

IL-21 promotes the anti-tumor effect of anti-CD47 chimeric antigen receptor-macrophages in ovarian cancer

Corresponding Author: Dr Yizhao Chen

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The manuscript by Chen et al. presents a CAR macrophage combination approach, by adding IL-21 cytokine sequence in the same vector as the CAR, to enhance CAR macrophage efficacy in ovarian cancer. While the concept of including IL-21 in adoptive cell therapy (ACT) has been used for CAR-iNKT cells (PMID: 38179061), CAR-NK cells (PMID: 38263004), as well as CAR-T cells (PMID: 32950390), IL-21 has not been tested in combination with CAR-M. It was demonstrated in this manuscript that including IL-21 in the CD47-targeted CAR-M led to improvement in therapeutic efficacy against ovarian cancer models. It is important to note however, that the authors used CAR-transduced THP-1 cells rather than primary macrophages. Some of the data presented here are scientifically adequate, however there are some major experimental control concerns, as well as clinical translational concerns that will need to be addressed. Below are the specific comments that will enhance this manuscript.

Major comments:

1. CAR-THP1 cells targeting CD47 was used in this study against CD47-expressing tumor cells. The authors showed CD47 expression on the tumor cells. Has CD47 expression been evaluated on the THP1 cells? In primary macrophages, CD47 is expressed (protein atlas & internal data), and therefore would raise concerns of fratricide when using primary macrophages for ACT in the clinic.
2. Throughout the experiments, the authors only compared vehicle control, CAR-M, and CAR-M (IL-21). Given IL-21 cytokine alone can have significant inhibitory effects on tumor growth and activate various immune cells including T and NK cells (PMID: 38638431), IL-21 alone should be tested in the tumor models that they used. This can be done either using THP-1 transduced with IL-21 alone, or addition of exogenous IL-21 cytokine (if doing in vitro to avoid toxicity in vivo).
3. Lines #219-222, Figures 1G, H are not accurate. The baseline GFP expression between CAR-M and CAR-M (IL-21) is not the same (see Fig. S2), there are more macs that do not express GFP/ CAR in the CAR-M alone group. Therefore, the higher numbers of the "Tumor cell survival" graphs could partly be attributed to the THP-1 cells not having the GFP/ CAR in them. Solution could be to mix some untransduced THP-1 cells with CAR-M (IL-21) to make the GFP expression more comparable to CAR-M group.
4. Figure 2: A control with CAR-M (+/- IL-21) and T cells, without tumor cells, would be important here to determine whether the effects seen were tumor cell/ antigen dependent, or if they were just the stimulatory effects of the adenovirus post transduction (Adenovirus alone has strong stimulatory effects on macrophages).

Minor comments:

1. Order of the supplementary figures is inconsistent. Please try to make sure that the figures come in order based on the results text.
2. Introduction line #57, please include study in glioblastoma that has shown some meaningful anti-tumor effects (PMID: 35130560).
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4. Please describe somewhere in the text and legends what vehicle control is, does it have GFP but without CAR?
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6. Fig 1D: x and y axis labelling needs to be made clear
7. Fig 2A-B: CD80/CD86 are both expressed in all macs at baseline, therefore using MFI rather than % would be better since

all the cells technically express those markers.

8. Fig 2C: An isotype control or non-transduced T cells would be a good control to have here to more accurately place the gating.

9. Fig 5F, Fig S1A-B: Need isotype control/ non-proliferating cell control to determine gating.

10. Fig 5H: It seems that there are not enough events in these flow plots to allow for statistical analysis.

Reviewer #2

(Remarks to the Author)

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- 1. Fig S1B , is this human patient tissue ? please correlate with the report summary part-Research involving human participants, their data, or biological material, which claimed there is No human participants, their data, or biological material were used. no scale bar included (this is for all figures presented.).
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- 3. Please clarify the origin of CD47 CAR sequence, where does the scFv sequence come from? Are they specifically recognize the human CD47? Please provide the solid data about the antibody recognition information, which is very crucial to evaluate the safety of CD47 CAR-M.
- 4. THP1 cell line is not a typical macrophage cell. Please provide primary cell derived CAR-M data.
- 5. tissue sections scale bar missing in supplemental data.
- 6. Fig S10 , some none-English words.

Main limits of this manuscript.

1. Experiments has to be performed to clarify the specificity of CAR-M. either using the CD47 antibody to block the CAR signal, or using CD47 KO tumor cells, or using CD 47 intracellular domain dead construct as a negative control.

2. Please provide the competition data of CD47 CAR and SIRPa, which all expressed on the THP-1 cells of CAR-CD47 THP-1

3. Please provide the IL21 only THP-1 function data, to compare with CD47 CAR, CD47 CAR-IL21 THP1 cells.

4. In discussion part, for the clinical trial CT-0508 (NCT04660929), initiated by CARISMA, there is no CR(complete response), four patients achieved SD(stable disease).

5. Normal macrophages has the ability to migrate to liver, whereas in this manuscript not the case, need to provide the imaging data. It could be THP1 cell line is not a typical macrophage cell, the in vivo behavior is different. Authors also have to provide IL21 only cells dynamic ,to see if the changed distribution pattern is due to IL21.

6.

In summary, this manuscript is poorly presented and designed. I don't think at this moment it would fit with stringent and high standard of COMMSBIO.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The authors have sufficiently addressed my concerns, and while not perfect, in my view the manuscript is now sufficient for publication.

Reviewer #2

(Remarks to the Author)

I have no further questions about this round of review.
the authors answered all the questions.

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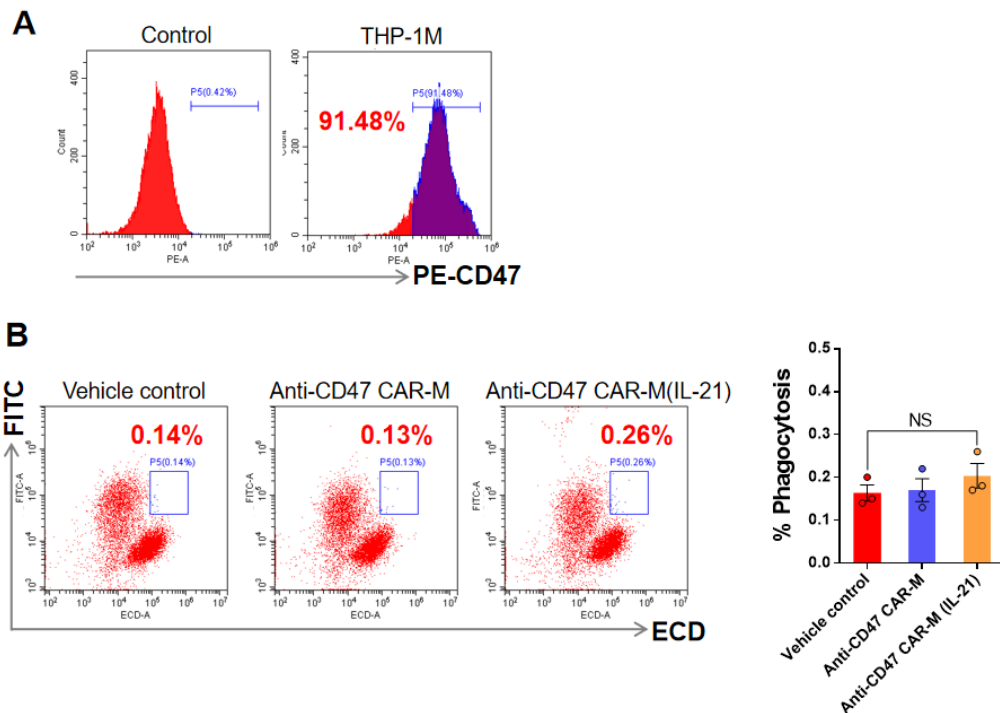
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Major comments:

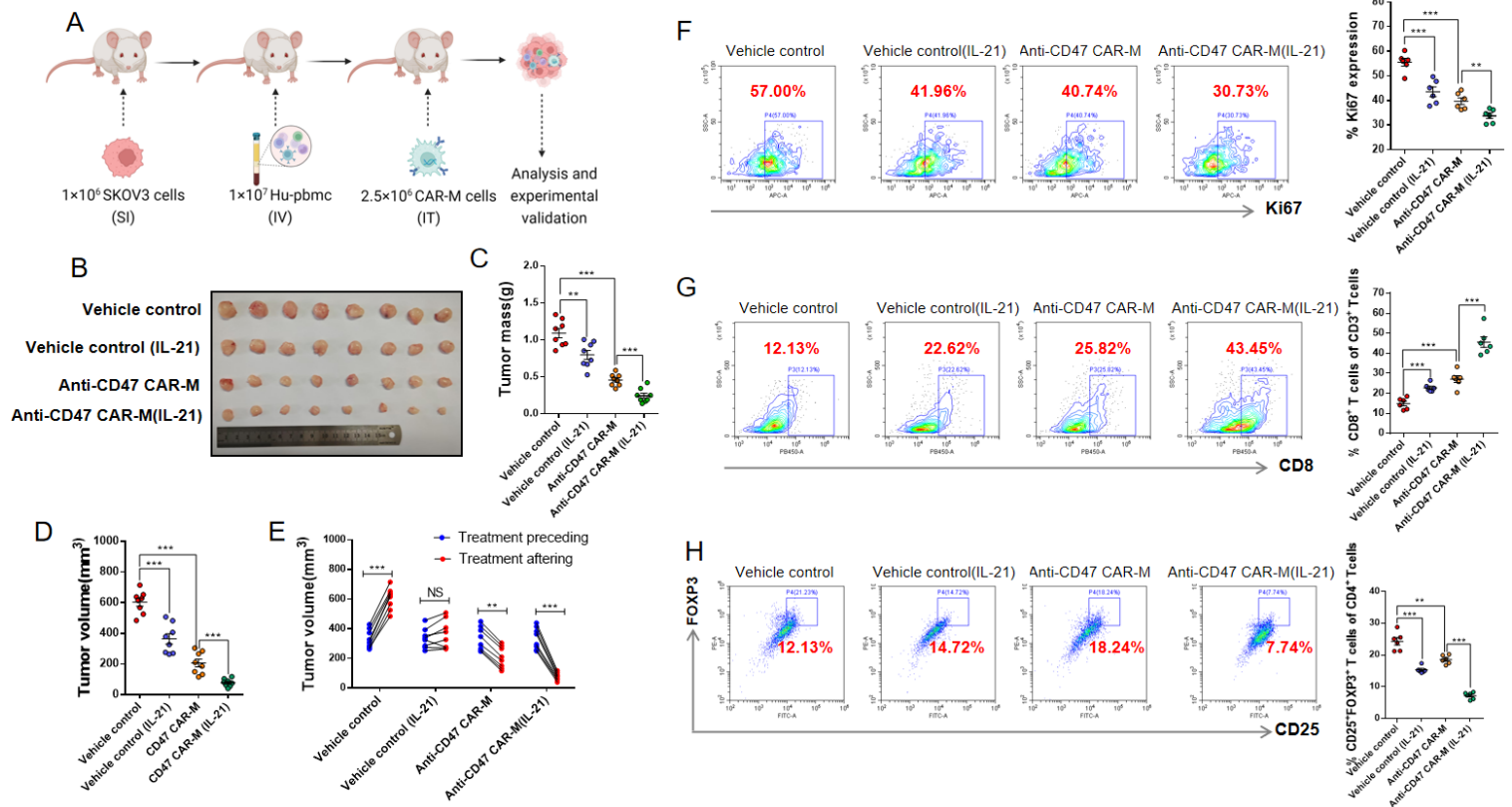
1. CAR-THP1 cells targeting CD47 was used in this study against CD47-expressing tumor cells. The authors showed CD47 expression on the tumor cells. Has CD47 expression been evaluated on the THP1 cells? In primary macrophages, CD47 is expressed (protein atlas & internal data), and therefore would raise concerns of fratricide when using primary macrophages for ACT in the clinic.

Response: We appreciate this valuable comment. We detected the expression of CD47 on THP-1-M cells and added the co-culture experiments between CAR macrophages and THP-1-M cells. The flow cytometry results indicated that CD47 was indeed highly expressed on the surface of THP-1M, but the results of co-culture showed that anti-CD47 CAR-M did not produce a strong fratricide against THP-1M, the underlying mechanism of this phenomenon needs to be further explored, and related work will be investigated in our follow-up study.



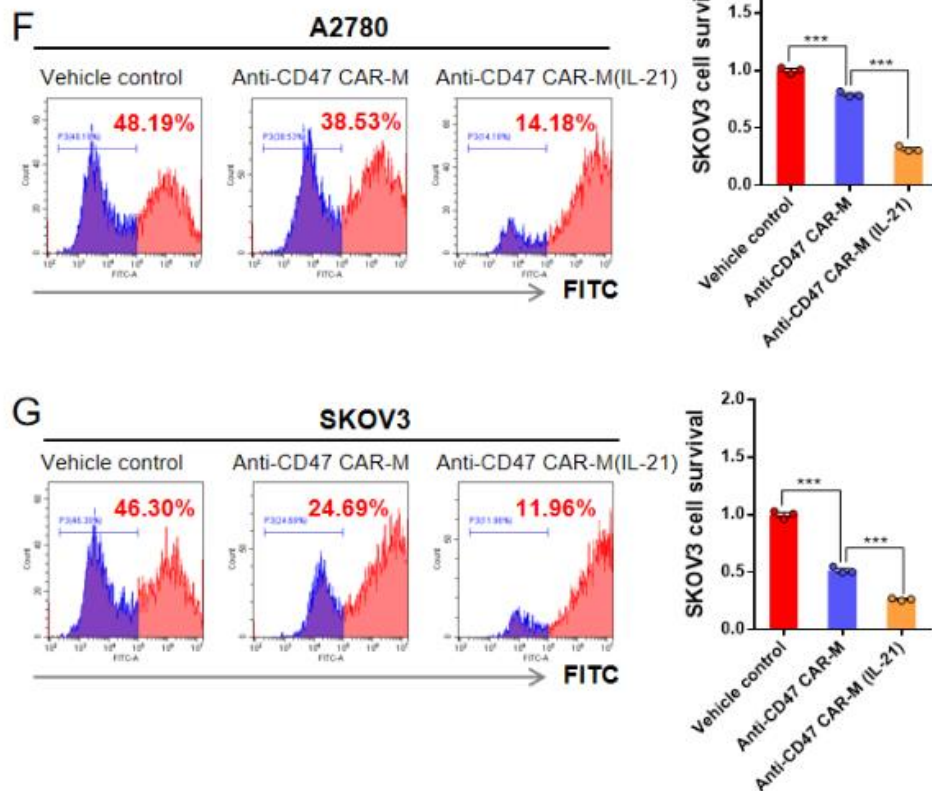
2. Throughout the experiments, the authors only compared vehicle control, CAR-M, and CAR-M (IL-21). Given IL-21 cytokine alone can have significant inhibitory effects on tumor growth and activate various immune cells including T and NK cells (PMID: 38638431), IL-21 alone should be tested in the tumor models that they used. This can be done either using THP-1 transduced with IL-21 alone, or addition of exogenous IL-21 cytokine (if doing in vitro to avoid toxicity in vivo).

Response: Thanks for this insightful suggestion. *In vivo* experiments was repeated by adding THP-1-M(IL-21) as a control group to evaluate the effects of IL-21 alone (**Figure 5**).



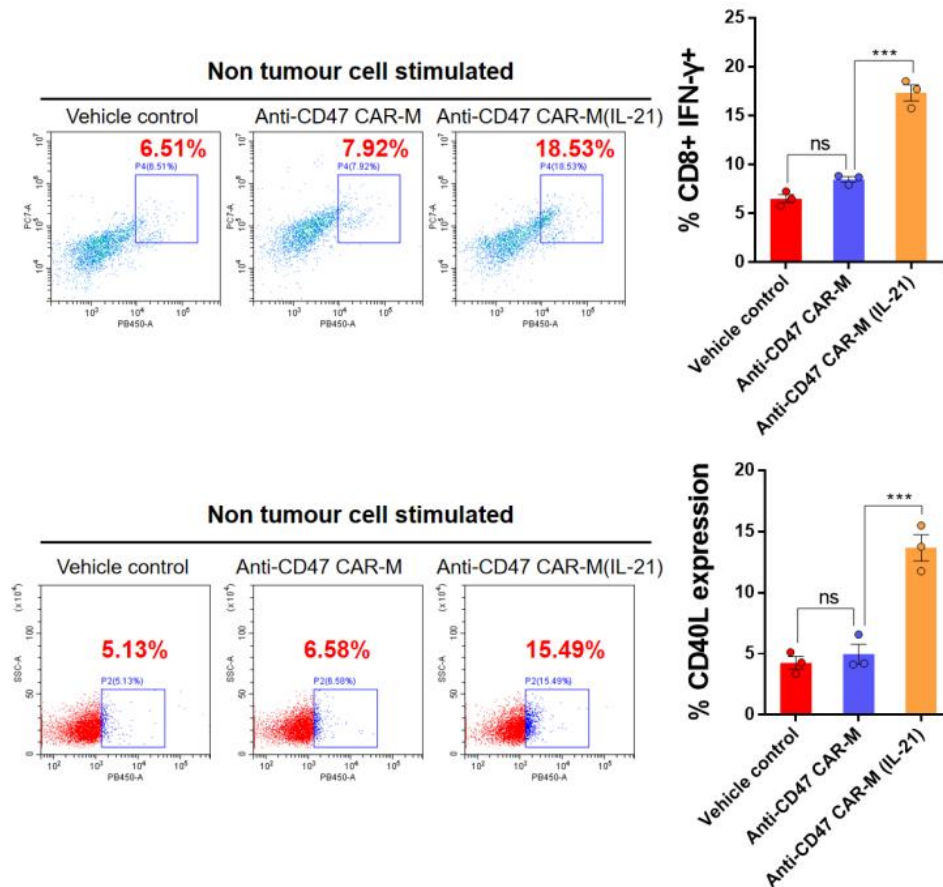
3. Lines #219-222, Figures 1G, H are not accurate. The baseline GFP expression between CAR-M and CAR-M (IL-21) is not the same (see Fig. S2), there are more macs that do not express GFP/ CAR in the CAR-M alone group. Therefore, the higher numbers of the “Tumor cell survival” graphs could partly be attributed to the THP-1 cells not having the GFP/ CAR in them. Solution could be to mix some untransduced THP-1 cells with CAR-M (IL-21) to make the GFP expression more comparable to CAR-M group.

Response: This is an important comment. As suggested, this experiment was conducted again by adding untransduced THP-1 to ensure consistency in the number of CAR cells (**Figure 1F and G**).



4. Figure 2: A control with CAR-M (+/- IL-21) and T cells, without tumor cells, would be important here to determine whether the effects seen were tumor cell/antigen dependent, or if they were just the stimulatory effects of the adenovirus post transduction (Adenovirus alone has strong stimulatory effects on macrophages).

Response: We appreciate this useful comment. A co-culture control group without tumor cells was added and determined that T cell activation is tumor cell-dependent. Together with the expression changes of CD40L, it suggested that there is indeed communication between CAR-M and tumor cells (Figure S4E and F).



Minor comments:

1. Order of the supplementary figures is inconsistent. Please try to make sure that the figures come in order based on the results text.

Response: We apologize about it. We have reorganized the order of supplementary materials to ensure consistency with the main text. Please check the revised manuscript.

2. Introduction line #57, please include study in glioblastoma that has shown some meaningful anti-tumor effects (PMID: 35130560).

Response: We have supplemented the relevant research progress, especially the excellent performance of CAR immune cell therapy in clinical studies.

3. Figure legends in the supplementary figures are not very informative. Please make sure that legends and figures can stand alone and be understandable/ informative even without the main text.

Response: We appreciate this comment. We have reorganized the legends of the supplementary

figures to ensure that the necessary information can be provided even without the main text.

4. Please describe somewhere in the text and legends what vehicle control is, does it have GFP but without CAR?

Response: Thanks for the comment. We provided more precise information in Figure 1 and figure legend. The vehicle control is an adenovirus vector that only contains fluorescent groups and does not include CARs to eliminate interference from adenovirus vector.

5. Fig 1A: The GFP part needs to be also added to the construct in the correct position

Response: The diagram has been modified to provide the position of GFP groups (**Figure 1A**).

6. Fig 1D: x and y axis labelling needs to be made clear

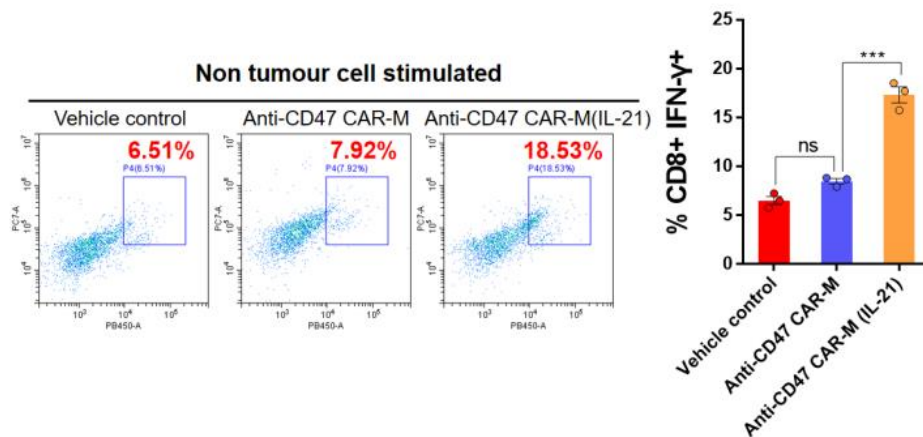
Response: The horizontal and vertical coordinates, as well as their corresponding fluorescence channels have been provided in Fig 1D.

7. Fig 2A-B: CD80/CD86 are both expressed in all macs at baseline, therefore using MFI rather than % would be better since all the cells technically express those markers.

Response: This is a useful suggestion. MFI has been used to evaluate the expression of CD80/CD86 in macs (**Figure 2A and B**).

8. Fig 2C: An isotype control or non-transduced T cells would be a good control to have here to more accurately place the gating.

Response: We agree with point. The results of T cells that were not co-cultured with tumour cell was added to provide better gating strategy (**Figure S4E**).



9. Fig 5F, Fig S1A-B: Need isotype control/ non-proliferating cell control to determine gating.

Response: The relevant gating strategy was modified in the supplementary materials.

10. Fig 5H: It seems that there are not enough events in these flow plots to allow for statistical analysis.

Response: We repeated the animal experiment and got similar results. We also thought that the number of events in this flowchart was not enough for statistical analysis, so the related data was deleted.

Reviewer #2 (Remarks to the Author):

Minor things.

- 1. Fig S1B, is this human patient tissue? please correlate with the report summary part-Research involving human participants, their data, or biological material, which claimed there is No human participants, their data, or biological material were used. no scale bar included (this is for all figures presented.).

Response: Thanks for this comment. The scale bars and ethical statement were provided.

- 2. Fig s2 A FITC signal of vehicle control is 80.14%, whereas CAR-M CD47 is 78.09%. indicating there is no expression of CAR-M.

Response: We appreciate this careful examination. Vehicle control is an empty vector that only contains fluorescent label and does not include CARs to eliminate interference from adenovirus vectors.

• 3. Please clarify the origin of CD47 CAR sequence, where does the scFv sequence come from? Are they specifically recognize the human CD47? Please provide the solid data about the antibody recognition information, which is very crucial to evaluate the safety of CD47 CAR-M.

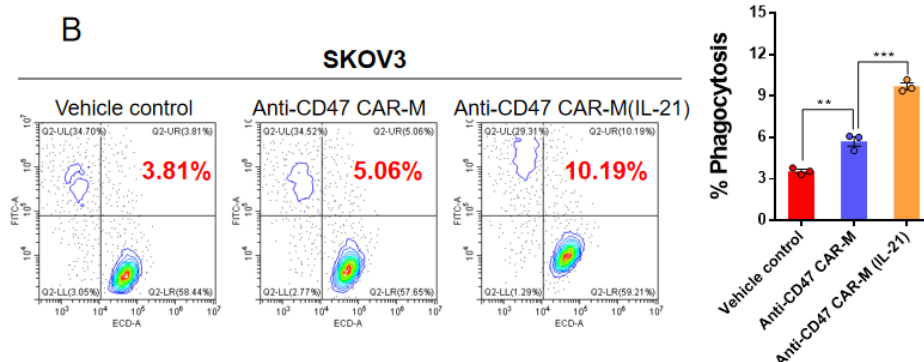
Response: The sequences of this study were obtained from available patents, and the specific sequences of anti-CD47 scFv was listed below and added to manuscript.

CAGGTGCAGCTCGTGCAGTCTGGCGCCGAGGTGAAGAAGCCTGGCGCCTCTGTGAAG
GTGTCTTGCAAGGCCTCTGGCTACACCTTCACAACTACAACATGCACTGGGTGAGAC
AGGCCCCAGGCCAGAGATTGGAGTGGATGGGCACCATTACCTGGCAACGACGACA
CATCTTACAACCAGAAGTTCAAGGACAGAGTGACCATCACAGCCGACACATCCGCCTC
CACCGCCTACATGGAGCTGTCTTCCCTGAGGTCTGAGGACACAGCCGTGTACTACTGC
GCACGAGGCGGCTACCGCGCGATGGACTACTGGGGCCAGGGCACCCCTCGTGACAGTG
TCCTCCGGCGGGCGGGCTCTGGCGGGCGGGCTCCGGCGGGCGGGCAGCGACATT
GTGATGACCCAGTCCCCACTGTCCCTGCCCCGTGACCCCAGGCGAGCCCGCCTCTATTTC
TTGCAGGTCTTCTCAGAGCATTGTGTACTCCAACGGCAACACATACCTGGGCTGGTACT
TGCAAAGCCAGGCCAGTCTCCTCAGCTGCTGATTTACAAGGTGTCTAACAGATTCTC
CGGCGTGCCCGACCGCTTCAGCGGCTCTGGCTCTGGCACCGACTTCACACTCAAGATT
AGCCGTGTGGAGGCCGAGGACGTGGGCGTGTACTACTGCTTCCAGGGCTCTCACGTGC
CATACACCTTCGGCCAGGGCACAAAGCTCGAAATTAAG

• 4. THP1 cell line is not a typical macrophage cell. Please provide primary cell derived CAR-M data.

Response: This is a valuable suggestion. To construct primary CAR-M, we isolated monocytes from the blood of healthy volunteers using magnetic bead sorting, and differentiated them into macrophages through GM-CSF stimulation. Subsequently, we delivered CAR genes using Ad5F35-based adenovirus. After completing the construction of primary CAR-M, it was co-cultured with SKOV3 cells to evaluate its targeted phagocytic activity. The experimental results supported our

conclusion that although the phagocytic activity of primary CAR-M was lower than that of CAR THP-1M, the groups showed similar trends (**Figure S3B**).



- 5. tissue sections scale bar missing in supplemental data.

Response: We apologize for the missing information. The scale bar was added (the lower right corner of each picture).

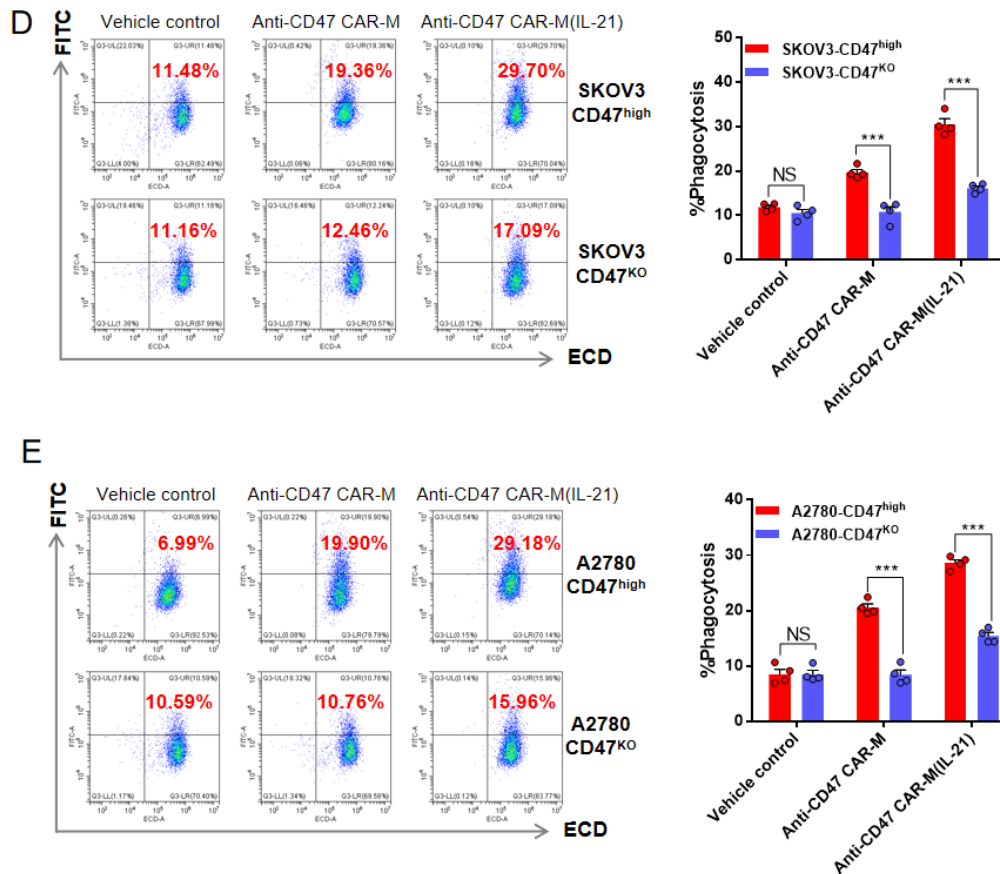
- 6. Fig S10, some none-English words.

Response: Thanks for the remainder. We have rechecked and polished the entire manuscript to avoid similar issues (**Figure S8**).

Main limits of this manuscript.

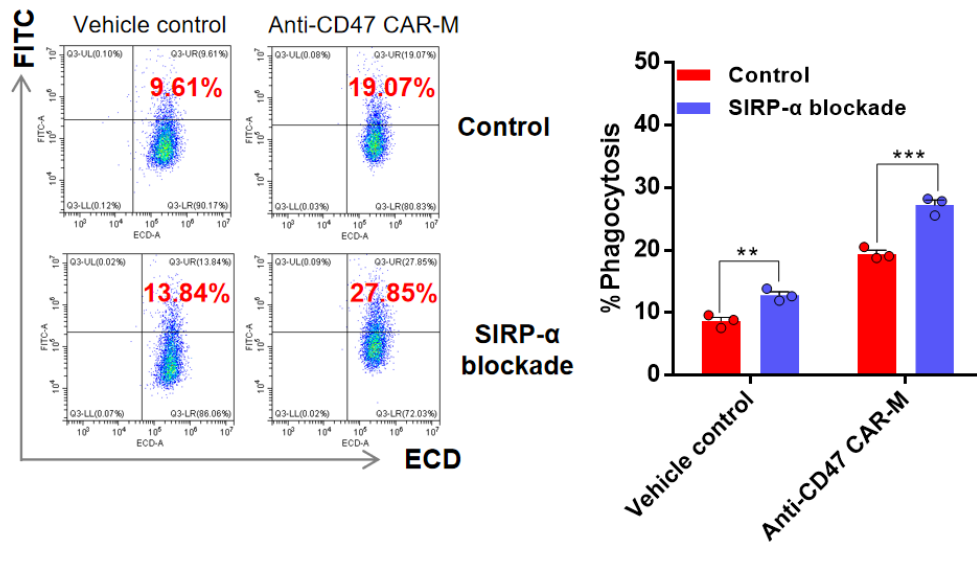
1. Experiments has to be performed to clarify the specificity of CAR-M. either using the CD47 antibody to block the CAR signal, or using CD47 KO tumor cells, or using CD 47 intracellular domain dead construct as a negative control.

Response: This is an insightful suggestion. The co-culture data after CD47 knockout was added to confirm the specificity of CAR molecules (**Figure 1D and E**).



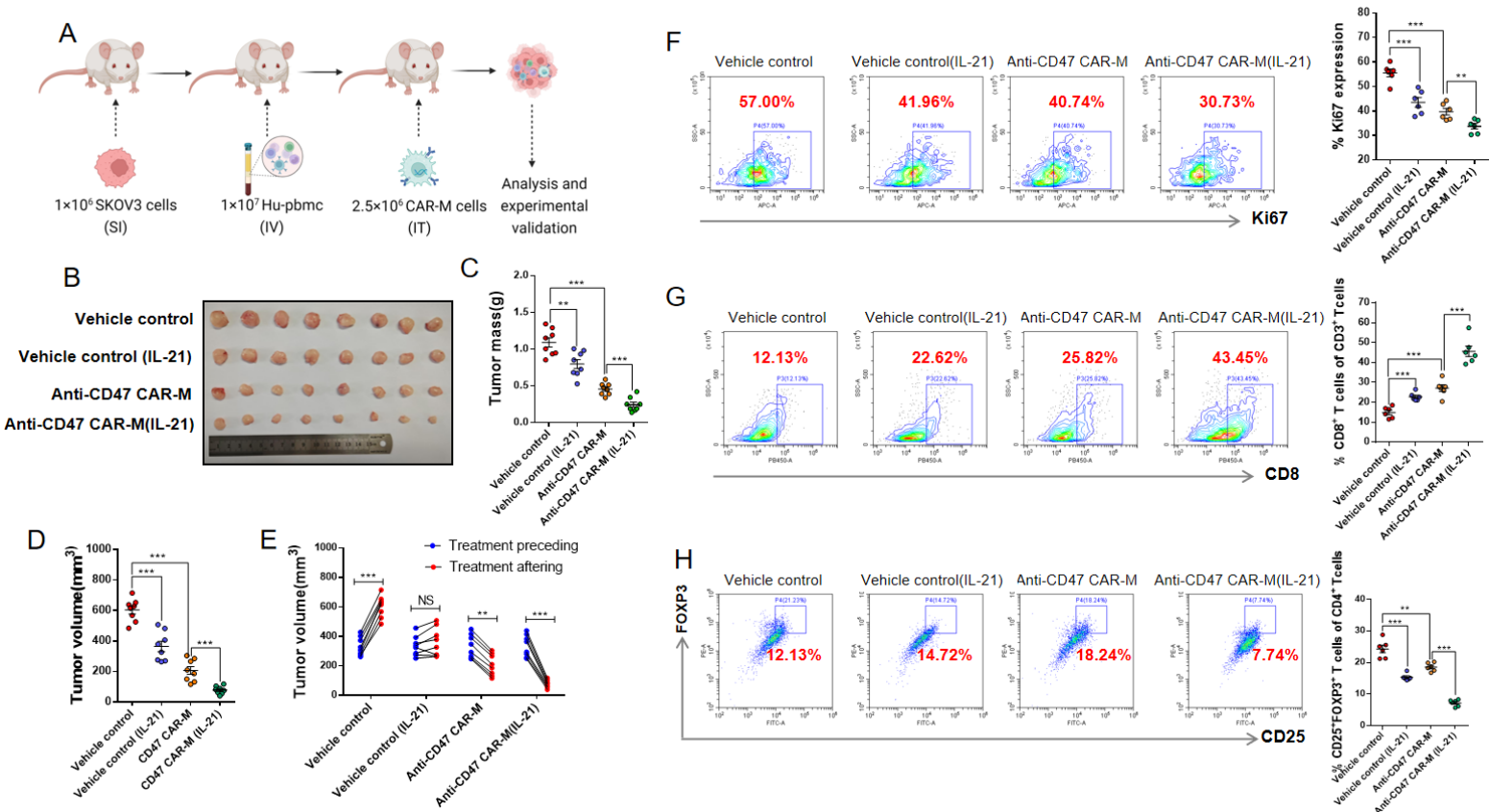
2. Please provide the competition data of CD47 CAR and SIRPa, which all expressed on the THP-1 cells of CAR-CD47 THP-1

Response: We appreciate this comment. We verified the competition between CD47 CAR and SIRP- α by blocking the SIRP- α . The experimental results confirmed that SIRP- α blockade enhanced the phagocytosis of CAR-M, indicating competitive binding of both to CD47.



3. Please provide the IL21 only THP-1 function data, to compare with CD47 CAR, CD47 CAR-IL21 THP1 cells.

Response: We appreciate this insightful comment. As suggested, the *in vivo* experimental data on GFP⁺ THP-1-M (IL-21) was shown as below (Figure 5).

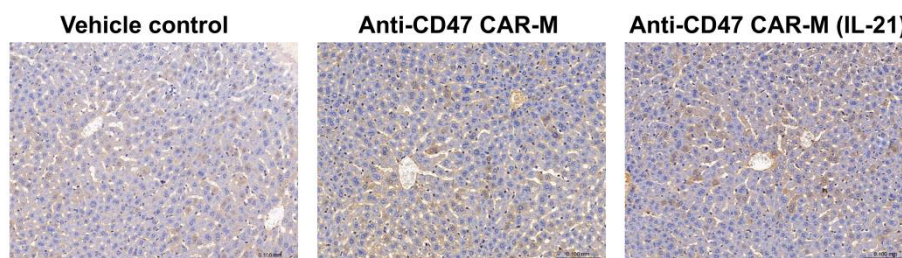


4. In discussion part, for the clinical trial CT-0508 (NCT04660929), initiated by CARISMA, there is no CR(complete response), four patients achieved SD(stable disease).

Response: We agree with this point and made modifications. Please check the revised manuscript.

5. Normal macrophages has the ability to migrate to liver, whereas in this manuscript not the case, need to provide the imaging data. It could be THP1 cell line is not a typical macrophage cell, the *in vivo* behavior is different. Authors also have to provide IL21 only cells dynamic ,to see if the changed distribution pattern is due to IL21.

Response: Thanks for this constructive comment. We have supplemented the liver staining images of mice that received intravenous tail vein injections of CAR-M, which revealed significant macrophage infiltration in the liver across all groups (**Figure S5D**). However, whether IL-21 can alter the *in vivo* distribution of CAR-M remains unknown. Addressing this question requires the establishment of more suitable animal models and long-term *in vivo* real-time monitoring. Unfortunately, our current technical platform is insufficient to support such experimental validation. Alternatively, an IHC staining results was added to support the enhancing effect of IL-21 on liver infiltration of anti-CD47 CAR-M.



6. In summary, this manuscript is poorly presented and designed. I don't think at this moment it would fit with stringent and high standard of COMMSBIO.

Response: We have revised this manuscript in accordance with the review comments. We really appreciate the reviewer's comments and suggestions, which are very important for improving the overall quality of our manuscript, I hope our response will satisfy you. At the same time, many comments also inspired us and pointed out the direction for our future work. We'd like to take this opportunity to sincerely thank you for your valuable time and input again!