nature portfolio

Peer Review File

Thermal-responsive activation of engineered bacteria to trigger antitumor immunity post microwave ablation therapy

Corresponding Author: Professor Zhuang Liu

Parts of this Peer Review File have been redacted as indicated to remove third-party material.

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

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
This manuscript provides a novel strategy in which VNP20009 is engineered to in situ release intratumoral IL-15&IL-15Rα in response to mildly elevated temperature induced by MWA treatment. Such 15&15R@VNP post intravenous injection could activate antitumor immune responses to effectively suppress tumor progress. They further design VNP20009 with thermal-responsive co-expression of both IL-15&IL-15Rα and soluble PD-1. Such sPD-1-15&15R@VNP could further reverse the functional suppression of immune cells driven by PD-1/PD-L1 interaction, thereby reinvigorating progenitor exhausted T (Tpex) cells and amplifying antitumor immune responses. Overall, the significance of the manuscript is sufficient and the experimental design is reasonable. Thus, I support the acceptance of this work for publication in Nature Communications, but the following concerns need to be addressed appropriately before this manuscript is published.

1. Although MWA has limitations in post-operation tumor recurrences due to incomplete tumor ablation, especially for those with large sizes and irregular shapes, it is still a widely used hyperthermia treatment strategy. However, MWA treatment has negligible therapeutic effect on tumor ablation in this manuscript. Is condition of MWA treatment optimal?

2. In the Abstract section, authors stated that VNP20009 was engineered to release IL-15&IL-15Rα in response to mildly elevated temperature at ~ 43 °C. However, in figure 1 and Method section, the bacteria cultures were incubated at 42 °C to induce protein expression. Please check it carefully.

3. Authors should provide the unprocessed western blot images used in the manuscript. In addition, restriction enzyme digestion map of plasmid pBV-sPD-1 should be displayed.

4. To induce IL-15&IL-15Rα expression, the bacteria cultures were incubated at 42 °C for different durations and then returned to 37 °C for an additional 6 hours. Why did bacteria return to 37 °C for another incubation? Is this step indispensable?

5. In figure 1L, western blot analysis of IL-15&IL-15Rα protein after heat induction for different durations should be evaluated. In addition, please check the legend of figure 1L carefully.

6. In figure 3, the control group of free IL-15&IL-15Rα should be set to demonstrate that the promoted therapeutic efficacy is attributed to the continuous in situ generation of intratumoral IL-15&IL-15Rα.

7. The description of y-axis in the figures about T-cell percentage should be unified. e.g., “CD8+ T cells” and “CD69+ T cells” were used in figure 4, yet “CXCR3+CD8+ T cell” was used in figure 5. In addition, the description of y-axis in figure S15B and S15C should be corrected to “CD8+ T cells (%)” and “NK in CD3- cells (%)”, respectively.

8. Gating strategy of flow cytometry data was shown in figure S19. However, the CD3-positive population is abnormal and CD45 gating is highly recommended to identify lymphocyte before CD3 gating. In addition, TCF7+Tim3- rather than TCF7+ cells are Tpex and TCF7-Tim3+ rather than Tim3+ cells are Tex. Authors should correct the gating strategy to obtain credible conclusion.

Reviewer #2

(Remarks to the Author)
The manuscript by Wu et al presets a new and innovative concept of combining tumor-infiltrating bacteria with local hypothermia treatment. To this end, the attenuated Salmonella strain VNP200009 is modified to produce pro-inflammatory proteins in a temperature-inducible way. They chose to express a fusion of IL-15 and the IL-15R alpha chain that can signal even in cell types not expressing the IL-15Ra. They later augment the bacteria with a soluble PD-1 to further increase the anti-tumor efficacy. The authors convincingly show the functionality of the thermal induction in the bacteria, as well as the selective colonialization of tumors in mice. The data on IL-15&IL-15Ra-production selectively in MWA-targeted tumor tissue is also convincing. Therapeutic tumor treatment in the H22 and the CT26 tumor models is satisfying. The authors also show that both CD8+ cells and NK cells contribute to tumor rejection. However the authors focus on the T cells and show a synergistic effect of PD-1 checkpoint inhibition, which they exploit by coexpressing soluble PD-1, which further increases treatment efficiency. They attribute this effect to the presence of T-pex. Then the authors switch to observatory results extracted from publically available data and various correlations between the presence of IL15 in different tumors, and the presence of activated T cells, NK cells, and clinical outcome. Unfortunately, this last part of the manuscript does not stand up to the high quality of all the previous figures and data. The interpretation of the correlations with so low correlation coefficients as causal correlations resulting in statements like: “undeniably validate the clinical translational value” and “undoubtedly serves as excellent alternative” is a massive over interpretation. This is unfortunate, because such exaggerations are unnecessary for the point of the otherwise very good manuscript.
Mayor comment:
The results presented in figure 7 should be interpreted with much more care, and the possibility of indirect correlations should be taken into account, especially in the discussion. Terms like “undeniable” and “undoubtedly” require proof beyond doubt and denial.

Minor comments
VPN + MWA has been tested before (Zhao et al PMID: 28498813) which should be mentioned.

Speaking of 20% of 5 mice is a bit overplaying. Saying “one out of five” would be more honest. (line 211)

It was not the authors that “discovered the presence of Tpex within tumors” (line 471)

The description of S2 is insufficient. It is not indicated how often der experiment was performed, and what was used as control. A suitable control would be heat inactivated IL-15&IL-15Rα to exclude that any bacterial components contaminating the product are responsible.

Figure S10: data point for condition V from panel A appears not present in panel B

Reviewer #3

(Remarks to the Author)
The manuscript presents an innovative approach combining the use of engineered bacteria with microwave ablation therapy to enhance antitumor immunity. The integration of IL-15&IL-15Rα and sPD-1 expression within a thermally-responsive bacterial system is a fascinating concept with potential for significant impact in cancer therapy. However, some similar articles about thermal-responsive activation of tumor-homing engineered bacteria for cancer immunotherapy have been published (Nat Commun. 2022. doi: 10.1038/s41467-022-31932-x.; Nat Commun. 2022;13(1):1585. doi: 10.1038/s41467-022-29065-2.). The manuscript lacks innovation. Specific comments to improve quality and clarity of the manuscript are listed as follows:

1. The manuscript effectively outlines the limitations of current ablation therapies and the potential of cytokine-based immunotherapy. However, the innovation in using engineered bacteria to overcome the challenges of direct cytokine therapy is limited. The authors need to provide more detailed description on the novelty of using thermally-responsive elements for controlling gene expression in vivo, especially in comparison to other temperature or environment-responsive systems (light, ultrasound).
2. IL-2 is more widely used as the immune factor in solid tumors, and positive results have been achieved in phase 3 clinical trials. This manuscript should explain why IL-15&IL-15Rα was chosen.
3. Figure 1B, how long did the expression of mCherry last during long-term incubation at 37 ℃.
4. Please provide the SDS-PAGE results of IL-15&IL-15Rα protein expressed in 15&15R@VNP under MWA treatment.
5. Does VNP20009 colonize all regions within the tumor, or does it specifically target hypoxic areas? Please provide the evidence to support your claim.
6. How long do the engineered bacteria colonize in the tumor and how long does the protein expression last after MWA treatment? It will determine whether to need to be microwaved again.
7. Figure 3D, the aim of the clinical application of MWA is to eradicate the tumor, but the treatment did not inhibit tumor progression. What parameters is used in this study?
8. Figure 3H, it is not appropriate for clinical use of microwave ablation of colonic carcinoma in situ. The authors need to provide more explanation about it.
9. Microwave ablation of liver cancer is often used in clinical practice, and it is necessary to provide the data on engineered

bacteria combined with microwave therapy for in-situ liver cancer.
10. The study focuses on early immune responses following treatment but does not address long-term tumor growth inhibition and potential adverse effects associated with the therapy. Are there data on the survival rates of treated mice, or any evidence of systemic inflammation or autoimmunity? These data are crucial for evaluating the clinical potential of this therapy.
11. The manuscript showed that the combination of MWA and 15&15R@VNP can enhance the maturation of DCs, activation of CD8+ T and NK cells, and increase IFN-γ production. However, it remains unclear how the engineered bacteria specifically support these immune responses. Does the expression of IL-15&IL-15Rα by the bacteria directly stimulate nearby immune cells, or are there intermediary factors involved? Further experiments, including in vitro studies, could provide deeper insights into the mechanism of action.
12. The authors found no substantial alteration in the quantity of CD4+ T cells following treatment but does not thoroughly discuss the implications of this finding. Given the important role of CD4+ T cells in the immune response to cancer, a discussion regarding why these cells may not be significantly affected by the treatment and how this might impact the overall efficacy and mechanism of the therapy would be beneficial.
13. Figure 5, while the authors did an excellent job about the role of Tpex cells in this therapeutic context, it would benefit to provide a deeper exploration into the mechanisms by which 15&15R@VNP and ICB therapy specifically promotes the expansion and function of these cells. Are there other cytokines or cellular interactions? Can the observed effects be replicated in vitro?
14. Figure 6, the engineered bacteria simultaneously co-express IL-15 and IL-15Rα and sPD-1, but it needs some time for IL-15 and IL-15Rα to promote T cell proliferation, and T cell exhaustion also needs some time. Will the expression of sPD-1 continue to exert its effect?
15. Although the authors successfully identified positive correlations between IL-15 expression, Tpex cell infiltration, and patient prognosis, the causal relationships remain speculative. In the aforementioned animal studies, does this conclusion also have a clear relevance?

Reviewer #4

(Remarks to the Author)
The manuscript presents an approach to engineer tumor-homing bacteria as temperature-triggerable system to produce immunotherapeutic payloads (IL15 & PD1). Combining with microwave ablation (MWA), the authors show antitumoral effect in several tumor models. While the authors have shown therapeutic efficacy in these subcutaneous models, the approach for engineering bacteria with temperature-sensitive constructs as well as photothermal therapy is not novel. Furthermore, there are many technical flows that makes the reviewer question the quality of the manuscript.

Major comments
- Thermos-sensitive promoters have been utilized extensively in the previous literature for bacterial cancer therapy. In fact, the hyperthermia induced gene expression system is obtained from a study by Abedi et al., Nature Comm 2022 where they've utilized this system to precisely control immunotherapeutic expression from bacterial cancer therapy.
- The details on how MWA treatment was done to mice are not described. As indicated above, previous work (including some work dating back to 2008, Chen et al., ACS Nano) had utilized variety of methods to locally heat the tumors.
- Figure 7 has very little to do with the rest of the paper. I would recommend taking it out.
- There are numbers of technical concerns in some critical data. Please see several examples below.
o Why does the temperature curve in (Fig. 2E and 3C) start from temperature that are lower than physiological temperature?
o Fig. 3B – I don't see the outline of a mouse in the picture, and hence I am not sure what I am seeing here.
o Fig. 4B & C – The data should be identical and be representative, but I don't see them matching. For example, Fig. 4B in group V is reported to be 17.3% and yet I don't see that datapoint in Fig. 4C.
o Fig. 4H – there are some extreme population cluster in both low and high levels of IFNg. I'd be cautious of using proportions to analyze this datasets as it might skew the interpretation.
o Fig. 4N – why did the author switched to a new model (B16) when showing the mechanisms of therapeutic effect from another model (H22 and CT26)?
o Figure S2: Did the authors control for other microbial factors that may contribute to T-cell proliferation? It is unclear if the bacteria supernatant itself without IL-15 is sufficient for proliferation

Minor comments
- Figure 1H-J: not clear what was done in this experiment. Did the bacteria receive 15min heat induction once, or everyday?
- Line 68: not sure if it has been shown that facultative anaerobes have intrinsic tropism towards hypoxia
- Line 73: Human studies have been conducted in these strains, but it is premature to state that the safety has been proven
- Line 77: I would not call Salmonella as a type of anaerobic bacteria
- Many figures are lacking statistical analysis. For example, Fig 2J looks like there are some increase in IL15 levels but it is hard to evaluate.
- Figure S6: It is very difficult to conclude from just images whether MWA treatment had any impact on bacterial survival in the tumor. I'd recommend measuring CFU pre & post treatment.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
My previous concerns have been addressed. No other comments available.

Reviewer #3

(Remarks to the Author)
Upon reviewing the revised manuscript, I am pleased to inform you that the changes have significantly improved the clarity and quality of the paper.Therefore, I am happy to recommend the acceptance of your paper for publication.

Reviewer #4

(Remarks to the Author)
The manuscript has addressed most of my concerns. I would suggest the authors to edit the texts to remove excessive or unnecessary wordings, such as “undoubtedly”, as suggested by other reviewers as well.

Responses to reviewer's comments

We sincerely thank the reviewers for their comments and suggestions. Below we have provided responses to the comments and have accordingly revised the manuscript. All modifications in this manuscript are highlighted with a yellow background.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author): with expertise in bacteria-based cancer therapy

This manuscript provides a novel strategy in which VNP20009 is engineered to in situ release intratumoral IL-15&IL-15Rα in response to mildly elevated temperature induced by MWA treatment. Such 15&15R@VNP post intravenous injection could activate antitumor immune responses to effectively suppress tumor progress. They further design VNP20009 with thermal-responsive co-expression of both IL-15&IL-15Rα and soluble PD-1. Such sPD-1-15&15R@VNP could further reverse the functional suppression of immune cells driven by PD-1/PD-L1 interaction, thereby reinvigorating progenitor exhausted T (Tpex) cells and amplifying antitumor immune responses. Overall, the significance of the manuscript is sufficient and the experimental design is reasonable. Thus, I support the acceptance of this work for publication in Nature Communications, but the following concerns need to be addressed appropriately before this manuscript is published.
Reply: We greatly appreciate your positive comments.

1. Although MWA has limitations in post-operation tumor recurrences due to incomplete tumor ablation, especially for those with large sizes and irregular shapes, it is still a widely used hyperthermia treatment strategy. However, MWA treatment has negligible therapeutic effect on tumor ablation in this manuscript. Is condition of MWA treatment optimal?

Reply: Thanks for your question. During microwave ablation, the rapid expansion of the ablation zone can lead to inadvertent ablation of adjacent tissues [1]. On the other side, for the boundary part of a large tumor or a tumor with irregular shape, the MW heating may not be sufficient to cause complete tumor cell ablation. In our experiment, we selected to conduct MWA at a mild condition by heating up the tumor to a temperature around 42 ℃ to 47 ℃, so as to mimic the incomplete MW ablation that may occur in some clinical practices and would lead to unsatisfactory therapeutic performance. With the presence of our engineered bacteria, tumors even under such mild heating condition could be effective suppressed owing to the activation of both the innate and adaptive immune responses.

In the revision, we have modified and added the following description:

"To mimic the incomplete MWA that may occur in some clinical practices and would lead to unsatisfactory therapeutic performance, we selected an ablation temperature around 42 ℃ to 47 ℃, a relatively mild temperature."

2. In the Abstract section, authors stated that VNP20009 was engineered to release IL-15&IL-15Rα in response to mildly elevated temperature at ~ 43 °C. However, in figure 1 and Method section, the bacteria cultures were incubated at 42 °C to induce protein expression. Please check it carefully.

Reply: Many thanks for your suggestion. The temperature was 42°C. We apologize for our oversight. We have revised these oversights and checked the whole manuscript carefully. We will be happy to edit the text further, based on helpful comments from you.

3. Authors should provide the unprocessed western blot images used in the manuscript. In addition, restriction enzyme digestion map of plasmid pBV-sPD-1 should be displayed.

Reply and revision: Thanks so much for your suggestion**.** We have provided the unprocessed western blot images used in the manuscript (**Figure S33**) and the restriction enzyme digestion map of plasmid pBV-sPD-1 in Supporting Information.

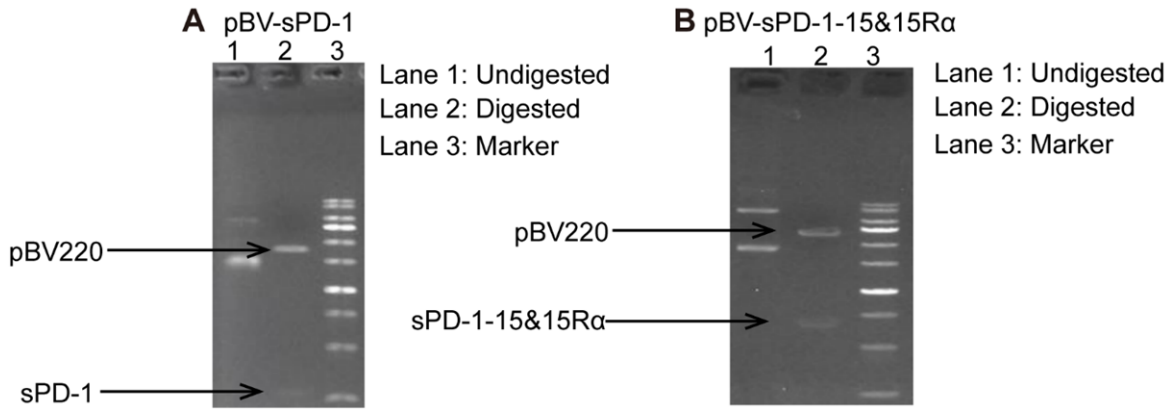


Figure S27. Restriction enzyme digestion maps of plasmids pBV-sPD-1 (A) and pBV-sPD-1-15&15R

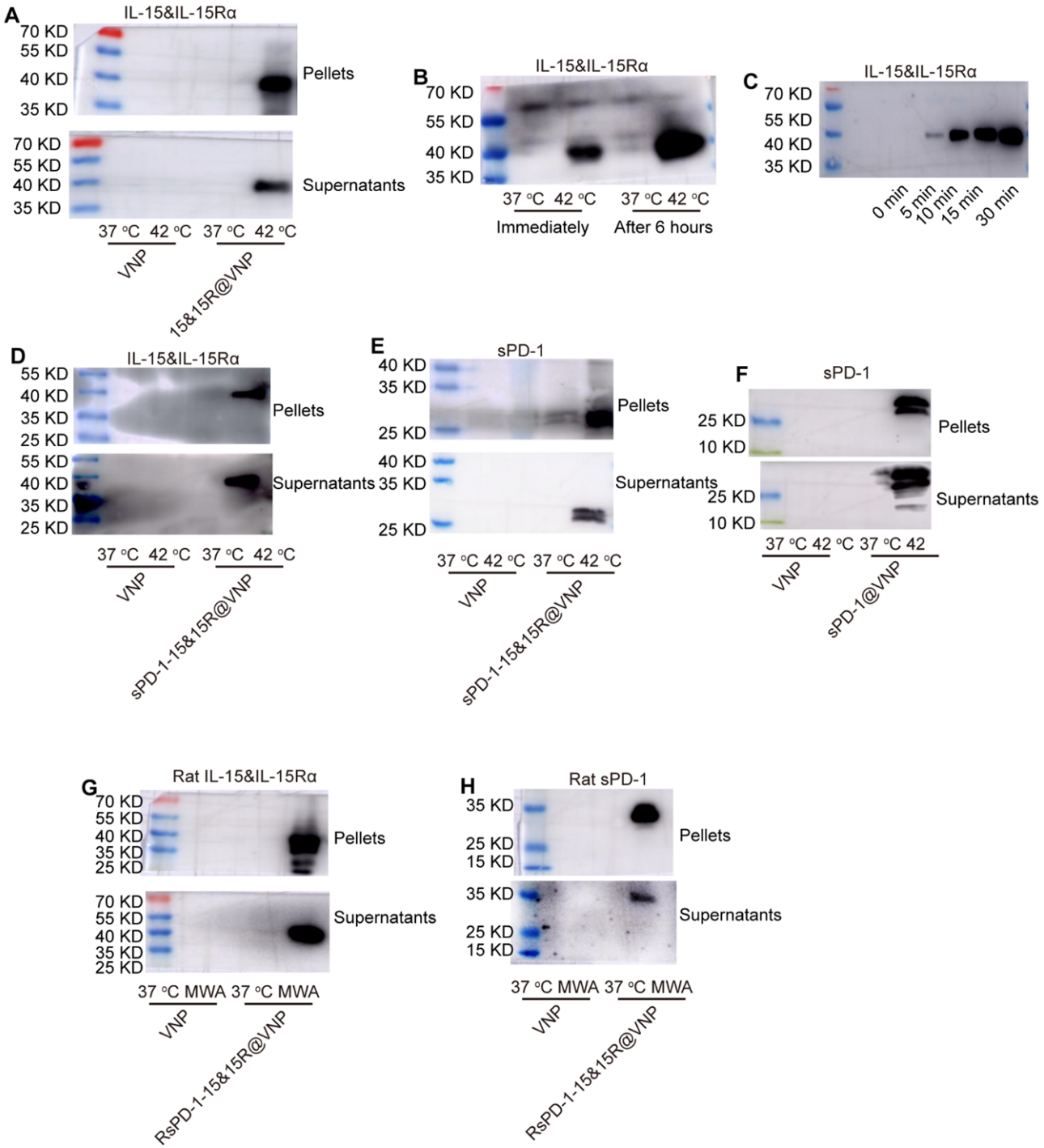


Figure S33. The unprocessed western blot images for Figure 1L (A), Figure S3A (B), Figure S3B (C), Figure S28A (D), Figure S28B (E), Figure S28C (F), Figure S31A (G), Figure S31B (H).

In the revision, we have modified and added the following description:

"The successful construction of sPD-1@VNP and sPD-1-15&15R@VNP vectors was confirmed through restriction enzyme digestion and DNA gel electrophoresis (**Figure S27**)."

"All unprocessed western blot images have been shown in **Figure S33.**"

4. To induce IL-15&IL-15Rα expression, the bacteria cultures were incubated at 42 °C for different durations and then returned to 37 °C for an additional 6 hours. Why did bacteria return to 37 °C for another incubation? Is this step indispensable?

Reply: Many thanks for your suggestion. In fact, we extended the cultivation period to 6 hours post-induction to enhance the protein yield. As illustrated in the figure below, there was a significant difference in the protein content secreted into the culture supernatant when comparing the engineered bacteria assessed immediately after 30 minutes of induction to those cultured at 37 ℃ for an additional 6 hours post-induction.

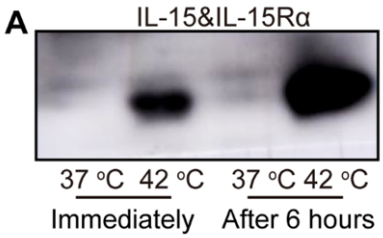


Figure S3. (A) Western blot images of IL-15&IL-15Rα protein in bacterial pellets and induced supernatants of 15&15R@VNP under different conditions, suggesting that the heat-activated bacteria would continuously produce the protein under 37℃.

In the revision, we have modified and added the following description:

"Similarly, the engineered 15&15R@VNP were subjected to heating at 42 ℃ for 30 minutes. The results demonstrated a significant increase in the protein content secreted into the culture supernatant when comparing the engineered bacteria assessed immediately after the 30-minute induction to those cultured at 37 ℃ for an additional 6 hours post-induction (**Figure S3A**). This suggested that heat-activated bacteria could continuously produce the protein at 37 ℃."

5. In figure 1L, western blot analysis of IL-15&IL-15Rα protein after heat induction for different durations should be evaluated. In addition, please check the legend of figure 1L carefully.

Reply and revision: Thanks for your suggestion. We have analyzed the western blot analysis of IL-15&IL-15Rα protein after heat induction for different durations according to your comments. We have also checked and the legend of Figure 1L.

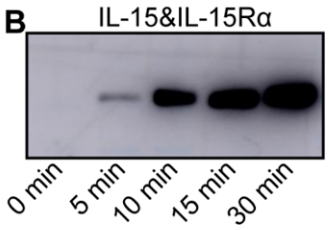


Figure S3. (B) Western blot images of IL-15&IL-15Rα protein in bacterial pellets and induced supernatants of 15&15R@VNP under 42°C for different durations.

In the revision, we have modified and added the following description:

"Next, the engineered 15&15R@VNP were subjected to varying durations of heating at 42 °C. The results demonstrated that with prolonged incubation time, the expression levels of IL-15&IL-15Rα gradually increased (**Figure S3B**)"

6. In figure 3, the control group of free IL-15&IL-15Rα should be set to demonstrate that the promoted therapeutic efficacy is attributed to the continuous in situ generation of intratumoral IL-15&IL-15Rα.

Reply: Many thanks for your suggestion. We indeed have conducted this experiment, and it was the observed effects that led us to choose IL-15&IL-15Rα in our study. The specific results are presented below.

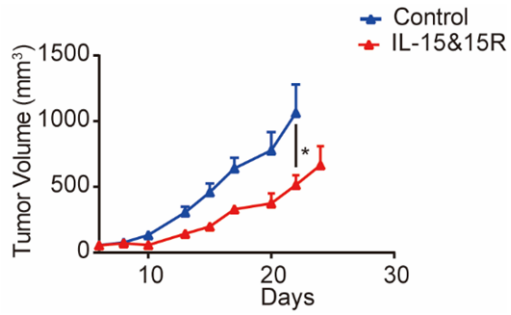


Figure S2. The tumor growth curve following intratumoral injection of IL-15&15Rα in the CT26 tumor-bearing model (n = 5). The P value was calculated by two-way ANOVA with Tukey's multiple comparisons, *p<0.05.

In the revision, we have modified and added the following description:

"To initiate our study, a murine tumor model was established using CT26 colon cancer cells to investigate the effects of intratumoral administration of the IL-15 and IL-15Rα complex on tumor growth. Notably, the results demonstrated a significant inhibition of tumor progression (**Figure S2**), providing compelling evidence for the potent antitumor activity of the IL-15 and IL-15Rα complex.**"**

7. The description of y-axis in the figures about T-cell percentage should be unified. e.g., "CD8+ T

cells" and "CD69+ T cells" were used in figure 4, yet "CXCR3+CD8+ T cell" was used in figure 5. In addition, the description of y-axis in figure S15B and S15C should be corrected to "CD8+ T cells (%)" and "NK in CD3- cells (%)", respectively.

Reply and revision: Many thanks for your suggestion. In fact, these descriptions refer to different subpopulations of T cells, $CD69^+$ T cells refer to activated T cells, and $CXCR3^+CD8^+$ T cells refer to $CD8^+$ T cells that express the CXCR3 receptor. As for other figures, we have adjusted all the descriptions.

8. Gating strategy of flow cytometry data was shown in figure S19. However, the CD3-positive population is abnormal and CD45 gating is highly recommended to identify lymphocyte before CD3 gating. In addition, TCF7+Tim3- rather than TCF7+ cells are Tpex and TCF7-Tim3+ rather than Tim3+ cells are Tex. Authors should correct the gating strategy to obtain credible conclusion.

Reply: Many thanks for your suggestion. We very much agree with your opinion. However, due to the limited channel number of our flow cytometry equipment and the many markers involved in these two types of cells, we could not stain CD45 at first. And we will avoid these omissions in the future. Usually, Tpex is defined based on the expression levels of PD-1, where $PD\text{-}1^{hi}$ $TCF7^+$ cells are classified as Tpex. However, upon entering the terminal exhaustion phase, Tpex cells lose Tcf7 expression and instead express TIM-3 [2, 3]. So, our definition is also reasonable.

9. Figure S10: data point for condition V from panel A appears not present in panel B

Reply and revision: Many thanks for your suggestion. We have since made the necessary corrections.

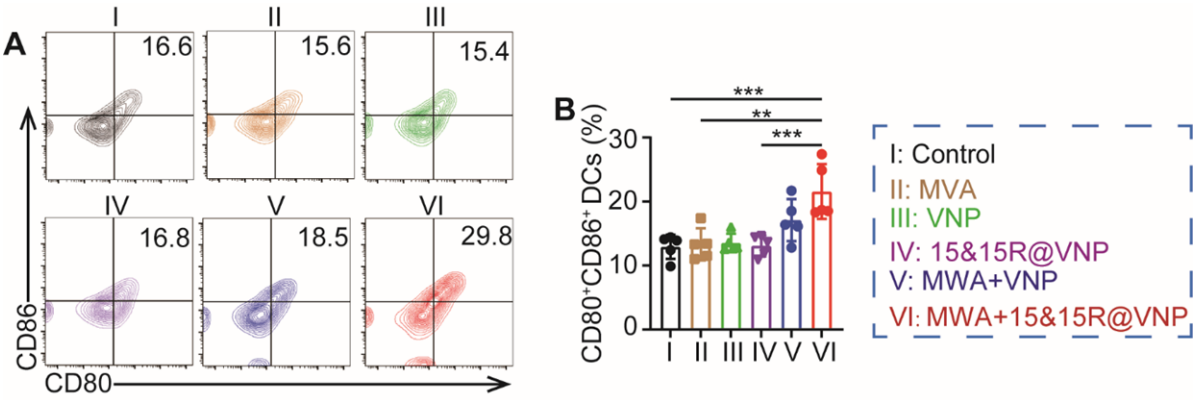


Figure S14. Flow cytometry analysis (A) and percentage (B) of mature DCs in TDLNs of mice post different treatments as indicated. Data in the figure were represented as the mean ± SD. *P* values were calculated by the one-way ANOVA. $^{**}p<0.01$, $^{***}p<0.001$.

Reviewer #2 (Remarks to the Author): with expertise in cancer immunology/therapy

The manuscript by Wu et al presets a new and innovative concept of combining tumor-infiltrating bacteria with local hypothermia treatment. To this end, the attenuated Salmonella strain VNP200009 is modified to produce pro-inflammatory proteins in a temperature-inducible way. They chose to express a fusion of IL-15 and the IL-15R alpha chain that can signal even in cell types not expressing the IL-15Ra. They later augment the bacteria with a soluble PD-1 to further increase the anti-tumor efficacy. The authors convincingly show the functionality of the thermal induction in the bacteria, as well as the selective colonialization of tumors in mice. The data on IL-15&IL-15Ra-production selectively in MWA-targeted tumor tissue is also convincing. Therapeutic tumor treatment in the H22 and the CT26 tumor models is satisfying. The authors also show that both CD8+ cells and NK cells contribute to tumor rejection. However the authors focus on the T cells and show a synergistic effect of PD-1 checkpoint inhibition, which they exploit by coexpressing soluble PD-1, which further increases treatment efficiency. They attribute this effect to the presence of T-pex. Then the authors switch to observatory results extracted from publically available data and various correlations between the presence of IL15 in different tumors, and the presence of activated T cells, NK cells, and clinical outcome. Unfortunately, this last part of the manuscript does not stand up to the high quality of all the previous figures and data. The interpretation of the correlations with so low correlation coefficients as causal correlations resulting in statements like: “undeniably validate the clinical translational value” and “undoubtedly serves as excellent alternative” is a massive over interpretation. This is unfortunate, because such exaggerations are unnecessary for the point of the otherwise very good manuscript.
Reply: We greatly appreciate your positive comments and valuable suggestion. In accordance with your and editor’s suggestion, we have removed the last part of the manuscript. The maintext has been revised accordingly based on helpful comments from you. We thank the reviewer for the help with our manuscript.

Mayor comment:
The results presented in figure 7 should be interpreted with much more care, and the possibility of indirect correlations should be taken into account, especially in the discussion. Terms like “undeniable” and “undoubtedly” require proof beyond doubt and denial.
Reply and revision: Many thanks for your suggestion. We have removed Figure 7 from our manuscript. Thanks again!

Minor comments

VPN + MWA has been tested before (Zhao et al PMID: 28498813) which should be mentioned.

Reply and revision: Thanks for your valuable suggestion. In the revision, we have mentioned this study. We have modified and added the following description:

"Encouraged by the aforementioned results, and MWA combined with VNP has also been shown to promote tumor control (37), we sought to explore the therapeutic efficacy of 15&15R@VNP combined with MWA in tumor-bearing mice."

Speaking of 20% of 5 mice is a bit overplaying. Saying "one out of five" would be more honest. (line 211)

Reply and revision: Many thanks for your suggestion. We have revised these oversights and checked the whole manuscript carefully.

In the revision, we have modified and added the following description:

"Moreover, this therapeutic regimen demonstrated a remarkable extension in the survival duration of mice, with one out of five tumor-bearing mice achieved complete remission (CR)."

"Notably, this combination therapy resulted in a significant extension in the overall survival of mice, reaching a CR rate of three out of six."

"This enhancement was evident not only in the suppression of tumor growth, but also in the prolonged survival of tumor-bearing mice with a notable CR rate of five out of eight."

"Moreover, this therapeutic approach realized significant inhibition of tumor growth and a noticeably prolonged survival rate, with a CR rate of six out of eight."

It was not the authors that "discovered the presence of Tpex within tumors" (line 471)

Reply and revision: Many thanks for your valuable suggestion. According to you and editor's suggestion, we have removed the result of Figure 7, along with this part of the description. Thanks again!

The description of S2 is insufficient. It is not indicated how often der experiment was performed, and what was used as control. A suitable control would be heat inactivated IL-15&IL-15Rα to exclude that any bacterial components contaminating the product are responsible.

Reply and revision: Many thanks for your valuable suggestion. Actually, we have considered this point and conducted our experiments using purified proteins of IL-15&IL-15Rα to eliminate the

possibility of contamination. In this process, IL-15&IL-15Rα with His tag protein will be adsorbed by nickel chelating column and then elute, thus avoiding bacterial protein contamination. In the revision, we have revised our manuscript based on your suggestions.

In the revision, we have modified and added the following description:

"To validate the activity maintenance of IL-15&IL-15Rα protein expressed by 15&15R@VNP on T cells, we purified the expressed fusion protein using a nickel chelated column (GenScript, Nanjing, China) according to the according to the manufacturer's instructions, and the concentration of the purified IL-15&IL-15Rα was measured with BCA kit (Thermo Scientific, USA). CFSE-labeled T cells were then stimulated with IL-15&IL-15Rα protein at a concentration of 20 ng·mL^{-1}. After 72 hours, T cell proliferation was assessed by flow cytometry."

We would like to express our gratitude to you once again.

Reviewer #3 (Remarks to the Author): with expertise in bacteria engineering, cancer therapy

The manuscript presents an innovative approach combining the use of engineered bacteria with microwave ablation therapy to enhance antitumor immunity. The integration of IL-15&IL-15Rα and sPD-1 expression within a thermally-responsive bacterial system is a fascinating concept with potential for significant impact in cancer therapy. However, some similar articles about thermal-responsive activation of tumor-homing engineered bacteria for cancer immunotherapy have been published (Nat Commun. 2022. doi: 10.1038/s41467-022-31932-x.; Nat Commun. 2022;13(1):1585. doi: 10.1038/s41467-022-29065-2.). The manuscript lacks innovation.

Reply: We greatly appreciate your comments. While we acknowledge the presence of previous research on the thermal-responsive activation of tumor-homing engineered bacteria, our study introduces several unique innovations that differentiate it from the referenced studies. **First**, while prior research may have focused on bacteria that respond to thermal stimuli, we expanded the application field and applied the system to MWA to induce localized hyperpyrexia, which is a clinically-relevant technology prioritizes translational feasibility. **Second**, in view of the core role of Tpex cells in tumor control [4, 5, 6], and IL-15 has been associated with the enhancement of Tpex cell function [7, 8].We utilized this system to express tumor-associated antigens while simultaneously promoting the maintenance of Tpex cells, enabling in situ ICB therapy. The combined use of IL-15 to drive Tpex and PD-1 inhibition to boost Tpex activity offers a powerful synergistic effect that has not been fully explored in previous studies involving tumor-homing bacteria. **Lastly**, our system uniquely combines IL-15&IL-15Rα with sPD-1, offering a dual mechanism for immune activation. This dual immunomodulatory approach represents a significant innovation in engineered bacterial cancer therapies.

The maintext has been revised accordingly based on helpful comments from you.

Specific comments to improve quality and clarity of the manuscript are listed as follows:

1. The manuscript effectively outlines the limitations of current ablation therapies and the potential of cytokine-based immunotherapy. However, the innovation in using engineered bacteria to overcome the challenges of direct cytokine therapy is limited. The authors need to provide more detailed description on the novelty of using thermally-responsive elements for controlling gene expression in

vivo, especially in comparison to other temperature or environment-responsive systems (light, ultrasound).

Reply: Many thanks for your suggestion.

During microwave ablation, the rapid expansion of the ablation zone can lead to inadvertent ablation of adjacent tissues [1]. On the other side, for the boundary part of a large tumor or a tumor with irregular shape, the MW heating may not be sufficient to cause complete tumor cell ablation. Therefore, the concept of this project and the clinical goal to be achieved in the future is to solve the problem of incomplete ablation of large tumors and irregularly shaped tumors by clinical thermal ablation. With the presence of our engineered bacteria, tumors could be effective suppressed owing to the activation of both the innate and adaptive immune responses.

Using engineered bacteria for therapeutic purposes, particularly to address the challenges of direct cytokine therapy, represents a significant innovation in biotechnology and medicine. For example, engineered bacteria specifically target sites of tumors, and programmed to produce cytokines locally, which reduces off-target effects and enhances the therapeutic efficacy compared to systemic cytokine administration. The incorporation of thermal priming into engineered bacteria effectively achieves a spatio-temporal synergy between thermal ablation therapy and cytokine therapy, thereby maximizing therapeutic efficacy while minimizing potential side effects.

As for the environment-responsive systems mentioned by you, as we know, the 408 nm blue light promoter vector (pDawn) has been used in related research fields, such as the treatment of DSS-induced colitis in mice [9]. The light-induced control element provides high spatio-temporal accuracy [10, 11], but is limited by the poor permeability of light to intact tissues [12]. Thus, we didn't choose this system.

As for the ultrasound mentioned by you, namely high-intensity focused ultrasound (HIFU) is another local hyperthermia modality by heating tumors with focused high-intensity ultrasound [13]. Therefore, HIFU can also achieve the same objectives with our engineered bacteria.

In the revision, we have modified and added the following description:

"Unlike chemical inducers, which are unable to target specific anatomical sites (25, 26), and light-induced control elements, which suffer from limited penetration (27), thermal-responsive control elements can provide spatiotemporal control at varying depths (28, 29)."

2. IL-2 is more widely used as the immune factor in solid tumors, and positive results have been achieved in phase 3 clinical trials. This manuscript should explain why IL-15&IL-15Rα was chosen.

Reply and revision: Many thanks for your suggestion. As for IL-2 mentioned by you, we have indeed taken it into consideration. However, there were many studies on T cell exhaustion and Treg activation caused by IL-2 [14, 15]. IL-2 strongly promotes the growth of regulatory T cells (Tregs) and T cell terminal exhaustion, which was verified in vitro in our experiment. While IL-15 does not have such concerns. Unlike IL-2, IL-15 does not significantly expand Tregs and promote T cell exhaustion, in contrast, IL-15 plays a pivotal role in the long-term survival and maintenance of T cells. IL-15 is often considered a more favorable alternative to IL-2 in cancer immunotherapy due to its ability to promote strong immune responses without expanding suppressive Tregs or inducing AICD [16]. This selective immune activation and improved safety profile make IL-15 particularly attractive for enhancing cytotoxic responses in cancer treatments.

Numerous clinical trials are also exploring IL-15 in combination therapies to boost cancer treatment outcomes. For example, N-803, an IL-15 superagonist, has shown significant efficacy in bladder cancer through its ability to enhance immune checkpoint inhibition. In the phase 2 QUILT-3.055 trial, N-803 combined with immune checkpoint inhibitors exhibited tolerability and impressive efficacy, achieving stable disease in 49% of patients. Additionally, it has been a promising option in preventing the need for radical surgeries, like cystectomies, in bladder cancer. In NSCLC, a phase 3 trial is assessing IL-15 combined with PD-1 inhibitors to enhance the efficacy of these therapies.

In the revision, we have modified and added the following description:

"Interleukin (IL) - 2, the first FDA-approved cytokine for metastatic renal cell carcinoma and advanced melanoma, expands NK and T cells but may also induce activation-induced cell death and Treg cell activation, while excessive levels in the TME can cause T cell exhaustion and reduced responsiveness to ICB therapy (8)."

3. Figure 1B, how long did the expression of mCherry last during long-term incubation at 37 ℃.

Reply: Thanks for your question. For bacteria after 30 min of induction, we assessed the persistence of mCherry expression over time. Notably, after one week of observation, the mCherry fluorescence remained detectable, as illustrated in **Figure 1H&I**. Furthermore, our findings were also consistent with previously published study [17].

4. Please provide the SDS-PAGE results of IL-15&IL-15Rα protein expressed in 15&15R@VNP under MWA treatment.

Reply: Many thanks for your question. According to your suggestion, we have added the SDS-PAGE results of IL-15&IL-15Rα protein expressed in 15&15R@VNP under MWA treatment. The relevant results are shown below.

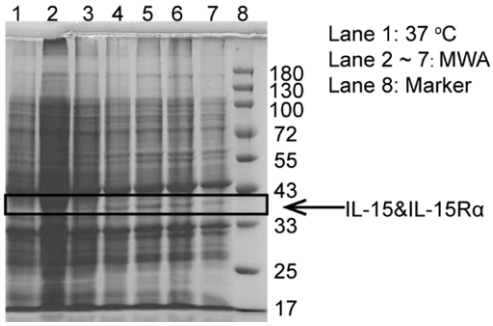


Figure S7. SDS-PAGE images of IL-15&IL-15Rα protein in bacterial pellets and induced supernatants of 15&15R@VNP under MWA treatment.

In the revision, we have modified and added the following description:

"SDS-PAGE gel electrophoresis analysis revealed that 15&15Rα was present in the lysates of bacteria induced by MWA for 30 minutes, and was absent in the lysates of bacteria incubated at 37 ℃ **(Figure S7**). This finding confirms the successful production of therapeutic 15&15Rα following MWA treatment."

5. Does VNP20009 colonize all regions within the tumor, or does it specifically target hypoxic areas? Please provide the evidence to support your claim.

Reply and revision: Many thanks for your question. We have carefully considered your suggestions and conducted immunofluorescence staining for hypoxia marker (HIF-1α) and bacterial outer membrane proteins in tumor tissues. The results indicate a higher colonization of bacteria in the hypoxic regions.

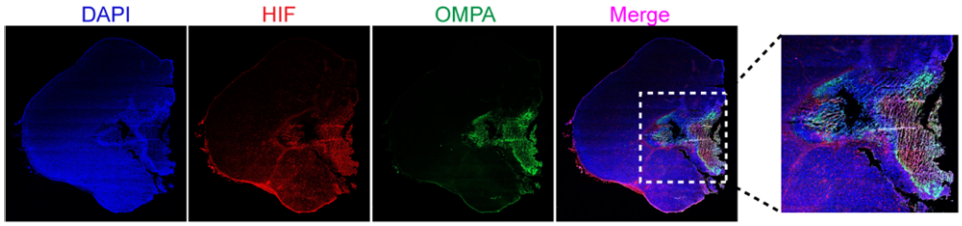


Figure S5. Immunofluorescence staining of whole tumor slices from non-hypoxic region and hypoxic regio. DAPI stands for the nuclei of tumor cells, red for HIF-1α highly expressed cells and green for

OMPA-stained salmonella typhimurium.

In the revision, we have modified and added the following description:
"Immunofluorescence staining assay confirmed that salmonella typhimurium showed high levels in the hypoxic regions of a tumor (overexpression of HIF-1α) (**Figure S5**). These results illustrated that the salmonella typhimurium could be efficiently accumulated in the tumor site and penetrated the tumor hypoxic region."

6. How long do the engineered bacteria colonize in the tumor and how long does the protein expression last after MWA treatment? It will determine whether to need to be microwaved again.

Reply and revision: Many thanks for your question. Regarding your first question, in fact, there have been relevant studies on tumor colonization of Salmonella, and our results are consistent with them[17, 18]. In the revision, we have taken your suggestions seriously and conducted a re-analysis of bacterial colonization in tumor tissues. Our results indicated that the engineered bacteria exhibited a gradual decline after day seven, but continued to colonize until day 21 (**Figure 2B**). Measurement of IL-15&IL-15Rα levels in the tumor tissues post-MWA revealed that IL-15&IL-15Rα concentration was elevated six hours following the treatment. Subsequently, due to the ongoing immune response, IL-15&IL-15Rα levels remain relatively high. On day 14, we observed a decrease in IL-15&IL-15Rα levels; however, IL-15&IL-15Rα still remained elevated compared to the untreated group (**Figure 2I**). Given that this time point falls within the later stages of treatment, we opted not to perform a second microwave application in our animal study. Nevertheless, your suggestions provide valuable strategies for our future research, and we will explore this aspect further.

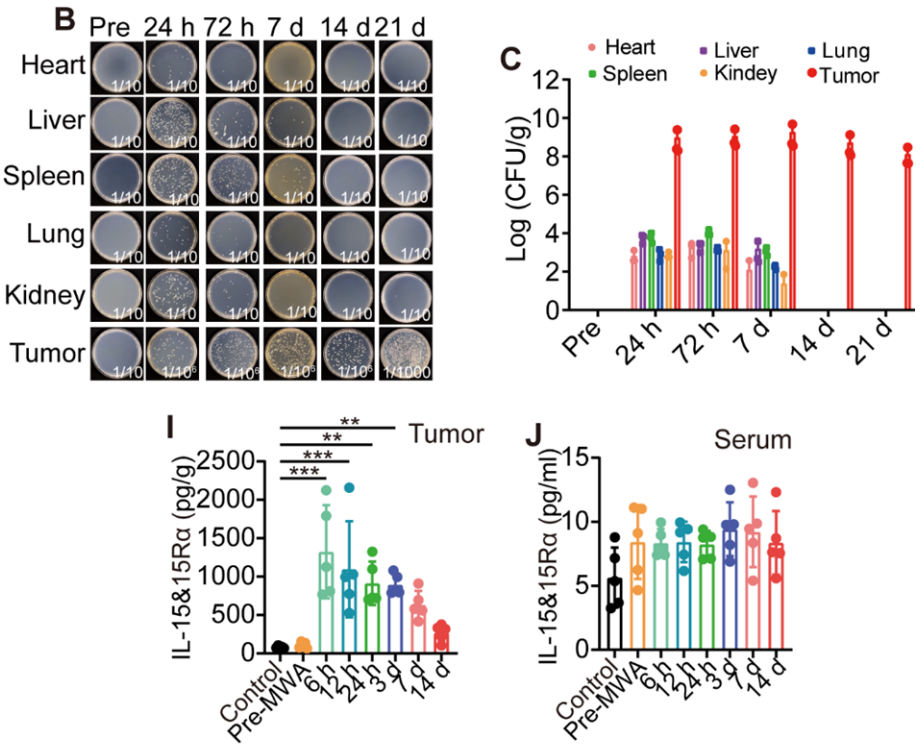


Figure 2. (B&C) Representative photographs (B) and quantification (C) of bacterial colonization in major organs and tumors obtained from CT26 tumor bearing mice at different time points after intravenous administration of engineered bacteria (n = 3). (I) Quantification of IL-15&IL-15Rα protein levels in tumor tissue homogenates at different time points following MWA in mice (n = 5). (J) Quantification of IL-15&IL-15Rα protein levels in peripheral serum at different time points following MWA in mice (n = 5).

In the revision, we have modified and added the following description:

"Significantly increased IL-15&IL-15Rα levels were found in tumor tissue at 6 hours post-MWA treatment, and persisted for 14 days, suggesting a sustained provision of IL-15&IL-15Rα within the TME by engineered bacteria."

7. Figure 3D, the aim of the clinical application of MWA is to eradicate the tumor, but the treatment did not inhibit tumor progression. What parameters is used in this study?

Reply: Many thanks for your question. We sincerely appreciate your suggestions. The parameters of MWA were adjusted to ensure local hyperpyrexia around 42 ℃ to 47 ℃ for 15 minutes. During microwave ablation, the rapid expansion of the ablation zone can lead to inadvertent ablation of adjacent tissues [1]. On the other side, for the boundary part of a large tumor or a tumor with irregular shape, the MW heating may not be sufficient to cause complete tumor cell ablation. In our experiment,

we selected to conduct MWA at a mild condition by heating the tumor at a temperature around 42 ℃ to 47 ℃, so as to mimic the incomplete MW ablation that may occur in some clinical practices and would lead to unsatisfactory therapeutic performance. With the presence of our engineered bacteria, tumors even under such mild heating condition could be effective suppressed owing to the activation of both the innate and adaptive immune responses.

In the revision, we have modified and added the following description:
"To mimic the incomplete MWA that may occur in some clinical practices and would lead to unsatisfactory therapeutic performance, we selected an ablation temperature around 42 ℃ to 47 ℃, a relatively mild temperature."

8. Figure 3H, it is not appropriate for clinical use of microwave ablation of colonic carcinoma in situ. The authors need to provide more explanation about it.

Reply: Many thanks for your question. We would like to express our gratitude for your suggestions. Most of our data were obtained from the H22 liver cancer model, and we also conducted in situ N1S1 liver cancer model in rat liver. Additionally, colorectal cancer is known to have a propensity for liver metastasis [19] [20], and liver metastases arising from colorectal cancer can also be treated with ablation [21].

9. Microwave ablation of liver cancer is often used in clinical practice, and it is necessary to provide the data on engineered bacteria combined with microwave therapy for in-situ liver cancer.

Reply and revision: Many thanks for your question. We sincerely appreciate your suggestion and find them highly meaningful. However, considering that the liver lobes of mice are quite small and may not withstand the damage caused by ablation, we opted to conduct MWA treatment in a rat model of primary liver cancer. Relevant treatment outcomes and data have been updated in the manuscript (**Figure 7A-H& Figure S31**).

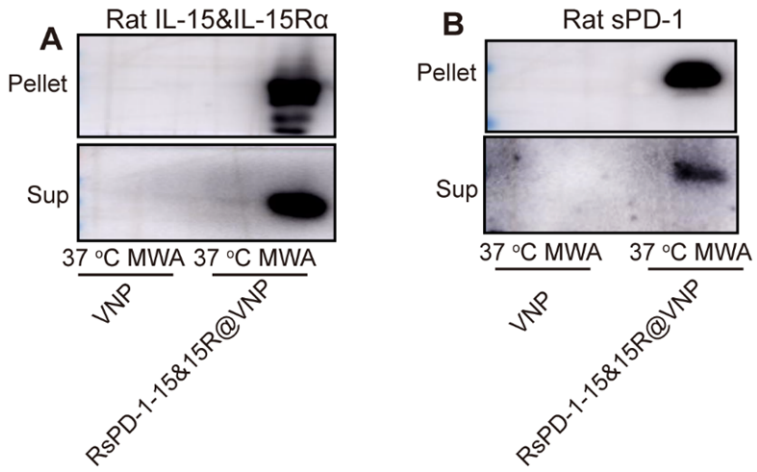
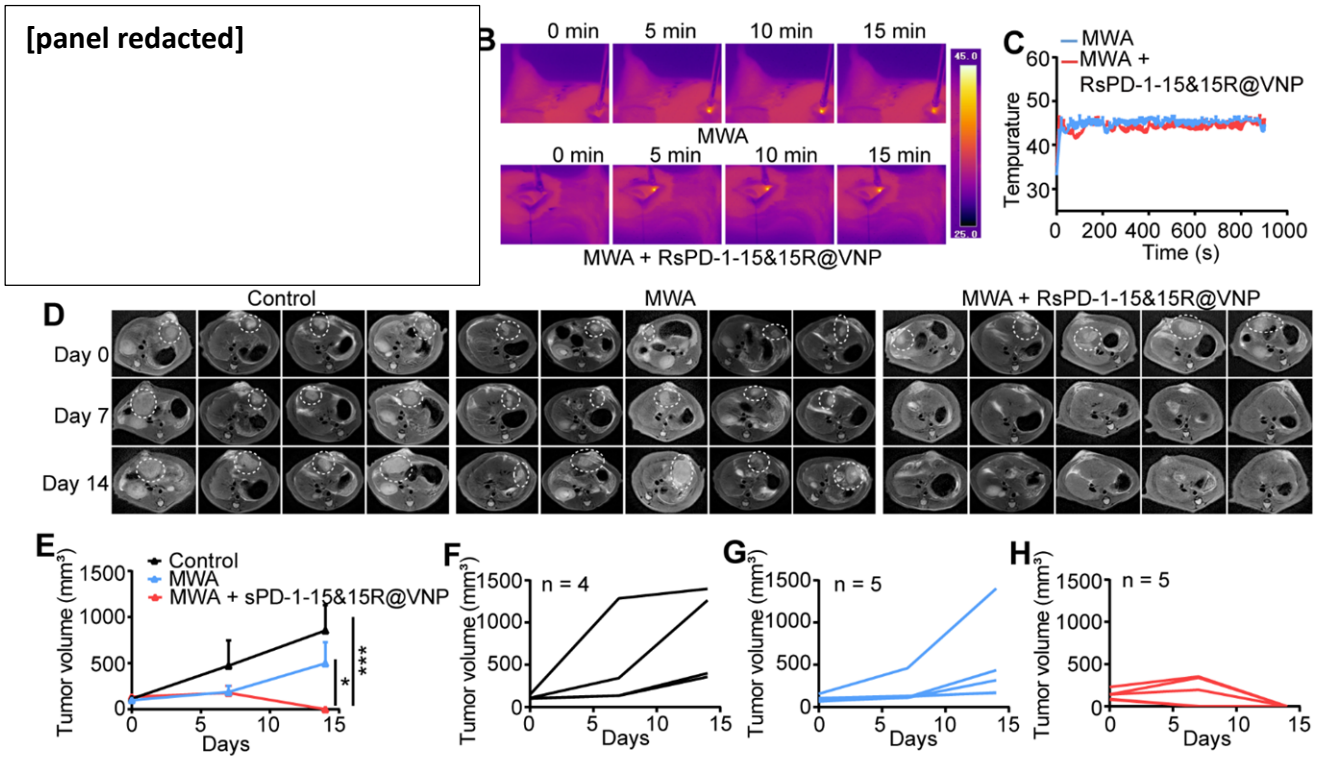


Figure 31. Western blot images of rat IL-15&IL-15Rα (A) and rat sPD-1 (B) protein in bacterial pellets and induced supernatants of sPD-1-15&15Rα@VNP under different temperature conditions.

Figure 7. (A) Schematic illustration of in vivo therapeutic schedule in N1S1 rat tumor model. (B-H) Thermal images (B), corresponding microwave heating profiles (C), corresponding T1 contrast-enhanced MRI scanning of N1S1 bearing rats with different treatments as indicated tumor curves (D), average (E) and individual (F-H) tumor growth curves of different groups of N1S1 bearing rats after various treatments as indicated.



In the revision, we have modified and added the following description:

"To further emulate clinical MWA for liver cancer, we constructed engineered bacteria RsPD-1-15&15R@VNP secreting rat IL-15&IL-15Ra and sPD-1 proteins. The engineered RsPD-1-15&15R@VNP could efficiently secrete rat IL-15&IL-15Ra and sPD-1 proteins under MWA (**Figure S31**). Next, the antitumor efficacy of RsPD-1-15&15R@VNP boosted MWA treatment was further confirmed in a highly malignant orthotropic N1S1 HCC rat tumor model. Briefly, 14 SD rats bearing N1S1 orthotopic tumors (~100 mm^3) were divided into three groups and treated as below: group I, Control (n = 4); group II, MWA (n = 5); group III, MWA+ RsPD-1-15&15R@VNP (n = 5). These SD rats of group III were intravenously injected with RsPD-1-15&15R@VNP at day 0 at a dose of 2 ×10^7 CFU (200 μL), while these SD rats of groups II and III were subjected to MWA treatment at day 3. At day 0 before different treatments and 7, 14 days post different treatment, these rats were then subjected to a 3.0-T MRI imaging system for recording the tumor volumes (**Figure 7A**). During MWA, the temperature of the tumor site was continuously monitored using an infrared thermometer, ensuring that it remained around 42 ℃ to 47 ℃ (**Figure 7B&C**). We found that engineered RsPD-1-15&15R@VNP combined with MWA showed highly effective tumor inhibitory effect, with all 5 of 5 rats cured at 14 days post corresponding treatment, much more effective than MWA alone (**Figure 7D - H**)."

10. The study focuses on early immune responses following treatment but does not address long-term tumor growth inhibition and potential adverse effects associated with the therapy. Are there data on the survival rates of treated mice, or any evidence of systemic inflammation or autoimmunity? These data are crucial for evaluating the clinical potential of this therapy.

Reply and revision: We sincerely appreciate your suggestion. Our data included the survival rates of mice after treatment, as shown in **Figure S29**. Previously, the survival time was not sufficient, but we have now recorded survival for up to 120 days. In the revision, we have conducted relevant experiments and the results showed that our engineered bacteria did not cause any risk of systemic inflammation or autoimmunity (**Figure 7I-O&S32**).

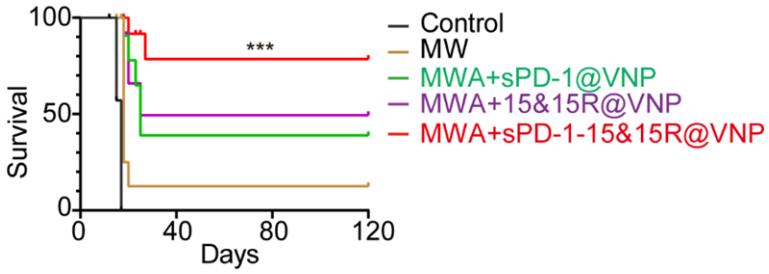


Figure S29. Corresponding mobility-free survival rate of CT26 tumor-bearing mice after different

treatments as indicated (n = 7 or 8). *P* value was calculated by the log-rank test. ***$p<0.001$.

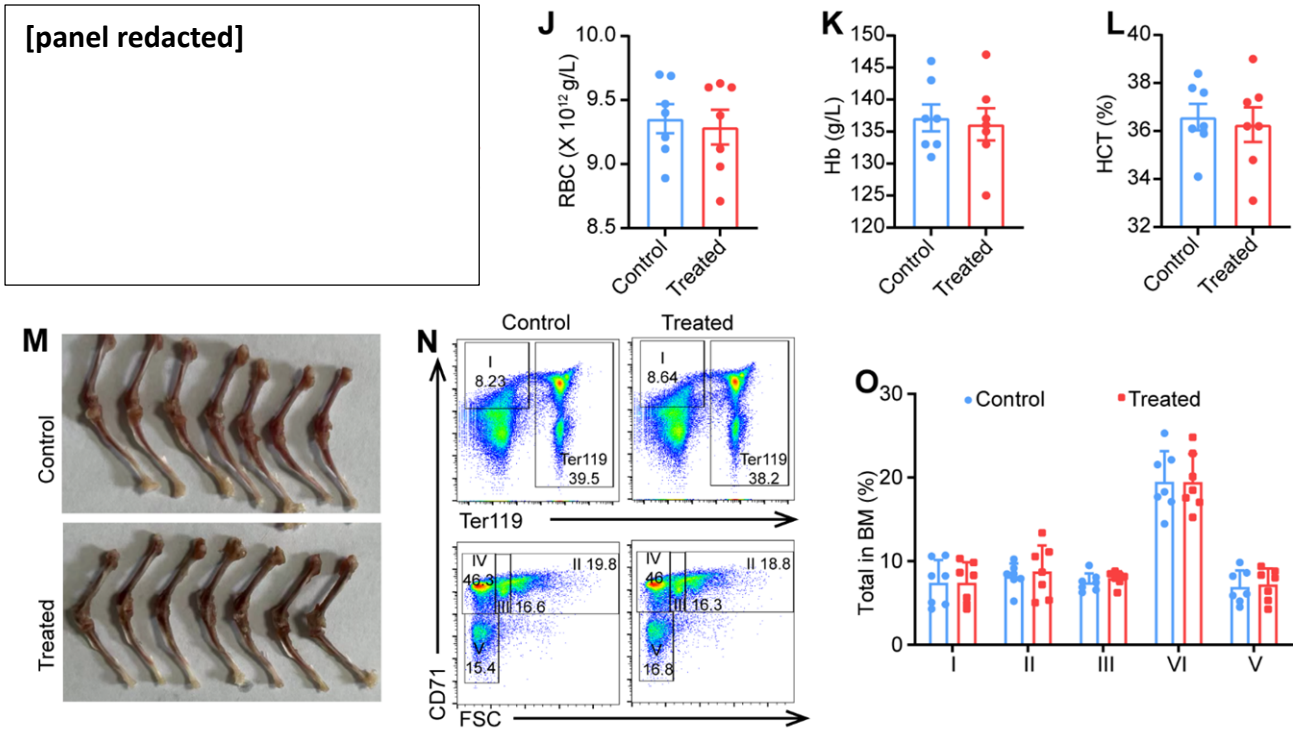


Figure 7I-O. (I) Schematic illustration of in vivo evaluation of irAE risk of engineered sPD-1-15&15R@VNP combined with MWA in H22 mouse tumor model. (J-L) The measured parameters included red blood cells (RBC, J), hemoglobin (Hb, K), and hematocrit (HCT, L) collected from these mice at day 30. (M) Generation of red cells in bone marrow. (N&O) Representative FACS profiles (N) and analysis (O) of depicting distribution of Ter119, CD71 and forward scatters (FSC-A) among bone marrow cells. The gating and % of cells at stage I–V are indicated.

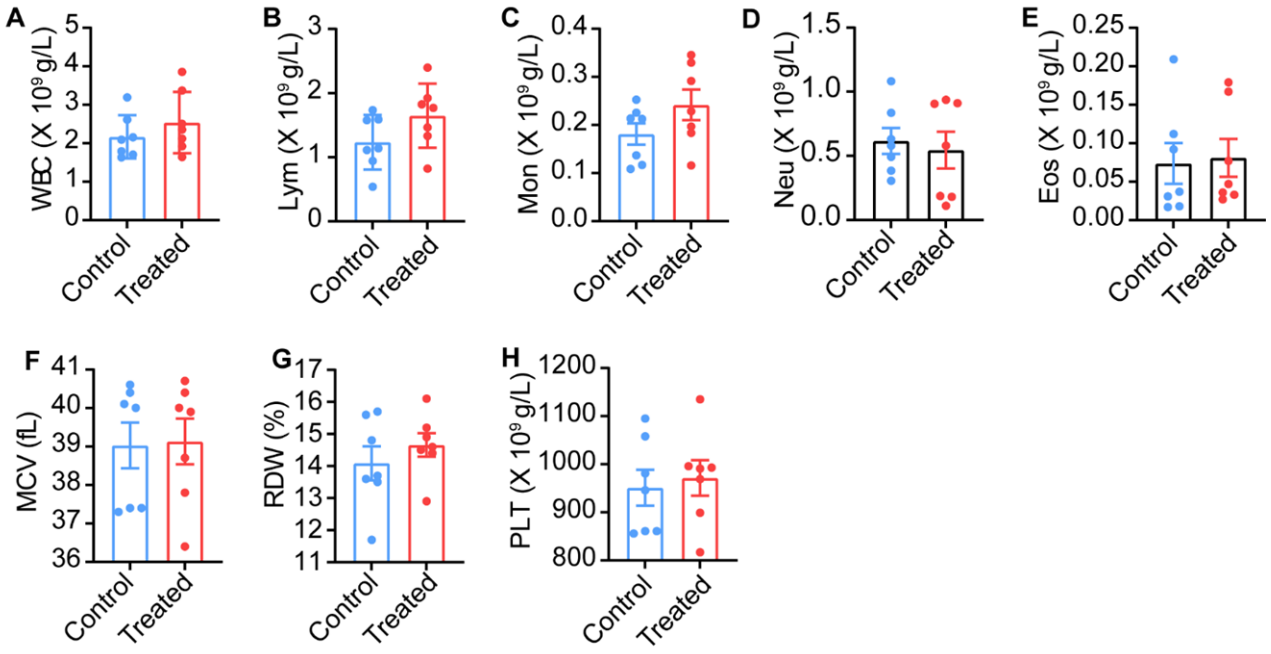


Figure S30. The measured parameters included white blood cells (WBC, A), lymphocyte (Lym, B), monocytes (Mon, C), neutrophil (Neu, D), eosinophil (Eos, E), mean corpuscular volume (MCV, F), red blood cell distribution width, (RDW, G) and blood platelet (PLT, H) collected from these mice at day 30. (M) Generation of red cells in bone marrow. (N&O) Representative FACS profiles (N) and analysis

In the revision, we have modified and added the following description:

"As accumulating reports have demonstrated that ICB therapy would cause potential systemic inflammation or autoimmunity concern (54), we then carefully evaluated the potential toxic effects our proposed cancer treatment strategy in a long-term experimental model. We treated 15 mice bearing H22 tumors according to the previous treatment regimen (**Figure 7I**). The seven cured mice were sacrificed for hematologic and histopathology alterations after 1 months. Notably, we observed no changes in red blood cells (RBC), total hemoglobin (Hb), blood hematocrit (HCT), platelets (PLT), hemoglobin (HGB), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC), as well as slightly decreased hematocrit (HCT), and mean corpuscular volume (MCV) among the majority of the mice treated with MWA + sPD-1-15&15R@VNP (**Figure 7J - L**). The presentation of leukocytes was largely normal (**Figure S32**). These data demonstrate that the combination of MWA and engineered sPD-1-15&15R@VNP did not cause significant hemolysis or inflammation. After necropsy, it is clear that erythrocyte production in the bone marrow was also not severely restricted, and the bone and bone marrow of MWA + sPD-1-15&15R@VNP treated mice were comparable to those of control mice (**Figure 7M**). To quantitate the

defects in the red cell lineage in the bone marrow, we analyzed the distribution of CD71 and Ter119 markers among the bone marrow cells as well as the cell sizes. These markers have been used to mark five stages of erythrocyte development: stage I, $CD71^{+}Ter119^{-}$; stage II, FSC-$A^{hi}CD71^{+}Ter119^{+}$; stage III, FSC-Ami$CD71^{+}Ter119^{+}$; stage IV, FSC-$A^{lo}CD71^{+}Ter119^{+}$; and stage V, $CD71^{-}Ter119^{+}$. As shown in Figure 7N, MWA + sPD-1-15&15R@VNP treated mice exhibited similar distribution and maturation states of erythrocytes in the bone marrow compared with control mice. Taken together, our results demonstrated that sPD-1-15&15R@VNP boosted MWA treatment could induce effective tumor suppression without imposing obvious side effects."

11. The manuscript showed that the combination of MWA and 15&15R@VNP can enhance the maturation of DCs, activation of CD8+ T and NK cells, and increase IFN-γ production. However, it remains unclear how the engineered bacteria specifically support these immune responses. Does the expression of IL-15&IL-15Rα by the bacteria directly stimulate nearby immune cells, or are there intermediary factors involved? Further experiments, including in vitro studies, could provide deeper insights into the mechanism of action.

Reply and revision: Thanks for your question. We think that you have raised a very professional question! Regarding the mechanism by which engineered bacteria stimulate DCs activation, it primarily involves the interaction of bacterial surface stimulatory molecules with receptors such as Toll-like receptors (TLRs). VNP20009, despite its modified LPS that avoids TLR4 activation, retains the ability to activate TLR5 receptors [22]. TLR5 is a pattern recognition receptor that recognizes flagellin and activates DCs [23, 24]. And we also conducted additional in vitro experiments that confirmed the ability of the bacteria to enhance DCs activation while promoting the secretion of CXCL10 (**Figure S20**), which, in turn, recruits $CXCR3^{+}$ T cells and NK cells. To investigate the role of IL-15&IL-15Rα in promoting immune cell functions, we performed transcriptome sequencing analysis of the lymphocytes derived from the tumor tissue after treatment. Gene Ontology (GO) enrichment analysis showed that the upregulated genes in the tumor tissues-derived lymphocytes of MWA+ IL-15&IL-15R@VNP treated mice were enriched in several immune-related pathways, including antigen binding, innate immune responses, adaptive immune responses, cytokine mediated signaling pathways, and positive regulation of NF-κB transcription factor activity, etc. (**Figure 5D&E**). Subsequent Gene Ontology (GO) enrichment analysis showed that upregulated genes were enriched in the pathways associated with IL-15&IL-15Rα-mediated T cell activation, specifically the MAPK, RAS, JAK-STAT, and NF-κB pathways, exhibited upregulation (**Figure 5H**).

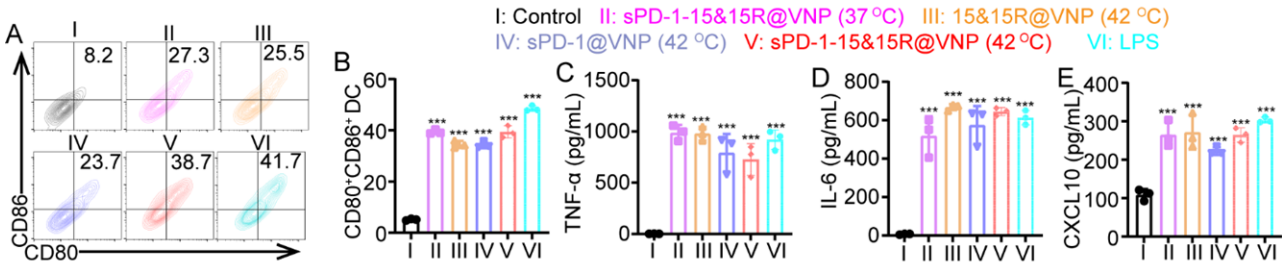


Figure S20. Flow cytometry analysis (A) and percentage (B) of mature BMDCs post different treatments as indicated. (C-E) Secretion levels of TNF-α (C), IL-6 (D) and CXCL10 (E) by BMDMs under different treatments.

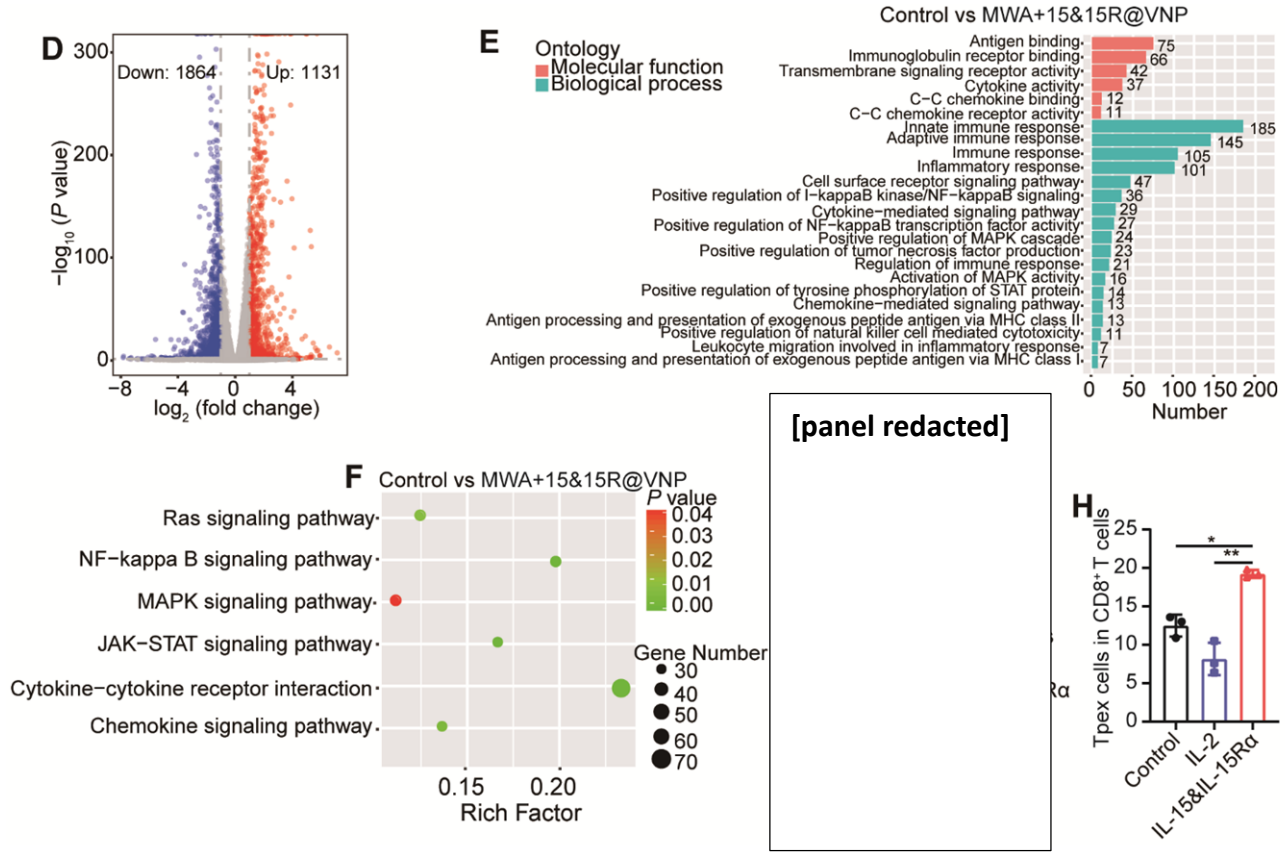


Figure 5. (D) On day 3 after treatment, control and MWA+15&15R@VNP-treated tumor tissues derived lymphocytes were taken for transcriptome sequencing. The overall distribution of regulated genes is represented by a volcano plot, in which the abscissa and ordinate represent the fold change of gene expression in different samples and statistical significance (*p* value) of the difference in gene expression, respectively. The red, blue and grey dots represent the upregulated, downregulated and no significant changes of genes, respectively. (E) GO enrichment analysis of the upregulated genes in tumor tissues- derived lymphocytes from the MWA+15&15R@VNP-treated mice. (F) The enrichment scatter plot to show KEGG enrichment analysis of a selection of immune-related KEGG terms (n = 3). (G) Schematic illustration of the sorting and treatment of tumor-derived $CD8^+$ T cells in tumor-bearing

mice. (H) The percentage of Tpex cells under different treatments.

In the revision, we have modified and added the following description:

"Additionally, we performed in vitro stimulation of DCs maturation using heat-inactivated bacteria. The results demonstrated that both the engineered bacteria prior to and following induction were capable of stimulating DCs maturation comparable to that induced by LPS. Furthermore, these mature DCs secreted substantial quantities of TNF-α, IL-6, and CXCL10 **(Figure S20)**."

"To investigate the role of IL-15&IL-15Rα in promoting immune cell functions, we performed transcriptome sequencing analysis of the lymphocytes from treated tumor tissues. This method identified 2995 differentially expressed genes, with 1131 transcripts upregulated and 1864 transcripts downregulated in tumor tissue-derived lymphocytes of MWA+ IL-15&IL-15R@VNP treated mice compared with those from the control mice (**Figure 5D**). Gene Ontology (GO) enrichment analysis showed that the upregulated genes were enriched in several immune-related pathways, including antigen binding, innate immune responses, adaptive immune responses, cytokine mediated signaling pathways, and positive regulation of NF-κB transcription factor activity, etc. (**Figure 5E**). These upregulated transcripts in MWA+ IL-15&IL-15R@VNP treated mice further indicate that our engineered bacteria in assisting MWA therapy could potently enhance immune responses. Subsequent sequencing also proved that genes involved in the pathways associated with IL-15&IL-15R-mediated T cell activation, specifically the RAS, NF-κB, MAPK, and JAK-STAT pathways, exhibited upregulation (**Figure 5H**). Most importantly, the expression of proliferation-related factor (Mki67), anti-apoptosis-related protein (Bcl2) and functional cytokines (Prf1, Grzb, IFN-γ) were also increased **(Figure S21A-F**). Subsequently, to further verify the mechanism, we sorted tumor-derived $CD8^+$ T cells and stimulated with IL-15&IL-15Rα (**Figure 5F**). The results showed that compared with IL-2, $CD8^+$ T cells treated with IL-15&IL-15Rα were mostly in the precursor exhaustion stage rather than the terminal exhaustion (**Figure 5G&S22**). It was further found that after the IL-15&IL-15Rα treatment, more PD-1^+CD8^+ T cells expressed Ki-67, a cell-proliferation marker, demonstrating that IL-15&IL-15R promotes the proliferation of PD-1^+CD8^+ T cells (**Figure S23**). These Tpex cells, when the PD-1-PD-L1 axis is blocked, can effectively play their tumor killing function. These data provide strong evidence for the implementation of ICB therapy. Collectively, we propose that the sustained activity of $CXCR3^+CD8^+$ T cells, particularly Tpex cells may be attributed to IL-15&IL-15Rα produced by 15&15R@VNP upon MWA treatment."

12. The authors found no substantial alteration in the quantity of CD4+ T cells following treatment but does not thoroughly discuss the implications of this finding. Given the important role of CD4+ T cells in the immune response to cancer, a discussion regarding why these cells may not be significantly affected by the treatment and how this might impact the overall efficacy and mechanism of the therapy would be beneficial.

Reply and revision: Many thanks for your suggestion. We strongly agree with your opinion. First of all, there are abundant subsets of $CD4^+$ T cells, including Th1 cells, Th2 cells, Th17 cells and Tregs, and each subgroup plays different roles and even has a mutually exclusive relationship [25]. For example, Th1 is often regarded as the main executor of anti-tumor immune response, while Th2 and Tregs are the key factors to promote tumor progression, the effect of Th17 varies according to different tumor movements [26]. According to our experimental results, $CD8^+$ T cells play a predominant role in our system. However, whether there are alterations in $CD4^+$ T cell subsets, as well as the dynamics among different $CD4^+$ T cell subsets, will be the focus of our subsequent investigations. We would like to once again express our sincere gratitude to the you for your professional opinion.

In the revision, we have modified and added the following description:

"This could be attributed to the predominant role of $CD8^+$ T cells in mediating the anti-tumor immune responses. However, whether there are alterations in $CD4^+$ T cell subsets, as well as the dynamics among different $CD4^+$ T cell subsets, will be the focus of our subsequent investigations (39)."

13. Figure 5, while the authors did an excellent job about the role of Tpex cells in this therapeutic context, it would benefit to provide a deeper exploration into the mechanisms by which 15&15R@VNP and ICB therapy specifically promotes the expansion and function of these cells. Are there other cytokines or cellular interactions? Can the observed effects be replicated in vitro?

Reply and revision: Many thanks for your suggestion. Related to your 11th question, first, the exhaustion of T cells is due to the continuous stimulation of T cells by antigens, and MWA treatment of damaged tumor cells will provide release of tumor antigens. Our results have shown that the proportion of Tpex cells in tumor tissues of MWA combined with mice in the 15&15R@VNP group was significantly up-regulated, suggesting that IL-15 is related to promoting the functions of Tpex cells, consistent to previous reports [7, 8]. Our subsequent analysis of the lymphocytes from treated tumor tissues confirmed that multiple pathways of anti-tumor immune responses were activated. Further sequencing also proved that the IL-15 regulated pathway was significantly activated, and the expression of proliferation-related factor (Mki67), anti-apoptosis-related protein (Bcl2) and functional

cytokines (Prf1, Grzb, IFN-γ) were increased. Several studies have confirmed that the Tpex cells are those primarily responsible for the antitumor effect in the ICB treatment. In our system, 15&15R@VNP provides a prerequisite for the maintenance of Tpex activity. Therefore, we performed 15&15R@VNP in combination with ICB.

To further verify the mechanism, we sorted tumor-derived CD8$^+$T cells and stimulated with IL-15&IL-15Rα in vitro. The results showed that compared with IL-2, CD8$^+$ T cells treated with IL-15&IL-15Rα were mostly in the precursor exhaustion stage rather than the terminal exhaustion. To investigate the origin of the PD-1$^+$CD8$^+$ T cell subset, we assessed Ki-67 expression, a marker of cell proliferation, in PD-1$^+$CD8$^+$ T cells. Following treatment with IL-15&IL-15Rα, an increased proportion of PD-1$^+$CD8$^+$ T cells expressed Ki-67, indicating that IL-15&IL-15Rα promote the proliferation of PD-1$^+$CD8$^+$ T cells. This result was consistent with previous research that IL-15&IL-15Rα could promote the proliferation of Tpex cells [8]. **These Tpex cells, when the PD-1-PD-L1 axis is blocked, can effectively play their tumor killing function**. These data provide strong evidence for the implementation of ICB therapy.

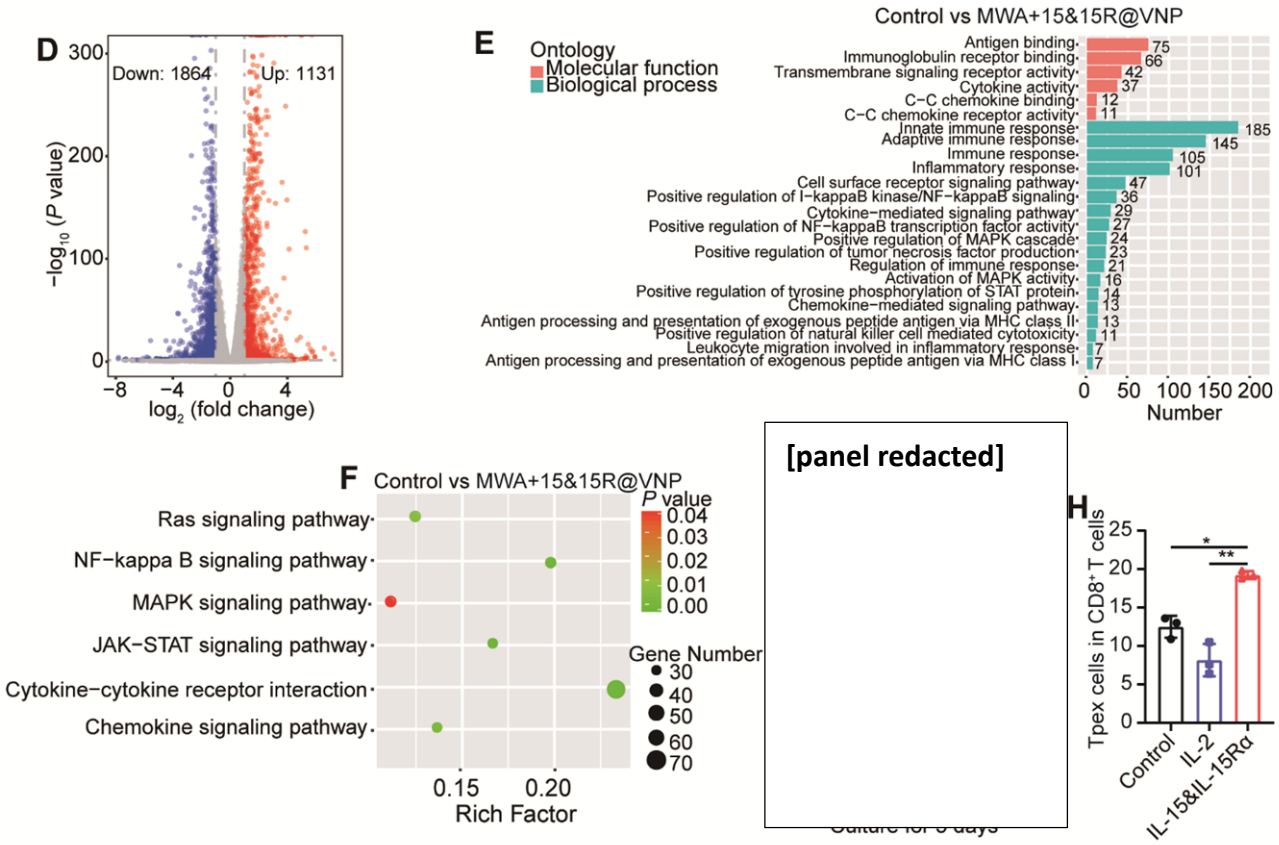


Figure 5. (D) On day 3 after treatment, control and MWA+15&15R@VNP-treated tumor tissues derived lymphocytes were taken for transcriptome sequencing. The overall distribution of regulated

genes is represented by a volcano plot, in which the abscissa and ordinate represent the fold change of gene expression in different samples and statistical significance (*p* value) of the difference in gene expression, respectively. The red, blue and grey dots represent the upregulated, downregulated and no significant changes of genes, respectively. (E) GO enrichment analysis of the upregulated genes in tumor tissues- derived lymphocytes from the MWA+15&15R@VNP-treated mice. (F) The enrichment scatter plot to show KEGG enrichment analysis of a selection of immune-related KEGG terms (n = 3). (G) Schematic illustration of the sorting and treatment of tumor-derived $CD8^+$ T cells in tumor-bearing mice. (H) The percentage of Tpex cells under different treatments.

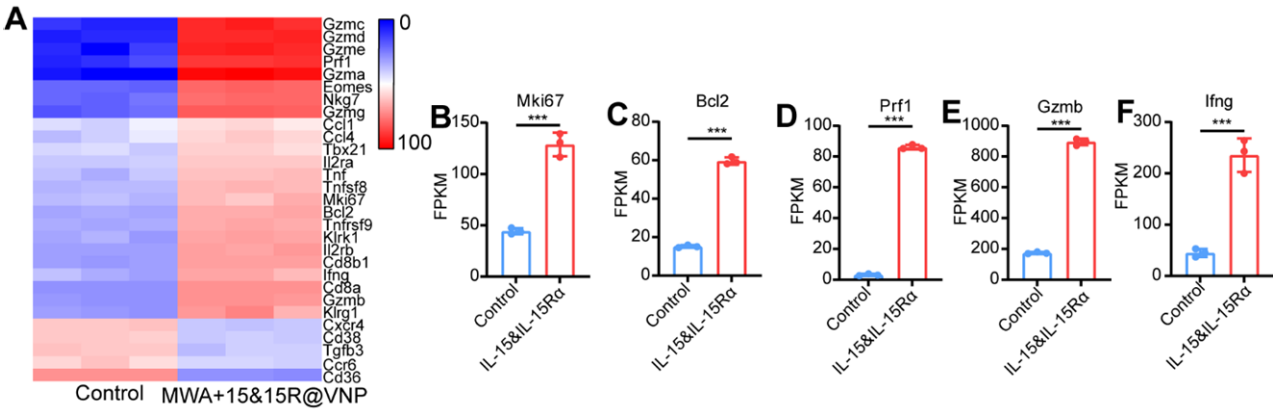


Figure S21. (A) Heatmap of genes involved in T cell proliferation, activation, and cytotoxic activity under different treatments. (B-F) Gene expression levels of Mki67 (B), Bcl2 (C), Prf1 (D), Gzmb (E), Ifng (F).

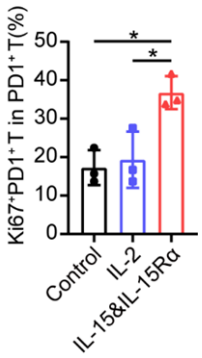


Figure S23. The percentage of $Ki67^+PD\text{-}1^+$ T cells in $PD\text{-}1^+$ T cells under different treatments.

In the revision, we have modified and added the following description:

"To investigate the role of IL-15&IL-15Rα in promoting immune cell functions, we performed transcriptome sequencing analysis of the lymphocytes from treated tumor tissues. This method identified 2995 differentially expressed genes, with 1131 transcripts upregulated and 1864 transcripts

downregulated in tumor tissue-derived lymphocytes of MWA+ IL-15&IL-15R@VNP treated mice compared with those from the control mice (**Figure 5D**). Gene Ontology (GO) enrichment analysis showed that the upregulated genes were enriched in several immune-related pathways, including antigen binding, innate immune responses, adaptive immune responses, cytokine mediated signaling pathways, and positive regulation of NF-κB transcription factor activity, etc. (**Figure 5E**). These upregulated transcripts in MWA+ IL-15&IL-15R@VNP treated mice further indicate that our engineered bacteria in assisting MWA therapy could potently enhance immune responses. Subsequent sequencing also proved that genes involved in the pathways associated with IL-15&IL-15R-mediated T cell activation, specifically the RAS, NF-κB, MAPK, and JAK-STAT pathways, exhibited upregulation (**Figure 5H**). Most importantly, the expression of proliferation-related factor (Mki67), anti-apoptosis-related protein (Bcl2) and functional cytokines (Prf1, Grzb, IFN-γ) were also increased **(Figure S21A-F**). Subsequently, to further verify the mechanism, we sorted tumor-derived $CD8^+$ T cells and stimulated with IL-15&IL-15Rα (**Figure 5F**). The results showed that compared with IL-2, $CD8^+$ T cells treated with IL-15&IL-15Rα were mostly in the precursor exhaustion stage rather than the terminal exhaustion (**Figure 5G&S22**). It was further found that after the IL-15&IL-15Rα treatment, more PD-1^+CD8^+ T cells expressed Ki-67, a cell-proliferation marker, demonstrating that IL-15&IL-15R promotes the proliferation of PD-1^+CD8^+ T cells (**Figure S23**). These Tpex cells, when the PD-1-PD-L1 axis is blocked, can effectively play their tumor killing function. These data provide strong evidence for the implementation of ICB therapy. Collectively, we propose that the sustained activity of $CXCR3^+CD8^+$ T cells, particularly Tpex cells may be attributed to IL-15&IL-15Rα produced by 15&15R@VNP upon MWA treatment."

14. Figure 6, the engineered bacteria simultaneously co-express IL-15&IL-15Rα and sPD-1, but it needs some time for IL-15 and IL-15Rα to promote T cell proliferation, and T cell exhaustion also needs some time. Will the expression of sPD-1 continue to exert its effect?

Reply and revision: Many thanks for your suggestion. First of all, we think this is a very good suggestion. In Figure 5, our immune evaluation was conducted on the third day of microwave ablation therapy, and the results showed that Tpex cells already existed at this time, and our engineered bacteria could express IL-15&IL-15Rα and sPD-1 at the same time. The subsequent IHC experiment also indicated part of the Block of PD-L1, and the immune evaluation in Figure 6 also indicated that this part of Tpex cells had a tendency to differentiate into Tex cells. Therefore, this strategy is feasible in our system. However, regarding your question, we think this is a very good suggestion. We think we will develop more intelligent systems in the future that enable different effector molecules to be

expressed at different time**.**

In the revised manuscript, we have added the following discussion:

"Considering it needs some time for IL-15&IL-15Rα to promote T cell proliferation, in the further study, sequential expressing system may be designed to further optimize the therapeutic outcomes."

15. Although the authors successfully identified positive correlations between IL-15 expression, Tpex cell infiltration, and patient prognosis, the causal relationships remain speculative. In the aforementioned animal studies, does this conclusion also have a clear relevance?

Reply and revision: First of all, there have been a lot of studies on the functions of IL-15&IL-15Rα in maintaining Tpex activity. IL-15&IL-15Rα mainly promotes the proliferation of T cells by binding to the shared receptors IL-2Rβ (CD122) and γc (CD132) with IL-2 on the surface of T cells. The process involves the activation of MAPK, RAS, JAK-STAT, and NF-κB pathways.

As for the verification of IL-15&IL-15Rα enhancement of Tpex function, we also conducted relevant analysis. For CD8+T cells derived from tumor tissues, $CD8^{+}$T cells treated with IL-15&IL-15Rα were mostly in the precursor exhaustion stage rather than the terminal exhaustion. Precursor exhaustion T cells are the cells corresponding to ICB therapy. Meanwhile, the expression of proliferation-related factor (Mki67), anti-apoptosis-related protein (Bcl2) and functional cytokines (Prf1, Grzb, IFN-γ) were increased. These data provide strong evidence for the implementation of ICB therapy.

And the editor suggested that we have deleted the patient's data.

Reviewer #4 (Remarks to the Author): with expertise in bacteria engineering, cancer therapy

The manuscript presents an approach to engineer tumor-homing bacteria as temperature-triggerable system to produce immunotherapeutic payloads (IL15 & PD1). Combining with microwave ablation (MWA), the authors show antitumoral effect in several tumor models. While the authors have shown therapeutic efficacy in these subcutaneous models, the approach for engineering bacteria with temperature-sensitive constructs as well as photothermal therapy is not novel. Furthermore, there are many technical flows that makes the reviewer question the quality of the manuscript.

Reply: We sincerely appreciate your comments and have carefully revised our manuscript in accordance with your suggestions. The maintext has been revised accordingly based on helpful comments from you.

Major comments

- Thermos-sensitive promoters have been utilized extensively in the previous literature for bacterial cancer therapy. In fact, the hyperthermia induced gene expression system is obtained from a study by Abedi et al., Nature Comm 2022 where they've utilized this system to precisely control immunotherapeutic expression from bacterial cancer therapy.

Reply: Many thanks for your question. While we acknowledge the presence of previous research on the thermal-responsive activation of tumor-homing engineered bacteria, our study introduces several unique innovations that differentiate it from the referenced works. **First**, while prior research may have focused on bacteria that respond to thermal stimuli, we expanded the application field and applied the system to MWA to induce localized hyperpyrexia, which is a clinically-relevant technology prioritizes translational feasibility. **Second**, considering IL-15 may help enhance Tpex cell function [7, 8], which plays a core role in tumor control [4, 5, 6]. We utilized this system to express tumor-associated antigens while simultaneously promoting the maintenance of Tpex cells, enabling in situ ICB therapy. The combined use of IL-15 to drive Tpex and PD-1 inhibition to boost Tpex activity offers a powerful synergistic effect that has not been explored in previous studies involving tumor-homing bacteria. **Lastly**, our system uniquely combines IL-15&IL-15Rα with sPD-1, offering a dual mechanism for immune activation. This dual immunomodulatory approach represents a significant innovation in engineered bacterial cancer therapies. Collectively, we believe that our concept shows high novelty, despite the existence of prior work in this area.

- The details on how MWA treatment was done to mice are not described. As indicated above, previous

work (including some work dating back to 2008, Chen et al., ACS Nano) had utilized variety of methods to locally heat the tumors.

Reply and revision: Many thanks for your question. To perform MWA, the MWA needle was first inserted vertically into the tumor. The operating frequency of the microwave irradiator was 2.45 GHz, as referenced in our previous work [27]. Temperature control during the microwave procedure was monitored using a thermal camera (Fotric 225). We have cited the relevant article, thanks again!

In the revision, we have modified and added the following description:

"To perform MWA, the MWA needle was inserted vertically into the tumor. The operating frequency of the microwave irradiator was 2.45 GHz, as referenced in our previous studies (56). The tumor surface temperature of the mice during microwave exposure was monitored using a thermal camera (Fotric 225)."

- Figure 7 has very little to do with the rest of the paper. I would recommend taking it out.

Reply and revision: Many thanks for your question. We have removed figure 7 from our manuscript.

- There are numbers of technical concerns in some critical data. Please see several examples below. Why does the temperature curve in (Fig. 2E and 3C) start from temperature that are lower than physiological temperature?

Reply: Many thanks for your question. We used infrared camera to measure the body surface temperature, which will be lower than the internal physiological temperature. Moreover, the body temperature of mice after being anesthetized would also be slightly lower than the normal state. This situation is not uncommon, and similar situations can be seen in many previous articles [28, 29].

o Fig. 3B – I don't see the outline of a mouse in the picture, and hence I am not sure what I am seeing here.

Reply and revision: Many thanks for your question. We enlarged the mouse, as shown in the figure below. We are very sorry for the inconvenience caused to you due to the limited space and small picture.

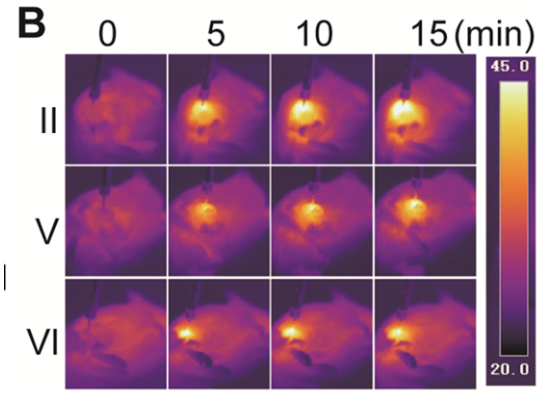


Figure 3B, Thermal images of mice after different treatments as indicated (n = 5 to 7).

o Fig. 4B & C – The data should be identical and be representative, but I don't see them matching. For example, Fig. 4B in group V is reported to be 17.3% and yet I don't see that datapoint in Fig. 4C.

Reply: Many thanks for your suggestion. We have made the necessary corrections.

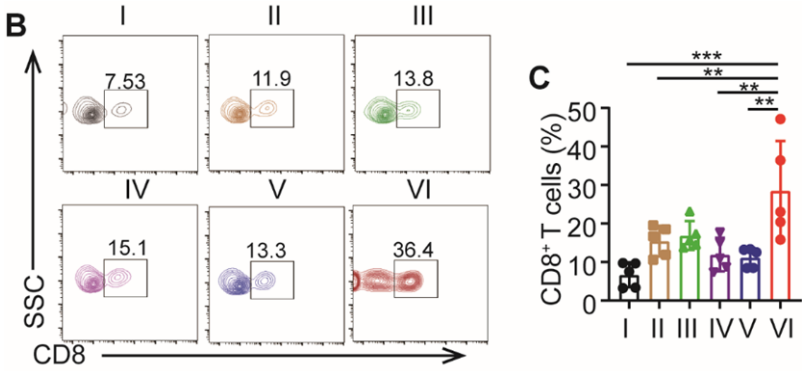


Figure 4. (B&C) Flow cytometric analysis (B) and proportions (C) of $CD8^+$ T cells ($CD45^+CD3^+CD8^+$) in tumor tissues after different treatments as indicated.

o Fig. 4H – there are some extreme population cluster in both low and high levels of IFNg. I'd be cautious of using proportions to analyze this datasets as it might skew the interpretation.

Reply and revision: Many thanks for your suggestion. We have revised it according to your suggestions.

o Fig. 4N – why did the author switched to a new model (B16) when showing the mechanisms of therapeutic effect from another model (H22 and CT26)?

Reply and revision: Many thanks for your question. The reason we employed the B16 model is that it serves as a valuable research tool, particularly because anti-NK1.1 is a well-defined antibody that effectively depletes NK cells. However, due to strain-specific limitations, this antibody cannot be

applied to BALB/C mice. However, we believe your suggestions are valid. Consequently, we have adopted their recommendations and utilized Anti-ASIO GM1 antibody to conduct a re-evaluation in the H22 model.

[panel redacted]

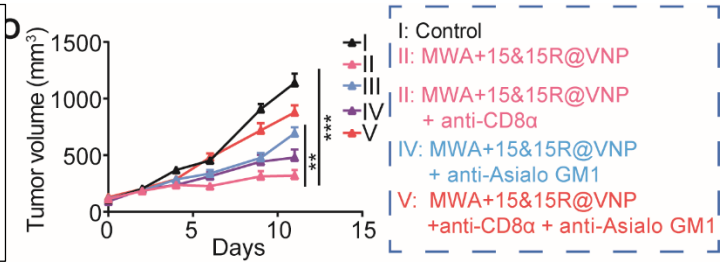


Figure 4. (N) Schematic illustration of the in vivo study schedule of the $CD8^+$ T and NK-cell depletion assay. (O) Tumor growth curves of H22 tumor-bearing mice after different treatments as indicated.

In the revision, we have modified the following description:

"Our immunological evaluation analysis mentioned above has confirmed that the anti-tumor immune responses triggered by the combined MWA with 15&15R@VNP primarily relies on $CD8^+$ T cells and NK cells. Next, a set of animal experiments was performed utilizing the H22 tumor model, wherein $CD8^+$ T cells and NK cells were depleted by corresponding antibodies. As shown in the **Figure 4N**, once the tumor volume reached approximately 100 mm^3, the mice were randomly divided into five groups. Specifically, with group I receiving PBS administration at the control, group II-V all received combined MWA and 15&15R@VNP treatment, together with intravenous injection of the control antibody anti-IgG (group II), anti-CD8α antibody to deplete $CD8^+$ T cells (group III), anti-ASIO GM1 antibody to deplete NK cells (group IV), and anti-CD8α + anti-ASIO GM1 antibodies to simultaneously deplete both $CD8^+$ T cells and NK cells (group V). The engineered bacteria were intravenously injected into the mice at day 0 with a dosage of 2×10^6 CFU per mouse. After 3 days, MWA treatment was carried out in groups II, III, IV, and V, heat up the tumors to a temperature of around 42 ℃ - 47 ℃ . Anti-CD8α and anti-ASIO GM1 antibodies were administered intravenously on days 3, 5, and 7 at a dose of $1mg \cdot kg^{-1}$ per mouse, respectively. Notably, up simultaneous depletion of $CD8^+$ T cells and NK cells, the tumor suppression effect by the combined MWA + 15&15R@VNP would no longer exist **(Figure 4O)**, while the single depletion of $CD8^+$ T cells or NK cells partially interfered with the synergistic therapeutic effects. Our results demonstrate the significant contribution of these two cell subsets in facilitating the therapeutic efficacy of 15&15R@VNP synergistic MWA therapy."

o Figure S2: Did the authors control for other microbial factors that may contribute to T-cell proliferation? It is unclear if the bacteria supernatant itself without IL-15 is sufficient for proliferation

Reply and revision: Many thanks for your question. Actually, we have considered this point and conducted our experiments using purified proteins of IL-15&IL-15Rα to eliminate the possibility of contamination. In this process, IL-15&IL-15Rα with His tag protein will be adsorbed by nickel chelating column and then elute, thus avoiding bacterial protein contamination. But our description is too brief, and we have revised our manuscript based on your suggestions. Thanks again!

In the revision, we have modified and added the following description:

"To validate the activity maintenance of IL-15&IL-15Rα protein expressed by 15&15R@VNP on T cells, we purified the expressed fusion protein using a nickel chelated column (GenScript, Nanjing, China) according to the according to the manufacturer's instructions, and the concentration of the purified IL-15&IL-15Rα was measured with BCA kit (Thermo Scientific, USA). CFSE-labeled T cells were then stimulated with IL-15&IL-15Rα protein at a concentration of 20 ng·mL^{-1}. After 72 hours, T cell proliferation was assessed by flow cytometry."

We would like to express our gratitude to you once again.

Minor comments

- Figure 1H-J: not clear what was done in this experiment. Did the bacteria receive 15min heat induction once, or everyday?

Reply: Many thanks for your question. The bacteria receive 15 min MWA heat induction once. This was an oversight on our part, which we have specifically explained in accordance with your comments. Thanks again!

- Line 68: not sure if it has been shown that facultative anaerobes have intrinsic tropism towards hypoxia

Reply and revision: Many thanks for your question. We have carefully considered your suggestions and conducted immunofluorescence staining for hypoxia marker (HIF-1α) and bacterial outer membrane proteins in tumor tissues. The results indicate a higher colonization of bacteria in the hypoxic regions.

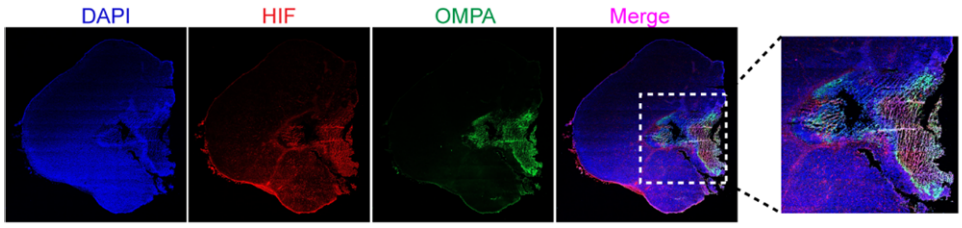


Figure S5. Immunofluorescence staining of whole tumor slices from non-hypoxic region and hypoxic regio. DAPI stands for the nuclei of tumor cells, red for HIF-1α highly expressed cells and green for OMPA-stained salmonella typhimurium.

In the revision, we have modified and added the following description:

"Immunofluorescence staining assay confirmed that salmonella typhimurium showed high levels in the hypoxic regions of a tumor (overexpression of HIF-1α) (**Figure. S5**). These results illustrated that the salmonella typhimurium could be efficiently accumulated in the tumor site and penetrated the tumor hypoxic region"

- Line 73: Human studies have been conducted in these strains, but it is premature to state that the safety has been proven

Reply and revision: Many thanks for your question. We strongly agree with your opinion, so we have carried out the relevant long-term safety test analysis. We have conducted relevant experiments and the results showed that our engineered bacteria did not cause any risk of systemic inflammation or autoimmunity (Figure 7I-O). However, we have revised the manuscript to make our article more rigorous. Thank you again！

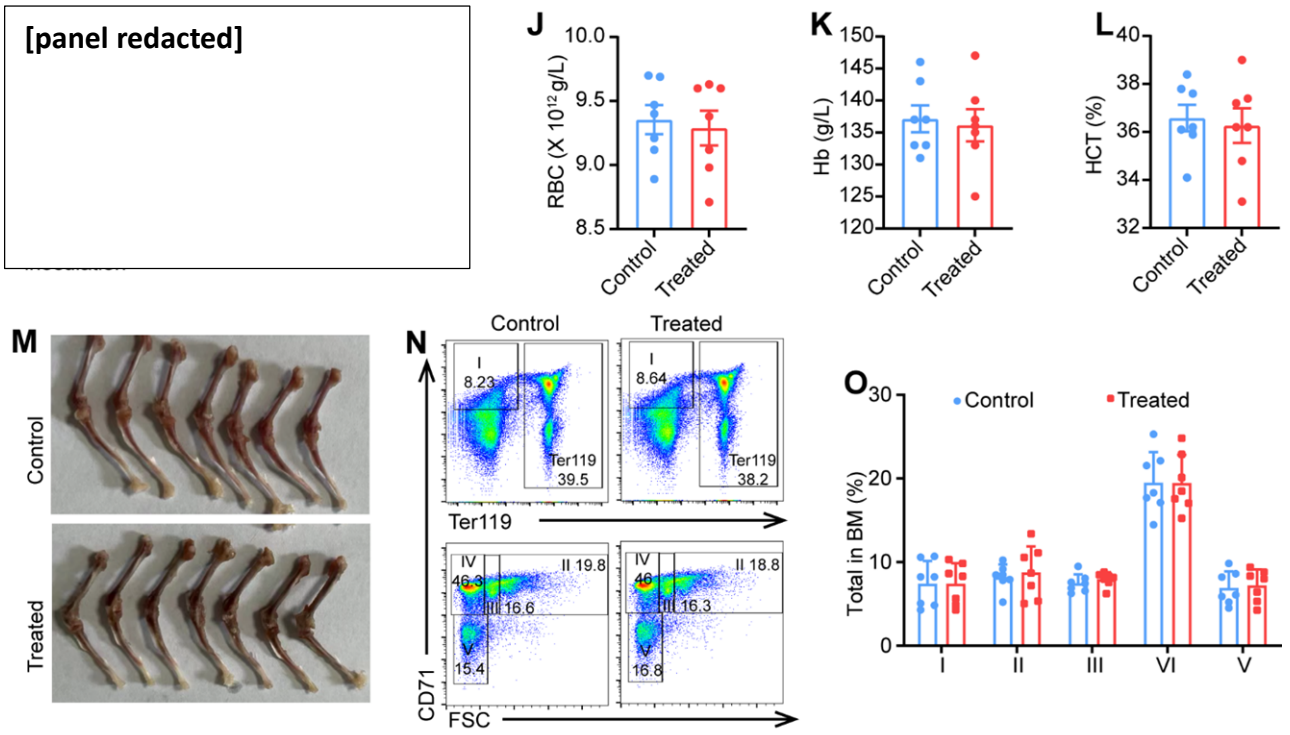


Figure 7I-O. (I) Schematic illustration of in vivo evaluation of irAE risk of engineered sPD-1-15&15R@VNP combined with MWA in H22 mouse tumor model. (J-L) The measured parameters included red blood cells (RBC, J), hemoglobin (Hb, K), and hematocrit (HCT, L) collected from these mice at day 30. (M) Generation of red cells in bone marrow. (N&O) Representative FACS profiles (N) and analysis (O) of depicting distribution of Ter119, CD71 and forward scatters (FSC-A) among bone marrow cells. The gating and % of cells at stage I–V are indicated.

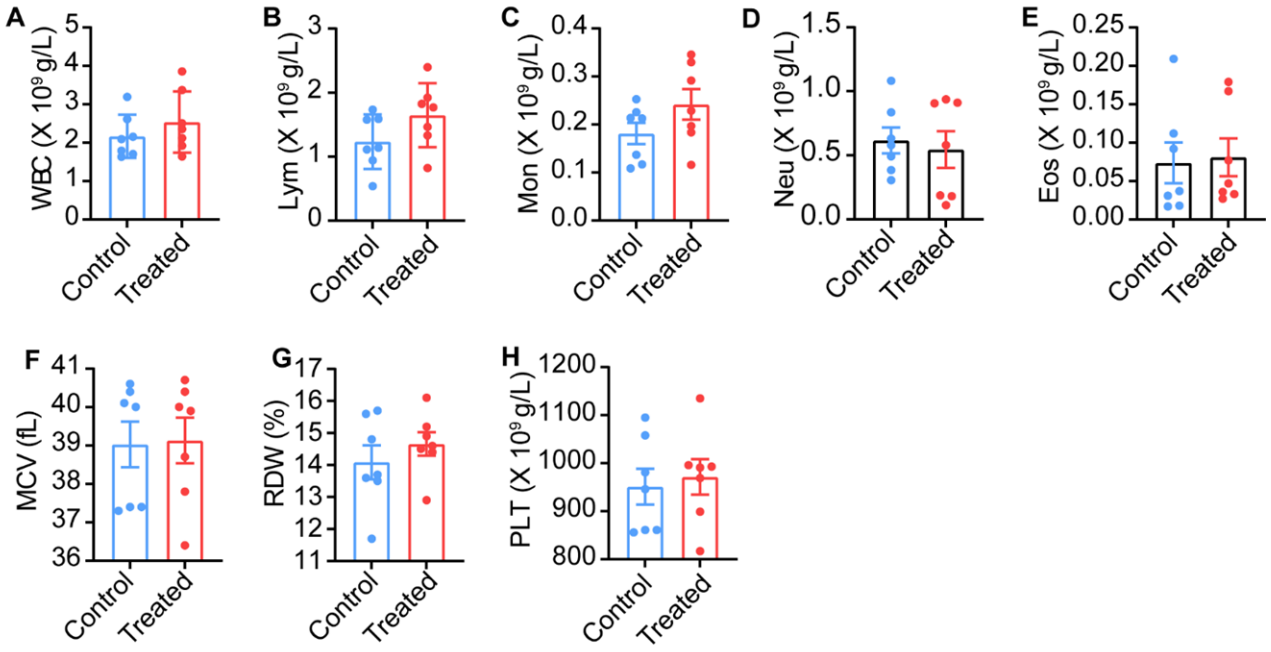


Figure S32. The measured parameters included white blood cells (WBC, A), lymphocyte (Lym, B), monocytes (Mon, C), neutrophil (Neu, D), eosinophil (Eos, E), mean corpuscular volume (MCV, F),

red blood cell distribution width, (RDW, G) and blood platelet (PLT, H) collected from these mice at day 30. (M) Generation of red cells in bone marrow. (N&O) Representative FACS profiles (N) and analysis

In the revision, we have modified and added the following description:

"As accumulating reports have demonstrated that ICB therapy would cause potential systemic inflammation or autoimmunity concern (54), we then carefully evaluated the potential toxic effects our proposed cancer treatment strategy in a long-term experimental model. We treated 15 mice bearing H22 tumors according to the previous treatment regimen (**Figure 7I**). The eight cured mice were sacrificed for hematologic and histopathology alterations after 1 months. Notably, we observed no changes in red blood cells (RBC), total hemoglobin (Hb), blood hematocrit (HCT), platelets (PLT), hemoglobin (HGB), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC), as well as slightly decreased hematocrit (HCT), and mean corpuscular volume (MCV) among the majority of the mice treated with MWA + sPD-1-15&15R@VNP (**Figure 7J - L**). The presentation of leukocytes was largely normal (**Figure S32**). These data demonstrate that the combination of MWA and engineered sPD-1-15&15R@VNP did not cause significant hemolysis or inflammation. After necropsy, it is clear that erythrocyte production in the bone marrow was also not severely restricted, and the bone and bone marrow of MWA + sPD-1-15&15R@VNP treated mice were comparable to those of control mice (Figure 7M). To quantitate the defects in the red cell lineage in the bone marrow, we analyzed the distribution of CD71 and Ter119 markers among the bone marrow cells as well as the cell sizes. These markers have been used to mark five stages of erythrocyte development: stage I, $CD71^{+}Ter119^{-}$; stage II, $FSC\text{-}A^{hi}CD71^{+}Ter119^{+}$; stage III, FSC-AmiCD71^{+}Ter119^{+}; stage IV, $FSC\text{-}A^{lo}CD71^{+}Ter119^{+}$; and stage V, $CD71^{-}Ter119^{+}$. As shown in Figure 7N, MWA + sPD-1-15&15R@VNP treated mice exhibited similar distribution and maturation states of erythrocytes in the bone marrow compared with control mice. Taken together, our results demonstrated that sPD-1-15&15R@VNP boosted MWA treatment could induce effective tumor suppression without imposing obvious side effects."

- Line 77: I would not call Salmonella as a type of anaerobic bacteria

Reply and revision: Many thanks for your suggestion. We have revised the manuscript according to your suggestion to make our article more rigorous.

In the revision, we have modified the following description:

"As a type of facultative anaerobic bacteria, such 15&15R@VNP upon intravenous injection would

selectively colonize in the tumor."

- Many figures are lacking statistical analysis. For example, Fig 2J looks like there are some increase in IL15 levels but it is hard to evaluate.

Reply and revision: Many thanks for your suggestion. We have added statistical analysis into these figures according to your suggestion to make our article more rigorous.

- Figure S6: It is very difficult to conclude from just images whether MWA treatment had any impact on bacterial survival in the tumor. I'd recommend measuring CFU pre & post treatment.

Reply and revision: Many thanks for your suggestion. We have measured CFU pre & post treatment according to your suggestion to make our article more rigorous. Thanks again!

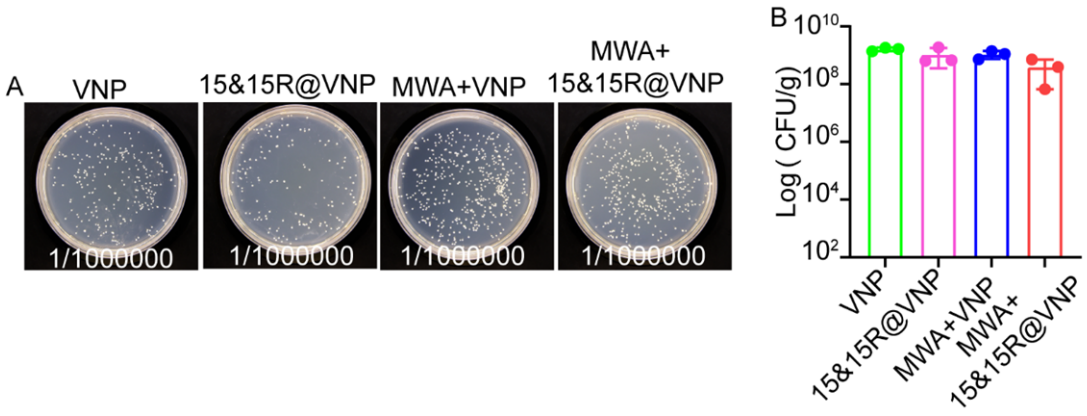


Figure S10. Representative photographs (A) and quantification (B) of bacterial colonization in tumor tissues post different treatments as indicated.

In the revision, we have modified and added the following description:

"Notably, we found that there was no significant change in the bacteria population within the tumor post-MWA (**Figure S10**), suggesting that MWA treatment at this mild condition would not significantly kill bacteria colonized in the tumor**".**

We thank the reviewers and editors for their comments, which greatly helped to improve the quality of our manuscript!

References

1. Zaidi, N. *et al.* Laparoscopic microwave thermosphere ablation of malignant liver tumors: An analysis of 53 cases. *Journal of surgical oncology* **113**, 130-134 (2016).

2. Miller, B.C. *et al.* Subsets of exhausted CD8(+) T cells differentially mediate tumor control and respond to checkpoint blockade. *Nature immunology* **20**, 326-336 (2019).

3. Paley, M.A. *et al.* Progenitor and terminal subsets of CD8+ T cells cooperate to contain chronic viral infection. *Science (New York, N.Y.)* **338**, 1220-1225 (2012).

4. Kallies, A., Zehn, D. & Utzschneider, D.T. Precursor exhausted T cells: key to successful immunotherapy? *Nature reviews. Immunology* **20**, 128-136 (2020).

5. Bonté, P.E. *et al.* Selective control of transposable element expression during T cell exhaustion and anti-PD-1 treatment. *Science immunology* **8**, eadf8838 (2023).

6. Meyran, D. *et al.* T(STEM)-like CAR-T cells exhibit improved persistence and tumor control compared with conventional CAR-T cells in preclinical models. *Science translational medicine* **15**, eabk1900 (2023).

7. Lee, J. *et al.* IL-15 promotes self-renewal of progenitor exhausted CD8 T cells during persistent antigenic stimulation. *Frontiers in immunology* **14**, 1117092 (2023).

8. Di Pilato, M. *et al.* CXCR6 positions cytotoxic T cells to receive critical survival signals in the tumor microenvironment. *Cell* **184**, 4512-4530.e4522 (2021).

9. Cui, M. *et al.* NIR light-responsive bacteria with live bio-glue coatings for precise colonization in the gut. *Cell reports* **36**, 109690 (2021).

10. Hartsough, L.A. *et al.* Optogenetic control of gut bacterial metabolism to promote longevity. *eLife* **9** (2020).

11. Liu, Z. *et al.* Programming Bacteria With Light-Sensors and Applications in Synthetic Biology. *Frontiers in microbiology* **9**, 2692 (2018).

12. Ash, C., Dubec, M., Donne, K. & Bashford, T. Effect of wavelength and beam width on penetration in light-tissue interaction using computational methods. *Lasers in medical science* **32**, 1909-1918 (2017).

13. Kennedy, J.E. High-intensity focused ultrasound in the treatment of solid tumours. *Nature reviews. Cancer* **5**, 321-327 (2005).

14. Liu, Y. *et al.* IL-2 regulates tumor-reactive CD8(+) T cell exhaustion by activating the aryl hydrocarbon receptor. *Nature immunology* **22**, 358-369 (2021).

15. Chinen, T. *et al.* An essential role for the IL-2 receptor in T(reg) cell function. *Nature immunology* **17**, 1322-1333 (2016).

16. Waldmann, T.A. The shared and contrasting roles of IL2 and IL15 in the life and death of normal and neoplastic lymphocytes: implications for cancer therapy. *Cancer immunology research* **3**, 219-227 (2015).

17. Abedi, M.H. *et al.* Ultrasound-controllable engineered bacteria for cancer immunotherapy. *Nature communications* **13**, 1585 (2022).

18. Zheng, J.H. *et al.* Two-step enhanced cancer immunotherapy with engineered Salmonella typhimurium secreting heterologous flagellin. *Science translational medicine* **9** (2017).

19. Zhao, S. *et al.* Tumor-derived exosomal miR-934 induces macrophage M2 polarization to promote liver metastasis of colorectal cancer. *Journal of hematology & oncology* **13**, 156 (2020).

20. Bertocchi, A. *et al.* Gut vascular barrier impairment leads to intestinal bacteria dissemination and colorectal cancer metastasis to liver. *Cancer cell* **39**, 708-724.e711 (2021).

21. Puijk, R.S. *et al.* Colorectal liver metastases: surgery versus thermal ablation (COLLISION) - a phase III single-blind prospective randomized controlled trial. *BMC cancer* **18**, 821 (2018).

22. Chen, J. *et al.* Salmonella flagella confer anti-tumor immunological effect via activating Flagellin/TLR5 signalling within tumor microenvironment. *Acta pharmaceutica Sinica. B* **11**, 3165-3177 (2021).

23. Means, T.K., Hayashi, F., Smith, K.D., Aderem, A. & Luster, A.D. The Toll-like receptor 5 stimulus bacterial flagellin induces maturation and chemokine production in human dendritic cells. *Journal of immunology (Baltimore, Md. : 1950)* **170**, 5165-5175 (2003).

24. Schmitt, S. *et al.* Fusion of Bacterial Flagellin to a Dendritic Cell-Targeting αCD40 Antibody Construct Coupled With Viral or Leukemia-Specific Antigens Enhances Dendritic Cell Maturation and Activates Peptide-Responsive T Cells. *Frontiers in immunology* **11**, 602802 (2020).

25. Wu, K. & Fong, L. CD4(+) T cells help myeloid-mediated killing of immune-evasive tumors. *Trends in cancer* **9**, 777-779 (2023).

26. Gao, S., Hsu, T.W. & Li, M.O. Immunity beyond cancer cells: perspective from tumor tissue. *Trends in cancer* **7**, 1010-1019 (2021).

27. Zhu, Y. *et al.* Metallo-alginate hydrogel can potentiate microwave tumor ablation for synergistic cancer treatment. *Science advances* **8**, eabo5285 (2022).

28. Chen, Y., Du, M., Yuan, Z., Chen, Z. & Yan, F. Spatiotemporal control of engineered bacteria

to express interferon-γ by focused ultrasound for tumor immunotherapy. *Nature communications* **13**, 4468 (2022).

29. Ma, W. *et al*. Bioactivable STING Nanoagonists to Synergize NIR-II Mild Photothermal Therapy Primed Robust and Long-Term Anticancer Immunity. *Advanced materials (Deerfield Beach, Fla.)* **35**, e2303149 (2023).

Responses to reviewer's comments

We sincerely thank the reviewers for their comments and suggestions. Below we have provided responses to the comments and have accordingly revised the manuscript.

REVIEWER COMMENTS

Reviewer #1. My previous concerns have been addressed. No other comments available.

Reply: We greatly appreciate your positive comments.

Reviewer #3. Upon reviewing the revised manuscript, I am pleased to inform you that the changes have significantly improved the clarity and quality of the paper.Therefore, I am happy to recommend the acceptance of your paper for publication.

Reply: We greatly appreciate your positive comments.

Reviewer #4. The manuscript has addressed most of my concerns. I would suggest the authors to edit the texts to remove excessive or unnecessary wordings, such as "undoubtedly", as suggested by other reviewers as well.

Reply: We greatly appreciate your positive comments. According to your suggestions, we have already removed words such as "undoubtedly", "new", "first", etc. Thank you again!

We thank the reviewers and editors for their comments, which greatly helped to improve the quality of our manuscript!