

CD133+PD-L1+ cancer cells confer resistance to adoptively transferred engineered macrophage-based therapy in melanoma

Corresponding Author: Professor Wei Lu

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

Parts of this Peer Review File have been redacted as indicated to maintain patient confidentiality.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The subject is certainly of interest given the important challenge of addressing the treatment resistant Cancer Stem Cells. The results do move the field further, and the results do support the claims and conclusions. The main issue for this reviewer is the unstructured and incoherent presentation of the structure of the research. The steps taken seem to have been taken on the fly and not as part of a pre-existing project plan. As a result the discussion/conclusion is summarizing the individual steps rather than the bigger picture. Having said that, I do like the results, but these are not properly discussed.

The use of hyperthermia is a good example, this seems to drop from the blue sky where one would have expected a proper introduction of a hypothesis towards the end of the introduction or at the start of the methods section. None of that, the authors simply present a row of different experiments without a rationale, or rather a rationale that suddenly emerges some place in the manuscript.

The description of the application of hyperthermia also lacks much relevant detail, the authors state they used a laser at a certain amount of Watt to achieve 45C for 30 minutes. No description whatsoever of how they measured temperature or whether they validated the method, or whether they copied that from earlier experiments of their lab or elsewhere.

The conclusion is apparently the section starting within summary on line 379. That is a decent summary, but I would have expected also thoughts on the logical next steps after finding these results. The real discussion/conclusion should start after line 389.

Detailed comments: the authors should check for typos, for instance line 148 'Vegfa' is apparently 'VEGF-alfa'

Reviewer #2

(Remarks to the Author)

Firstly, I congratulate the authors of this manuscript for the interesting investigations and results. The experiments implemented to validate the hypothesis are appropriate, I do not have concerns related to their performance and presentation.

As a minor suggestion, I recommend to improve the explanations related to the nature of the "adoptive macrophage transfer" from the beginning of the results section (beginning of figure 1). I understand that these issues are clear for the authors, but the reader has to "go back and forth" to understand the performance of these experiments. After this, I consider appropriate the rest of the text and figures.

As major concerns:

1- I recognize that hyperthermia results are important and very clear in Figure 7, and I agree with the authors about the

potential use of hyperthermia and/or NPs for melanoma in the clinic. However, the application of these approaches could be not so straight-forward for other cancer types ("deeper location with difficult access"). A discussion about these challenges and possible solutions is needed. Some results and/or discussion about the translation of these results to other tumor types is also lacking.

2 - As an additional major concern, I miss some data related to the use of TGFb inhibitors, to validate the hypothesis. The clear role for TGFb has been demonstrated in the manuscript, and several inhibitors of TGFb are now in clinical trials. Thus, some experiments using drug combinations, following the rational of this manuscript, could present a great potential for the treatment of solid tumors.

Reviewer #3

(Remarks to the Author)

This study focusses on CD133+ PD-L1high CSCs and the specific role in adoptive macrophage therapy. Several main concerns:

1. It is now well accepted that CD133 does not serve as a reliable single CSC marker due to its wide expression tumor cells.
2. So far, M1 and M2 (TAM, tumor-associated macrophages, immune suppressive) have identified which demonstrated opposite antitumor activity. Without specialize what macrophages are adoptively transferred is very much problematic.
3. Utilizing clinical melanoma samples adds some value to the study. However, the data were confusing and not conclusive due to the lack of systematic patients characters, such stages, metastases, type of melanoma, et al.
4. It is crucial to quantify the presence of CD133+ PD-L1high cells in primary tumor versus relapsed tumors. This data may reveal important trends in tumor behavior and resistance.
5. It is also important to compare CD133+PD-L1high cells with CD133+PD-L1low cells across melanoma progression stages. Highlighting differences, especially in relation to the TGF-beta pathway, may unveil pivotal insights into melanoma's evolution.

Reviewer #4

(Remarks to the Author)

In this manuscript, Xu et al. identify a subpopulation of cancer stem cells (CSC) with upregulated programmed death-ligand 1 expression (PD-L1+ CSCs) in mouse melanoma. PD-L1+ CSCs were resistant to phagocytoses by macrophages exposed to copper sulfide nanoparticles (NPs-Mo). Compared to other cancer cells PD-L1+ CSCs expressed high levels of TGFb, which increased matrix stiffness, increased the expression of PDL-1 and reduced CD8 T cell activation. The authors found that hyperthermia increased the expression of calreticulin in PD-L1+ CSCs, which was associated with enhanced therapeutic efficacy of transferred NPs-Mo.

The authors also found by using human melanoma cell line and human macrophages similar results in NSGS mice.

The manuscript seems to be addressing important points. While I think that the manuscript may be of interest to the Nature Communications readership, at its current stage, it requires a major revision in terms of data presentation and discussion. Indeed, some sentences are very difficult to interpret. Other important points are indicated below.

Figure 1B is missing statistical analysis.

Figure 1F, it is not clear why the untreated mice still show NPs-Mo in the tumors.

Figures 2F and 2G, it is not clear why the authors are not assessing the role of TGFb in these functional assays.

Figure 3G, it is not clear if the indicated macrophage in the hNPs-Mo group has phagocytized a human cell.

Figure 3H, it is not clear why CD8 T cells are getting activated. Which are the antigens that are presented in MHC-I? Why are these CD8 T cells recognizing specially these antigens?

Is this activation mediated by antigen recognition?

The single cell RNA sequencing in Figure 4 seems of interest. However, the authors should better explain what comparisons they are referring to in their analysis. Also, groups and n values should be better explained.

Figure 5D, the authors should change the color of cluster 11.

Line 253, what is "the data of bubble diagram [...]" referring to?

It is not clear how the authors are designing CSC in Figure 5E. Because the dots seem to be less than those identified in clusters 10 and 11 in Figure 5D. In scRNA data analysis it is often necessary to use cluster analysis to refer to cell types, not only single marker expression.

In Figure 6, is anti-PD-L1 reducing the therapeutic efficacy of NPs-Mo?

Could the authors perform an experiment employing anti-PD1 blockade to overpass the proposed by the authors degradation of anti-PD-L1 caused by transferred macrophages?

Figure E, in the figure it is missing which of the shown cells belong to the spleen.

The authors tested in vivo the combination of adoptive macrophage transfer and hyperthermia involving a laser treatment that reached 51°C at the tumor site. Previous reports suggested that mild hyperthermia prompts a measured apoptotic response. In contrast, excessive hyperthermia leads to a dysregulated and more forceful apoptotic process, engaging the complete executioner caspase repertoire (e.g. Mantso et al. Sci Rep. 2018). Moreover, heat shock proteins (HSPs) released from tumor cells have been described to promote anti-tumor immunity and to reach maximum activity at milder temperatures as well. Can the authors comment on this?

Adjuvant hyperthermia seems a promising approach to overcome the resistance to the macrophage therapy. The authors could consider reproducing some of the key features observed upon monotherapy in B16F10 and A375 models also upon combination with hyperthermia. For example, is fibrosis reduced? Is the number of PD-L1 CRC cells reduced?

The role of CD8 T cells is not clear. It would be interesting, depleting CD8 T cells in the B16F10 model to investigate the contribution of the adaptive immunity to the therapeutic effect. In this line, the authors could also try to rechallenge the surviving mice with tumors to observe whether adaptive immunity plays a role in tumor relapse.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The authors have properly addressed the issues I raised for the original version of this manuscript and I now advise acceptance of the manuscript

Reviewer #2

(Remarks to the Author)

I congratulate the authors for the excellent revision of the manuscript. The major and minor concerns have been addressed. New in vivo experiments have been performed adding relevant results. A better explanation of key issues for clinical translation is now provided, improving the impact of the manuscript. Therefore, I recommend the manuscript for publication without further delay.

Reviewer #3

(Remarks to the Author)

The authors did not address the issues raised in the first round review satisfactorily.

Reviewer #4

(Remarks to the Author)

Reviewer #5

(Remarks to the Author)

This paper claims that a subpopulation of melanoma cancer stem cells is responsible for resistance to car-macrophage therapy. the topic would be very interesting, if the experiments would have been conducted with appropriate rational and technique. I am not an expert in macrophages neither adoptive cell transfer, so I will limit my comments to the melanoma CSC and therapeutic approach in general, rather than on details in Car therapy or thermal therapy approach.

I found the paper challenging to follow and share some of the concerns raised by other reviewers. It appears that the manuscript sometimes feels like a collection of different projects rather than a cohesive study. Moreover, the results section includes extensive discussion on CD133, which might be more appropriately placed in the introduction or discussion sections. I also agree with Reviewer 3's observation that CD133 is not a reliable marker and that the authors seem to have overlooked conflicting literature regarding their proposed marker, as well as general CSC features. They could revise their opinion about those cells being CSC (changing definition and so) and study them in relation to the therapeutic approach their propose, staying focused on the focus of the manuscript (and providing appropriate experiments to support their conclusions).

Furthermore, the study's reliance on a single melanoma cell line to support their conclusions is a significant concern. Specifically my main concerns are:

1. CD133 simply can't be used as a CSC marker, particularly for cell lines if not supported by functional studies to claim that

those cells are true CSCs. In CSC community, there is overall consensus about the necessity of functional assays to claim that a cell is a CSC.

Additionally, there are several important elements missing from the characterization of these cells (whether they express PDL1 or not):

- a. The manuscript lacks clonogenic assays and/or self-renewal assays in stem cell medium.
- b. There is no assessment of tumor initiation potential.
- c. Self-renewal assays are absent.
- d. There are no gene expression profiles provided to support their CSC features, such as Nanog, Oct4, or EMT signatures.
- e. The source and methodology behind the "fold increase" markers indicated in multiple figures are not explained. Are these results from flow cytometry or gene expression? Additionally, it would be beneficial to use flow cytometry for cytokines or ELISA for production.
- f. The statement about "greater proliferation potential in CSCs" is unclear, as it contradicts the consensus that CSCs are slow cycling.
- g. It is also unclear how the cells in Supplementary Figure 6 are shown to be 100% CD133 positive.

2. I find it challenging to understand the rationale for selecting these cells due to several points:

- a. It is unclear whether this population increases after therapy. The markers do not appear in the gene expression analysis, and the flow cytometry data in Figure 1 is not convincing. Additionally, the number of mice used and other experimental details are not clearly presented.
- b. It is not clear whether this subpopulation can regenerate the tumor after therapy.
- c. It is uncertain whether these cells are found in patients before, during, or after treatment. Moreover, it is not specified whether these cells are present in primary versus metastatic tumors, high-grade versus low-grade tumors, or pre- versus post-relapse conditions.
- d. It is also unclear whether these cells are specifically spared by CAR-macrophages or how they survive and drive relapse. What happens if the therapy cycle is repeated?

2. They claim that PDL-1 therapy doesn't change the outcome of the car-macrophage transfer, so what is the rational to claim that this population is responsible for resistance/ relapse/progression?

3. Why don't they silence CD133 (they cite ref 45 for indicating CD133 as target) and see the change in therapy outcome? That would be more interesting and linking the therapy to their "CSC" section.

4. Does TGFB treatment directly affect melanoma cells or the therapy works through broader/ indirect effect (more CD8 cytotoxic, effect on TME)? The simple increase in TGFB is not enough to claim that that is the mechanism to target CSC.

5. Not clear the link with stiffened matrix, relapse and thermal therapy, the matrix data re "lost" in the last part of the paper with the therapy effect, what happens after therapy to the matrix (and during therapy?)

6. I have some concerns regarding the immunofluorescence (IF) on patient samples:

a. It appears that all patient samples show cells that are positive for both CD133 and PDL1. While some CD133 expression is weak, this consistent co-expression suggests a potential technical issue, as it is unusual to see almost no PDL1+ cells that are negative for CD133 (and vice versa).

b. The scarcity of PDL1+ cells in patients 1, 2, 4, and 5 is quite unusual.

c. There is a lack of detailed information on patient lesions. It is unclear whether the samples are from early or late-stage tumors, primary tumors, metastasis, or therapy-naive cases. Additionally, information on whether the patients received therapy, and if so, what kind of therapy, is missing. This would help explain the increased expression in advanced cases. The criteria for patient selection and the claims being made are also not clearly presented

Version 2:

Reviewer comments:

Reviewer #5

(Remarks to the Author)

I thank the author for taking my suggestion in a pro-active way, revising the language and rewiring their statement. I think that now the paper has more clarity, value and is more realistic in the conclusions.

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RESPONSE TO REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author): with expertise in cancer therapy, hyperthermia

The subject is certainly of interest given the important challenge of addressing the treatment resistant Cancer Stem Cells. The results do move the field further, and the results do support the claims and conclusions. The main issue for this reviewer is the unstructured and incoherent presentation of the structure of the research. The steps taken seem to have been taken on the fly and not as part of a pre-existing project plan. As a result the discussion/conclusion is summarizing the individual steps rather than the bigger picture. Having said that, I do like the results, but these are not properly discussed.

RE: We greatly appreciate your positive and suggestive comments. Following your suggestion, we have adjusted the Results section and restructured the Discussion section for coherent presentation.

First, we have added the introduction of hyperthermia to the end of the Introduction section, that may help to better understand the hypothesis of combinatorial adoptive macrophage therapy and hyperthermia.

In the Results section, the main findings were described under the following subtitles. **(1)** Stiffened tumor ECM restricts the trafficking of transferred macrophages (Fig. 1). **(2)** PD-L1⁺ CSCs inhibit adoptive macrophages by TGF- β signaling pathway (Fig. 2). **(3)** TGF- β -enriched and stiffened TME impedes adoptive human macrophages activity (Fig. 3 and 4). **(4)** Human PD-L1⁺ CSCs exhibit activated TGF- β signaling pathway (Fig. 5). **(5)** TGF- β inhibitor but not PD-L1 blockade improves the adoptive macrophage therapy (Fig. 6). **(6)** Hyperthermia reverses resistance of PD-L1⁺ CSCs to adoptive macrophages (Fig. 7).

The Discussion section was restructured as follows. **Paragraph 1:** A summary of major findings of this work as suggested by you. **Paragraph 2:** Discussion about the TGF- β signaling pathway activated in the PD-L1⁺ CSCs responsible for the formation of the macrophage therapy-resistant tumor niche. **Paragraph 3:** Discussion about role of the inducible PD-L1 expression in the PD-L1⁺ CSCs resistant to the adoptive macrophage therapy. **Paragraph 4:** Discussion about the clinical

implications of the identified PD-L1⁺ CSCs. **Paragraph 5:** Discussion about the reasons why immune checkpoint blockade with antibodies is not appropriate for the adoptive macrophage therapy. **Paragraph 6:** Discussion about the possible mechanisms of adjuvant mild hyperthermia (newly added). **Paragraph 7:** Discussion about the potential application of combinational adjuvant hyperthermia and adoptive macrophage therapy (newly added).

We hope that you will be satisfied with the revised manuscript structure.

The use of hyperthermia is a good example, this seems to drop from the blue sky where one would have expected a proper introduction of a hypothesis towards the end of the introduction or at the start of the methods section. None of that, the authors simply present a row of different experiments without a rationale, or rather a rationale that suddenly emerges some place in the manuscript.

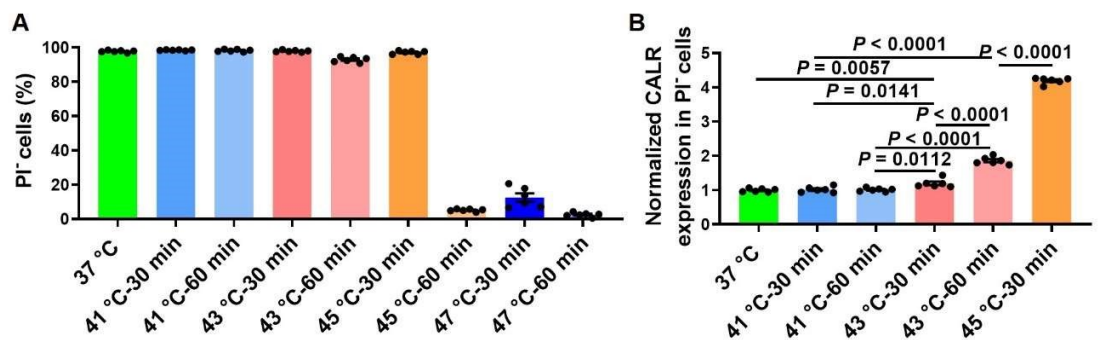
RE: Thank you for the constructive suggestion. We have added the below description of hyperthermia to the Introduction section in **Page 4, Line 85-93**.

Clinically, hyperthermia as an adjuvant therapy has been applied to cancer treatments in combination with radiotherapy and chemotherapy, particularly to superficial tumors^{1,2}. Adjuvant hyperthermia with radiotherapy significantly enhances the therapeutic efficacy in patients with recurrent or metastatic melanoma^{2,3}. The mechanism involves the induction of apoptotic responses of tumor cells, activation of immune system, and transformation from the immunosuppressive TME to the immunogenic TME^{4,5}. In addition, hyperthermia has been reported as a promising approach to eliminating therapy-resistant CSCs⁶. We thus propose an adjuvant hyperthermia to reverse the stiffened and immunosuppressive TME and to block the CSCs. Our results show that reversal of the PD-L1⁺ CSCs resistance to the adoptive macrophages with hyperthermia improves the efficacy of the adoptive macrophage therapy.

The description of the application of hyperthermia also lacks much relevant detail, the authors state they used a laser at a certain amount of Watt to achieve 45C for 30 minutes. No description

whatsoever of how they measured temperature or whether they validated the method, or whether they copied that from earlier experiments of their lab or elsewhere.

RE: We appreciate this valuable comment. We apologize for the confusion. For the cell experiment, we incubated the cells in water bath to control the temperature of hyperthermia. Whereas, for the *in vivo* experiment, we illuminated the tumor with an NIR laser. Previous studies have shown that hyperthermia with the temperature range of 41-43 °C induced immunogenic cell death of tumor cells⁷. This process led to the exposure of calreticulin (CALR) on the plasma membrane of tumor cells, which acted as an ‘eat me’ signal to facilitate the macrophage-mediated phagocytosis. Here, we performed additional experiment to treat the CD133⁺PD-L1⁺ tumor cells with temperatures ranging from 41 °C to 47 °C for 30 min or 60 min. The hyperthermia treatment was conducted by placing the cell culture plates in a water bath under the automated temperature control. The temperatures were verified using a thermometer. The results of flow cytometric analysis showed the highest CALR expression on the cell membrane at 45 °C for 30 min, which was about 4.19-fold of the control group at 37 °C (Supplementary Fig. 12). We have added the above results to **Page 16, Line 366-369** and the experiment methods to **Supplementary Information, Page 6, Line 110-120**.



Supplementary Fig. 12. CALR expression in the CD133⁺PD-L1⁺ tumor cells under hyperthermia. (A) The percentage of propidium iodide (PI⁺) cells under different hyperthermia treatment. (B) The surface CALR expression of the PI⁺ cells was analyzed. The cells incubated at

37 °C were used as control. One-way ANOVA with Tukey's post-test ($n = 6$). Data are expressed as mean $\pm$ s.e.m. $P < 0.05$ is considered statistically significant.

The conclusion is apparently the section starting within summary on line 379. That is a decent summary, but I would have expected also thoughts on the logical next steps after finding these results. The real discussion/conclusion should start after line 389.

RE: Thank you for the constructive suggestion. Following your suggestion, we have moved the summary paragraph to the beginning of the Discussion section in **Page 18, Line 416-426**.

Detailed comments: the authors should check for typos, for instance line 148 'Vegfa' is apparently 'VEGF-alfa'

RE: Thank you for the kind suggestion. Both 'Vegfa' for mouse gene and 'VEGFA' for human gene were written as 'VEGF- α ' in the manuscript. Similarly, 'Tgfb' for mouse gene and 'TGFB' for human gene were written as 'TGF- β '. Accordingly, we have changed 'Vegfa' with 'VEGF- α ' in **Page 8, Line 178** (Line 148 of the original manuscript).

References

1. Wust, P. et al. Hyperthermia in combined treatment of cancer. *Lancet Oncol.* **3**, 487-497 (2002).
2. Overgaard, J. The current and potential role of hyperthermia in radiotherapy. *Int. J. Radiat. Oncol., Biol., Phys.* **16**, 535-549 (1989).
3. Overgaard, J. et al. Randomised trial of hyperthermia as adjuvant to radiotherapy for recurrent or metastatic malignant melanoma. *Lancet* **345**, 540-543 (1995).
4. Skitzki, J.J., Repasky, E.A., and Evans, S.S. Hyperthermia as an immunotherapy strategy for cancer. *Curr. Opin. Investig. Drugs* **10**, 550-558 (2009).
5. Yang, X. et al. Hyperthermia combined with immune checkpoint inhibitor therapy in the treatment of primary and metastatic tumors. *Front. Immunol.* **13**, 969447 (2022).
6. Oei, A.L. et al. Targeting therapy-resistant cancer stem cells by hyperthermia. *Int. J. Hyperthermia* **33**, 419-427 (2017).
7. Yang, Z. et al. Thermal immuno-nanomedicine in cancer. *Nat. Rev. Clin. Oncol.* **20**, 116-134 (2023).

Reviewer #2 (Remarks to the Author): with expertise in tumor-associated macrophages, nanomedicine

Firstly, I congratulate the authors of this manuscript for the interesting investigations and results. The experiments implemented to validate the hypothesis are appropriate, I do not have concerns related to their performance and presentation.

RE: We greatly appreciate your positive comments.

As a minor suggestion, I recommend improve the explanation related to the nature of the "adoptive macrophage transfer" from the beginning of the results section (beginning of figure 1). I understand that it is clear for the authors, but the reader has to "go back and forth" to understand the performance of this experiments. After this, I consider appropriate the rest of the text and figures.

RE: Thank you for this valuable comment. Following your suggestion, we have added a schematic diagram to illustrate the preparation of NPs-MΦ and the signaling pathway to induce the M1 polarization of adoptively transferred NPs-MΦ (Fig. 1A). Besides, we have added the following description in **Page 5, Line 97-100**.

In this study, the adoptive NPs-MΦ were prepared by incubating the mouse bone marrow-derived macrophages (BMDMs) with CuS-NPs. The phagocytized nanoparticles elevated the intracellular production of the reactive oxygen species (ROS) by slow release of Cu ions and induced the M1 polarization through the classical IKK-dependent NF-κB activation (Fig. 1A).

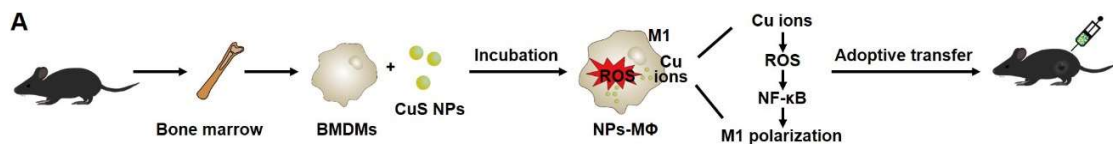


Fig. 1. (A) Schematic illustration of the preparation of NPs-MΦ and the mechanism of CuS NPs-induced M1 polarization.

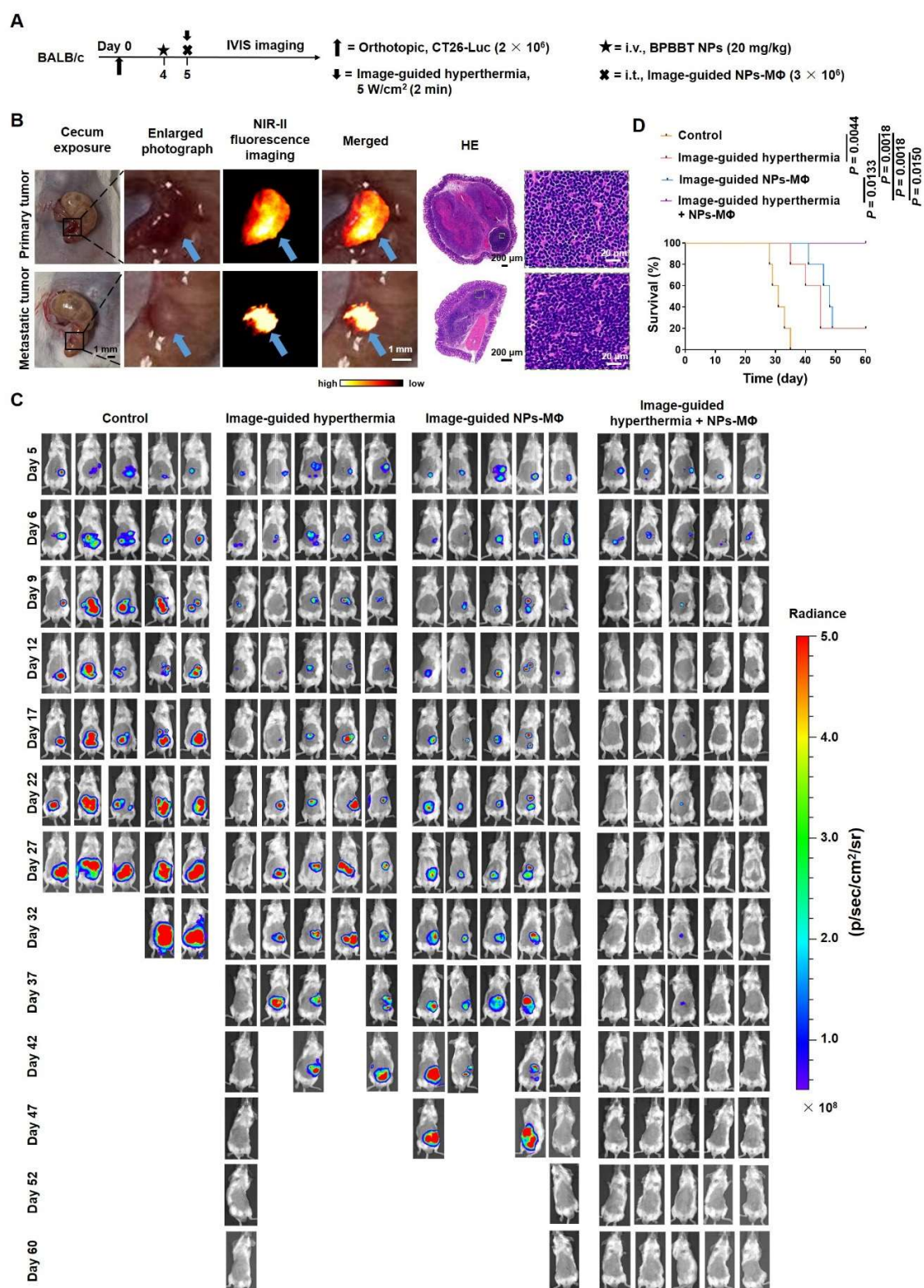
As major concerns:

1- I recognize that hyperthermia results are important and very clear in Figure 7, and I agree with the authors about the potential use of hyperthermia and/or NPs for melanoma in the clinic. However, the application of these approaches could be not so straight-forward for other cancer types ("deeper location with difficult access"). A discussion about these challenges and possible solutions is needed. Some results or discussion about the translation of these results to other tumor types is also lacking.

RE: Thank you for this critical comment. We have conducted an additional experiment to test the feasibility of hyperthermia with the adoptive macrophage therapy of tumor in deep location. The mice bearing orthotopic CT26-Luc colon cancer model and the intraoperative near-infrared window-II (NIR-II) fluorescence imaging with the theranostic probes BPBBT NPs was adopted following our previously reported protocol¹. Following the i.v. injection with BPBBT NPs, both primary and metastatic tumor foci of mice were detected under the intraoperative NIR-II fluorescence imaging (Supplementary Fig. 15A, B). The hyperthermia with an NIR laser illumination (5 W/cm², 2 min, 808 nm) was applied to both primary and metastatic tumor under the imaging guidance, followed by the image-guided i.t. injection of NPs-MΦ. The intraoperatively NIR-II fluorescence image-guided hyperthermia alone and adoptive transfer with NPs-MΦ alone extended the median survival times of mice to 45 days and 48 days, respectively (Supplementary Fig. 15C, D). By contrast, the intraoperatively image-guided combination therapy resulted in a complete cure of mice free of tumor within the 60 days of observation. We have added the above description in **Page 17, Line 403-413**.

Moreover, we have added the following discussion to **Page 21, Line 500-512**. Besides the melanoma treatment, the combinatorial hyperthermia with the macrophage transfer may show promising for the treatment of other superficial tumors such as head and neck cancer in clinical practice. Regarding the deep-seated tumors, because the tissue penetration of NIR light is limited to centimeters in depth, a surgery or interventional therapy is additionally required, through which an intraoperative or interventional diagnosis can be applied to visualizing the tumor localization to

ensure the treatment accuracy of hyperthermia. Our previous studies have demonstrated that the intraoperative NIR-II fluorescence imaging enables the detection of microtumor of size as small as $0.5\text{ mm} \times 0.3\text{ mm}$, and substantially improved the accuracy of photothermal therapy under the image-guidance in the orthotopic colon tumor model¹. Here, by using the same imaging agents, we showed that the combinatorial intraoperative NIR-II fluorescence image-guided hyperthermia and adoptive NPs-M Φ transfer achieved a complete cure of mice. Since the widely used colonoscopy for colon cancer diagnosis and treatment in clinic, the NIR-II fluorescence image-guided hyperthermia in combination with adoptive macrophage transfer under the colonoscopy shows promise for clinical application to colon cancer treatment.



Supplementary Fig. 15. Intraoperative fluorescence image-guided hyperthermia and adoptive transfer of NPs-MΦ for treatment of mice bearing orthotopic CT26-Luc colon

cancer. (A) Treatment regimens. (B) Intraoperative NIR-II fluorescence imaging of tumors. Left four columns, intraoperative photograph and NIR-II fluorescence images of the exposed mouse cecum with primary or metastatic tumor. Arrows, tumor. Right two columns, H&E staining of the resected tumors. (C) *In vivo* bioluminescence imaging of CT26-Luc tumors after treatment. (D) Kaplan-Meier survival curves, log-rank analysis ($n = 5$). $P < 0.05$ is considered statistically significant.

2 - As an additional major concern, I miss some data related to the use of TGFb inhibitors, to validate the hypothesis. The clear role for TGFb has been demonstrated in the manuscript, and several inhibitors of TGFb are now in clinical trials. Thus, some experiments using drug combinations, following the rational of this manuscript, could present a great potential for the treatment of solid tumors.

RE: We appreciate this valuable comment. TEW-7197 as a TGF- β signaling inhibitor has completed phase I clinical study in patients with advanced solid tumors (NCT02160106) including melanoma^{2,3}. TEW-7197 was selected to evaluate whether the adoptive macrophage therapy would benefit from the combinatorial TGF- β signaling inhibitors against the tumor resistance. The results showed that the adoptive NPs-M Φ combined with TEW-7197 treatment significantly delayed the tumor growth in comparison with TEW-7197 alone or NPs-M Φ transfer alone (Fig. 6E). The medium survival time of mice receiving TEW-7197 alone or NPs-M Φ alone was 26 days or 38 days. By contrast, a combinatorial TEW-7197 with NPs-M Φ transfer therapy was prolonged to 45 days (Supplementary Table 1). Besides, the combinatorial treatment ameliorated immune-suppressive TME, evidenced by the significant reduction of the inhibitory cell subsets of regulatory T (T_{reg}) cells and myeloid-derived suppressor cells (MDSCs), as well as the increase in the infiltration of IFN- γ -producing and GranB-producing CD8⁺ T effector cells into tumor (Fig. 6F). These results demonstrated that the TGF- β signaling inhibitor enhanced the antitumor effect of NPs-M Φ . However, the combination therapy was unable to eradicate the tumor with recurrence. We have added the above description in **Page 15, Line 349-361**.

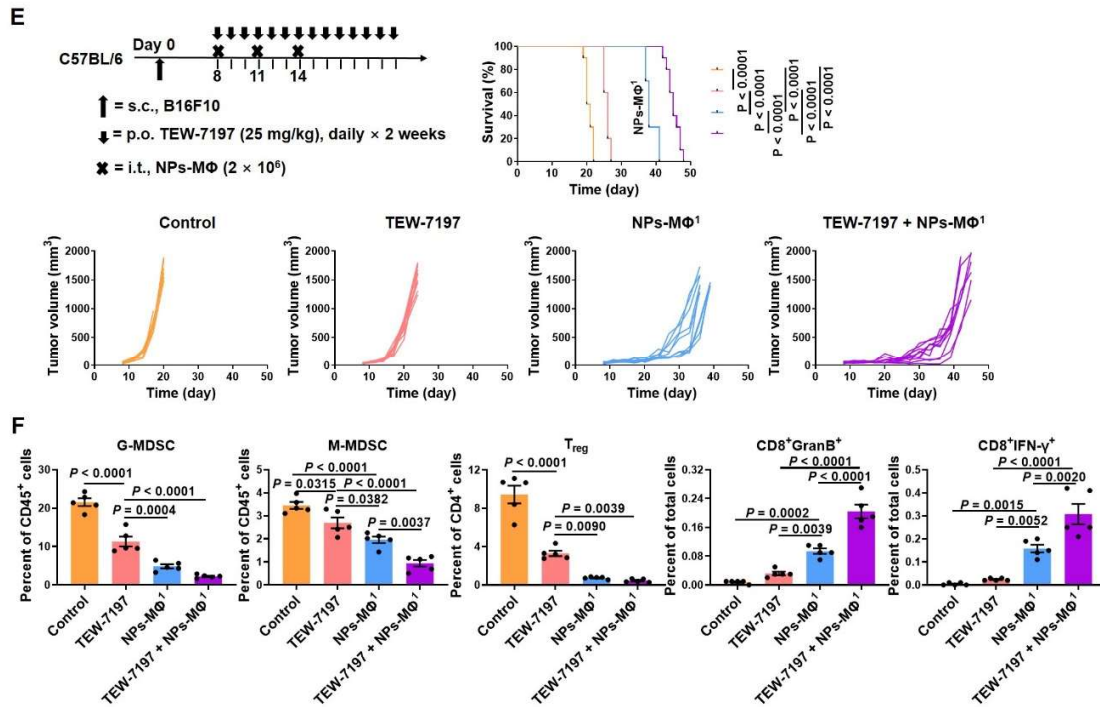


Fig. 6. (E) Combinatorial TGF- β inhibitor and adoptive macrophage transfer therapy (NPs-M Φ^1) for B16F10 melanoma-bearing mice. Upper, the treatment regimens and the corresponding Kaplan-Meier survival curves. Lower, individual tumor growth curves, log-rank analysis ($n = 10$). (F) The changes of immune cell populations in tumor after combinatorial TGF- β inhibitor and the adoptive NPs-M Φ^1 therapy in the B16F10 mouse model at day 22. CD11b⁺Gr-1⁺ MDSC subsets (CD11b⁺Gr-1^{high} granulocytic (G-MDSC) and CD11b⁺Gr-1^{int} monocytic (M-MDSC)), T_{reg} cells (CD4⁺CD25⁺Foxp3⁺) as well as granzyme B-positive CD8⁺ T cells (CD8⁺GranB⁺) and IFN- γ -positive CD8⁺ T cells (CD8⁺IFN- γ ⁺) in the tumor were analyzed. One-way ANOVA with Tukey's post-test ($n = 5$). Data are expressed as mean $\pm$ s.e.m. $P < 0.05$ is considered statistically significant.

Supplementary Table 1. The median survival times of mice bearing B16F10 tumor following the combinatorial TEW-7197 and adoptive macrophage therapy.

Group	Median survival time
Control	20.5 ($n = 10$)
TEW-7197	26 ($n = 10$)
NPs-M Φ^1	38 ($n = 10$)
TEW-7197 + NPs-M Φ^1	45 ($n = 10$)

References

1. Gao, S. et al. Albumin tailoring fluorescence and photothermal conversion effect of near-infrared-II fluorophore with aggregation-induced emission characteristics. *Nat. Commun.* **10**, 2206 (2019).
2. Kim, B.-G., Malek, E., Choi, S.H., Ignatz-Hoover, J.J., and Driscoll, J.J. Novel therapies emerging in oncology to target the TGF- β pathway. *J. Hematol. Oncol.* **14**, (2021).
3. Colak, S., and Ten Dijke, P. Targeting TGF- β signaling in cancer. *Trends Cancer* **3**, 56-71 (2017).

Reviewer #3 (Remarks to the Author): with expertise in cancer immunology, cancer stem cells

1. It is now well accepted that CD133 does not serve as a reliable single CSC marker due to its wide expression tumor cells.

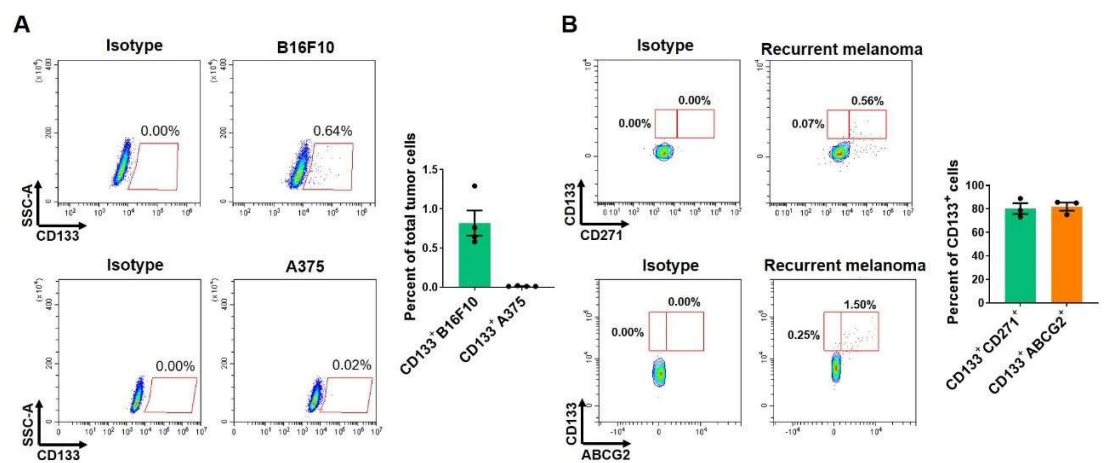
RE: Thank you for this insightful comment. Previous studies have demonstrated that the CD133⁺ CSCs characterized by increased proliferation capacity and tumorigenic potential were functionally distinct from bulk tumor population, contributing to tumor resistance and poor prognosis in various malignancies including melanoma¹⁻⁵. CD133 has been reported as a single CSC marker for melanoma². Targeting CSCs via CD133 is an effective strategy for anti-melanoma therapy⁶. Our results demonstrated that the CD133⁺ mouse B16F10 and the CD133⁺ human A375 melanoma cell lines accounted for only about 0.82% and 0.01% of the melanoma bulk population cells, respectively (Supplementary Fig. 4A). Therefore, CD133 is not widely expressed in these two melanoma cells. These numbers are consistent with the previously reported data of ~3.40% of CD133 expression in the B16F10 cell line¹ and the nearly undetectable CD133 expression in the human A375 cell line⁷.

Besides CD133, melanoma cells express other CSC markers such as CD271 and ABCG2^{8,9}. The co-expression of CSC markers was usually investigated to identify distinct CSC subpopulations with specific biological functions^{7,8}. For example, the CD133⁺CD271⁺ subset of melanoma cells were featured by tumor invasion and metastasis¹⁰. The CD133⁺ABCG2⁺ melanoma cells were featured by multidrug resistance and lymphoangiogenic properties^{8,11}. These subpopulations have been shown to contribute to tumor resistance to antitumor therapy, particularly chemotherapy^{10,11}. Herein, we performed the coexpression analysis of CD133 with these CSC markers in the recurrent B16F10 melanoma samples. Approximately 80.28% and 81.94% of CD133⁺ melanoma cells co-expressed CD271 and ABCG2, respectively (Supplementary Fig. 4B), suggesting that the majority of CD133⁺ recurrent melanoma cells exhibited the biologic functions of CD271 and ABCG2. However, because the recurrent tumor in our study was specifically

resistant to the adoptive macrophage therapy, the CD133⁺ tumor cells did not show 100% positivity for these two markers. We have added the above description to the Results section in **Page 7, Line 140-159**.

Alternatively, in this study, we identified a CSC subpopulation of CD133⁺PD-L1⁺ in the relapsed tumor, which was in relation to the TGF- β signaling pathway and conferred the resistance to adoptive macrophage therapy.

In summary, because the low population of CD133⁺ tumor cells were observed in both melanoma cell lines and clinical melanoma samples from our findings and the literature reports, we think CD133 is appropriate for the CSC marker of melanoma. Furthermore, our results of mechanism and pharmacologic study demonstrated that the identified CD133⁺PD-L1⁺ CSC subpopulation are responsible for the resistance to adoptive macrophage therapy.



Supplementary Fig. 4. Identification of CD133 and other CSC markers in melanoma cells.

(A) The CD133 expression in mouse B16F10 and the human A375 cell lines. Left, representative dot plots of the flow cytometric analysis ($n = 4$). Right, corresponding percentage of CD133⁺ gated in total tumor cells. (B) The co-expression of CD133 with other CSC markers of CD271 and ABCG2 in the recurrent B16F10 melanoma, respectively. The recurrent tumor samples were collected from B16F10-bearing mice at day 35 following the NPs-M Φ^1 treatment. Left, representative dot plots of the flow cytometric analysis ($n = 3$). Right, the corresponding percentage of CD133⁺CD271⁺ and CD133⁺ABCG2⁺ CSCs gated in CD133⁺ CSCs, respectively. Data are expressed as mean $\pm$ s.e.m.

2. So far, M1 and M2 (TAM, tumor-associated macrophages, immune suppressive) have identified which demonstrated opposite antitumor activity. Without specialize what macrophages are adoptively transferred is very much problematic.

RE: We appreciate this critical comment. Our previous study showed that NPs-M Φ were reprogramed by copper sulfide nanoparticles and characterized by the M1 phenotype¹². The nanoparticles elevated the intracellular production of the reactive oxygen species (ROS) by slow release of the Cu ions for the M1 polarization through classical IKK-dependent NF- κ B activation. Compared with the M Φ without CuS NPs (Un-M Φ), NPs-M Φ significantly decreased the expression of classical M2 marker CD206 (Fig. R1A), and downregulated the expression of M2-related gene set by bulk RNA-seq analysis (Fig. R1B). The transferred NPs-M Φ (F4/80⁺DiD⁺) decreased the expression of CD206 in tumor (Fig. R1C). The classical IKK-dependent NF- κ B activation mediated by the intracellular Cu ions was evidenced by the increased phosphorylation of both IKK β and NF- κ B p65 (Fig. R1D).

In this study, the CuS NPs-engineered human macrophages (NPs-hM Φ) significantly increased the intracellular ROS level, compared with the human macrophages without CuS NPs (Un-hM Φ) (Fig. 3B). NPs-hM Φ significantly reduced the expression of classical human M2 marker CD163 (Fig. 3C), confirmed by the down-regulated expression of the M2-related genes (Fig. 3D). Besides, we have added a schematic diagram to illustrate the preparation of NPs-M Φ and the mechanism of CuS NPs-induced M1 polarization (Fig. 1A).

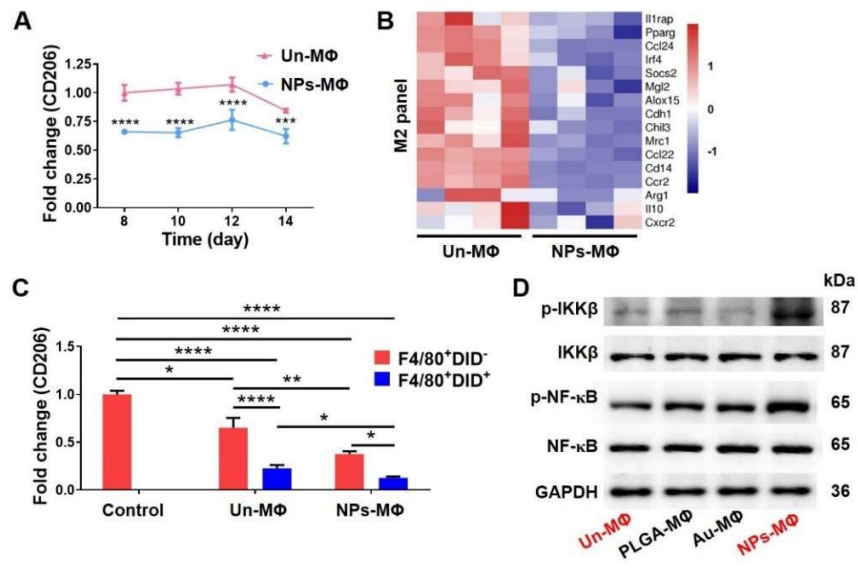


Fig. R1. Intracellular ROS directs NPs-MΦ polarization toward M1 phenotype by classical IKK-dependent NF-κB activation¹². (A) The classical M2 marker of CD206 expression in Un-MΦ or NPs-MΦ over time *in vitro*, normalized with the corresponding Un-MΦ at day 8. Two-way ANOVA with Sidak's post-test ($n = 3$). (B) Heat maps of the cluster of genes associated with the M2-related markers of NPs-MΦ in comparison with Un-MΦ ($n = 4$). (C) The CD206 expression in either the transferred (F4/80⁺DiD⁺) or tumor-associated (F4/80⁺DiD⁻) macrophages at day 3 after single dosage transfer of DiD-labeled NPs-MΦ or Un-MΦ, normalized by control group without treatment. One-way ANOVA with Tukey's post-test ($n = 5-6$). (D) Immunoblot of IKKβ, NF-κB, and the corresponding phosphorylated (p-) IKKβ, NF-κB, and GAPDH (loading control). Data are expressed as mean $\pm$ s.e.m. ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

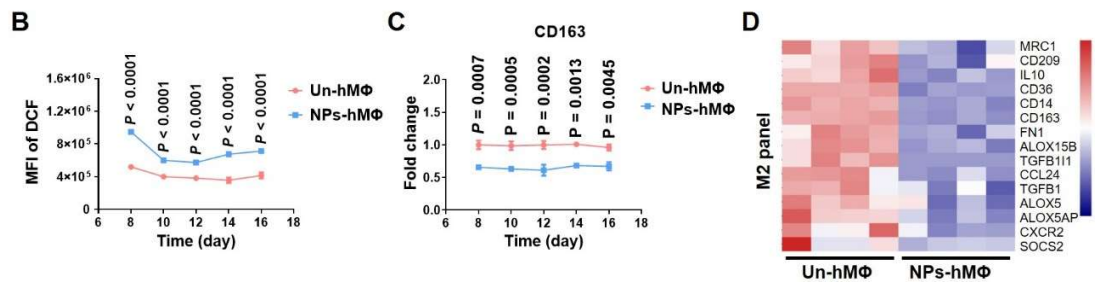


Fig. 3. (B) Intracellular ROS level of copper sulfide nanoparticle-engineered human macrophage (NPs-hMΦ) over time. NPs-hMΦ or Un-hMΦ were prepared at day 8. The cellular ROS stained with 2',7'-dichlorodihydrofluorescein diacetate (H2-DCFDA) was measured by flow cytometry. Two-way ANOVA with Sidak's post-test ($n = 4$). (C) Cellular expression of CD163 measured by flow cytometry, normalized with the corresponding Un-hMΦ at day 8. Two-way ANOVA with

Sidak's post-test ($n = 3-4$). **(D)** Heatmaps of the genes associated with the M2-related markers of NPs-hMΦ in comparison with those of Un-hMΦ. The macrophages were collected at day 8 ($n = 4$). Data are expressed as mean $\pm$ s.e.m. $P < 0.05$ is considered statistically significant.

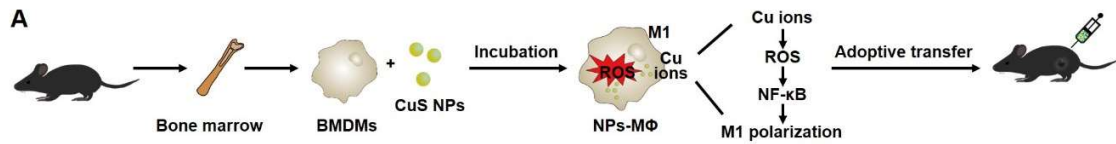


Fig. 1. (A) Schematic illustration of the preparation of NPs-MΦ and the mechanism of CuS NPs-induced M1 polarization.

3. Utilizing clinical melanoma samples adds some value to the study. However, the data were confusing and not conclusive due to the lack of systematic patients characters, such stages, metastases, type of melanoma, et al.

RE: We apologize for this confusion. The patient characters were summarized in Supplementary Table 3.

[Redacted]

4. It is crucial to quantify the presence of CD133⁺ PD-L1⁺ high cells in primary tumor versus relapsed tumors. This data may reveal important trends in tumor behavior and resistance.

RE: Thank you for this valuable comment. We compared the CD133⁺PD-L1⁺ CSC population in mice between relapsed tumor following the NPs-MΦ¹-treatment and the untreated tumor. The tumor in both groups had similar volume of approximately 500 mm³. The data demonstrated that the CD133⁺PD-L1⁺ CSCs existed predominantly in the relapsed tumor, responsible for the tumor resistance against the NPs-MΦ therapy (Fig. 2C).

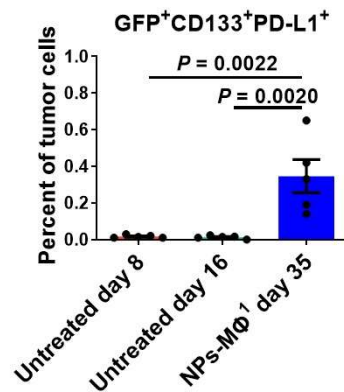


Fig. 2. (C) Identification of CD133⁺PD-L1⁺ from the B16-GFP tumor cells of mice receiving the adoptive NPs-MΦ therapy by FACS-based cell sorting. Mice before treatment at day 8, following the NPs-MΦ¹-treatment at day 35, or without treatment at day 16 were included. One-way ANOVA with Tukey's post-test ($n = 5$). Data are expressed as mean $\pm$ s.e.m. $P < 0.05$ is considered statistically significant.

5. It is also important to compare CD133⁺PD-L1^{high} cells with CD133⁺PD-L1^{low} cells across melanoma progression stages. Highlighting differences, especially in relation to the TGF-beta pathway, may unveil pivotal insights into melanoma's evolution.

RE: Thank you for this constructive comment. We classified the B16-GFP melanoma into 3 stages after the NPs-MΦ¹ therapy based on the tumor volume, i.e. an early stage with 100-250 mm³, a middle stage with 500-1000 mm³, and a late stage with 1500-1800 mm³. No significant differences were observed in the distribution of CD133⁺PD-L1⁻ or CD133⁺PD-L1⁺ CSCs numbers across various stages of tumor (Fig. 2F). The CD133⁺PD-L1⁺ CSCs exhibited higher TGF-β level than CD133⁺PD-L1⁻ CSCs across all the stages of tumor (Fig. 2G, left). The intracellular TGF-β level correlated well with the increased in the tumor volume in both CD133⁺PD-L1⁻ and CD133⁺PD-L1⁺ CSC population (Fig. 2G, right). We have added the above description in **Page 8, Line 183-190**.

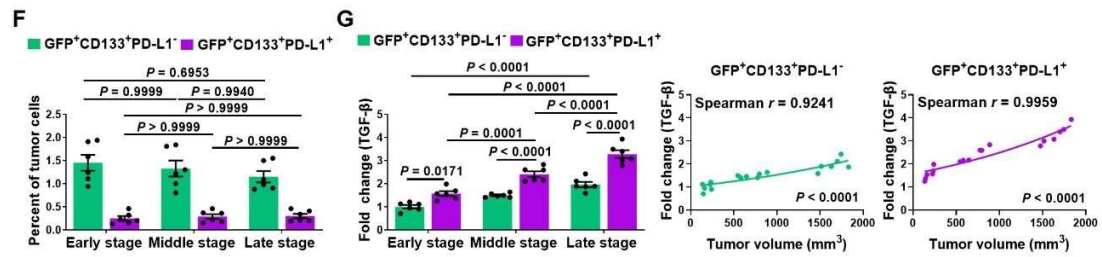


Fig. 2. (F) The percentage of GFP⁺CD133⁺PD-L1⁻ or GFP⁺CD133⁺PD-L1⁺ tumor cells in the tumor of mice following the NPs-M Φ ¹ treatment. Two-way ANOVA with Sidak's post-test ($n = 6$). (G) The TGF- β level in GFP⁺CD133⁺PD-L1⁻ or GFP⁺CD133⁺PD-L1⁺ tumor cells following the NPs-M Φ ¹ treatment. Two-way ANOVA with Sidak's post-test ($n = 6$). Right, the corresponding correlation between the tumor volume and the TGF- β level in GFP⁺CD133⁺PD-L1⁻ or GFP⁺CD133⁺PD-L1⁺ tumor cells following the NPs-M Φ ¹ treatment. The TGF- β level was normalized with the average of the values in the GFP⁺CD133⁺PD-L1⁻ tumor cells of tumor in the early stage (left). An exponential growth fit curve is shown. Two-tailed Spearman correlation was used to evaluate the association ($n = 18$). In (F) and (G), mice with tumor volume of 100-250 mm³ (early stage), 500-1000 mm³ (middle stage) or 1500-1800 mm³ (late stage) were included. Data are expressed as mean $\pm$ s.e.m. $P < 0.05$ is considered statistically significant.

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Reviewer #4 (Remarks to the Author): with expertise in cancer, macrophages, bio-engineering

The manuscript seems to be addressing important points. While I think that the manuscript may be of interest to the Nature Communications readership, at its current stage, it requires a major revision in terms of data presentation and discussion. Indeed, some sentences are very difficult to interpret. Other important points are indicated below.

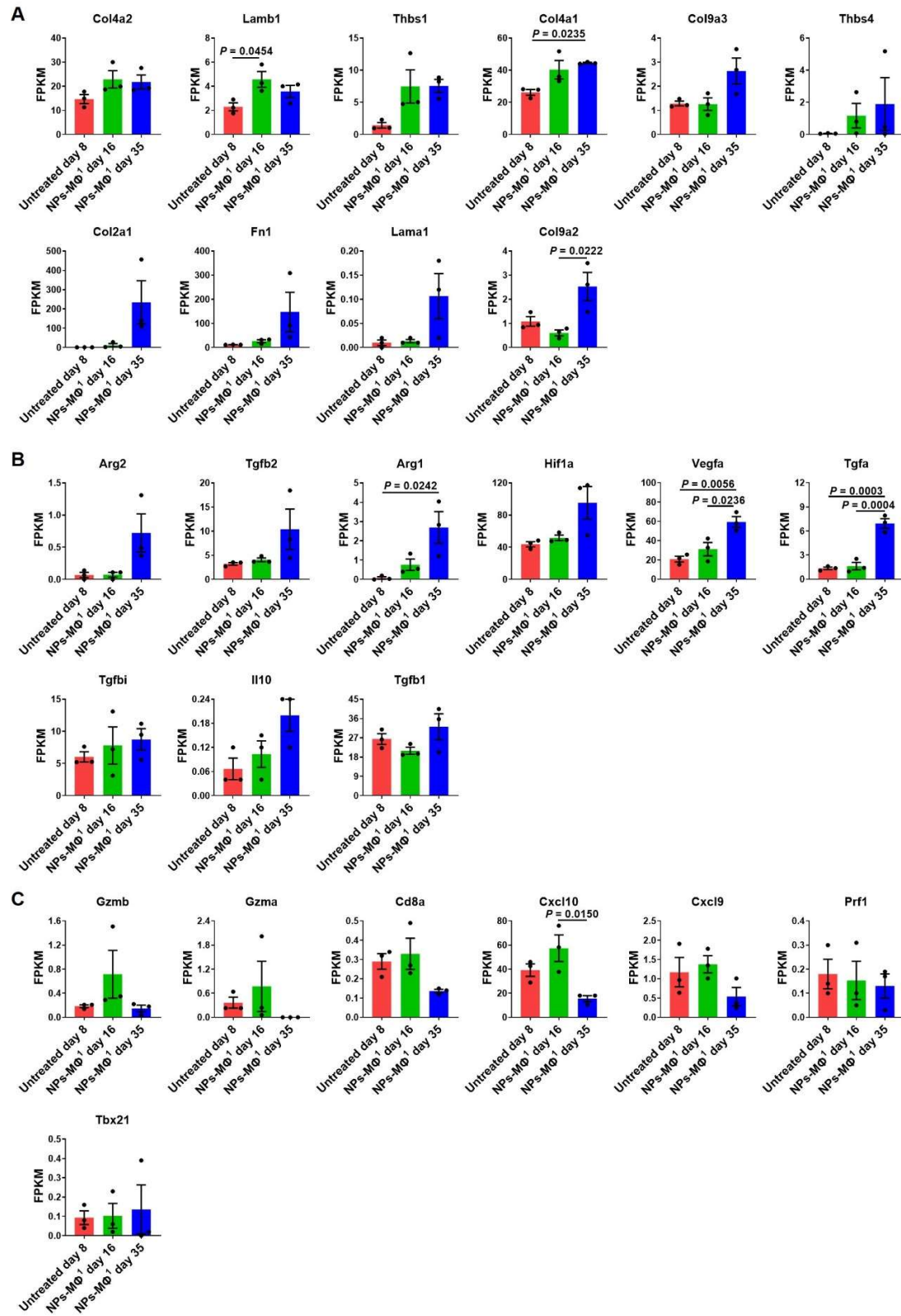
RE: We appreciate your encouraging and constructive comments and apologize for bringing the confusion regarding the data interpretation. We have performed additional experiment and revised the manuscript according to your suggestions. We hope that you will be satisfied with our revision.

Figure 1B is missing statistical analysis.

RE: Thank you for this constructive comment. Following your suggestion, we have performed the statistical analysis of the FPKM values of genes (Supplementary Fig.1). Bulk transcriptome RNA sequencing (bulk RNA-seq) analysis of the tumor samples following the NPs-M Φ^1 treatment at day 35 revealed an upregulation of genes Col4a1 and Col9a2 associated with the tumor ECM compared with that before the treatment at day 8 or after the NPs-M Φ^1 treatment at days 16, respectively (Fig. 1C, Supplementary Fig.1). Besides, a significantly increased expression of immunosuppressive genes Vegfa and Tgfa was observed in the relapsed tumor at day 35 than the other two groups. We have added the above description to **Page 5, Line 105-110**.

The stiffened ECM of the relapsed tumor was validated by BioAFM analysis (Fig. 1D, E). The enhanced stiffness of tumor ECM correlated well with the increase in the CAFs characterized by the myofibroblast marker α -smooth muscle actin (α -SMA), as well as the main ECM components collagen IV and collagen II (Fig. 1F, Supplementary Fig. 2). Moreover, the TGF- β level was significantly upregulated in the relapsed tumor after the macrophage transfer at day 35 (Fig. 1G), showing a positive correlation with the increased CAFs and the stiffened ECM (Fig. 1C-F).

Compared with the tumor of mice following the NPs-M Φ treatment at day 14, the infiltration of various types of immune cells was significantly reduced in the tumor at day 23 (Supplementary Fig. 3A). This result demonstrated the inhibited antitumor functions of CD8⁺ T cells in the tumor relapse stage. Please see the above description in **Page 5, Line 110-118**.



Supplementary Fig. 1. Quantitative analysis of the gene expression of the cluster (Fig. 1C (Fig. 1B of the original manuscript)) associated with the ECM (A), the key immunosuppressive molecules (B), or the CD8⁺ T effector cells (C) in the B16F10 tumor of C57BL/6 mice before the treatment at day 8, or after 1 cycle-treatment (NPs-MΦ¹) at days 16 or 35. FPKM, fragments per kilobase of transcript per million mapped reads. One-way ANOVA with Tukey's post-test ($n = 3$). Data are expressed as mean $\pm$ s.e.m. $P < 0.05$ is considered statistically significant.

Figure 1F, it is not clear why the untreated mice still show NPs-Mo in the tumors.

RE: We apologize for this confusion. The DiD-labeled NPs-MΦ were i.t. injected into the tumors following the NPs-MΦ¹ therapy at day 35. For comparison, the mice without the NPs-MΦ¹ therapy were i.t. injected with the DiD-labeled NPs-MΦ for evaluation of tumor trafficking at day 8 (Without NPs-MΦ¹, day 8) or day 16 (Without NPs-MΦ¹, day 16). We have added the above description to the legend of Fig. 1H (Fig. 1F of the original manuscript) in **Page 27, Line 716-719**. For clarity, the labels of 'Untreated day 8' and 'Untreated day 16' in Fig. 1H (Fig. 1F of the original manuscript) were replaced with 'Without NPs-MΦ¹, day 8' and 'Without NPs-MΦ¹, day 16', respectively.

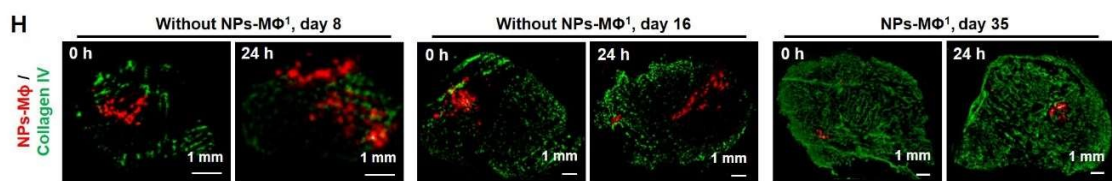


Fig. 1. (H) Fluorescence images of the injected DiD-labeled NPs-MΦ (red) and the immuno-stained collagen IV (green) in tumor sections. Samples were collected at 0 h and 24 h post-administration, respectively. The DiD-labeled NPs-MΦ were i.t. injected into the tumors following the NPs-MΦ¹ treatment at day 35. For comparison, the mice without the NPs-MΦ¹ treatment were i.t. injected DiD-labeled NPs-MΦ for evaluation of tumor trafficking at days 8 (Without NPs-MΦ¹, day 8) or 16 (Without NPs-MΦ¹, day 16).

Figures 2F and 2G, it is not clear why the authors are not assessing the role of TGFb in these functional assays.

RE: Thank you for this valuable suggestion. The role of TGF- β in redirecting the macrophages toward an M2 phenotype^{1,2} and suppressing CTL-mediated tumor cytotoxicity^{1,3} has been well-documented. Following your suggestion, we performed additional experiment to validate the effect of TGF- β on phagocytosis and tumor lysis. The CD133⁺PD-L1⁻ tumor cells appeared resistant to the phagocytosis by NPs-M Φ in the presence of the immunosuppressive factors TGF- β , IL-10 or VEGF- α (Fig. 2H (Fig. 2F of the original manuscript), right). Notably, the combo treatment with these three factors resulted in only approximately 8.70% of the tumor cells being phagocytized. The presence of TGF- β , IL-10 and VEGF- α further prevented the tumor cells from the lysis (Fig. 2I (Fig. 2G of the original manuscript), right). We have added the above description to **Page 9, Line 193-196**, and **Page 9, Line 197-198**. These results proved that the inhibitory effect of TGF- β on the phagocytosis capability of NPs-M Φ and the tumor lysis ability of splenocytes.

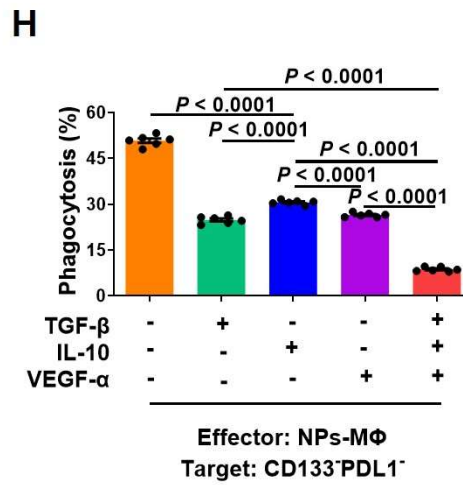


Fig. 2. (H) *In vitro* phagocytosis assay quantified by flow cytometry. The CFSE-labeled tumor cells were cocultured with the DiD-labeled NPs-M Φ in a 96-well ultra-low-adherent plate at a ratio of 1:1 for 2 h. The CD133⁺PD-L1⁻ tumor cells as the target cells and the NPs-M Φ pretreated with TGF- β , IL-10, VEGF- α or the combination overnight before the phagocytosis assay as the effector cells. The percentage of phagocytosis was measured by DiD⁺CFSE⁺ gated in the DiD⁺ NPs-M Φ . One-way ANOVA with Tukey's post-test ($n = 6$). Data are expressed as mean $\pm$ s.e.m. $P < 0.05$ is considered statistically significant.

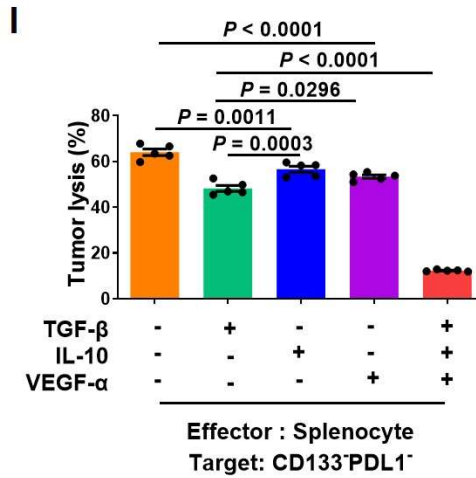


Fig. 2. (I) Tumor cell lysis by MTT assay. The stimulated splenocytes as the effector cells were incubated with the CD133⁺PD-L1⁻ tumor cells at an effector-to-target ratio of 1:1 for 48 h, and the coinubation was conducted in the presence of TGF-β, IL-10, VEGF-α or the combination. The data of tumor lysis was normalized with the control without addition of the effector cells. One-way ANOVA with Tukey's post-test ($n = 5$). Data are expressed as mean $\pm$ s.e.m. $P < 0.05$ is considered statistically significant.

Figure 3G, it is not clear if the indicated macrophage in the hNPs-Mo group has phagocytized a human cell.

RE: Thank you for this comment. We have repeated the experiment. The white arrow in the DIC image indicates a phagocytized tumor cell with undetected tumor cell nucleus and the DiD-labeled fragmented cell membrane (Fig. 3G, lower panel).

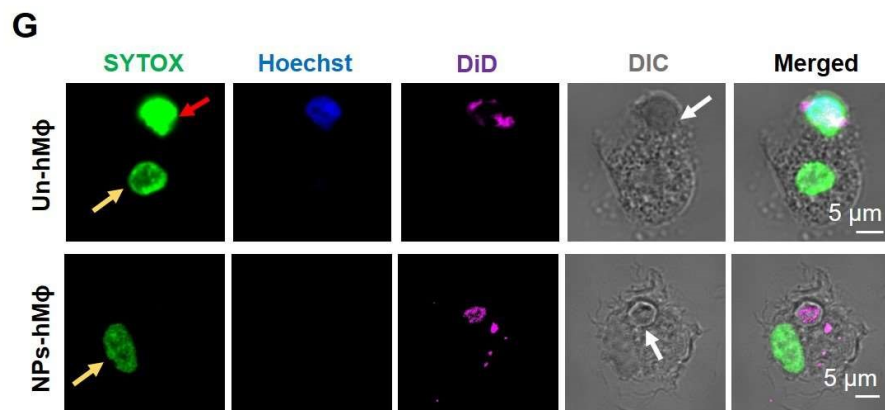


Fig. 3. (G) Digestion of the phagocytized A375 tumor cells by NPs-hMΦ or Un-hMΦ. The macrophages were cocultured with Hoechst 33342 (nucleus, blue) and DiD (membrane, magenta) dual-labeled A375 cells at a ratio of macrophages to tumor cells 1:2 for 1 h. After 2 h, the cells were fixed with 4% paraformaldehyde and counterstained with SYTOX (nuclei, green). Red arrow, the nucleus of the phagocytized tumor cell; yellow arrows, the nuclei of the macrophages; white arrows, differential interference contrast (DIC) images of the phagocytized tumor cells.

Figure 3H, it is not clear why CD8 T cells are getting activated. Which are the antigens that are presented in MHC-I? Why are these CD8 T cells recognizing specially these antigens? Is this activation mediated by antigen recognition?

RE: Thank you for this comment. We apologize for bringing this confusion. Macrophages are recognized as professional antigen-presenting cells capable of cross-presenting phagocytized tumor antigens via MHC I to activate CD8⁺ T cells^{4,5}. To test such capability, both CD14⁺ monocytes and CD8⁺ T cells were collected from the same healthy donor. NPs-hMΦ or Un-hMΦ prepared from the CD14⁺ monocytes were co-incubated with A375 tumor cells for 24 h to allow the phagocytosis of the tumor cells and the tumor antigen processing. Then, these macrophages were co-cultured with the HLA-matched CD8⁺ T cells, enabling the cross-presentation of tumor antigens to prime CD8⁺ T cell activation. We have added the above description to **Page 10, Line 216-221**.

In short, the antigens presented by the macrophages were derived from the whole tumor cells. The recognition of these antigens by CD8 T cells for activation were HLA-dependent because both NPs-hMΦ and CD8⁺ T cells were from the same donor.

The single cell RNA sequencing in Figure 4 seems of interest. However, the authors should better explain what comparisons they are referring to in their analysis. Also, groups and n values should be better explained.

RE: Thank you for this constructive comment. In Fig. 4B, six human A375 tumors were pooled for generation single cells for scRNA-seq analysis. In Fig. 4C and D, $n = 9650$ is referred to as a total of 9650 single cells from the tumors for the scRNA-seq analysis. In Fig. 4E, the comparison was made between CAFs versus other cell types, including mouse-derived neutrophils, macrophages and endothelial cells ($n = 1955$ for CAFs, $n = 2725$ for other cells). In Fig. 4F, the displayed stiffness associated ECM genes were from the pathway of ECM-receptor interaction in Fig. 4E. So, the data in Fig. 4E had the same comparison and n value as Fig. 4E. In Fig. 4G and H, $n = 4680$ was for TME cells, including mouse-derived neutrophils, fibroblasts, endothelial cells and macrophages. $n = 4485$ was for A375 tumor cells. $n = 485$ was for the transferred NPs-MΦ. In Fig. 4I, the correlation analysis was made in the phagocytosis checkpoint pair between calreticulin (CALR) in the tumor cells and low lipoprotein receptor-related protein 1 (LRP1) in the remaining NPs-hMΦ. $n = 4485$ was for the A375 tumor cells. $n = 485$ was for the transferred NPs-MΦ. We have added the description to the Fig. 4 legend in **Page 34, Line 789-804**.

Figure 5D, the authors should change the color of cluster 11.

RE: Thank you for the kind suggestion. We have changed the color of cluster 11 in the revised Fig. 5D.

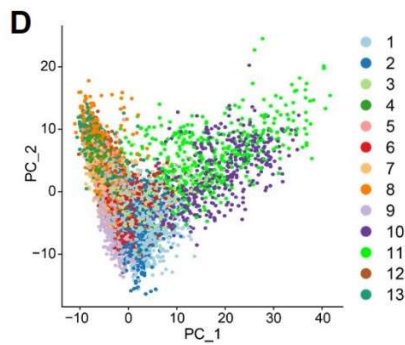


Fig. 5. (D) Principal component analysis of gene expression of the thirteen clusters based on the scRNA-seq data.

Line 253, what is "the data of bubble diagram [...]" referring to?

RE: We apologize for this confusion. The data of bubble diagram in Fig. 5G represented the expression of genes such as CDK1, MCM2, MKI67, PCNA, TOP2A, AURKA, CENPF, and CCNB1 in the different tumor cell populations.

It is not clear how the authors are designing CSC in Figure 5E. Because the dots seem to be less than those identified in clusters 10 and 11 in Figure 5D. In scRNA data analysis it is often necessary to use cluster analysis to refer to cell types, not only single marker expression.

RE: Thank you for this constructive comment. We used an unsupervised inference method to analyze the tissue stem cells based on the differentiation state according to previously reported method^{6,7}. The CytoTRACE scores were given not based on a single marker expression. The clusters 10 and 11 were attained with the highest two CytoTRACE scores among all the clusters (Fig. 5B, C). The two clusters of cells represented the lowest differentiation state and the highest degree of stemness, which can be classified into the CSC populations in tumors^{6,7}. In addition, we performed the principal component analysis (PCA) of the clusters and the results supported that the clusters 10 and 11 were positioned in close proximity to each other, but separated from the other clusters (Fig. 5D). We then consolidated the clusters 10 and 11 into one new population and

performed differential analysis with the other tumor cells. Together, the CSCs were classified by CytoTRACE scores, which was confirmed by PCA. We have revised the description in **Page 12, Line 277-292.**

In addition, the cell number of clusters 10 and 11 was 432 and 400, respectively, which gave 832 in total (Fig. 5D, Fig. R2). This number was equal to the sum of the number of PD-L1⁺ CSCs and PD-L1⁻ CSCs (832, Fig.5 E, F).

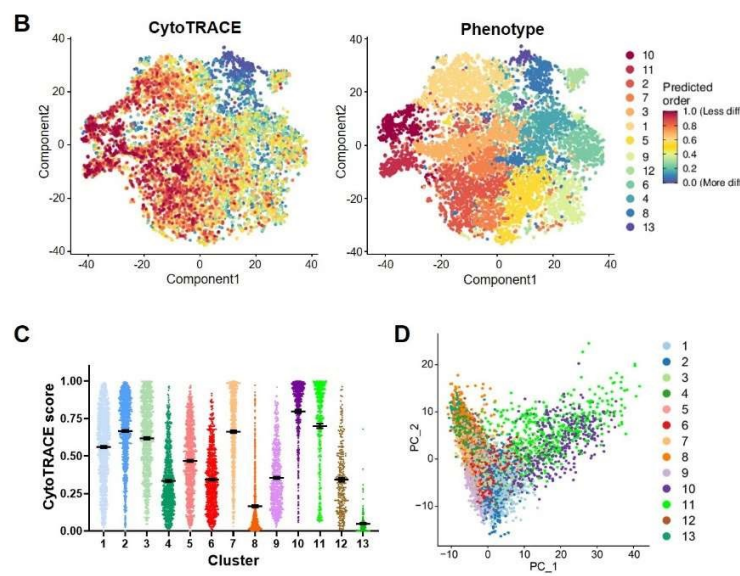


Fig. 5. (B) Heatmap for CytoTRACE scores of the scRNA-seq data overlaid on the t-SNE map. **(C)** Scatter plots of CytoTRACE score in each cluster. **(D)** Principal component analysis of gene expression of the thirteen clusters based on the scRNA-seq data.

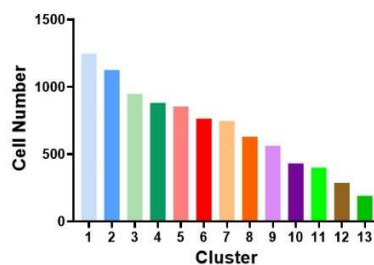


Fig. R2. Numbers of the corresponding thirteen clusters. $n = 432$ for cluster 10. $n = 400$ for cluster 11.

In Figure 6, is anti-PD-L1 reducing the therapeutic efficacy of NPs-Mo?

RE: Thank you for this constructive comment. Following your suggestion, we have evaluated the anti-tumor effect of NPs-MΦ¹ alone. The median survival time of the mice receiving NPs-MΦ¹ plus the PD-L1 blockade was not improved in comparison with that treated with the αPD-L1 alone (25 days versus 26.5 days), and significantly shorter than that with NPs-MΦ¹ alone (25 days versus 38 days) (Fig. 6A, Supplementary Table 1). These results indicated that anti-PD-L1 reduced the therapeutic efficacy of NPs-MΦ. We have revised the description in **Page 14, Line 323-327**.

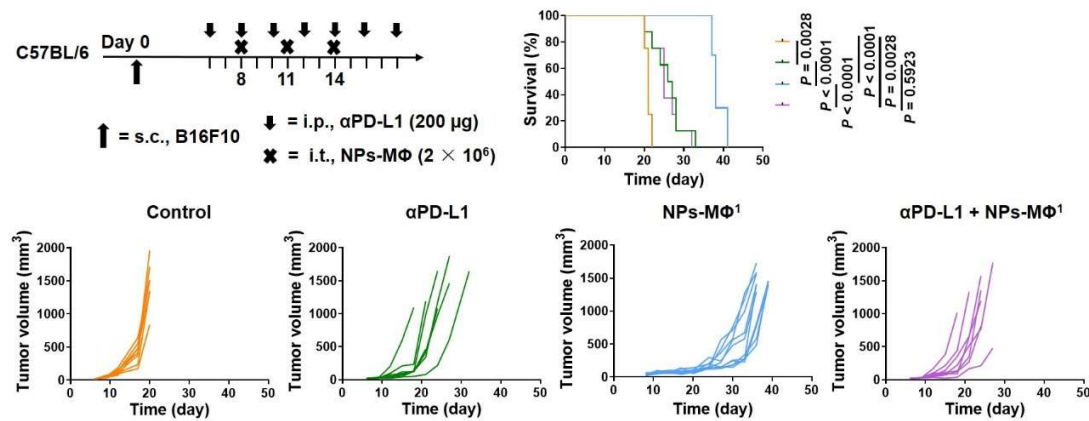


Fig. 6. (A) Combinatorial αPD-L1 and the adoptive macrophage transfer therapy (NPs-MΦ¹) for B16F10 melanoma-bearing mice. Upper, the treatment regimens. Lower, individual tumor growth curves and the corresponding Kaplan-Meier survival curves, log-rank analysis ($n = 8-10$). $P < 0.05$ is considered statistically significant.

Supplementary Table 1. The median survival times of mice bearing B16F10 tumor following αPD-L1 and adoptive macrophage therapy.

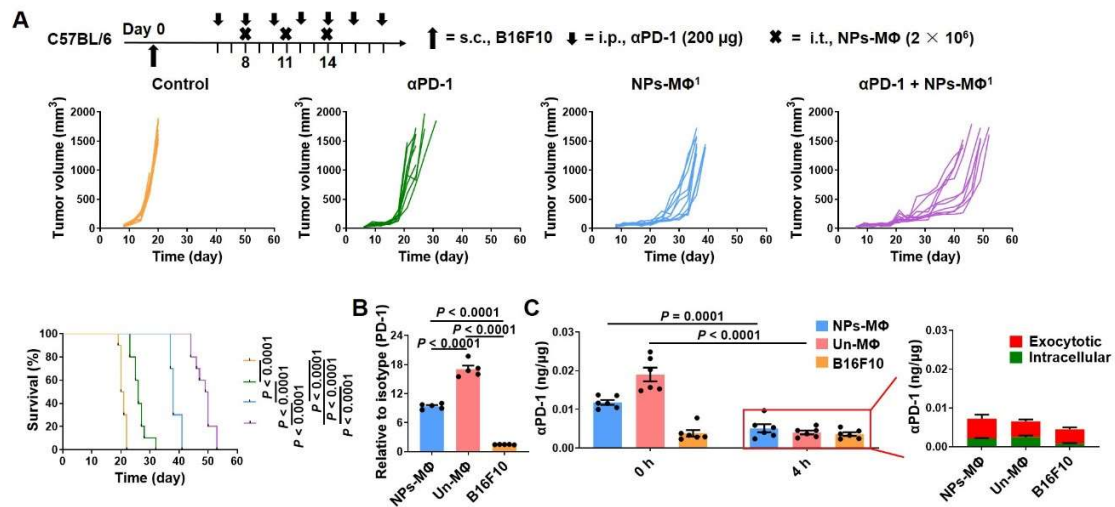
Group	Median survival time
Control	21 ($n = 8$)
αPD-L1	26.5 ($n = 8$)
NPs-MΦ ¹	38 ($n = 10$)
αPD-L1 + NPs-MΦ ¹	25 ($n = 8$)

Could the authors perform an experiment employing anti-PD1 blockade to overpass the proposed by the authors degradation of anti-PD-L1 caused by transferred macrophages?

RE: We appreciate this insightful comment. Following your suggestion, to investigate whether anti-PD-1 (α PD-1) blockade could overpass the degradation of anti-PD-L1 caused by transferred macrophages, a combinatorial α PD-1 antibody and NPs-M Φ transfer therapy was conducted. The combo treatment significantly reduced B16F10 tumor burden of mice in comparison with the α PD-1 alone or the NPs-M Φ transfer alone (Supplementary Fig. 11A). The medium survival time of mice in the combinatorial group was 49.5 days, significantly longer than that of PD-1 blockade alone of 26 days or NPs-M Φ transfer alone of 38 days (Supplementary Table 1). Compared with the Un-M Φ , the PD-1 expression by NPs-M Φ was reduced to approximately 55.02%, resulting in the lower uptake of α PD-1 by the NPs-M Φ (Supplementary Fig. 11B). After 4 h, the α PD-1 content in NPs-M Φ was reduced to approximately 43.20% of the initial level, while the α PD-1 in B16F10 cells was decreased to about 93.98% (Supplementary Fig. 11C). Despite the degradation of α PD-1 by NPs-M Φ , the tumor-bearing mice benefited from the combinatorial PD-1 blockade and NPs-M Φ therapy. However, the tumor recurrence still existed. We have added the above description to **Page 14, Line 337-348**.

Although the degradation of α PD-1 by the transferred NPs-M Φ , the PD-1 blockade plus adoptive NPs-M Φ transfer showed the enhanced antitumor effect. This may be attributed to the following reasons. First, compared to PD-L1, the expression of PD-1 by the NPs-M Φ was significantly lower, resulting in decreased internalization of the surface-bound α PD-1 (Supplementary Fig. 11B, C). Second, besides the PD-1 blockade effect on the tumor cells, the α PD-1 additionally blocked the PD-1 on CD8⁺ T cells, to activate the CD8⁺ T cells and promote the antitumor immune responses. However, the PD-1 blockade plus adoptive NPs-M Φ transfer experienced tumor relapse. Previous study reported the antibody transfer ability of TAMs through

a non-specific mechanism. The administered α PD-1 captured from the PD-1⁺ T cell surface was transferred to the PD-1⁻ TAMs by Fc γ receptor, leading to the resistance to the macrophage-mediated α PD-1 therapy⁸. Our results and the previous research data collectively indicated that immune checkpoint blockade with antibodies may not be appropriate for the adoptive macrophage transfer due to their nature of avid phagocytosis and digestion. We have added the above description to **Page 20, Line 470-482**.



Supplementary Fig. 11. Combinatorial α PD-1 and adoptive macrophage therapy for B16F10 melanoma. (A) Treatment regimens, individual tumor growth curves, and the corresponding Kaplan-Meier survival curves, log-rank analysis ($n = 10$). (B) Expression of PD-1 in the B16F10 cells, Un-M Φ or NPs-M Φ *in vitro*, relative to isotype control. One-way ANOVA with Tukey's post-test ($n = 5$). (C) Uptake and degradation of α PD-1 by Un-M Φ , NPs-M Φ or the tumor cells. The amount of α PD-1 within the cells after a 1-h incubation with α PD-1 was measured by ELISA, and designated as '0 h'. After replacement with the fresh medium and incubation for another 4 h, the total amount of α PD-1, designated as '4 h', in the red box was calculated as the sum of the intracellular and exocytotic measurements listed in the right. Two-way ANOVA with Sidak's post-test ($n = 6$). Data are expressed as mean $\pm$ s.e.m. $P < 0.05$ is considered statistically significant.

Supplementary Table 1. The median survival times of mice bearing B16F10 tumor following α PD-1 and adoptive macrophage therapy.

Group	Median survival time
Control	20.5 (n = 10)
α PD-1	26 (n = 10)
NPs-M Φ ¹	38 (n = 10)
α PD-1 + NPs-M Φ ¹	49.5 (n = 10)

Figure E, in the figure it is missing which of the shown cells belong to the spleen.

RE: Thank you for this comment. In Supplementary Fig. 13 (Fig. 7E of the original manuscript), the investigated immune cell populations, including CD11b⁺Gr-1⁺ MDSC subsets (CD11b⁺Gr-1^{high} granulocytic (G-MDSC) and CD11b⁺Gr-1^{int} monocytic (M-MDSC)), T_{reg} cells (CD4⁺CD25⁺Foxp3⁺) as well as granzyme B-positive CD8⁺ T cells (CD8⁺GranzB⁺) and effector memory T cells (CD8⁺CD44⁺CD62L⁻) all belonged to spleen.

The authors tested in vivo the combination of adoptive macrophage transfer and hyperthermia involving a laser treatment that reached 51°C at the tumor site. Previous reports suggested that mild hyperthermia prompts a measured apoptotic response. In contrast, excessive hyperthermia leads to a dysregulated and more forceful apoptotic process, engaging the complete executioner caspase repertoire (e.g. Mantso et al. Sci Rep. 2018). Moreover, heat shock proteins (HSPs) released from tumor cells have been described to promote anti-tumor immunity and to reach maximum activity at milder temperatures as well. Can the authors comment on this?

RE: Thank you for the insightful comment. Mild hyperthermia primarily provokes apoptotic responses to elicit immune response rather than to induce complete necroptotic cell death⁹. The molecular mechanisms involve attenuation of the density of tumor ECM, recruitment of the immune cells, inhibition of the TGF- β pathway, as well as the production and release of heat shock proteins (HSPs)¹⁰⁻¹³. In general, excessive hyperthermia is referred to as thermal ablation that produces tumor cell necrosis at the temperatures exceeding 50 °C for more than 6 min, while mild

hyperthermia typically ranging from 40-45 °C for time duration between 15 min and 72 h may cause cell injury instead of death^{14,15}. Regarding the hyperthermia treatment in our study, we used an 808-nm laser to irradiate the tumor of mice for 2 min. With an infrared thermal imaging camera, a temperature of 51°C was recorded on the surface of skin above the tumor at the site of laser exposure. The temperature of the subcutaneous tumor especially the deep and marginal regions of tumor was expected to be below 51°C, due to the skin tissue absorption of the light. Since the tumor recurrence was observed in the mice bearing B16F10 or A375 melanoma treated with the hyperthermia alone (Fig. 7D, F), the hyperthermia treatment under such condition may bring sublethal or partially irreversible tumor damage. Therefore, the thermally injured tumor cells are susceptible to other treatment including the phagocytosis by the adoptively transferred macrophages. The released heat shock proteins can chaperone tumor antigens to either DCs or the adoptive macrophages for antigen presentation¹⁵. We will compare the effects of different hyperthermia conditions on the immunogenic response in the future study. We have added the above description to the Discussion section in **Page 20, Line 483-499**.

Adjuvant hyperthermia seems a promising approach to overcome the resistance to the macrophage therapy. The authors could consider reproducing some of the key features observed upon monotherapy in B16F10 and A375 models also upon combination with hyperthermia. For example, is fibrosis reduced? Is the number of PD-L1⁺ CRC cells reduced?

RE: We appreciate this valuable comment. Following your suggestion, to investigate whether hyperthermia would attenuate TGF- β production in PD-L1⁺ CSCs and tumor stiffness in the relapsed B16F10 melanoma model following the NPs-M Φ transfer, the hyperthermia treatment was given following the NPs-M Φ ¹ treatment at day 25, and the tumor samples were collected at 2 days after hyperthermia treatment. The number of CD133⁺PD-L1⁺ CSCs did not change in the relapsed B16F10 tumor after hyperthermia treatment, while a significant decrease in the TGF- β level of CD133⁺PD-L1⁺ CSCs was observed (Fig. 7E, left). The adjuvant hyperthermia treatment led to a

significant reduction in the CAFs characterized by the α -SMA, as well as in the main ECM components collagen IV and collagen II in the relapsed B16F10 tumor (Fig. 7E, right). We have added the above description to **Page 16, Line 380-387**.

Hyperthermia treatment in the relapsed A375 tumor significantly reduced both the number of CD133⁺PD-L1⁺ CSCs, and the TGF- β expression in the CD133⁺PD-L1⁺ CSCs (Fig. 7G, left). Adjuvant hyperthermia treatment also resulted in a significant reduction in the CAFs and tumor ECM components in A375 tumor (Fig. 7G, right). These results demonstrated adjuvant hyperthermia may potentially overcome the resistance to the adoptive NPs-M Φ therapy by increasing the susceptibility to the macrophage phagocytosis, decreasing the TGF- β level in PD-L1⁺ CSCs and tumor stiffness. We have added the above description to **Page 17, Line 397-402**.

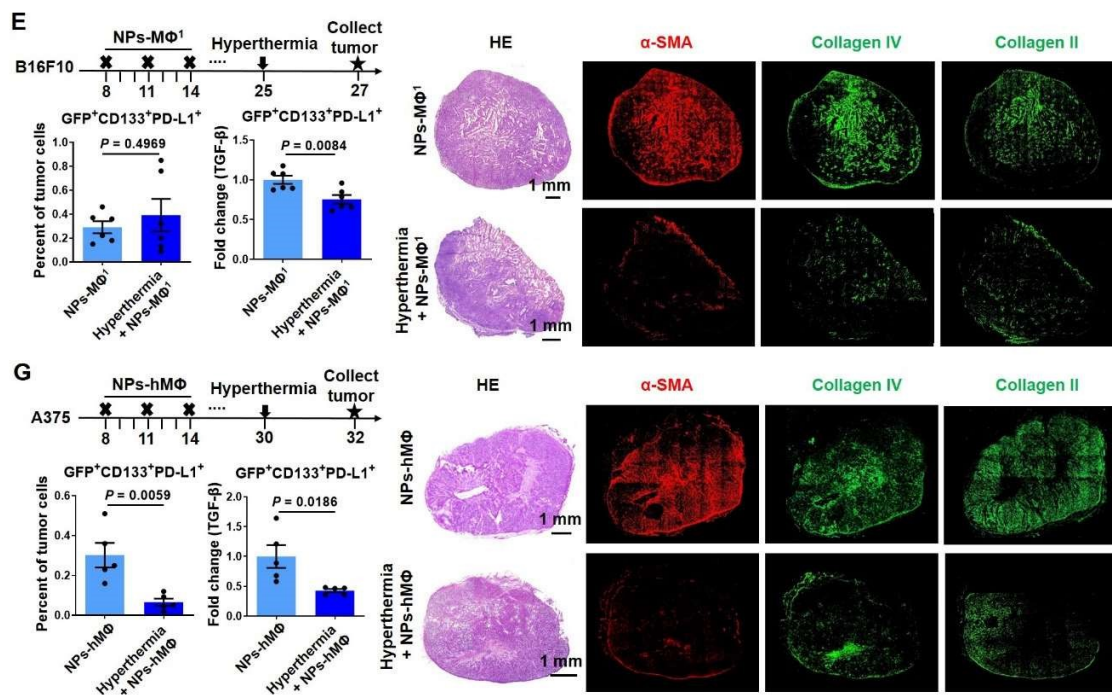


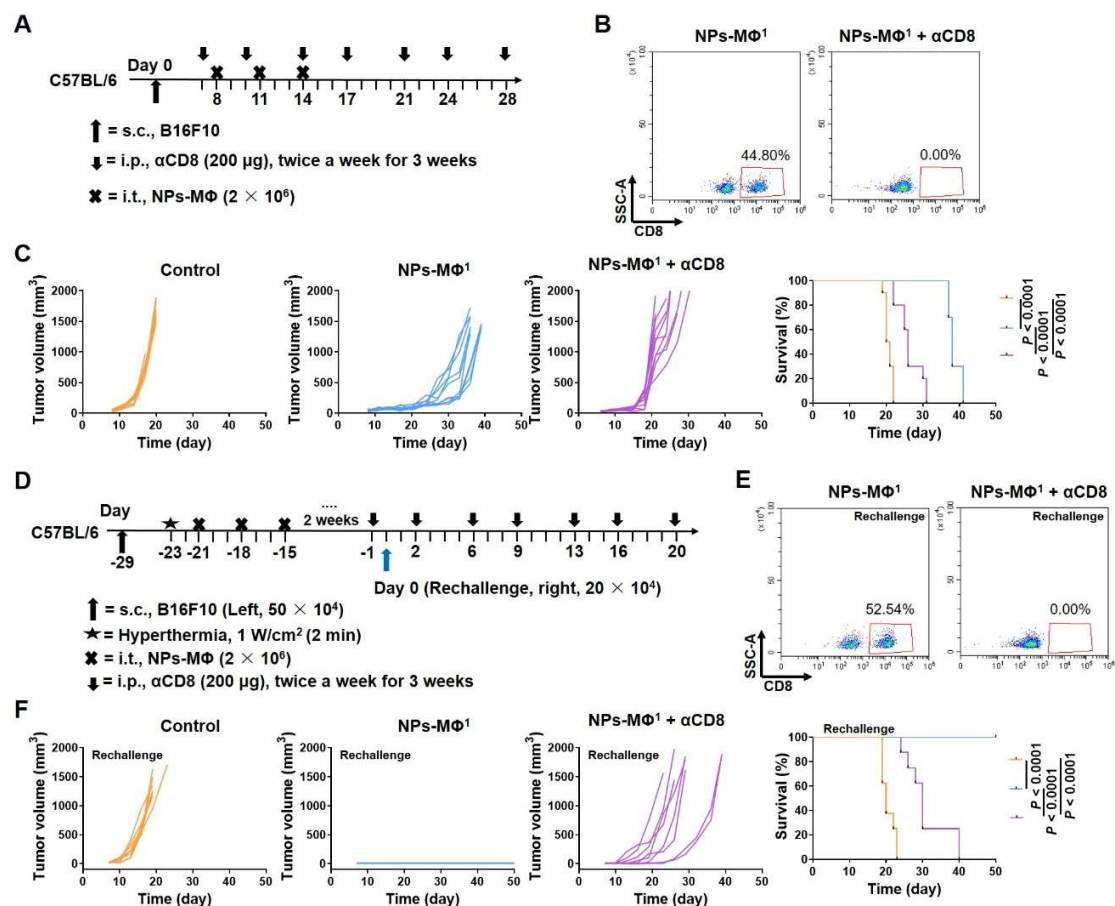
Fig. 7. (E) Hyperthermia attenuated TGF- β production in PD-L1⁺ CSCs and tumor stiffness in the relapsed B16F10 melanoma following the NPs-M Φ^1 treatment. Left, the changes in the number and the intracellular TGF- β level in GFP⁺CD133⁺PD-L1⁺ CSCs. The TGF- β level was normalized with that in the GFP⁺CD133⁺PD-L1⁺ tumor cells without hyperthermia. Unpaired two-tailed *t*-test (*n* = 6). Right, micrographs of H&E staining and immunofluorescence staining of α -SMA and

collagens. (G) Hyperthermia attenuated TGF- β production in PD-L1⁺ CSCs and tumor stiffness in relapsed A375 melanoma model following the NPs-hM Φ transfer. Left, the changes in the number and the intracellular TGF- β level in GFP⁺CD133⁺PD-L1⁺ CSCs. The TGF- β level was normalized with that in the GFP⁺CD133⁺PD-L1⁺ tumor cells without hyperthermia. Unpaired two-tailed *t*-test (*n* = 5). Right, micrographs of H&E staining and immunofluorescence staining of α -SMA and collagens. Data are expressed as mean $\pm$ s.e.m. *P* < 0.05 is considered statistically significant.

The role of CD8 T cells is not clear. It would be interesting, depleting CD8 T cells in the B16F10 model to investigate the contribution of the adaptive immunity to the therapeutic effect. In this line, the authors could also try to rechallenge the surviving mice with tumors to observe whether adaptive immunity plays a role in tumor relapse.

RE: Thank you for this constructive comment. Following your suggestion, we depleted CD8⁺ T cells using anti-CD8 antibody twice a week for 3 weeks, to elucidate the role of CD8⁺ T cells in mediating the antitumor effect and tumor rejection in mice. The efficiency of depletion was confirmed by flow cytometry analysis of peripheral blood. The results showed the depletion of CD8⁺ T cells dramatically reduced the therapeutic efficacy of the adoptive NPs-M Φ transfer. In the cured mice following the combination therapy with hyperthermia and NPs-M Φ , the depletion of CD8⁺ T cells led to growth of the rechallenged tumor (Supplementary Fig. 14). We have added the above description to **Page 17, Line 388-393**.

These results demonstrated the crucial role of CD8⁺ T cells in the adaptive immunity elicited by the adoptive NPs-M Φ transfer.



Supplementary Fig. 14. CD8 $^+$ T cells enhanced the antitumor efficacy of the NPs-M Φ transfer. (A-C) The combinatorial antitumor effect of CD8 $^+$ T cell depletion and adoptive NPs-M Φ ^{1} -treatment. Treatment regimens (A). The percentage of CD8 $^+$ T cells in blood following the *in vivo* depletion with anti-CD8 antibody (α CD8) (B). Individual tumor growth curves and the corresponding Kaplan-Meier survival curves, log-rank analysis ($n = 10$) (C). (D-F) The combinatorial effect of CD8 $^+$ T cell depletion and adoptive NPs-M Φ ^{1} -treatment on tumor rejection. Treatment regimens (D). The percentage of CD8 $^+$ T cells in blood following the *in vivo* depletion with α CD8 (E). Individual tumor growth curves and the corresponding Kaplan-Meier survival curves, log-rank analysis ($n = 8$) (F). $P < 0.05$ is considered statistically significant.

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RESPONSE TO REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

The authors have properly addressed the issues I raised for the original version of this manuscript and I now advise acceptance of the manuscript

RE: Thank you for your constructive comments. Your suggestions and insights greatly helped us to enhance the clarity and quality of our manuscript.

Reviewer #2 (Remarks to the Author):

I congratulate the authors for the excellent revision of the manuscript. The major and minor concerns have been addressed. New in vivo experiments have been performed adding relevant results. A better explanation of key issues for clinical translation is now provided, improving the impact of the manuscript. Therefore, I recommend the manuscript for publication without further delay.

RE: We sincerely appreciate your positive comments and the time spent in reviewing our manuscript and responses. Your valuable comments remarkably helped us to improve the quality of our manuscript.

Reviewer #3 (Remarks to the Author):

The authors did not address the issues raised in the first round review satisfactorily.

RE: Thank you for your valuable concern. After taking further literature search, we have found the conflicting reports on the marker CD133. We agree with you and Reviewer #5 that CD133 may not be a reliable marker for CSCs. Having discussed with and approved by the editor, we changed the manuscript title with “**CD133⁺PD-L1⁺ cancer cells confer resistance to adoptive macrophage therapy through transforming growth factor- β pathway**”. The CD133⁺ cancer cells are not defined as the CSCs in the revision. Alternatively, we have performed additional functional assays

suggested by Reviewer #5. The results have demonstrated that CD133⁺ cancer cells exhibit some stem-like characteristics.

Reviewer #4 (Remarks to the Author):

RE: We greatly appreciate your constructive and positive comments. Your suggestions significantly helped us to improve our work.

Reviewer #5 (Remarks to the Author): with expertise in melanoma, cancer stem cells

This paper claims that a subpopulation of melanoma cancer stem cells is responsible for resistance to car-macrophage therapy. the topic would be very interesting, if the experiments would have been conducted with appropriate rational and technique. I am not an expert in macrophages neither adoptive cell transfer, so I will limit my comments to the melanoma CSC and therapeutic approach in general, rather than on details in Car therapy or thermal therapy approach.

I found the paper challenging to follow and share some of the concerns raised by other reviewers. It appears that the manuscript sometimes feels like a collection of different projects rather than a cohesive study. Moreover, the results section includes extensive discussion on CD133, which might be more appropriately placed in the introduction or discussion sections. I also agree with Reviewer 3's observation that CD133 is not a reliable marker and that the authors seem to have overlooked conflicting literature regarding their proposed marker, as well as general CSC features. They could revise their opinion about those cells being CSC (changing definition and so) and study them in relation to the therapeutic approach their propose, staying focused on the focus of the manuscript (and providing appropriate experiments to support their conclusions).

Furthermore, the study's reliance on a single melanoma cell line to support their conclusions is a significant concern.

RE: We would like to thank you for the expert comments on cancer stem cells (CSCs) and greatly appreciate your insightful suggestion on refocusing the manuscript. After taking further literature search, we have found the conflicting reports on the marker CD133. We agree with you that CD133 may not be a reliable marker for CSCs. Therefore, to define the CD133⁺ cancer cells as CSCs may not be appropriate in our study.

Following your suggestion and having discussed with and approved by the editor, we changed the manuscript title with “**CD133⁺PD-L1⁺ cancer cells confer resistance to adoptive macrophage therapy through transforming growth factor- β pathway**”. The CD133⁺ cancer cells are not defined as the CSCs in this revision. Alternatively, we have performed additional functional assays suggested by you. The results have demonstrated that CD133⁺ cancer cells exhibit some stem-like characteristics. Since our major findings in this study are the discovery of the CD133⁺PD-L1⁺ cancer cells responsible for the resistance to adoptive macrophage therapy through the TGF- β pathway, and adjuvant thermal therapy as an effective strategy to counteract the resistance, we think that the claim of CD133⁺PD-L1⁺ cancer cells with some stem-like features, instead of CD133⁺PD-L1⁺ CSCs, will be more rigorous and less controversial, and not dampen the conclusion or novelty. Accordingly, we have restructured the manuscript to emphasize the resistance role of CD133⁺PD-L1⁺ cancer cells. The current results from mouse B16F10 and human A375 melanoma cell models support this conclusion. Given the conflicting literature report on CD133 as a CSC marker, and the CD133⁺ cancer cells not defined as CSCs in the revised manuscript, the discussion on CD133 marker in the Results section (Page 7, Line 139-144 of the original manuscript) has been removed. Instead, the results of the function assays of the CD133⁺ cancer cells have been added to **Page 7, Line 149-170**.

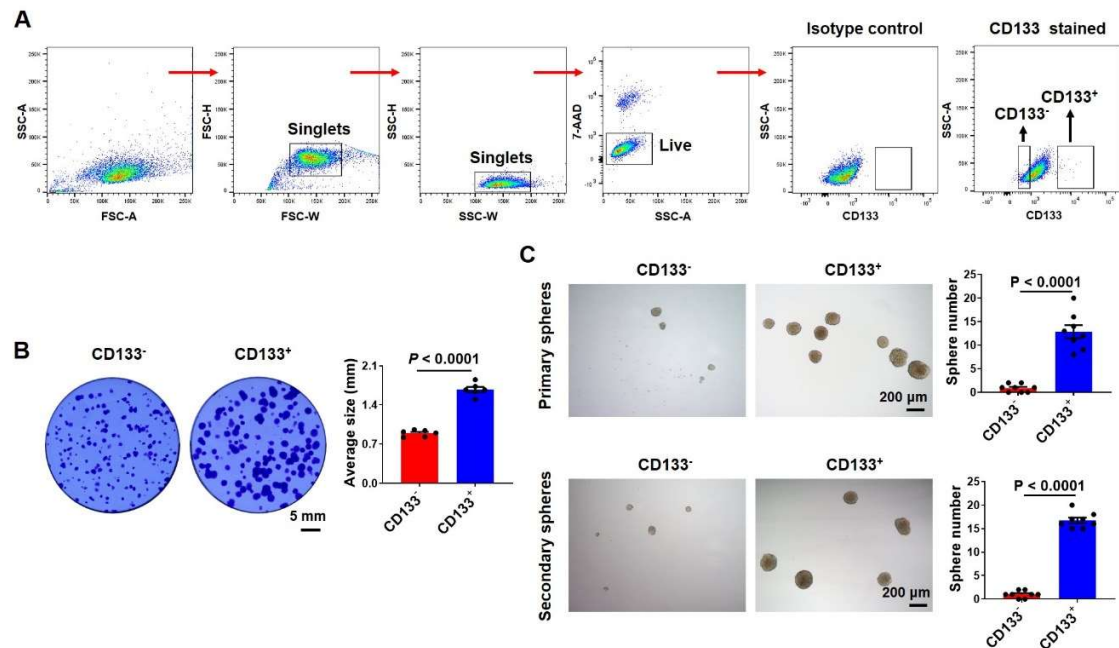
Specifically my main concerns are:

1. CD133 simply can't be used as a CSC marker, particularly for cell lines if not supported by functional studies to claim that those cells are true CSCs. In CSC community, there is overall consensus about the necessity of functional assays to claim that a cell is a CSC.

Additionally, there are several important elements missing from the characterization of these cells (whether they express PDL1 or not):

a. The manuscript lacks clonogenic assays and/or self-renewal assays in stem cell medium.

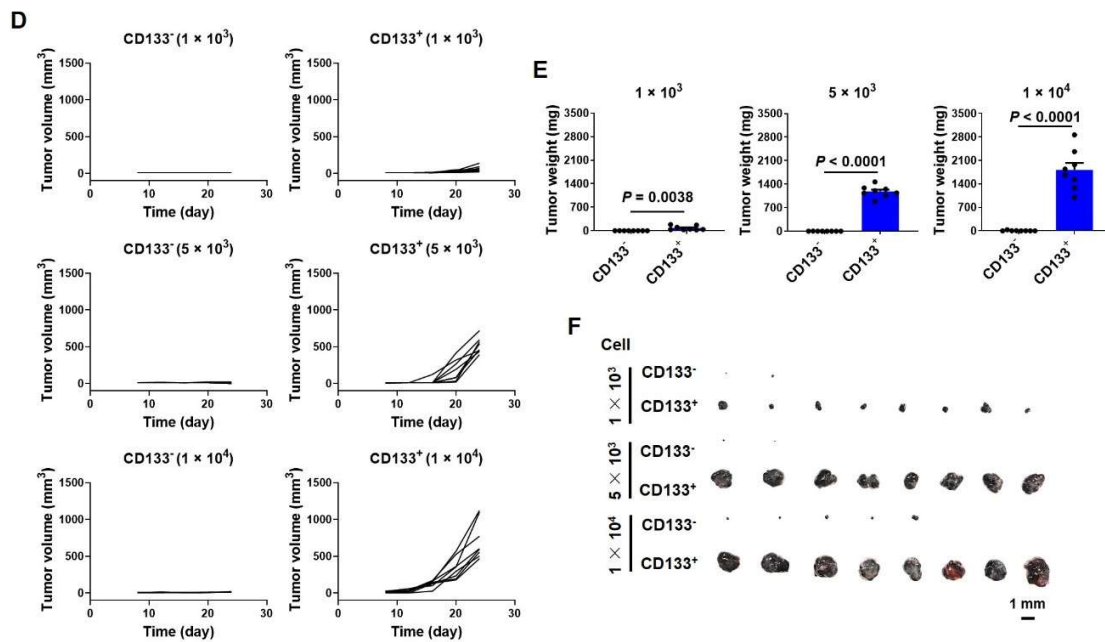
RE: Thank you for this constructive suggestion. Following your suggestion, we sorted CD133⁺ and CD133⁻ B16F10 cancer cells, respectively, for clonogenic assays and self-renewal assays (Supplementary Fig. 5A). In clonogenic assays, the CD133⁺ cancer cells were observed to form significantly larger colonies than the CD133⁻ cancer cells (Supplementary Fig. 5B). In the self-renewal assays, the sizes of primary tumorspheres from the CD133⁺ cancer cells were significantly larger than those from the CD133⁻ cancer cells (Supplementary Fig. 5C, upper). Furthermore, we passaged the single cells derived from the primary spheres to culture the secondary tumorspheres. The sizes of the secondary tumorspheres from of the CD133⁺ cancer cells were also significantly bigger than those from the CD133⁻ cancer cells (Supplementary Fig. 5C, lower). We have added the above results to **Page 7, Line 149-157**. These results demonstrated that the CD133⁺ B16F10 cancer cells possess a greater capacity for clonogenicity and tumorsphere formation compared to the CD133⁻ cancer cells.



Supplementary Fig. 5. CD133⁺ B16F10 melanoma cancer cells exhibit some stem-like features. (A) FACS gating strategy for sorting of CD133⁺ and CD133⁻ fractions. SSC-A, side scatter area; FSC-A, forward scatter area; FSC-H, forward scatter height; FSC-W, forward scatter width; SSC-H, side scatter height; SSC-W, side scatter width. (B) Clonogenic assay. The sorted CD133⁺ and CD133⁻ cancer cells were seeded in 6-well plates at a density of 200 cells per well for 8 days, respectively. The average size of colonies was quantified by Nano Measurer software. Unpaired two-tailed *t*-test (*n* = 6). (C) Sphere formation assays. The sorted CD133⁺ and CD133⁻ cancer cells were cultured in 24-well ultra-low-adherent plates at a density of 500 cells per well for 10 days, respectively. Spheres with a diameter over 70 μm were counted. For secondary tumorsphere formation, the primary tumorspheres were dissociated into single cells, following by replated in 24-well ultra-low-adherent plates at 500 cells per well. Unpaired two-tailed *t*-test (*n* = 8). Data are expressed as mean ± s.e.m. *P* < 0.05 is considered statistically significant.

b. There is no assessment of tumor initiation potential.

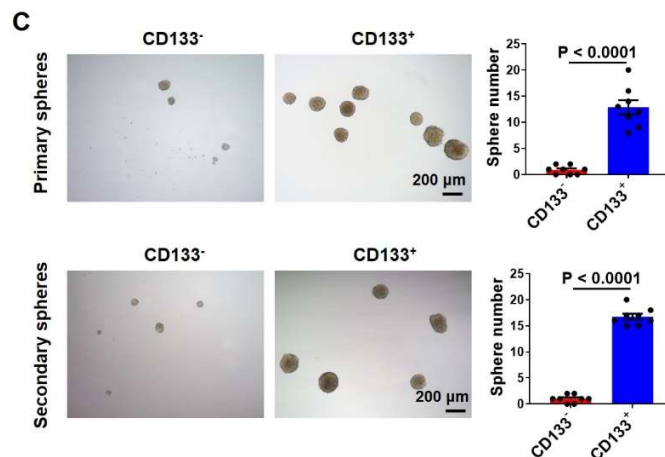
RE: We appreciate this valuable comment. Following your suggestion, the sorted CD133⁺ and CD133⁻ B16F10 cancer cells were subcutaneously (s.c.) injected into the left flank of C57BL/6 mice, respectively. The mice injected with the CD133⁻ cancer cells did not generate palpable tumors at the injection dose of 1×10^3 , 5×10^3 or 1×10^4 tumor cells (Supplementary Fig. 5D). By contrast, the tumor growth was observed in the mice injected with the CD133⁺ cancer cells at the dose of 5×10^3 or 1×10^4 tumor cells. The growth of tumor was confirmed by the tumor weight and photographs following the resection (Supplementary Fig. 5E, F). We have added the above results to **Page 7, Line 157-163**. These findings showed the significantly enhanced tumor initiation potential of CD133⁺ B16F10 cancer cells than that of the CD133⁻ counterparts.



Supplementary Fig. 5. (D-F) *In vivo* tumorigenicity of CD133⁺ B16F10 cancer cells. (D) Individual tumor growth curves of mice s.c. injected with the sorted CD133⁺ or CD133⁻ cancer cells at the dose of 1×10^3 , 5×10^3 or 1×10^4 cells. **(E, F)** The weights (E) and photographs (F) of the dissected tumors in (D) at day 24 post injection. Unpaired two-tailed *t*-test ($n = 8$). Data are expressed as mean $\pm$ s.e.m. $P < 0.05$ is considered statistically significant.

c. Self-renewal assays are absent.

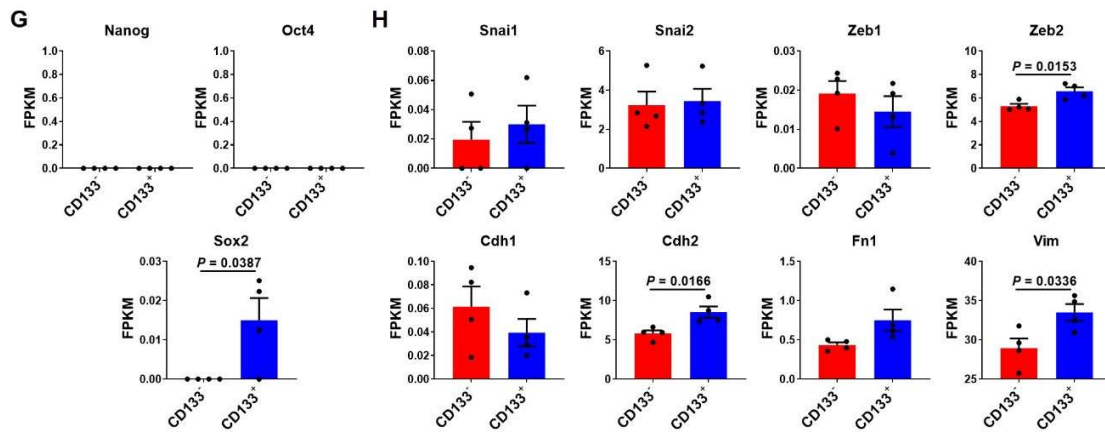
RE: Thank you for the constructive suggestion. Following your suggestion, we have performed self-renewal assays of the tumorsphere formation. The sizes of primary tumorspheres from the CD133⁺ cancer cells were significantly larger than those from the CD133⁻ cancer cells (Supplementary Fig. 5C, upper). Furthermore, we passaged the single cells derived from the primary spheres to culture the secondary tumorspheres. The sizes of the secondary tumorspheres from of the CD133⁺ cancer cells were also significantly bigger than those from the CD133⁻ cancer cells (Supplementary Fig. 5C, lower). We have added the above results to **Page 7, Line 152-157**. These results demonstrated that the CD133⁺ B16F10 cancer cells possess a greater capacity for tumorsphere formation compared to the CD133⁻ cancer cells.



Supplementary Fig. 5. (C) Sphere formation assays. The sorted CD133⁺ and CD133⁻ cancer cells were cultured in 24-well ultra-low-adherent plates at a density of 500 cells per well for 10 days, respectively. Spheres with a diameter over 70 μm were counted. For secondary tumorsphere formation, the primary tumorspheres were dissociated into single cells, following by replated in 24-well ultra-low-adherent plates at 500 cells per well. Unpaired two-tailed *t*-test (*n* = 8). Data are expressed as mean ± s.e.m. *P* < 0.05 is considered statistically significant.

d. There are no gene expression profiles provided to support their CSC features, such as Nanog, Oct4, or EMT signatures.

RE: Thank you for this constructive comment. Following your suggestion, we have conducted the microRNA-seq analysis of the sorted CD133⁺ and CD133⁻ B16F10 cancer cells, respectively. The expression of CSC marker Sox2 was significantly higher in the CD133⁺ cancer cells compared to that in the CD133⁻ cancer cells, while the other two markers Nanog and Oct4 were not significantly changed (Supplementary Fig. 5G). In addition, the gene profiles of epithelial-to-mesenchymal transition (EMT) signatures such as Zeb2, Cdh2 and Vim were significantly increased in the CD133⁺ cancer cells than the CD133⁻ cancer cells, while Snai1, Snai2, Zeb1, Cdh1 and Fn1 were not significantly changed (Supplementary Fig. 5H). We have added the above results to **Page 7, Line 163-168**. Collectively, the CD133⁺ B16F10 cancer cells exhibited some CSC features. This result also supported that to define the CD133⁺ B16F10 cancer cells as CSCs may not be rigorous since not all the above CSC markers were upregulated.



Supplementary Fig. 5. (G-H) Quantitative analysis of the gene expression profiles associated with the CSC features (G) or EMT signatures (H). FPKM, fragments per kilobase of transcript per million mapped reads. Unpaired two-tailed *t*-test ($n = 4$). Data are expressed as mean $\pm$ s.e.m. $P < 0.05$ is considered statistically significant.

e. The source and methodology behind the "fold increase" markers indicated in multiple figures are not explained. Are these results from flow cytometry or gene expression? Additionally, it would be beneficial to use flow cytometry for cytokines or ELISA for production.

RE: We apologize for this confusion. All the “fold change” related to the analysis of CD133 cells in Figs. 2D, 2G, 7E, 7G and Supplementary Fig. 8D are the results from flow cytometry. We have described the methods in the corresponding figure legends. Specially, the expression of TGF- β , IL-10 or VEGF- α were normalized with the corresponding GFP⁺CD133⁺PD-L1⁻ cancer cells (Fig. 2D, **Page 29, Line 710-711**). The TGF- β level was normalized with that in the GFP⁺CD133⁺PD-L1⁻ cancer cells of tumor in the early stage (Fig. 2G, **Page 29, Line 719-720**). The TGF- β level was normalized with that in the GFP⁺CD133⁺PD-L1⁺ cancer cells without hyperthermia (Fig. 7E, **Page 40, Line 841-842**; Fig. 7G, **Page 40, Line 849-850**). The expression of TGF- β , IL-10 or VEGF- α were normalized with the corresponding CD133⁺PD-L1⁻ cancer cells (Supplementary Fig. 8D, **Supplementary Information, Page 21, Line 270-271**).

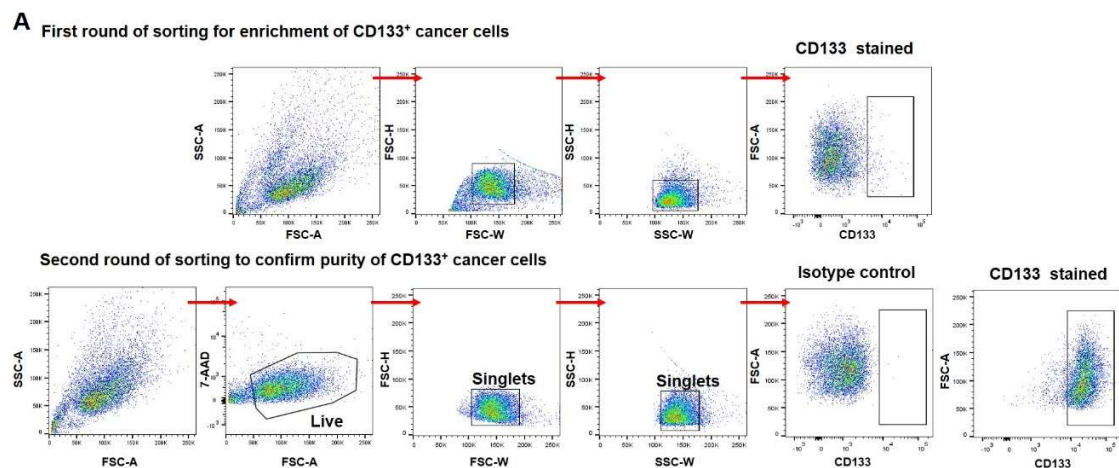
f. The statement about “greater proliferation potential in CSCs” is unclear, as it contradicts the consensus that CSCs are slow cycling.

RE: Thank you for this valuable comment. Since the CD133⁺ cancer cells are not defined as the CSCs in this revision, we have deleted the following description in the Results section of Page 7, Line 140-142 of the original manuscript. “The CD133⁺ CSCs characterized by increased proliferation capacity and tumorigenic potential were functionally distinct from bulk tumor population, contributing to tumor resistance and poor prognosis in various malignancies including melanoma³⁶⁻⁴⁰.”

Since the additional experiments have shown the CD133⁺ cancer cells with some stem-like features including increased self-renewal and tumorigenic potential (Supplementary Fig. 5), we revised the statement that “In summary, these results demonstrated that the CD133⁺ cancer cells expressed some CSC markers, and presented some stem-like functions including self-renewal capacity and tumorigenic potential” in the Results section (**Page 8, Line 168-170**).

g. It is also unclear how the cells in Supplementary Figure 6 are shown to be 100% CD133 positive.

RE: We apologize that we did not describe the experimental procedure clearly. We have reanalyzed the CD133 gating strategy data related to the FACS sorting process. As shown in Supplementary Figure 8A, the CD133⁺ cells were first sorted by flow cytometry following the CD133 immunostaining. These sorted CD133⁺ cells were then subjected to a second round of sorting to confirm their purity. The result showed approximately 100% of the CD133 positivity rate. We have added the above description to the legend of Supplementary Fig. 8A in **Supplementary Information, Page 20, Line 260-266**.



Supplementary Fig. 8. (A) FACS gating strategy in the first round of sorting for enrichment of the CD133⁺ B16F10 cancer cells (Upper) and second round of sorting to confirm the purity of CD133⁺

B16F10 cancer cells (Lower). Doublets were excluded on the basis of FSC and SSC. The 7-AAD⁻ live cancer cells were selected. The CD133⁺ cells were first sorted by flow cytometry following the CD133 immunostaining. These sorted CD133⁺ cells were then subjected to a second round of sorting to confirm their purity, showing approximately 100% of the CD133 positivity rate.

2. I find it challenging to understand the rationale for selecting these cells due to several points:

a. It is unclear whether this population increases after therapy. The markers do not appear in the gene expression analysis, and the flow cytometry data in Figure 1 is not convincing. Additionally, the number of mice used and other experimental details are not clearly presented.

RE: Thank you for this valuable comment. As shown in Figure 2C, the number of CD133⁺PD-L1⁺ cancer cells in tumor samples following the NPs-MΦ¹ treatment at day 35 were significantly increased compared with that without treatment at day 8 or day 16. Therefore, we conclude that the population of CD133⁺PD-L1⁺ cancer cells increase after the therapy.

Since Figure 1 did not include the flow cytometry data, we assume that the flow cytometry data mentioned here is Figure 2C. This experiment was performed to measure the protein levels of both CD133 and PD-L1 expression in cancer cells by flow cytometry using B16-GFP melanoma model. Tumors were collected from the NPs-MΦ¹-treated mice at day 35. Mice before the treatment at day 8, and without treatment at day 16 were used as control, respectively. Of note, in the mice without treatment at day 16, the tumor volume reached the similar size (~500 mm³) to that of mice following the NPs-MΦ¹ treatment at day 35. We have added the above detailed description to the legend of Fig. 2C, **Page 29, Line 703-707**. The data were analyzed from 5 repeated mice each group, and the images on the right of Figure 2C are the representative dot plots of the flow cytometric analysis. The data of the other 4 mice per group were added to Supplementary Fig. 6 in **Supplementary Information**.

In a parallel experiment, CD133⁺PD-L1⁺ and CD133⁺PD-L1⁻ cancer cells were additionally sorted and subjected to microRNA-seq analysis, respectively (Supplementary Fig. 7A). The higher expression of PD-L1 gene (Cd274) in CD133⁺PD-L1⁺ cancer cells was measured compared with that in CD133⁺PD-L1⁻ cancer cells (Supplementary Fig. 7D). However, the gene Cd133 was not detected in either CD133⁺PD-L1⁺ or CD133⁺PD-L1⁻ cancer cells. This could be attributed to the low signals since the ultra-low input RNA samples were used in the microRNA-seq. Another possible explanation is that there was no relationship observed between the CD133 protein evaluated by flow cytometry and mRNA expression level due to a complex post-transcriptional regulation of the membrane expression of CD133¹. Although the gene expression of Cd133 was not detectable by microRNA-seq, the expression of CD133 in protein level has been confirmed not only in the mouse melanoma model (Fig. 2, Supplementary Fig. 6-7), but also in the melanoma patient samples especially the patients with metastasis tumors (Fig. 5H, Supplementary Fig. 12).

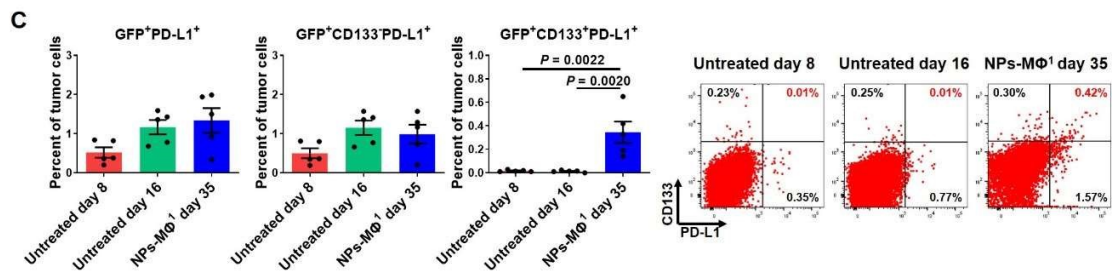
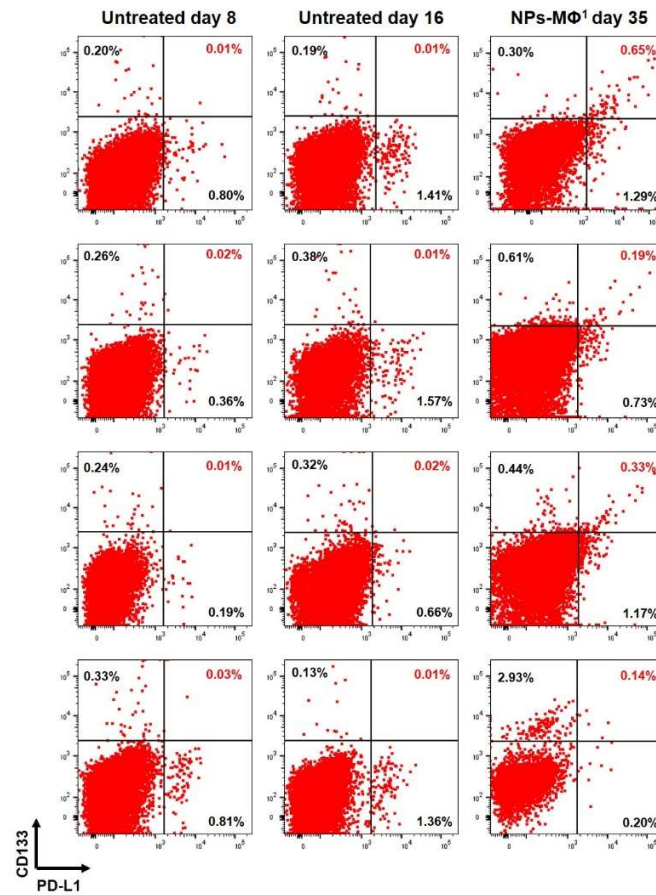


Fig. 2. (C) Identification of CD133⁺PD-L1⁺ from the B16-GFP cancer cells of mice receiving the adoptive NPs-MΦ therapy. Tumors were collected from the NPs-MΦ¹-treated mice at day 35. Mice before the treatment at day 8, and without treatment at day 16 were used as control, respectively. Of note, in the mice without treatment at day 16, the tumor volume reached the similar size (~500 mm³) to that of mice following the NPs-MΦ¹ treatment at day 35. One-way ANOVA with Tukey's post-test ($n = 5$). Right, representative dot plots of the flow cytometric analysis. Data are expressed as mean $\pm$ s.e.m. $P < 0.05$ is considered statistically significant.

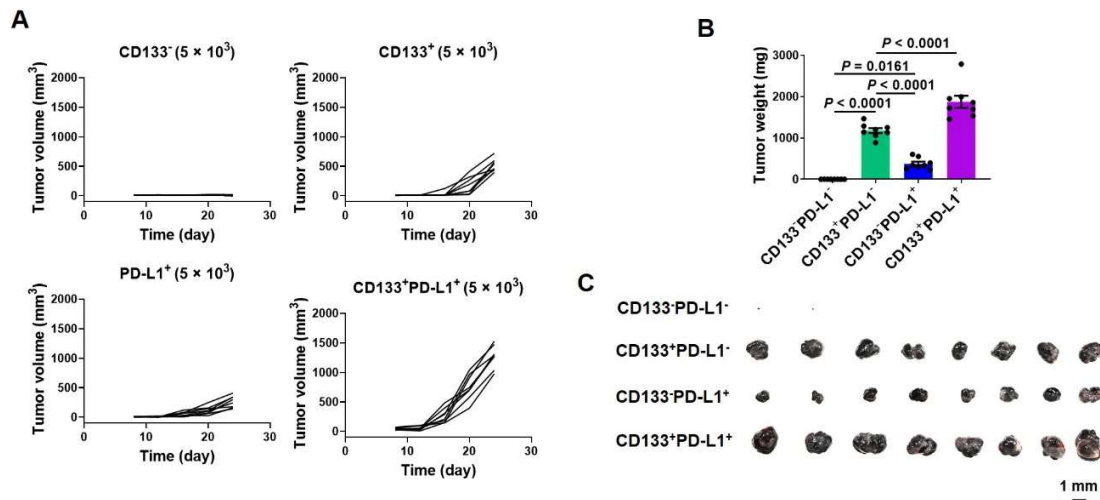


Supplementary Fig. 6. Individual dot plots of the other four samples of each group by the flow cytometric analysis in Fig. 2C.

b. It is not clear whether this subpopulation can regenerate the tumor after therapy.

RE: Thank you for the insightful comment. To assess the tumor generation capability of the CD133⁺PD-L1⁺ cancer cells, we induced and sorted the CD133⁺PD-L1⁺, CD133⁺PD-L1⁻, CD133⁻PD-L1⁺ and CD133⁻PD-L1⁻B16F10 cancer cells *in vitro*, respectively. Each subpopulation was s.c. injected into the left flank of mice. The results demonstrated that the tumor grew the fastest in the CD133⁺PD-L1⁺ cancer cells groups among all the four groups, resulting in the highest tumor

weights at day 24 post tumor cell inoculation (Supplementary Fig. 9). We have added the above results to **Page 9, Line 211-215**.



Supplementary Fig. 9. Tumorigenesis of CD133⁺PD-L1⁺ B16F10 cells in C57BL/6 mice. (A) Individual tumor growth curves of mice following s.c. injection of 5×10^3 of CD133⁻PD-L1⁻, CD133⁺PD-L1⁻, CD133⁻PD-L1⁺ or CD133⁺PD-L1⁺ B16F10 cancer cells into the left flank of mice. (B, C) The weights (B) and photographs (C) of the dissected tumors in (A). One-way ANOVA with Tukey's post-test ($n = 8$). Data are expressed as mean $\pm$ s.e.m. $P < 0.05$ is considered statistically significant.

c. It is uncertain whether these cells are found in patients before, during, or after treatment. Moreover, it is not specified whether these cells are present in primary versus metastatic tumors, high-grade versus low-grade tumors, or pre- versus post-relapse conditions.

RE: Thank you for this insightful comment. Following your suggestion, we have checked the medical history of the patients and reanalyzed the specimens. As shown in Supplementary Table 3, all the six clinical melanoma specimens were classified as high-grade tumors. Among them, the specimens #1 and #2 were primary tumors at early stage before treatment in the pre-relapse

conditions. In these samples, a population of PD-L1⁺ cells but with CD133-negative staining were observed (Fig. 5H, upper row, green arrows, Supplementary Figure 12A, B). Only a few CD133⁺PD-L1⁺ cells were detected (Fig. 5H, upper row, yellow arrows, Supplementary Figure 12A, B). The specimens #3-#6 were metastatic tumors at late stage after receiving various treatment in the post-relapse conditions. Compared to the specimens #1 and #2, the CD133⁺PD-L1⁺ cells in the tumor of specimens #3-#6 were significantly increased (Fig. 5H, lower row, yellow arrows, Supplementary Figure 12C-F), indicating that the CD133⁺PD-L1⁺ cells were increased in the metastatic melanoma. We have added the above description to the Results section in **Page 13, Line 306-316**.

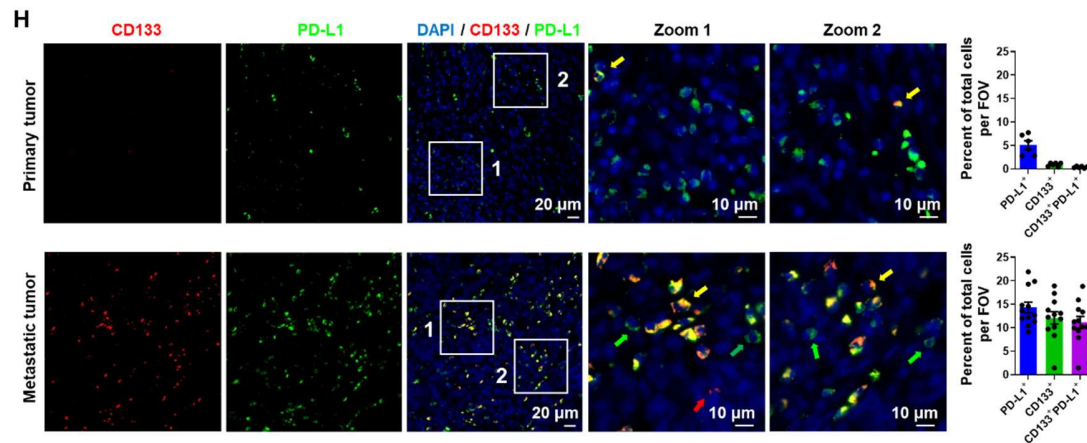


Fig. 5. (H) Representative immunofluorescence micrographs of the expression of CD133 (red) and PD-L1 (green) in primary or metastatic tumor of human melanoma specimens. Blue, cell nuclei. Right, the number of CD133⁺, PD-L1⁺ or CD133⁺PD-L1⁺ cells within the field of view (FOV) was analyzed by Fiji ImageJ software. Data are presented from 2 primary melanoma specimens and 4 metastatic melanoma specimens. Three FOVs from each sample were selected for the analysis. Data are expressed as mean $\pm$ s.e.m. Yellow arrow, CD133⁺PD-L1⁺ cells; green arrows, CD133⁻PD-L1⁺ cells; red arrows, CD133⁺PD-L1⁻ cells. The individual fluorescence micrographs of each specimen were presented in Supplementary Figure 12.

[Redacted]

d. It is also unclear whether these cells are specifically spared by CAR-macrophages or how they survive and drive relapse. What happens if the therapy cycle is repeated?

RE: Thank you for this constructive comment. Our data showed that the CD133⁺PD-L1⁺ cancer cells appeared after one cycle of the adoptive NPs-MΦ therapy (i.e. 3 repeated doses) in the B16F10 model, while the untreated tumor nearly did not have this population of cancer cells (Fig. 2C). Moreover, the CD133⁺PD-L1⁺ cancer cells resisted macrophage phagocytosis and CD8⁺ T cell-killing, contributing to tumor survival and relapse following the NPs-MΦ therapy (Fig. 2H, I). To exclude the dose effect on the adoptive NPs-MΦ therapy, we increased the NPs-MΦ treatment to three cycles (i.e. 9 repeated doses in total) and found that the tumor resistance still occurred (Fig. 1B, Supplementary Table 1). Accordingly, we think it might not be necessary to investigate the CD133⁺PD-L1⁺ cancer cells following the repeated therapy cycles since this cell population have been observed and investigated after 1-cycle treatment (3 repeated doses) at day 35 of tumor relapse stage. We hope you will be satisfied with the explanation.

2. They claim that PDL-1 therapy doesn't change the outcome of the car-macrophage transfer, so what is the rational to claim that this population is responsible for resistance/ relapse/progression?

RE: Thank you for the comment. Our results showed that the combined αPD-L1 with the adoptive NPs-MΦ transfer unable to produce a synergetic effect in treating melanoma, was ascribed to the antigen-specific degradation of αPD-L1 by the PD-L1⁺ macrophages (Fig. 6A-D) and the attritted PD-L1 blockade in the presence of TGF-β². The CD133⁺PD-L1⁺ cancer cells responsible for resistance/relapse/progression were attributed to the overexpressed TGF-β and the related signals in these cells (Fig. 2C-E). The CD133⁺ PD-L1⁺ cancer cells exhibited higher TGF-β level than CD133⁺ PD-L1⁻ cancer cells across all the stages of tumor (Fig. 2G, left). The intracellular TGF-β level in CD133⁺ PD-L1⁺ cancer cells correlated well with the increased tumor volume (Fig. 2G,

right). Moreover, the CD133⁺PD-L1⁺ cancer cells resisted macrophage phagocytosis and CD8⁺ T cell-killing, contributing to tumor survival and relapse following the NPs-MΦ therapy (Fig. 2H, I). In addition to the above collected results, the assessment of tumor initiation potential has also confirmed the tumor generation capacity of the CD133⁺PD-L1⁺ cancer cells *in vivo* (Supplementary Fig. 9). Therefore, the rational of the CD133⁺PD-L1⁺ cancer cells responsible for resistance/relapse/progression involves the TGF-β signaling pathway.

3. Why don't they silence CD133 (they cite ref 45 for indicating CD133 as target) and see the change in therapy outcome? That would be more interesting and linking the therapy to their "CSC" section.

RE: We appreciate this comment. In our study, we claim that the CD133⁺PD-L1⁺ cancer cells are responsible for the resistance to adoptive macrophage therapy through TGF-β signaling pathway. Silencing CD133 may not be specific for the CD133⁺PD-L1⁺ cancer cells. After searching the literature, therapeutic strategies targeting the CD133⁺ cancer cells are typically to use the anti-CD133 antibody-toxin conjugates or anti-CD133 CAR T cells³⁻⁵. However, our lab has technical difficulties in preparing such CD133 therapeutics. Alternatively, we have proved that TGF-β inhibitor (TEW-7197) improved the therapeutic efficacy of adoptive macrophage therapy (Fig. 6E, F), supporting the above claim. Since the CD133⁺PD-L1⁺ cancer cells are not defined as CSCs in this revision, and the discussion including the citation of CD133 as target in Result section of the original manuscript has been removed, we think that targeting TGF-β signaling pathway may be more suitable to prove the role of CD133⁺PD-L1⁺ cancer cells against tumor resistance.

4. Does TGFB treatment directly affect melanoma cells or the therapy works through broader/indirect effect (more CD8 cytotoxic, effect on TME)? The simple increase in TGFB is not enough to claim that that is the mechanism to target CSC.

RE: Thank you for this constructive comment. Our results have shown that TGF- β can directly increase the capability of CD133⁺PD-L1⁻ B16F10 cancer cells to resist macrophage phagocytosis and CD8⁺ T cell-killing (Fig. 2H right, I right). On the other hand, the CD133⁺PD-L1⁺ cancer cells even in absence of TGF- β have shown more effective to resist the macrophage phagocytosis than the CD133⁻PD-L1⁻ cancer cells in presence of TGF- β (Fig. 2H, purple column in the left v.s. green column in the right). Besides, the CD133⁺PD-L1⁺ cancer cells were identified to express the highest levels of TGF- β (Fig. 2D). This cell population was confirmed with the upregulated TGF- β signaling pathway (Fig. 2E). Thus, the TGF- β pathway represents an effective target for the CD133⁺PD-L1⁺ cancer cells.

In terms of the therapeutic efficacy evaluation, our data demonstrated that the TGF- β signaling inhibitor enhanced the antitumor effect of NPs-M Φ (Fig. 6E). Besides the direct effect on tumor cells, the TGF- β signaling inhibitor reversed the immunosuppressive TME, evidenced by the significant reduction of the inhibitory cell subsets of regulatory T (T_{reg}) cells and myeloid-derived suppressor cells (MDSCs), as well as increased the infiltration of IFN- γ -producing and GranB-producing CD8⁺ T effector cells into tumor (Fig. 6F).

Collectively, although the existence of indirect effect of TGF- β on TME, the upregulation of TGF- β signaling pathway in the CD133⁺PD-L1⁺ cancer cells compared with CD133⁻PD-L1⁻ cancer cells suggested that TGF- β is an appropriate mechanism to target CD133⁺PD-L1⁺ cancer cells.

5. Not clear the link with stiffened matrix, relapse and thermal therapy, the matrix data re “lost” in the last part of the paper with the therapy effect, what happens after therapy to the matrix (and during therapy?)

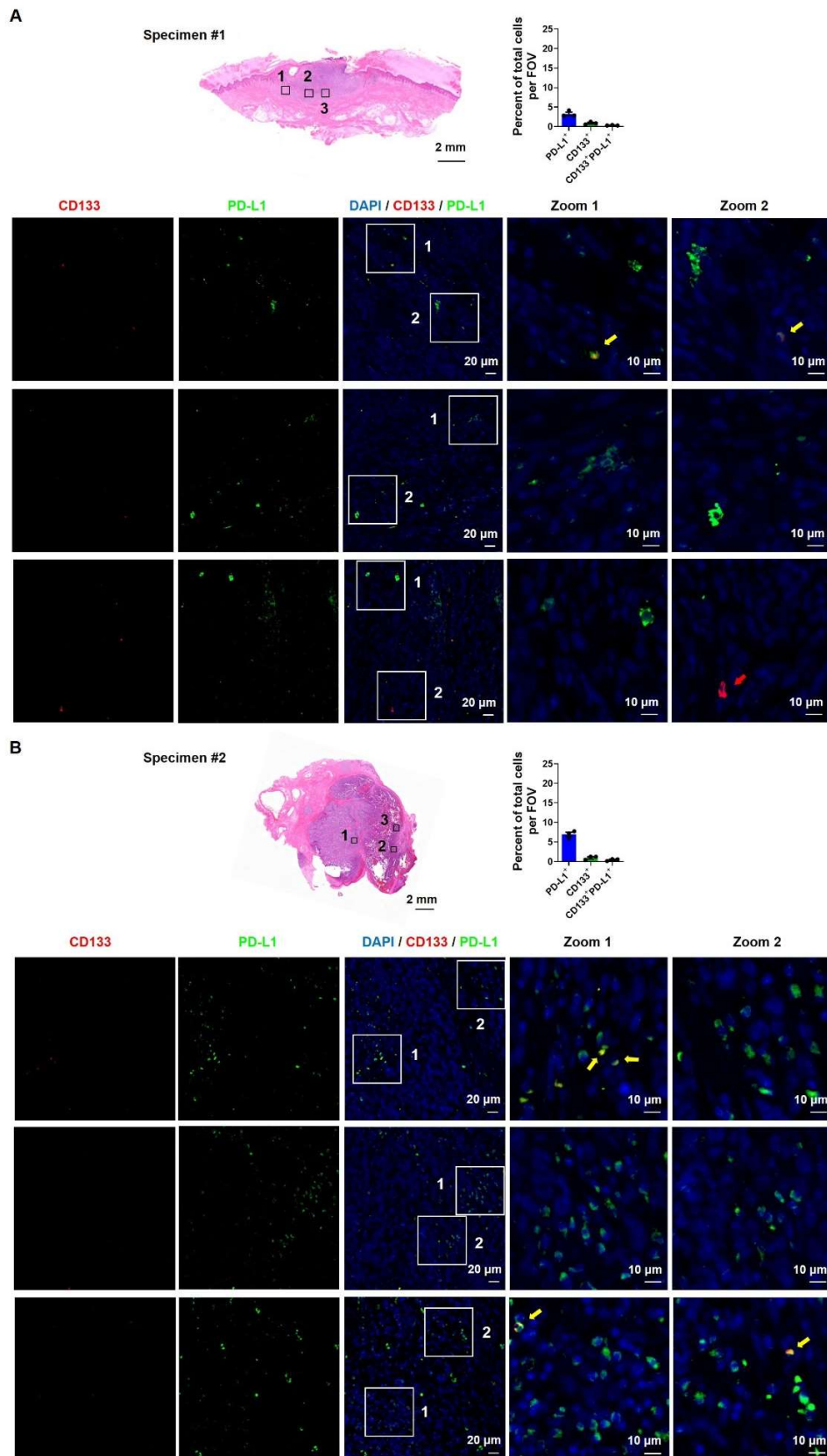
RE: We apologize that we did not describe the experimental procedure clearly. The relapsed tumor following the adoptive transfer of NPs-hMΦ exhibited the characteristics of stiffened extracellular matrix (Fig. 1D-F, Supplementary Fig. 2). Our results showed the thermal therapy improved the efficacy of the adoptive macrophage therapy, in which the hyperthermia treatment was performed 2 days before the macrophage transfer (Fig. 7D, F). To investigate whether hyperthermia attenuated tumor stiffness, we first established the relapsed tumor model with enhanced stiffness following the NPs-MΦ¹-treatment. In this condition, hyperthermia treatment was applied at the tumor relapse stage. We then evaluated the matrix of TME at 2 days after the thermal therapy (Fig. 7E, G). The results demonstrated that the thermal therapy led to a significant reduction in the cancer-associated fibroblasts characterized by the myofibroblast marker α -smooth muscle actin, as well as in the main extracellular matrix components collagen IV and collagen II in the relapsed B16F10 tumor (Fig. 7E, right) and the relapsed A375 tumor (Fig. 7G, right), respectively.

6. I have some concerns regarding the immunofluorescence (IF) on patient samples:

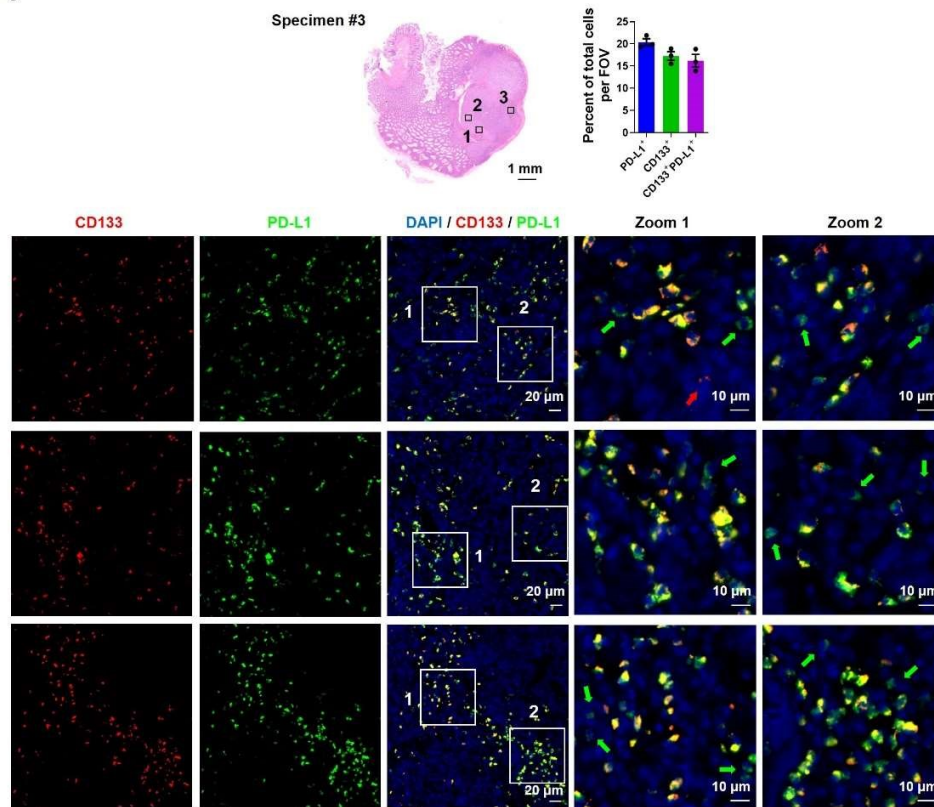
a. It appears that all patient samples show cells that are positive for both CD133 and PDL1. While some CD133 expression is weak, this consistent co-expression suggests a potential technical issue, as it is unusual to see almost no PDL1⁺ cells that are negative for CD133 (and vice versa).

RE: Thank you for this constructive comment. Our previous presentation primarily focused on the display of CD133⁺PD-L1⁺ double-positive cells, we apologize for this confusion. We have reanalyzed the specimens. In the primary melanoma, the majority of PD-L1⁺ cells were negative for CD133 expression (Fig. 5H, Supplementary Fig. 12A, B). By contrast, in the metastatic

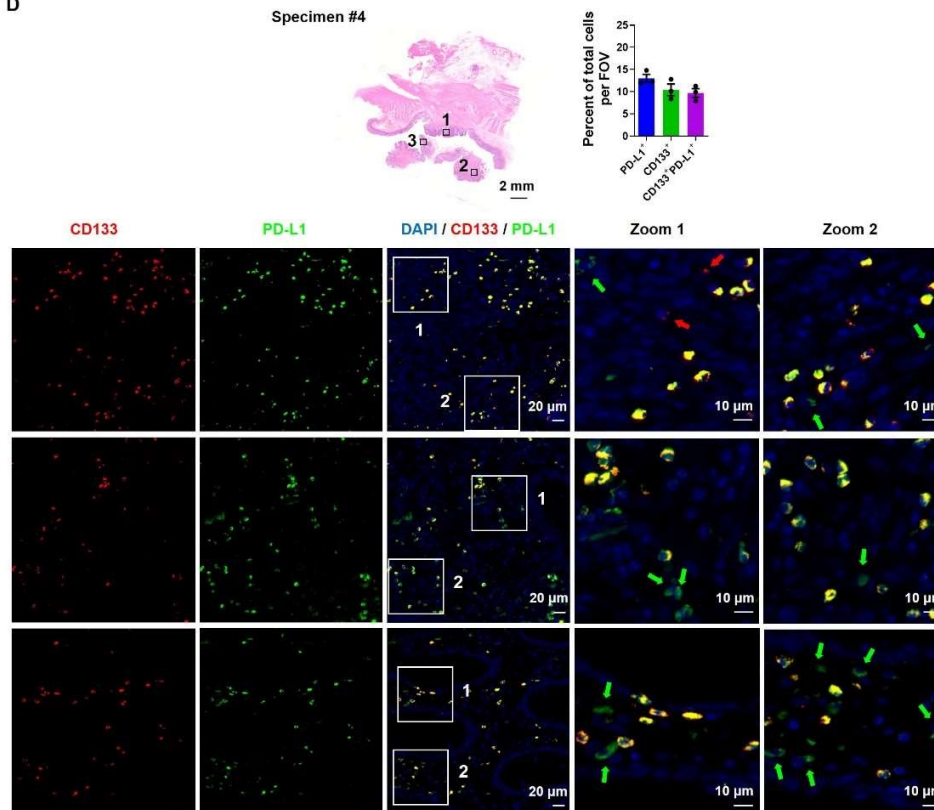
melanoma, a significant portion of the PD-L1⁺ cells co-expressed CD133 (Fig. 5H, Supplementary Fig. 12 C-F). Besides, we also found a small subset of PD-L1⁺ cells without co-expression of CD133 (Fig. 5H, Supplementary Fig. 12 C-F, green arrows), as well as a minor population of CD133⁺ cells without PD-L1 expression (Fig. 5H, Supplementary Fig. 12 C-F, red arrows).



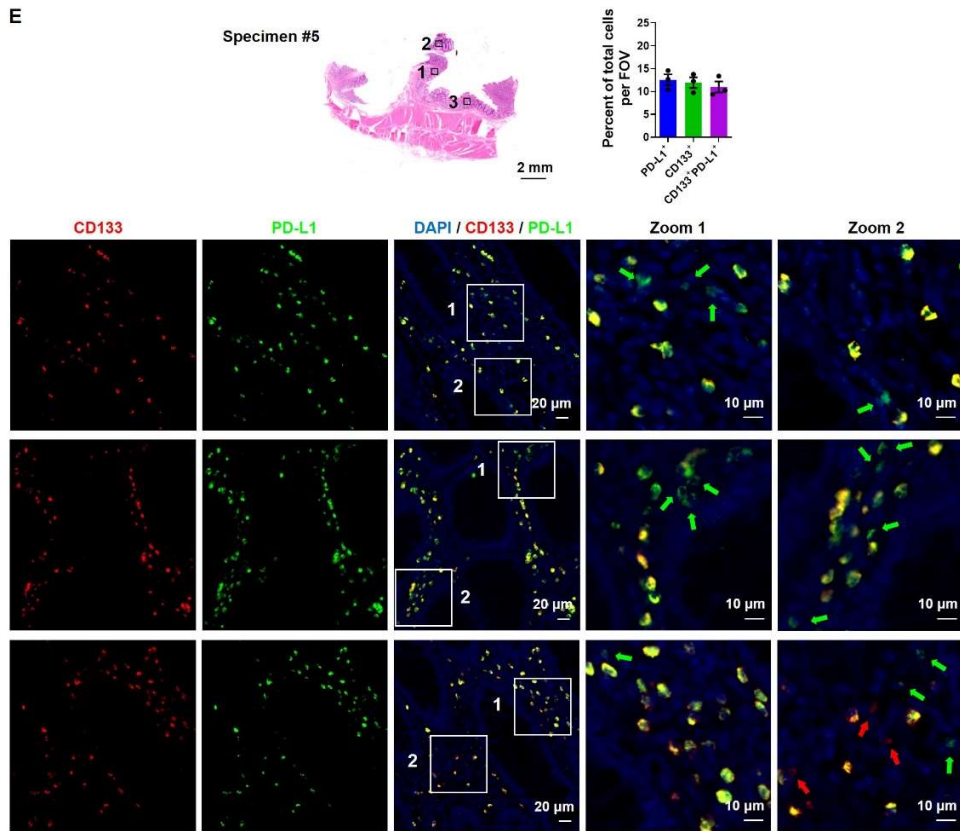
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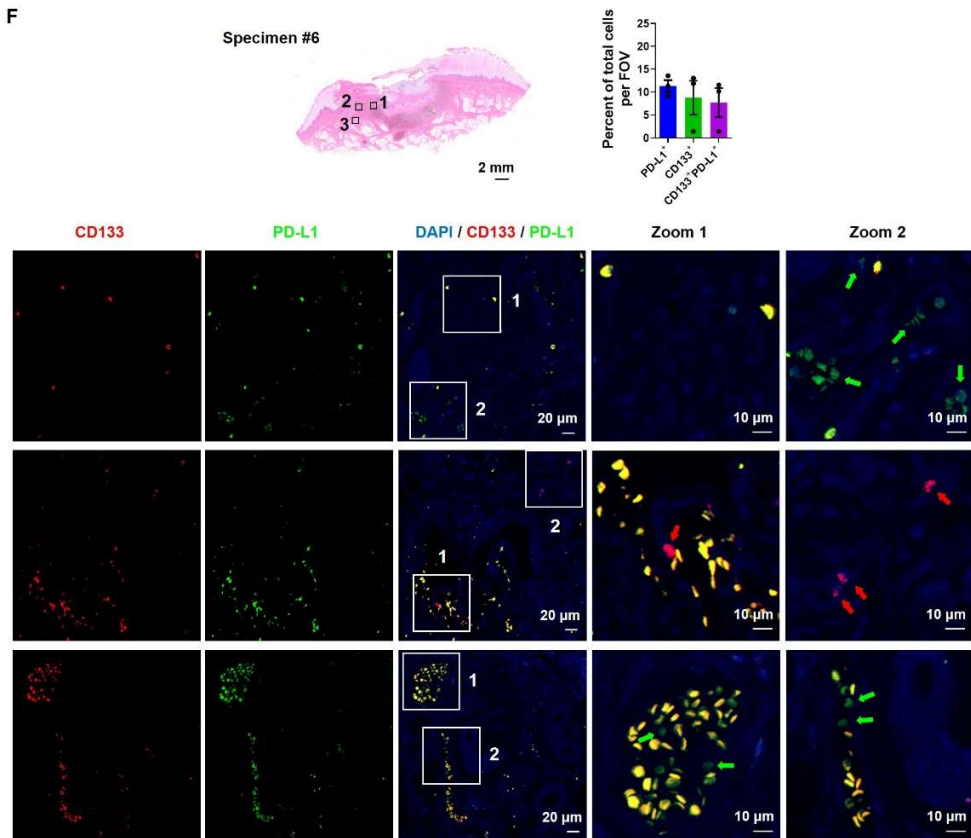
D



E



F



Supplementary Fig. 12. Identification of the CD133⁺PD-L1⁺ cancer cells in human primary and metastatic melanoma specimens. Two specimens of primary melanoma (#1 and #2) (**A** and **B**) and four specimens of metastatic melanoma (#3-#6) (**C-F**) were stained with CD133 (red) and PD-L1 (green), respectively. The nuclei were counterstained with DAPI (blue). The number of CD133⁺, PD-L1⁺ or CD133⁺PD-L1⁺ cancer cells within the field of view (FOV) was analyzed by Fiji ImageJ software. Three FOVs from each sample were selected for the analysis. Data are expressed as mean $\pm$ s.e.m. Yellow arrow, CD133⁺PD-L1⁺ cells; green arrows, CD133⁻PD-L1⁺ cells; red arrows, CD133⁺PD-L1⁻ cells. The detailed information of the specimens was presented in Supplementary Table 3.

b. The scarcity of PDL1⁺ cells in patients 1, 2, 4, and 5 is quite unusual.

RE: Thank you for this constructive comment. We have reanalyzed the number of CD133⁺, PD-L1⁺ and CD133⁺PD-L1⁺ cancer cells. The CD133⁻PD-L1⁺ cells were clearly identified in all of the tumor samples (Fig. 5H, Supplementary Fig. 12). In the primary tumor of specimens #1 and #2, the PD-L1⁺ cells including CD133⁻PD-L1⁺ cells and CD133⁺PD-L1⁺ cells accounted for approximately 5.12% within the field of view (FOV) (Fig. 5H, upper row, Supplementary Fig. 12A, B). Among them, most of the PD-L1⁺ cells were CD133⁻PD-L1⁺ cells. By contrast, the percentage of PD-L1⁺ cells was increased to approximately 14.32% in the metastatic tumor of specimens #3-#6 (Fig. 5H, lower row, Supplementary Fig. 12C-F). Among them, the population of CD133⁺PD-L1⁺ cells significantly increased. This result indicated that the CD133⁺PD-L1⁺ cells were increased in the metastatic melanoma.

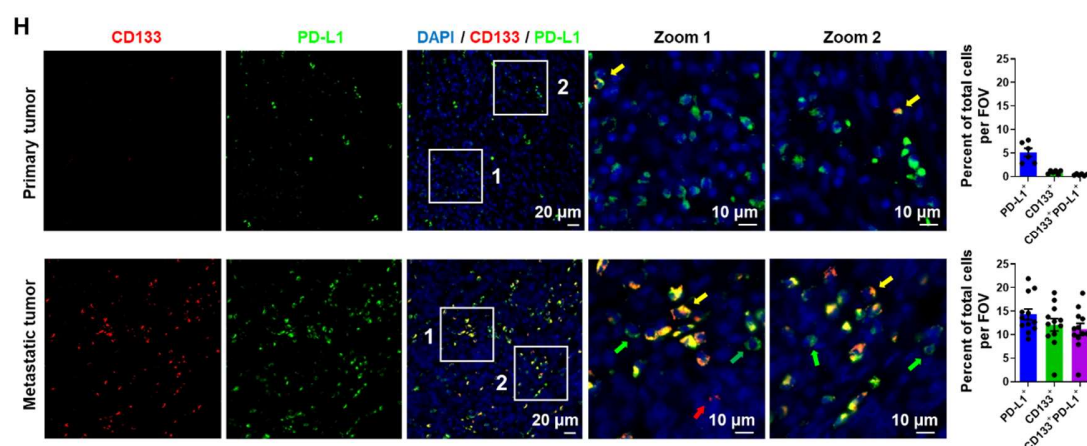


Fig. 5. (H) Representative immunofluorescence micrographs of the expression of CD133 (red) and PD-L1 (green) in primary or metastatic tumor of human melanoma specimens. Blue, cell nuclei. Right, the number of CD133⁺, PD-L1⁺ or CD133⁺PD-L1⁺ cells within the field of view (FOV) was analyzed by Fiji ImageJ software. Data are presented from 2 primary melanoma specimens and 4 metastatic melanoma specimens. Three FOVs from each sample were selected for the analysis. Data are expressed as mean $\pm$ s.e.m. Yellow arrow, CD133⁺PD-L1⁺ cells; green arrows, CD133⁻PD-L1⁺ cells; red arrows, CD133⁺PD-L1⁻ cells. The individual fluorescence micrographs of each specimen were presented in Supplementary Figure 12.

c. There is a lack of detailed information on patient lesions. It is unclear whether the samples are from early or late-stage tumors, primary tumors, metastasis, or therapy-naïve cases. Additionally, information on whether the patients received therapy, and if so, what kind of therapy, is missing. This would help explain the increased expression in advanced cases. The criteria for patient selection and the claims being made are also not clearly presented.

RE: Thank you for this constructive comment. Following your suggestion, we have provided a summary information of human melanoma specimens in Supplementary Table 3. The specimens #1 and #2 were from early-stage, primary and therapy-naïve cases. Whereas, the specimens #3-#6 were from late-stage and metastasis, who all had received treatment. Specifically, patients of specimens #3 to #6 underwent surgery, which received radical resection depending on tumor location, or plus immunoregulatory agents. Patients of specimens #4 and #5 additionally received continuous treatment with anti-PD-1 antibody Camrelizumab. In this study, human tumor specimens with a diagnosis of melanoma were randomly selected from the hospital biobank. Overall, these information supported that the increased expression of CD133⁺PD-L1⁺ cells in patients with advanced cases (late-stage tumors and metastatic tumors) (Fig. 5H, Supplementary Fig. 12A-F).

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RESPONSE TO REVIEWERS' COMMENTS

Reviewer #5 (Remarks to the Author):

I thank the author for taking my suggestion in a pro-active way, revising the language and rewiring their statement. I think that now the paper has more clarity, value and is more realistic in the conclusions.

RE: We sincerely appreciate your expertise in melanoma and cancer stem cells and positive comments on our responses. Your insightful suggestions have tremendously helped us to strengthen the quality of our work.