reviewers and should be highly commended for their efforts.

One note is that it might be helpful to edit the new title. The new title is presently:
IL-4 Drives CART Cell Exhaustion in CD8+ Cells

but it might be easier for it to read as:
IL-4 Drives Exhaustion of CD8+ CAR T Cells

(to avoid "CART cell" and "CD8+ T cells" as separate entities)

REVIEWER COMMENTS

Reviewer #2 (Remarks to the Author):

Prior comments addressed.

Thank you for your time spent reviewing this manuscript and all of your helpful suggestions.

Reviewer #3 (Remarks to the Author):

The authors have fully responded to the comments of all reviewers and should be highly commended for their efforts.

Thank you so much for your time spent reviewing this manuscript and for the positive feedback.

One note is that it might be helpful to edit the new title. The new title is presently:
IL-4 Drives CART Cell Exhaustion in CD8+ Cells

but it might be easier for it to read as:
IL-4 Drives Exhaustion of CD8+ CAR T Cells

(to avoid "CART cell" and "CD8+ T cells" as separate entities)

Thank you for this comment. We have changed the title based on your suggestion.