EMBO Molecular Medicine

Early induction of cytokine release syndrome by rapidly generated CAR T cells in preclinical models

Christian Buchholz, Arezoo Jamali, Naphang Ho, Angela Braun, Elham Adabi, and Frederic Thalheimer

Corresponding author(s): Christian Buchholz (Christian.Buchholz@pei.de)

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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)

23rd Nov 2023

Dear Prof. Buchholz,

Thank you for the submission of your manuscript to EMBO Molecular Medicine. We have now received feedback from the three reviewers who agreed to evaluate your manuscript. As you will see from the reports below, the referees acknowledge the interest of the study and are overall supporting publication of your work pending appropriate revisions.

Addressing the reviewers' concerns in full will be necessary for further considering the manuscript in our journal, and acceptance of the manuscript will entail a second round of review. EMBO Molecular Medicine encourages a single round of revision only and therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript. For this reason, and to save you from any frustrations in the end, I would strongly advise against returning an incomplete revision.

If you would like to discuss further the points raised by the referees, I am available to do so via email or video. Let me know if you are interested in this option.

We are expecting your revised manuscript within three months, if you anticipate any delay, please contact us.

When submitting your revised manuscript, please carefully review the instructions that follow below. We perform an initial quality control of all revised manuscripts before re-review; failure to include requested items will delay the evaluation of your revision.

We require:

1) A .docx formatted version of the manuscript text (including legends for main figures, EV figures and tables). Please make sure that the changes are highlighted to be clearly visible.

2) Individual production quality figure files as .eps, .tif, .jpg (one file per figure). For guidance, download the 'Figure Guide PDF' (https://www.embopress.org/page/journal/17574684/authorguide#figureformat).

3) At EMBO Press we ask authors to provide source data for the main figures. Our source data coordinator will contact you to discuss which figure panels we would need source data for and will also provide you with helpful tips on how to upload and organize the files.

4) A .docx formatted letter INCLUDING the reviewers' reports and your detailed point-by-point responses to their comments. As part of the EMBO Press transparent editorial process, the point-by-point response is part of the Review Process File (RPF), which will be published alongside your paper.

5) A complete author checklist, which you can download from our author guidelines (https://www.embopress.org/page/journal/17574684/authorguide#submissionofrevisions). Please insert information in the checklist that is also reflected in the manuscript. The completed author checklist will also be part of the RPF.

6) It is mandatory to include a 'Data Availability' section after the Materials and Methods. Before submitting your revision, primary datasets produced in this study need to be deposited in an appropriate public database, and the accession numbers and database listed under 'Data Availability'. Please remember to provide a reviewer password if the datasets are not yet public (see https://www.embopress.org/page/journal/17574684/authorguide#dataavailability).

In case you have no data that requires deposition in a public database, please state so in this section. Note that the Data Availability Section is restricted to new primary data that are part of this study.

7) For data quantification: please specify the name of the statistical test used to generate error bars and P values, the number (n) of independent experiments (specify technical or biological replicates) underlying each data point and the test used to calculate p-values in each figure legend. The figure legends should contain a basic description of n, P and the test applied. Graphs must include a description of the bars and the error bars (s.d., s.e.m.). Please provide exact p values.

8) Our journal encourages inclusion of *data citations in the reference list* to directly cite datasets that were re-used and obtained from public databases. Data citations in the article text are distinct from normal bibliographical citations and should directly link to the database records from which the data can be accessed. In the main text, data citations are formatted as follows: "Data ref: Smith et al, 2001" or "Data ref: NCBI Sequence Read Archive PRJNA342805, 2017". In the Reference list, data citations must be labeled with "[DATASET]". A data reference must provide the database name, accession number/identifiers and a resolvable link to the landing page from which the data can be accessed at the end of the reference.

Further instructions are available at .

9) We replaced Supplementary Information with Expanded View (EV) Figures and Tables that are collapsible/expandable online. A maximum of 5 EV Figures can be typeset. EV Figures should be cited as 'Figure EV1, Figure EV2" etc... in the text and their respective legends should be included in the main text after the legends of regular figures.
- For the figures that you do NOT wish to display as Expanded View figures, they should be bundled together with their legends in a single PDF file called *Appendix*, which should start with a short Table of Content. Appendix figures should be referred to in the main text as: "Appendix Figure S1, Appendix Figure S2" etc.
- Additional Tables/Datasets should be labeled and referred to as Table EV1, Dataset EV1, etc. Legends have to be provided in a separate tab in case of .xls files. Alternatively, the legend can be supplied as a separate text file (README) and zipped together with the Table/Dataset file.
See detailed instructions here:

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- the results obtained and
- their clinical impact.
This may be edited to ensure that readers understand the significance and context of the research. Please refer to any of our published articles for an example.

11) For more information: There is space at the end of each article to list relevant web links for further consultation by our readers. Could you identify some relevant ones and provide such information as well? Some examples are patient associations, relevant databases, OMIM/proteins/genes links, author's websites, etc...

12) Author contributions: CRediT has replaced the traditional author contributions section because it offers a systematic machine readable author contributions format that allows for more effective research assessment. Please remove the Authors Contributions from the manuscript and use the free text boxes beneath each contributing author's name in our system to add specific details on the author's contribution. More information is available in our guide to authors.

13) Disclosure statement and competing interests: We updated our journal's competing interests policy in January 2022 and request authors to consider both actual and perceived competing interests. Please review the policy https://www.embopress.org/competing-interests and update your competing interests if necessary.

14) Every published paper now includes a 'Synopsis' to further enhance discoverability. Synopses are displayed on the journal webpage and are freely accessible to all readers. They include a short stand first (maximum of 300 characters, including space) as well as 2-5 one-sentences bullet points that summarizes the paper. Please write the bullet points to summarize the key NEW findings. They should be designed to be complementary to the abstract - i.e. not repeat the same text. We encourage inclusion of key acronyms and quantitative information (maximum of 30 words / bullet point). Please use the passive voice. Please attach these in a separate file or send them by email, we will incorporate them accordingly.

Please also suggest a striking image or visual abstract to illustrate your article as a PNG file 550 px wide x 300-600 px high.

15) As part of the EMBO Publications transparent editorial process initiative (see our Editorial at http://embomolmed.embopress.org/content/2/9/329), EMBO Molecular Medicine will publish online a Review Process File (RPF) to accompany accepted manuscripts.
In the event of acceptance, this file will be published in conjunction with your paper and will include the anonymous referee reports, your point-by-point response and all pertinent correspondence relating to the manuscript. Let us know whether you agree with the publication of the RPF and as here, if you want to remove or not any figures from it prior to publication.
Please note that the Authors checklist will be published at the end of the RPF.

EMBO Molecular Medicine has a "scooping protection" policy, whereby similar findings that are published by others during review or revision are not a criterion for rejection. Should you decide to submit a revised version, I do ask that you get in touch after three months if you have not completed it, to update us on the status.

I look forward to receiving your revised manuscript.

Yours sincerely,

Lise Roth

Lise Roth, PhD
Senior Editor
EMBO Molecular Medicine

***** Reviewer's comments *****

Referee #1 (Comments on Novelty/Model System for Author):

This is a very interesting paper exploring the murine models for assessing CRS in CAR T treatment and their data provide important clues. However, the controls used in the experiments were not ideal. This paper could improve significantly if this caveat is solved.

Referee #1 (Remarks for Author):

The authors assessed the risk of cytokine release syndrome (CRS) from short-term (st) CAR T cells in a murine model. Their data recapitulated the early onset of CRS in the murine model and emphasized the importance of assessing the stCAR T cells used in clinical trials.
Compared with standard CAR T cells, stCAR T cells showed a similar stable expression level of CARs day seven post-transduction and elicited comparable killing of target cells in vitro. However, stCAR T cells had a higher percentage of naïve T cells with a less differentiated phenotype. Infused into mice bearing NALM6 tumor, stCAR T cells triggered typical CRS-related symptoms in mice, which was probably exaggerated by monocytes. It was also very interesting that in this murine model, stCAR T cells could trigger the CRS symptoms in the absence of target tumor cells.
These findings are very interesting as there are few accepted murine models to assess the CRS, especially regarding stCAR T cells. Their data could provide helpful information for developing short-term CAR T therapies. I recommended publishing this paper with minor revisions.

Minor revisions:

1. It is very interesting that stCAR T cells alone could trigger CRS symptoms in mice without recognizing NALM6 target cells. As the authors discussed, "the high amounts of VSV-G protein on stCAR T cells trigger the innate immune response similarly to a viral infection." Therefore, it is critical to make a well-controlled experiment to elucidate this question. In Figure 2 and S2, the ideal control for the mice experiment should be mock transduced T cells using the same virus backbone in addition to untransduced activated T cells. This control could answer this fundamental question.
2. Similarly, in Figure 3, it might be possible that stCAR T cells could release CRS-related cytokines in vitro without target cells. Therefore, the ideal controls should include mock transduced T cells and effector T cells alone. The expected experiment groups should be: 1) stCAR T cell no Monocytes no NALM6, 2) stCAR T cells no Monocytes + NALM6, 3) stCAR T cells + Monocytes no NALM6, 4) stCAR T cells + Monocytes + NALM6; 5) Mock T cell no Monocytes no NALM6, 6) Mock T cells no Monocytes + NALM6, 7) Mock T cells + Monocytes no NALM6, 8) Mock T cells + Monocytes + NALM6.
3. The authors thought stCAR T cells migrate more to the bone marrow, which caused the low number of stCAR T cells in peripheral blood. It would be wonderful if the authors could run a flow with stCAR and CAR T cells using a panel of chemokine receptors and integrins. Certain chemokine receptors, such as CXCR4, have a significant role in cell migration to bone marrow.
4. In Figure S2, D and E showed the human T cells and NALM6 cell in the bone marrow. I thought the two groups were mice treated with T cells and stCAR T cells. Why was there only one histogram?
5. In Figure S9, E, there were much fewer stCAR T cells than CAR T cells in bone marrow. Could the authors discuss this regarding the data in Figure 3?
6. The calculation equation for cytotoxicity was not correctly written. It should be:
% Specific lysis = 100 X (1 - (% viable target cells in the presence of CAR T cells)/(% viable target cells in the presence of untransduced T cells))

Referee #2 (Comments on Novelty/Model System for Author):

CAR-T therapy is effective but very costly and stressful for the patient. There is significant toxicity, and there is shortage of capacity. Shorter manufacture is a possible solution, but has risks which this animal study documents carefully

Referee #2 (Remarks for Author):

This animal model revealing greater toxicity due to greater cytokine levels might be used to optimise the therapy of CRS, and its prevention by optimising anticytokine therapy. This is too difficult to do in humans, but might comparing therapies to CRS in these mice be useful for patients? AntiIL6 receptor is standard of care but its never been compared to antiTNF or antiIFNg or ant combinations.

Referee #3 (Remarks for Author):

In the manuscript entitled "Early induction of cytokine release syndrome by rapidly generated CAR T cells in a preclinical mouse model", Ho et. al. seek to observe the safety of short-term expanded CAR T (st) focusing specifically on residual vector particle expression and induction of cytokine release syndrome (CRS). Given the push of the field to use shorter expansion times for cell products, this study offers important insight for the advancement of CAR T cells in the clinic. While the study is fairly easy to follow and has great potential, there are some concerns with the presented work.

Major revisions:

1. How do CAR T compare to stCAR T in the in vitro model with Nalm6 and monocytes? Are there differences in the cytokine expression or do these look similar to the in vivo model? Given that the NSG-SGM3 strain expresses human GM-CSF, it could be hiding potential differences of this cytokine between groups. This could be explored using the in vitro model. T cells alone compared to stCAR T are not necessarily helpful to compare.

2. The authors show that CAR T are twice as prevalent in the bone marrow of non-tumor-bearing mice compared to stCAR T cells. How does their expression compare in the presence of Nalm6 tumor in NSG-SGM3 mice? This would be more relevant to treatment of cancer-bearing patients.

3. How does the murine innate cell compartment (as shown in Fig. 2F) differ between Nalm6-bearing mice treated with CAR T versus stCAR T?

Minor revisions:

1. Fig. 1C: Almost 80% of CAR T cells are double negative (DN)--is this data correct? I would expect more CAR+ by this point in culture.

2. Labeling is off in the text for Fig. 1E-I.

3. Data is not presented in order--i.e. some of Suppl. Fig. 3 is discussed in the text prior to jumping back to Supp. Fig. 2. This made it difficult to follow and would flow better if the data was introduced in the correct order.

4. In Supp. Fig. 8, changing the axis of the middle plot (CAR T) to a max of 8 to match the other two graphs will make your data more striking. As it's currently graphed, it appears that the severity of CRS is comparable between stCAR T and CAR T.

5. Interferon gamma is written as both IFN-g and INF-g in the manuscript--please choose one and make consistent.

***** Reviewer's comments *****

Referee #1 (Comments on Novelty/Model System for Author):

This is a very interesting paper exploring the murine models for assessing CRS in CAR T treatment and their data provide important clues. However, the controls used in the experiments were not ideal. This paper could improve significantly if this caveat is solved.

Thank you very much for this positive feedback. We have added further data and controls as described below.

Referee #1 (Remarks for Author):

The authors assessed the risk of cytokine release syndrome (CRS) from short-term (st) CAR T cells in a murine model. Their data recapitulated the early onset of CRS in the murine model and emphasized the importance of assessing the stCAR T cells used in clinical trials. Compared with standard CAR T cells, stCAR T cells showed a similar stable expression level of CARs day seven post-transduction and elicited comparable killing of target cells in vitro. However, stCAR T cells had a higher percentage of naïve T cells with a less differentiated phenotype. Infused into mice bearing NALM6 tumor, stCAR T cells triggered typical CRS-related symptoms in mice, which was probably exaggerated by monocytes. It was also very interesting that in this murine model, stCAR T cells could trigger the CRS symptoms in the absence of target tumor cells.
These findings are very interesting as there are few accepted murine models to assess the CRS, especially regarding stCAR T cells. Their data could provide helpful information for developing short-term CAR T therapies. I recommended publishing this paper with minor revisions.

Thank you for this positive feedback, much appreciated.

Minor revisions:
1. It is very interesting that stCAR T cells alone could trigger CRS symptoms in mice without recognizing NALM6 target cells. As the authors discussed, "the high amounts of VSV-G protein on stCAR T cells trigger the innate immune response similarly to a viral infection." Therefore, it is critical to make a well-controlled experiment to elucidate this question. In Figure 2 and S2, the ideal control for the mice experiment should be mock transduced T cells using the same virus backbone in addition to untransduced activated T cells. This control could answer this fundamental question.

We totally agree, that your proposed experimental setting will be a valuable control, however, it will not be possible to perform this additional animal experiment in due course. We therefore decided to focus on the *ex vivo* experiment you suggested below, which confirmed that the presence of CAR plus the VSV glycoprotein were causative for the cytokine release we observed. The data were added as additional main figure (Fig. 6).

2. Similarly, in Figure 3, it might be possible that stCAR T cells could release CRS-related cytokines in vitro without target cells. Therefore, the ideal controls should include mock transduced T cells and effector T cells alone. The expected experiment groups should be:

1) stCAR T cell no Monocytes no NALM6,
2) stCAR T cells no Monocytes + NALM6,
3) stCAR T cells + Monocytes no NALM6,
4) stCAR T cells + Monocytes + NALM6;
5) Mock T cell no Monocytes no NALM6,
6) Mock T cells no Monocytes + NALM6,
7) Mock T cells + Monocytes no NALM6,
8) Mock T cells + Monocytes + NALM6.

Thank you for suggesting this experimental setup, which we followed with slight adaptations. The obtained data were clear-cut in showing that release of CRS-related cytokines does not occur when the vector particles delivered GFP or were empty (VLP). In contrast, massive cytokine release was determined with stCAR T cells, which further increased in presence of monocytes. The presence of tumor cells made some difference for the secretion of particular cytokines. The data are now shown in an additional main figure (Fig. 6) and mentioned in the Discussion (line 292 and following).

3. The authors thought stCAR T cells migrate more to the bone marrow, which caused the low number of stCAR T cells in peripheral blood. It would be wonderful if the authors could run a flow with stCAR and CAR T cells using a panel of chemokine receptors and integrins. Certain chemokine receptors, such as CXCR4, have a significant role in cell migration to bone marrow.

We are sorry if we were not precise enough in our statement on the bone marrow migration. In fact, there were more CAR T cells in bone marrow after administration of conventional than of stCAR T cells. We have now added a data set quantifying CAR T cells in bone marrow of the mouse study shown in Fig. 4. Also here, there were about twice as many conventional CAR T cells in BM than stCAR T cells (Fig. EV3). The misleading statement has been rephrased (line 191-195).

4. In Figure S2, D and E showed the human T cells and NALM6 cell in the bone marrow. I thought the two groups were mice treated with T cells and stCAR T cells. Why was there only one histogram?

This shows the cells migrated to BM for the stCAR T group only, since these mice had to be sacrificed on day one after cell administration whereas the group having received T cells was sacrificed after 12 days. Therefore, we have do not have values for the amounts of T cells on day one.

5. In Figure S9, E, there were much fewer stCAR T cells than CAR T cells in bone marrow. Could the authors discuss this regarding the data in Figure 3?

We are sorry for explaining this improperly. As mentioned above, we have now added the comparison stCART and conventional CART in BM for the mouse study in Fig. 4 (Fig. EV3A). The comparison related to the mouse study in Fig. 5 is now shown in Fig. EV5 (previously Fig. S9). Both data sets are in full agreement showing

about half as many stCART in BM than conventional CART. We are now better explaining this (line 191-195).

6. The calculation equation for cytotoxicity was not correctly written. It should be:
% Specific lysis = 100 X (1 - (% viable target cells in the presence of CAR T cells)/(% viable target cells in the presence of untransduced T cells))

Thank you for pointing this out. The formula has been corrected.

Referee #2 (Remarks for Author):

This animal model revealing greater toxicity due to greater cytokine levels might be used to optimise the therapy of CRS, and its prevention by optimising anticytokine therapy. This is too difficult to do in humans, but might comparing therapies to CRS in these mice be useful for patients? AntiIL6 receptor is standard of care but its never been compared to antiTNF or antiIFNg or ant combinations.

Thank you very much for the positive feedback and the suggestion on using the mouse model. We do now mention this in the final sentence of the Discussion.

Referee #3 (Remarks for Author):

In the manuscript entitled "Early induction of cytokine release syndrome by rapidly generated CAR T cells in a preclinical mouse model", Ho et. al. seek to observe the safety of short-term expanded CAR T (st) focusing specifically on residual vector particle expression and induction of cytokine release syndrome (CRS). Given the push of the field to use shorter expansion times for cell products, this study offers important insight for the advancement of CAR T cells in the clinic. While the study is fairly easy to follow and has great potential, there are some concerns with the presented work.

Major revisions:
1. How do CAR T compare to stCAR T in the in vitro model with Nalm6 and monocytes? Are there differences in the cytokine expression or do these look similar to the in vivo model? Given that the NSG-SGM3 strain expresses human GM-CSF, it could be hiding potential differences of this cytokine between groups. This could be explored using the in vitro model. T cells alone compared to stCAR T are not necessarily helpful to compare.

Thank you for your positive and constructive feedback. In the revised manuscript we have added data of an additional *in vitro* experiment designed according to the suggestion by reviewer #1. The data provided in main Fig. 6 demonstrate that the cytokine release requires the presence of residual vector and the CAR. Reporter gene encoding LVs or empty LVs do not induce cytokine release. Monocytes enhance cytokine release.

2. The authors show that CAR T are twice as prevalent in the bone marrow of non-tumor-bearing mice compared to stCAR T cells. How does their expression compare in the presence

of Nalm6 tumor in NSG-SGM3 mice? This would be more relevant to treatment of cancer-bearing patients.

The requested data are now provided in Fig. EV3A.

3. How does the murine innate cell compartment (as shown in Fig. 2F) differ between Nalm6-bearing mice treated with CAR T versus stCAR T?

In Fig. EV3B we do now show quantifications of the murine immune cell populations in tumor bearing mice compared to tumor-free mice ("wo target"). There were increased levels of immune cells especially macrophages detected. We are now mentioning this on line 191-195.

Minor revisions:

1. Fig. 1C: Almost 80% of CAR T cells are double negative (DN)--is this data correct? I would expect more CAR+ by this point in culture.

The data are correct. This is a very early time point compared to a 10-21 days expansion for clinical applications. As you can see in Fig. 1E the amount of CAR positive cells is increasing over the next few days to around 60%, which is well in range of CART cells manufactured for clinical applications.

2. Labeling is off in the text for Fig. 1E-I.

Thank you for making us aware of this error. We have corrected the wrong figure references.

3. Data is not presented in order--i.e. some of Suppl. Fig. 3 is discussed in the text prior to jumping back to Supp. Fig. 2. This made it difficult to follow and would flow better if the data was introduced in the correct order.

We have corrected this.

4. In Supp. Fig. 8, changing the axis of the middle plot (CAR T) to a max of 8 to match the other two graphs will make your data more striking. As it's currently graphed, it appears that the severity of CRS is comparable between stCAR T and CAR T.

Thank you, we have revised as suggested (now Fig. EV5B).

5. Interferon gamma is written as both IFN-g and INF-g in the manuscript--please choose one and make consistent.

We have corrected this all over.

16th Feb 2024

Dear Prof. Buchholz,

Thank you for submitting your revised manuscript. We have now received the report from the referees who re-reviewed your manuscript. As you will see below, they are satisfied with the revisions, and I will therefore be able to accept your manuscript once the following editorial points will be addressed:

1/ Manuscript text:
- Please accept the previous changes, and only keep in track changes mode any new modification.
- We can accommodate a maximum of 5 keywords, please adjust accordingly.
- Materials and Methods:
o Cells: please indicate whether the cells were tested for mycoplasma contamination.
o Mice: please check your sentence "mice were purchased from Jackson Laboratory and". Please provide age, gender, housing and husbandry conditions of the mice.
o Antibodies: please provide dilutions/concentrations.
o Statistics: please provide a statement on sample size, exclusion/inclusion criteria, randomization, and blinding.
- Data availability: Please replace your statement by "This study includes no data deposited in external repositories."
- Author contributions: CRediT has replaced the traditional author contributions section because it offers a systematic machine readable author contributions format that allows for more effective research assessment. Please remove the Authors Contributions from the manuscript and use the free text boxes beneath each contributing author's name in our system to add specific details on the author's contribution. More information is available in our guide to authors.
- Please replace "Declaration of interests" by "Disclosure statement and competing interests". We updated our journal's competing interests policy in January 2022 and request authors to consider both actual and perceived competing interests. Please review the policy https://www.embopress.org/competing-interests and update your competing interests if necessary.
- References: this section should be placed before the figure legends, and references should be listed in alphabetical order.

2/ Figures and Appendix:
- Please make sure all exact p values are provided in the figures or their legends, including for ns, non-significant.
- Appendix: please add a table of content with page numbers. The nomenclature should be corrected to "Appendix Figure S1" etc, and "Appendix Table S1".
- Please address the following queries from our data editors:
Figure legends:
1. Please note that a separate 'Data Information' section is required in the legends of figures 1c-i; 2d-g; 3b-d; 5b-d; EV 1d; EV 5c-g.
2. Please note that the figure 5b does not contain any statistical parameter, kindly rectify the statistical test related information in the figure legend appropriately.
3. Please note that the statistical test related information for the legend for figure EV 5c is incorrectly labelled as 5a in the legend. This needs to be rectified.
4. Please note that in figures 3d; there is a mismatch between the annotated p values in the figure legend and the annotated p values in the figure file that should be corrected.
5. Please note that for the figures 3d, p-values and statistical tests are indicated in the legends. However, comparison for the same, "*" has not been represented in the figures. Please rectify this in the figures or legends as applicable.
6. Please note that information related to n is missing in the legends of figures 4c-d; EV 3b.
7. Although 'n' is provided, please describe the nature of entity for 'n' in the legends of figures 5b-d; EV 1d-e.
8. Please note that the error bars are not defined in the legends of figures 4c-d; 5b-d; EV 1d; EV 3b; EV 5c-g.

3/ Thank you for providing Source Data. Please upload them as one file per figure, and provide the completed checklist. Source data for Figure 2G is missing.

4/ Thank you for providing The Paper Explained. Please note that this section should be included in the manuscript and should contain the following sub-sections: Problem - Results - Impact. (You may refer to previously published articles for reference).

5/ I introduced minor modifications in your synopsis text, please let me know if you agree or amend as you see fit:

To make CAR T cells available to all patients, various strategies facilitating the manufacturing process are followed. Short-term (st) CAR T cells are administered shortly after exposure to lentiviral vector (LV) particles. Here, their preclinical safety was assessed ex vivo and in vivo.
• stCAR T cells contain residual vector components on their surface, in particular the vesicular stomatitis virus (VSV) glycoprotein
• NSG-SGM3 mice develop severe CRS-like symptoms rapidly after stCAR T cell administration

• Release of CRS-typical cytokines occurs in absence of tumor cells and is enhanced by monocytes
• The interplay between CAR and LV proteins is the main trigger for cytokine release

6/ As part of the EMBO Publications transparent editorial process initiative (see our Editorial at http://embomolmed.embopress.org/content/2/9/329), EMBO Molecular Medicine will publish online a Review Process File (RPF) to accompany accepted manuscripts.
This file will be published in conjunction with your paper and will include the anonymous referee reports, your point-by-point response and all pertinent correspondence relating to the manuscript. Let us know whether you agree with the publication of the RPF.
Please note that the Authors checklist will be published at the end of the RPF.

I look forward to receiving your revised manuscript.

Yours sincerely,

Lise Roth

Lise Roth, PhD
Senior Editor
EMBO Molecular Medicine

***** Reviewer's comments *****

Referee #1 (Comments on Novelty/Model System for Author):

This is a very interesting paper exploring the murine models for assessing CRS in CAR T treatment and their data provide important clues. However, the controls used in the experiments were not ideal. This paper could improve significantly if this caveat is solved.

Referee #1 (Remarks for Author):

All the questions are well addressed and I recommend publishing this paper.

Referee #3 (Remarks for Author):

Thank you to the authors for addressing my comments. I have no further concerns at this time.

All editorial and formatting issues were resolved by the authors.

4th Mar 2024

Dear Prof. Buchholz,

Thank you for sending the revised files. I am pleased to inform you that your manuscript is accepted for publication and is now being sent to our publisher to be included in the next available issue of EMBO Molecular Medicine!

Your manuscript will be processed for publication by EMBO Press. It will be copy edited and you will receive page proofs prior to publication. Please note that you will be contacted by Springer Nature Author Services to complete licensing and payment information.

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Should you be planning a Press Release on your article, please get in contact with embo_production@springernature.com as early as possible in order to coordinate publication and release dates.

If you have any questions, please do not hesitate to contact the Editorial Office.

Thank you for your contribution to EMBO Molecular Medicine.

With kind regards,

Lise Roth

Lise Roth, Ph.D
Senior Editor
EMBO Molecular Medicine

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EMBO Press Author Checklist

Corresponding Author Name: Christian Buchholz
Journal Submitted to: EMBO Molecular Medicine
Manuscript Number: EMM-2023-18905

USEFUL LINKS FOR COMPLETING THIS FORM
The EMBO Journal - Author Guidelines
EMBO Reports - Author Guidelines
Molecular Systems Biology - Author Guidelines
EMBO Molecular Medicine - Author Guidelines

Reporting Checklist for Life Science Articles (updated January
This checklist is adapted from Materials Design Analysis Reporting (MDAR) Checklist for Authors. MDAR establishes a minimum set of requirements in transparent reporting in the life sciences (see Statement of Task: 10.31222/osf.io/9sm4x). Please follow the journal's guidelines in preparing your
Please note that a copy of this checklist will be published alongside your article.

Abridged guidelines for figures
1. Data
The data shown in figures should satisfy the following conditions:
- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- ideally, figure panels should include only measurements that are directly comparable to each other and obtained with the same assay.
- plots include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical
- if n<5, the individual data points from each experiment should be plotted. Any statistical test employed should be justified.
- Source Data should be included to report the data underlying figures according to the guidelines set out in the authorship guidelines on Data

2. Captions
Each figure caption should contain the following information, for each panel where they are relevant:
- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements.
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.
- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple $\chi 2$ tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Please complete ALL of the questions below.
Select "Not Applicable" only when the requested information is not relevant for your study.

Materials

Newly Created Materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
New materials and reagents need to be available; do any restrictions apply?	Not Applicable	

Antibodies	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
For **antibodies** provide the following information: - Commercial antibodies: RRID (if possible) or supplier name, catalogue number and or/clone number - Non-commercial: RRID or citation	Yes	Material and methods, Flow cytometry

DNA and RNA sequences	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Short novel DNA or RNA including primers, probes: provide the sequences.	Not Applicable	

Cell materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Cell lines: Provide species information, strain. Provide accession number in repository **OR** supplier name, catalog number, clone number, and/**OR** RRID.	Yes	Material and methods, under Primary cells and cell lines title
Primary cultures: Provide species, strain, sex of origin, genetic modification status.	Yes	Material and methods, under Primary cells and cell lines title
Report if the cell lines were recently **authenticated** (e.g., by STR profiling) and tested for mycoplasma contamination.	Yes	Material and methods, under Primary cells and cell lines title

Experimental animals	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Laboratory animals or Model organisms: Provide species, strain, sex, age, genetic modification status. Provide accession number in repository **OR** supplier name, catalog number, clone number, **OR** RRID.	Yes	Material and methods, under CRS tumor mouse model title
Animal observed in or captured from the field: Provide species, sex, and age where possible**.**	Not Applicable	
Please detail **housing and husbandry conditions**.	Yes	Material and methods, under CRS tumor mouse model title

Plants and microbes	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Plants: provide species and strain, ecotype and cultivar where relevant, unique accession number if available, and source (including location for collected wild specimens).	Not Applicable	
Microbes: provide species and strain, unique accession number if available, and source.	Not Applicable	

Human research participants	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If collected and within the bounds of privacy constraints report on age, sex and gender or ethnicity for all study participants.	Not Applicable	

Core facilities	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If your work benefited from core facilities, was their service mentioned in the acknowledgments section?	Yes	Material and Methods

Design

Study protocol	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If study protocol has been **pre-registered, provide DOI in the manuscript**. For clinical trials, provide the trial registration number **OR** cite DOI.	Not Applicable	
Report the **clinical trial registration number** (at ClinicalTrials.gov or equivalent), where applicable.	Not Applicable	

Laboratory protocol	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Provide DOI OR other citation details if **external detailed step-by-step protocols** are available.	Not Applicable	

Experimental study design and statistics	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Include a statement about **sample size** estimate even if no statistical methods were used.	Yes	Material and Methods
Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. **randomization procedure**)? If yes, have they been described?	Yes	Material and Methods, Figures legend
Include a statement about **blinding** even if no blinding was done.	Yes	Material and Methods (in animal distribution)
Describe **inclusion/exclusion criteria** if samples or animals were excluded from the analysis. Were the criteria pre-established? If sample or data points were omitted from analysis, report if this was due to attrition or intentional exclusion and provide justification.	Yes	Material and Methods, Figures legend
For every figure, are **statistical tests** justified as appropriate? Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it. Is there an estimate of variation within each group of data? Is the variance similar between the groups that are being statistically compared?	Yes	Figures legend

Sample definition and in-laboratory replication	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
In the figure legends: state number of times the experiment was **replicated** in laboratory.	Yes	
In the figure legends: define whether data describe **technical or biological replicates**.	Yes	

Ethics

Ethics	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Studies involving **human participants**: State details of **authority granting ethics approval** (IRB or equivalent committee(s), provide reference number for approval.	Yes	Materials and Methods, Primary Cells
Studies involving **human participants**: Include a statement confirming that **informed consent** was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	Not Applicable	
Studies involving **human participants:** For publication of **patient photos**, include a statement confirming that consent to publish was obtained.	Not Applicable	
Studies involving experimental **animals**: State details of **authority granting ethics approva**l (IRB or equivalent committee(s), provide reference number for approval. Include a statement of compliance with ethical regulations.	Yes	Material and Methods under the CRS tumor mouse model title
Studies involving **specimen and field samples:** State if relevant **permits** obtained, provide details of authority approving study; if none were required, explain why.	Not Applicable	

Dual Use Research of Concern (DURC)	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Could your study fall under dual use research restrictions? Please check biosecurity documents and list of **select agents and toxins** (CDC): https://www.selectagents.gov/sat/list.htm	Not Applicable	
If you used a select agent, is the security level of the lab appropriate and reported in the manuscript?	Not Applicable	
If a study is subject to dual use research of concern regulations, is the name of the **authority granting approval and reference number** for the regulatory approval provided in the manuscript?	Not Applicable	

Reporting

The MDAR framework recommends adoption of discipline-specific guidelines, established and endorsed through community initiatives. Journals have their own policy about requiring specific guidelines and recommendations to complement MDAR.

Adherence to community standards	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
State if relevant guidelines or checklists (e.g., **ICMJE, MIBBI, ARRIVE, PRISMA**) have been followed or provided.	Not Applicable	
For **tumor marker prognostic studies,** we recommend that you follow the **REMARK** reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	Not Applicable	
For **phase II and III randomized controlled trials**, please refer to the **CONSORT** flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	Not Applicable	

Data Availability

Data availability	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Have **primary datasets** been deposited according to the journal's guidelines (see 'Data Deposition' section) and the respective accession numbers provided in the Data Availability Section?	Yes	Data Availability Section
Were **human clinical and genomic datasets** deposited in a public access-controlled repository in accordance to ethical obligations to the patients and to the applicable consent agreement?	Not Applicable	
Are **computational models** that are central and integral to a study available without restrictions in a machine-readable form? Were the relevant accession numbers or links provided?	Not Applicable	
If publicly available data were reused, provide the respective **data citations in the reference list**.	Not Applicable	