nature portfolio

Peer Review File

Reprogramming the tumor immune microenvironment using engineered dual-drug loaded Salmonella

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author), with expertise in nanomedicine for cancer immunotherapy:

In this work, the authors reported the targeted immunotherapy using an attenuated Salmonella typhimurium (SAM-FC) engineered to release two payloads: cytolysin A (ClyA) and Vibrio vulnificus flagellin B (FlaB). They found that the localized secretion of ClyA from SAM-FC could induce immunogenic cancer cell death and promote the release of tumor-specific antigens and damage-associated molecular patterns, while the localized secretion of FlaB promoted phenotypic and functional remodeling of intratumoral macrophages for inhibiting the tumor metastasis in mice bearing tumors of mouse and human origin. This work may be considered for publication after addressing the following issues:

1. The characterization of the obtained SAM-FA system is insufficient. Major steps presented in Fig. 1a and Supplementary Fig. 1 should be fully supported experimentally.

2. The morphological changes of SAM-FA before and after payload release should be characterized by microscopic techniques.

3. (1) The interactions between the two payloads in Salmonella and between the payloads and Salmonella and (2) release mechanisms of the payloads from Salmonella should be further studied.

4. Immunofluorescence staining should be conducted to support the immune response in vivo.

Reviewer #2 (Remarks to the Author), with expertise in Salmonella mediated cancer therapy:

Please see the attached PDF file.

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

The authors established multi-drug loaded Salmonella delivery system based on their inducible bidirectional Tet system for reprogramming the tumor immune microenvironment by robust ICD and macrophage-activating capacity. Localized secretion of ClyA from SAM-FC induced immunogenic cancer cell death and promoted release of tumor-specific antigens and damage-

associated molecular patterns, which establish long-term antitumor memory. Localized secretion of FlaB promoted phenotypic and functional remodeling of intratumoral macrophages that then markedly inhibited tumor metastasis in mice bearing tumors of mouse and human origin. But the results do not explain the necessity of combing ClyA and FlaB. Results demonstrated that the SAM-FC induced functional reprogramming of tumor immune microenvironment by activating both the innate and adaptive arms of the immune system. However, a few questions should be carefully solved before further consideration for publishing.

1. For the cancer metastasis inhibition evaluation via immunotherapy, immunocompetent BALB/c and immunocompromised BALB/c athymic nu−/nu− mice were chosen as animal model, why not choose the C57BL/6 mouse?

2. As shown in Fig 4b, the expression of leukocytes (CD45+) in both SAM-FC(-) and SAM-FC(+) treated groups are significantly increased compared to PBS group. But it shows that relevant difference in SAM-FC(-) group (△=4.9%) is more obvious than SAM-FC(+) group (△=4%), does it means that SAM itself will contribute the process of inhibition of cancer metastasis, not just ClyA and FlaB?

3. In section 'SAM-FC enhances the HMGB1-mediated cross-presentation of TSAs', result data demonstrated that SAM-FC(+) could promote the activation of DCs, CD4+ T cells, CD8+ T cells, and Th1 response synchronously via HMGB1. However, whether, which one (APC process, or T cell activation process) is core process should be further explored.

4. For bilateral tumor model by unilateral intratumoral injection, although increased frequency of proliferating CD4+ and CD8+ T cells were observed in SAM-DC(+) group, but the bioluminescent signals of SAM-FC(+) were observed in both treated and untreated tumors after intratumoral injection, which means that the terminal tumor inhibition was contributed by SAM-FC(+), not immunotherapy, please carefully clarify the contribution.

5. Besides bilateral tumor model, pls employing the unilateral tumor model for SAM-FC(+) treatment, after the primary tumor was eliminated, then inoculated the distant tumor model for ICD-mediated immunotherapy evaluation.

Reviewer #4 (Remarks to the Author), with expertise in Bacteria, immunology:

In their manuscript, Nguyen et al describe the use of an attenuated Salmonella Typhimurium strain that has been equipped with inducible bacterial genes to target transplantable murine tumors. The therapeutic genes are encoding cytolysin A and the flagellar protein FlaB. They provide extensive data on the characterization and in vitro as well as in vivo function. Although, this work is in general interesting to my mind the novelty of the findings is rather limited. Cytolysin A and FlaB expressed

by tumor targeting bacteria has been described before. The combined expression shows only additive effects. The effects on the immune cells from various tissues are due the particular bacterial protein, has been by enlarge described before, and therefore were expectable. Together with some inconsistencies within the experimental strategy. I find this manuscript not acceptable and more prone for publication in a specialized journal. There are some major and some minor point. I did nt separate them. In detail:

1. Title: multi-drug loaded is an overstatement. Only two are described beside the luciferase.

2. Introduction: it would be helpful to shortly introduce the TAAs and TSAs in general and in particular.

3. Line 129: it is not entirely true that bacteria alone, i.e. without recombinant anti-tumor compound did not lead to an entire eradication in preclinical models especially of CT 26. Should be corrected.

4. Results: It would be helpful if the components of the plasmids would be named before the abbreviation is used.

5. Line 170-171 is misleading should be corrected.

6. “Bom” as a component of the plasmids should be introduced here and the function explained as it appears to be an important function of the plasmid.

7. Line 197: anti-tumor payload is meant. Should be corrected.

8. My impression is that the experiments were carried out only once. Experiment should be reproduced. Of course, I can see that quite a large number of mice was included in most of the experiments and two or three tumor models were employed.

9. Line 205 and followed: the results of the control of SAM-E should be mentioned here. It is only found in the figure.

10. Line 253:tetramers against a peptide from MuLV gp70 was used as TAA. No references is given. Why was peripheral blood analyzed and not TILs and draining LN cells and spleen as in the other cases. The flow cytometry data should be shown because the tetramer staine shown on suppl. Fig. 10i is not convincing.

11. Line 270 and follow: the T cells in the draining LN were only characterized be general surface molecules. It is therefore not clear whether they were tumor specific or possibly specific for Salmonella. Why were the TILs not analyzed.

12. The metastasis part is to my mind the weakest part. Why was 4T1 not applied orthotopic. It is usually injected into the fat pad of female mice. The findings can simply be explained by assuming that the reduction of the cell number is sufficient to delay metastasis formation. More characterizations have to be done to allow this statement. For instance, is there any influence on the premetastatic niche etc.

13. Why was a solid sc tumor implanted besides the intrasplenic injection of MC38.

14. Why was the liver not analyzed for colonization by bacteria. The fast growing tumor colonies in the liver should already display a size that allows colonization by bacteria at the time of bacterial injection.

15. Line 314: what does mean “liver directed SAM-FC treatment?

16. Line 377: similar experiments with similar results have been carried out and published several years ago. They should be quoted.

17. Line 447: “to the best of our knowledge….” Is an overstatement. The parts of the system have been described before and the dual expression has been published by the authors. Please remove.

18. The authors show extensive data of imaging. The panels are so small that nothing can be recognized especially when using hard copies. Similarly, nothing can be recognized on the micrographs. It is also missing how many field of view were analyzed.

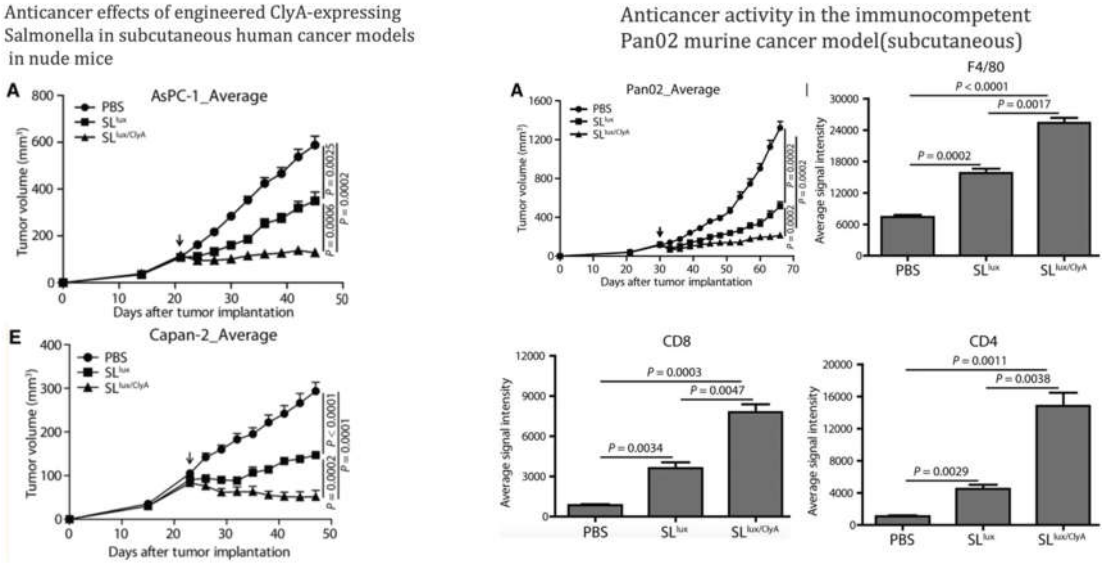
19. Legend Suppl. Fig 8b. the description is confusing. Eradicated might have to be added.

20. Fig. 1: An effect by the bacteria carrying empty vector is seen. –why due the mice not survive longer.

21. Suppl. Fig. 2c: the +/- of SAM-FC is mixed up.

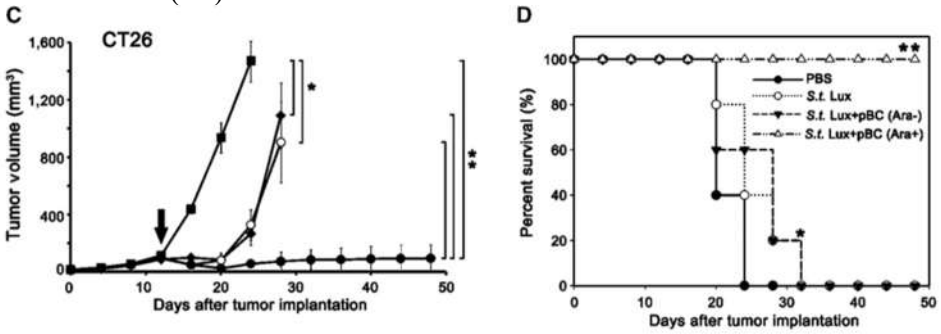
In the field of bacterial therapy for tumors, taking advantage of tumor-targeting Salmonella typhimurium to deliver various therapeutic cargo has always been an attractive area. Among these therapeutic cargos, toxins or immunomodulators are common choice. For example, toxin ClyA and FlaB used in this manuscript. This research team has done a lot of work about using ClyA and FlaB, separately:

In the paper entitled as "Targeting of pancreatic cancer cells and stromal cells using engineered oncolytic *Salmonella typhimurium*" by the same research team, they demonstrated that Salmonella typhimurium expressing ClyA can almost completely inhibit the growth of subcutaneous pancreatic cancer models in Nude mice and immunocompetent C57BL/6 mice, as shown in F1.



F1. ClyA expressing Salmonella suppressed subcutaneous pancreatic tumors in nude mice and C57BL/6 mice

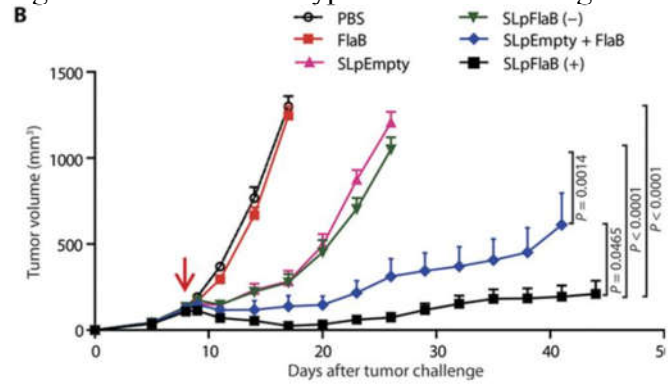
In another article entitled as "Genetically engineered Salmonella typhimurium as an imageable therapeutic probe for cancer", this research team also showed that Salmonella typhimurium expressing ClyA alone can almost completely inhibit the subcutaneous tumor growth of colon cancer CT26 (F2).



F2. Balb/c mice bearing subcutaneous CT-26 tumor were treated with ClyA expressing ppGpp-defective *S. typhimurium*

In addition, this research team also found that Salmonella typhimurium expressing FlaB can effectively inhibit the growth of subcutaneous colon cancer MC38 by activating TLR5 pathway

(F3). This relevant article is entitled as “Two-step enhanced cancer immunotherapy with engineered Salmonella typhimurium secreting heterologous flagellin”



F3. C57BL/6J mice bearing subcutaneous MC38 tumor were treated with FlaB expressing Salmonella

Based on their past work mentioned above, this research team explored the effect of co-expressing two therapeutic cargoes and explored its underlying mechanism in this manuscript. Although using ClyA or FlaB alone already has good effects, combination therapy has indeed shown a slight but significant enhancement in therapeutic efficacy, which has some scientific significance. For this work, we have the following questions:

Major:

1. From lines 281-291, the authors validated the anti-metastatic ability of SAM in the 4T1 spontaneous model, and found that SAM_FC(+) suppressed metastasis in both immunocompetent Balb/c mice and immunocompromised Nude mice.

1) We are extremely curious about the anti-tumor effects on primary 4T1 tumor growth, rather than just metastasis. This is because the 4T1 cancer model is known to be more aggressive than CT26 and MC38 models, which typically respond well to various therapies. Therefore, it is essential to study the impact of anti-tumor therapies on primary 4T1 tumors, which typically respond poorly to such treatments.

2) authors found SAM-Fc(+) still suppressed 4T1 metastasis in nude mice, together with the results that SAM-Fc(+) shapes macrophages from M2-to-M1 type, then they concluded that M2-to-M1 polarization alone is sufficient for the SAM-FC-mediated inhibition of tumor. However, this conclusion has a serious logical flaw.

Compared to Balb/c mice, Nude mice just don’t have mature T cells, but still have B cells, NK cells, as well as other myeloid cells, such as macrophages and neutrophils, etc. NK cells and neutrophils are well studied to control metastasis, and also definitely up-regulated after Salmonella treatment. Therefore, it is questionable for the authors to attribute the observed anti-tumor effects solely to M2-to-M1 polarization. If the authors want to confirm this hypothesis, they should conduct experiments using macrophage-deficient mice or macrophage depletion in vivo.

2. in the line of 311,“ Moreover, liver-directed SAM-FC treatment also increased infiltration of T cells into subcutaneous tumors significantly”. Please claim the meaning of “liver-directed”. Are the SAM-Fc intrasplenic or portal vien injected? Or just the same as in other figures using intravenous injection?

3. in the line of 315: “without any systemic toxicity (Supplementary Fig. 9d)”, the authors claimed that their treatment had low toxicity by demonstrating that there was no significant change in body weight. However, the way they presented the data is questionable. The Y-axis range for body weight changes was from 0 to 30 grams, which is not appropriate. For example, if a 20-gram mouse loses 20% of its weight, which is a significant loss and should be a cause for concern, its body weight would only drop to 16 grams, which would appear as a minor disturbance in the curve. Therefore, body weight changes should be presented as a percentage loss with a more appropriate Y-axis range. The same issue applies to Supplementary Figure 3f.

4. one more issue regarding the safety of SAM-FC. Safety concerns are the one of the biggest hurdles in the clinical application of bacterial therapies. Therefore, the authors should provide some data showing the toxicity of SAM-FC(+). This may include organ counts of the bacteria (spleen and liver may be enough), weight loss, and splenomegaly data from a small cohort at 3 time points, say week 1, week 2 and week 3 post-infection of 2*10^7 CFU SAM-Fc(+).

5. in lines 317 to 345, the authors neutralized HMGB1 and observed that its neutralization decreased the anti-tumor effect of SAM-FC(+) to a level close to that of the SAM-FC(-) group (Fig. 5b). Moreover, neutralization of HMGB1 reduced the ratio of active CD4+ T cells (Fig. 5d,i), activated DC cells (Fig. 5h), and active CD8+ T cells (Fig. 5j) to levels similar to those observed in the SAM-FC(-) group. However, it is important to note that HMGB1 seems to only contribute to the additional gain of anti-tumor immunity observed with the expression of ClyA/FlaB on the basis of SAM, rather than compared to the PBS-treated group. Salmonella without ClyA/FlaB expression (SAM-FC(-) group) still exhibited some basic anti-tumor immunity compared to the PBS control group, which is not dependent on HMGB1.

Therefore in the lines 343 to 345, the statement “indicating that SAM-FC promotes the Th1 response, which requires HMGB1. Collectively, these data demonstrate that SAM-FC induced a robust T-cell-mediated anti- tumor immune response by promoting TSA cross-presentation, a process that relies heavily on HMGB1” is misleading and inaccurate, as it can lead people to believe that HMGB1 mediates the entire anti-tumor immunity observed with ClyA/FlaB-expressing Salmonella.

6. In the lines 415 to 418, the authors argued that the immunological memory induced by ClyA-secreting Salmonella is tumor-specific by re-challenging mice cured from CT26 with 4T1 cancer cells. In other words, authors suggested that the growth of 4T1 tumors is due to the immunological memory is CT26 specific, rather than nonspecific, thus no therapeutic response to 4T1 tumors. However, the authors did not rule out the possibility that the 4T1 tumor intrinsically does not respond to the SAM-Fc(+) treatment. If this is the case, the development of 4T1 tumors is not due to a lack of immunological memory specific to 4T1.

Therefore, rechallenging cured mice from MC38 with Pan02 in the background of C57BL/6 mice or rechallenging cured mice from CT26 with a Balb/c derived cell line that originally responds to SAM-Fc(+) may provide stronger evidence.

7. in most figure legend, the authors only claimed sample size, didn’t mention whether it is a representative experiment from at least two independent experiments or merged results from

different batches of experiments. Fig2.b/c/d/e/f/g/i/j/k have a sample size of 5, Fig2h has a sample size of 3; Fig3b/c/d has a sample size of 3~4; Fig.3f/g/h has a sample size of 3~4; Fig.3i-j has a sample size of 5; all figures in Fig.4 have a sample size of 3; most figures in Fig.5 also have sample size of 3~5； Fig.6a/b/c have a sample size of 3 and Fig.6d-e have sample size of 5. For all mentioned figures, it seems that the authors only performed the experiments once with a small sample size, please justify this issue and provide repeated experiment results if they really did.

Minor:

1. supplementary figure 6o-r, intratumoral cytokine quantification, the unit should be pg/gram, not pg/ml.
2. in the line of 289-290: " This suggests that an increase in M2-to-M1 polarization (Fig. 3d, Supplementary Fig. 9b), is sufficient for the SAM-FC-mediated inhibition of tumor". Neither Fig3d or Supplementary Fig.9b was referenced to M2-to-M1 polarization. It should be typo?

3. the legend of Fig3e-f about MC38 model are not clear enough:
"e Experimental scheme relating to generation of the C57BL/6 mice MC38 liver metastatic model. MC38 cells were administered to mice by **subcutaneously implantation (5×10^6 cells) (SC), or simultaneously via both the subcutaneous (5×10^6 cells) and intrasplenic (1×10^6 cells) routes (SC + liver).** f Average growth curves of subcutaneous tumors. SC + liver refers to mice with both MC38 subcutaneous and liver tumors. h Metastatic scores of intrasplenic tumors in MC38 dual tumor-bearing mice. Metastatic nodules on the liver surface on Day 25 were counted. n = 3–4;"

Since authors used sc or sc+liver, we assumed there should be sc results and sc+liver results separately, but it seems that Fig3.g-h are results from sc+liver model. Supplementary 9f labels better and easy to read.

4. M1 markers here used is CD80, not the same as in Fig2.b and Fig.3c. Is there any consideration?

5. there seems to be a typo in Supplementary 10e, the second row of samples should be SAM-Fc(-), rather than SAM-FC(+)?

6. Supplementary Figure 8a is the only direct data in this paper shows that the anti-tumor immunity induced by SAM-Fc(+) is T cell mediated, it might be included in Figure 2.

7. there seems to be a typo in the legend of Supplementary 9c, which obviously are C57BL/6 mice, not "(c) Individual images of lung metastasis in 4T1-Luc2 tumor-bearing BALB/c athymic nu−/nu− mice (n = 5)."

Responses to the reviewer's comments

We would like to express our sincere appreciation to the editor and reviewers for their valuable comments and feedback on our study. We have carefully examined and responded to the comments on a point-by-point basis below. The changes that we made in response to the reviewer comments and an English editor are highlighted by red-colored font in the revised manuscript.

Reviewer #1 (Remarks to the Author), with expertise in nanomedicine for cancer immunotherapy:

In this work, the authors reported the targeted immunotherapy using an attenuated Salmonella typhimurium (SAM-FC) engineered to release two payloads: cytolysin A (ClyA) and Vibrio vulnificus flagellin B (FlaB). They found that the localized secretion of ClyA from SAM-FC could induce immunogenic cancer cell death and promote the release of tumor-specific antigens and damage-associated molecular patterns, while the localized secretion of FlaB promoted phenotypic and functional remodeling of intratumoral macrophages for inhibiting the tumor metastasis in mice bearing tumors of mouse and human origin. This work may be considered for publication after addressing the following issues:

Q1. *The characterization of the obtained SAM-FC system is insufficient. Major steps presented in Fig. 1a and Supplementary Fig. 1 should be fully supported experimentally.*

Author's response: As shown in Fig. 1a and Supplementary Fig. 1, SAM bacteria transformed with pJH18-based expression vectors constitutively expressed the TetR repressor under the control of P_{araB} in Doxy-free culture condition. The TetR repressor binds to the P_{tet} promoter, switching off gene expression of downstream payloads. In the presence of Doxy, TetR is released from P_{tet} and payload genes are expressed. We measured TetR expression in bacteria carrying pJH18-based expression vectors in Doxy-free culture conditions. In the absence of Doxy, TetR was detected in the transformed SAMs (SAM-E, SAM-FR, SAM-CR, and SAM-FC) but not in

non-transformed SAM by western blotting. The new data has been added as Supplementary Fig. 2a to the revised manuscript. We also added the sentence "TetR repressor (23 kDa) was expressed only in SAM transformed with pJH18 (Supplementary Fig. 2a),…" to revised manuscript (Page 6 line 169-170).

Q2. *The morphological changes of SAM-FC before and after payload release should be characterized by microscopic techniques.*

Author's response: We examined for morphological changes in SAM-FC bacteria. The bacteria were cultured in the absence or presence of Doxy overnight and were observed under a transmission electron microscopy (TEM). There was no significant change in bacterial morphology in both conditions. The relevant data have been added as Supplementary Fig. 2d to the revised manuscript. We also added the sentence "… but did not change bacterial morphology (Supplementary Fig. 2c, d)." to the revised manuscript (page 6, line 173).

Q3. *(1) The interactions between the two payloads in Salmonella and between the payloads and Salmonella and (2) release mechanisms of the payloads from Salmonella should be further studied.*

Author's response:

In response to (1), we measured the functional expression of the two payloads in SAM-FC, SAM-CR, and SAM-FR. The expression of ClyA and FlaB in SAM-FC were similar to those in SAM-CR and SAM-FR, respectively. The activity of each payload in SAM-FC was also similar to that in SAM-CR or SAM-FR. These results indicate that the expression and activity of one payload did not affect those of the other. The relevant data have been added to Supplementary Fig. 2g-i in the revised manuscript. We also added the following sentence "The amounts and activities of ClyA and FlaB secreted from SAM-FC(+) were similar to those secreted from SAM-CR(+) or SAM-FR(+), indicating that the expression of one payload did not affect the expression of the other (Supplementary Fig. 2e-i)." to the revised manuscript (page 6, line 176 – 179).

In response to (2), there are previous reports describing the release mechanisms of these payloads. ClyA is naturally secreted (References: 33, 34, 35, 36, 37) and FlaB is secreted via the *pelB* leader sequence (References: 32, 39, 40). We have added these references including the

original reference 34 to the end of the sentence "ClyA (34 kDa) and FlaB (43 kDa) were secreted from SAM-FC pellets only after Doxy induction (Supplementary Fig. 2b), as previously shown [32-37,39,40]" in the revised paper (page 6, lines 170-171). We have added these references to the reference list of the revised manuscript (page 30, lines 937-955).

Q4. *Immunofluorescence staining should be conducted to support the immune response in vivo.*

Author's response: We already showed the immunofluorescence staining images of CD8+ T cells in MC38 tumor tissues (Fig. 4h) and infiltrating immune cells including M2-like ($F4/80^+$ $CD206^+$) and M1-like ($F4/80^+$ $CD86^+$) macrophages, Treg ($FOXP3^+$), CD4 T cells ($CD4^+$), and CD8 T cells ($CD8^+$) in CT26 tumor tissues (Supplementary Fig. 6k-n). However, we have improved the quality of images in the revised manuscript.

Reviewer #2 (Remarks to the Author), with expertise in Salmonella mediated cancer therapy:

In the field of bacterial therapy for tumors, taking advantage of tumor-targeting Salmonella typhimurium to deliver various therapeutic cargo has always been an attractive area. Among these therapeutic cargoes, toxins or immunomodulators are common choice. For example, toxin ClyA and FlaB used in this manuscript. This research team has done a lot of work about using ClyA and FlaB, separately:

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In addition, this research team also found that Salmonella typhimurium expressing FlaB can effectively inhibit the growth of subcutaneous colon cancer MC38 by activating TLR5 pathway

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Based on their past work mentioned above, this research team explored the effect of coexpressing two therapeutic cargoes and explored its underlying mechanism in this manuscript. Although using ClyA or FlaB alone already has good effects, combination therapy has indeed shown a slight but significant enhancement in therapeutic efficacy, which has some scientific significance. For this work, we have the following questions:

Q1. *From lines 281-291, the authors validated the anti-metastatic ability of SAM in the 4T1 spontaneous model, and found that SAM_FC(+) suppressed metastasis in both immunocompetent Balb/c mice and immunocompromised Nude mice.*

1) *We are extremely curious about the anti-tumor effects on primary 4T1 tumor growth, rather than just metastasis. This is because the 4T1 cancer model is known to be more aggressive than CT26 and MC38 models, which typically respond well to various therapies. Therefore, it is essential to study the impact of anti-tumor therapies on primary 4T1 tumors, which typically respond poorly to such treatments.*

Author's response: According to reviewer's suggestion, we additionally evaluated the 4T1 antitumor effects of SAM-FC in BALB/c mice that had been inoculated with 4T1 cells into the mammary fat pad (orthotopic 4T1 tumor and metastasis). Similar to CT26 tumors, 4T1 primary tumors were optimally suppressed by SAM-FC, followed by SAM-CR, SAM-FR, and SAM-E, although the therapeutic efficacy against 4T1 tumors was slightly less than that against CT26 tumors. In general, the antitumor immune effect of SAM-FC was similar for CT26 and 4T1 primary tumors. Macrophages, $CD8^+$ T, Treg cells, and macrophage-mediated anti-tumor cytokines (IL-1α, TNF-α) exhibited similar changes in both tumors after SAM-FC treatment. We have added these data to Fig. 3a-e and Supplementary Fig. 9e-m in the revised manuscript. We have also added the sentences "Firstly, immunocompetent BALB/c mice with 4T1 tumors and spontaneous lung metastasis were generated by inoculating them with 4T1 cells into either the mammary fat pad … more potent in inhibiting metastasis than ClyA-secreting bacteria." in revised manuscript (page 10, lines 295-309) and "We next analyzed the immunologic phenotypes in orthotopic 4T1 primary tumors and lung metastases treated with SAMs..... modulating Treg

and CD8$^+$ T cell responses in 4T1 primary tumors" to revised manuscript (page 11, lines 337 - 350).

2) Authors found SAM-FC(+) still suppressed 4T1 metastasis in nude mice, together with the results that SAM-FC(+) shapes macrophages from M2-to-M1 type, then they concluded that M2-to-M1 polarization alone is sufficient for the SAM-FC-mediated inhibition of tumor. However, this conclusion has a serious logical flaw.

Compared to Balb/c mice, Nude mice just don't have mature T cells, but still have B cells, NK cells, as well as other myeloid cells, such as macrophages and neutrophils, etc. NK cells and neutrophils are well studied to control metastasis, and also definitely up-regulated after Salmonella treatment. Therefore, it is questionable for the authors to attribute the observed antitumor effects solely to M2-to-M1 polarizati on. If the authors want to confirm this hypothesis, they should conduct experiments using macrophage-deficient mice or macrophage depletion in vivo.

Author's response: We have added new data to the revised manuscript showing that macrophages play a predominant role in the inhibition of 4T1 metastasis in mice treated with SAM-FC bacteria, although we cannot exclude the possibility that other immune cells also inhibit 4T1 metastasis.

Firstly, in BALB/c mice inoculated with 4T1 tumor cells in the mammary fat pad, the new data showed that the M1(F4/80$^+$CD86$^+$)/M2(F4/80$^+$CD206$^+$) macrophage ratio was similarly increased by SAM-FC(+) and SAM-FR(+) and that this increase correlated with inhibition of 4T1 metastasis. SAM-E and SAM-CR(+) exhibited modest suppression of metastasis. These results indicate that FlaB- but not ClyA-secreting bacteria played a predominant role in macrophage polarization toward the M1 phenotype. We have added the data to Fig. 3c-e in the revised manuscript and added the sentences "We assessed whether the anti-metastasis effect of FlaB-secreting bacteria was mediated by functional macrophage polarization … in the metastatic lung of mice treated with FlaB-secreting bacteria (Fig. 3d, e)" to the revised manuscript (page 10, lines 310-315).

Also, we performed macrophage neutralization with αCSF-1R antibody in the same mice innoculated with 4T1 tumor cells. The SAM-FC(+) plus αCSF-1R group showed a significant decrease in the M1/M2 ratio in lung metastasis compared with the SAM-FC(+) group alone. αCSF-1R increased metastasis of control mouse (PBS), but SAM-FC(+) reversed the αCSF-1R effect, indicating that macrophage neutralization abolished the suppression of metastasis by SAM-FC(+). We have added the new data to Fig. 3a-e and Supplementary Fig. 9e-g. The sentences "To further explore the role of macrophages in the inhibition of 4T1 metastasis, we neutralized macrophages using the specific antibody αCSF-1R… suppressed primary tumor growth and metastasis in the SAM-FC(+)-treated group (Fig. 3a-c, Supplementary Fig. 9e, f)" have been added to the revised manuscript (page 11 line 324-329).

Finally, we assessed a role of macrophages in the suppression of metastasis in BALB/c athymic nu^-/nu^- mice (nude mice) treated with SAM-FC. In addition to the results of macrophage analysis of primary tumor and metastasis of nude mice bearing subcutaneous 4T1-Luc2 tumors in the original manuscript (Supplementary Fig. 9b), we analyzed macrophages in lung metastasis of nude mice innoculated with human MDA-MB-231-FLuc-GFP luciferase tumor cells into mammary fat pad. After bacterial treatment, SAM-FC(-) suppressed lung metastasis more effectively than PBS but less effectively than SAM-FC(+), indicating that other immune cells, not T cells, were required for the inhibition of metastasis by SAM-FC (Fig. 3f). We also conducted phagocytosis assays using macrophages from lung metastasis of the same mice. The highest phagocytic activity was observed in mice treated with SAM-FC(+), followed by those treated with SAM-FC(-) and PBS (Fig. 3g). The new data have been added as Fig. 3f, g. We also added the following description "Notably, SAM-FC(+) was able to significantly reduce metastasis in immunodeficient BALB/c athymic nu^-/nu^- mice,... bacteria induce functional M2-to-M1 macrophage polarization, which is essential for the inhibition of metastasis by phagocytosis." to the revised manuscript (page 11, lines 329-336).

Q2. *in the line of 311," Moreover, liver-directed SAM-FC treatment also increased infiltration of T cells into subcutaneous tumors significantly". Please claim the meaning of "liver-directed". Are the SAM-FC intrasplenic or portal vien injected? Or just the same as in other figures using intravenous injection?*

Author's response: We changed "liver-directed SAM-FC treatment" to "SAM-FC(+) treatment" in the revised manuscript (page 12, line 379). SAM-FC was intravenously injected into the mice bearing both MC38 subcutaneous tumor and liver metastasis (SC + liver).

Q3. *In the line of 315: "without any systemic toxicity (Supplementary Fig. 9d)", the authors claimed that their treatment had low toxicity by demonstrating that there was no significant change in body weight. However, the way they presented the data is questionable. The Y-axis range for body weight changes was from 0 to 30 grams, which is not appropriate. For example, if a 20-gram mouse loses 20% of its weight, which is a significant loss and should be a cause for concern, its body weight would only drop to 16 grams, which would appear as a minor disturbance in the curve. Therefore, body weight changes should be presented as a percentage loss with a more appropriate Y-axis range. The same issue applies to Supplementary Figure 3f.*

Author's response: We changed the figure format of Supplementary Fig. 3f and Supplementary Fig. 9d and their legends in original manuscript to "Change in average body weight. Body weight changes relative to those on day 0 were calculated" in Supplementary Fig. 3j and "Changes in average body weights in SC + liver MC38 mice (n = 3). Body weight changes at the indicated day were calculated relative to those on day 0" in supplementary Fig. 10f, respectively, in the revised manuscript, as advised by the reviewer.

Q4. *One more issue regarding the safety of SAM-FC. Safety concerns are the one of the biggest hurdles in the clinical application of bacterial therapies. Therefore, the authors should provide some data showing the toxicity of SAM-FC(+). This may include organ counts of the bacteria (spleen and liver may be enough), weight loss, and splenomegaly data from a small cohort at 3 time points, say week 1, week 2 and week 3 post-infection of 2*10^7 CFU SAM-FC(+).*

Author's response: We previously provided data on body weight changes, but we have changed the figure format in the revised manuscript as described in our answer to Q3 above (Supplementary Fig. 3j). To further support the safety of bacterial treatment, we have added new data from CT26 tumor-bearing mice treated with SAM-FC(+). We observed that liver and spleen weights increased on day 7 in mice treated with SAM-FC(+), decreased on day 14, and returned to the weights of control mice (PBS) on day 21. After bacterial injection (2 x 10^7 cfu), the numbers of viable bacteria were 10^4 cfu/gram in spleen and 10^2 cfu/gram in liver on day 7, and

no viable bacteria could be detected on day 14. These results demonstrated that the treatment of mice with SAM-FC(+) was safe without evident systemic toxicity. The new data have been added to Supplementary Fig. 3e-h in the revised manuscript.

Q5. *In lines 317 to 345, the authors neutralized HMGB1 and observed that its neutralization decreased the anti-tumor effect of SAM-FC(+) to a level close to that of the SAM-FC(-) group (Fig. 5b). Moreover, neutralization of HMGB1 reduced the ratio of active CD4+ T cells (Fig. 5d,i), activated DC cells (Fig. 5h), and active CD8+ T cells (Fig. 5j) to levels similar to those observed in the SAM-FC(-) group. However, it is important to note that HMGB1 seems to only contribute to the additional gain of anti-tumor immunity observed with the expression of ClyA/FlaB on the basis of SAM, rather than compared to the PBS-treated group. Salmonella without ClyA/FlaB expression (SAM-FC(-) group) still exhibited some basic anti-tumor immunity compared to the PBS control group, which is not dependent on HMGB1.*

Therefore, in the lines 343 to 345, the statement "indicating that SAM-FC promotes the Th1 response, which requires HMGB1. Collectively, these data demonstrate that SAM-FC induced a robust T-cell-mediated anti- tumor immune response by promoting TSA cross-presentation, a process that relies heavily on HMGB1" is misleading and inaccurate, as it can lead people to believe that HMGB1 mediates the entire anti-tumor immunity observed with ClyA/FlaBexpressing Salmonella.

Author's response: In the revised manuscript, we changed the statement "Collectively, these data demonstrate that SAM-FC … that relies heavily on HMGB1." to "Collectively, these data demonstrate that SAM-FC induced a robust T-cell-mediated anti-tumor immune response by promoting TSA cross-presentation, a process in which HMGB1 plays a primary immunoadjuvant role in the tumor-specific immune response" (page 14 line 429-432).

Q6. *In the lines 415 to 418, the authors argued that the immunological memory induced by ClyAsecreting Salmonella is tumor-specific by re-challenging mice cured from CT26 with 4T1 cancer cells. In other words, authors suggested that the growth of 4T1 tumors is due to the immunological memory is CT26 specific, rather than nonspecific, thus no therapeutic response to 4T1 tumors. However, the authors did not rule out the possibility that the 4T1 tumor*

intrinsically does not respond to the SAM-FC(+) treatment. If this is the case, the development of 4T1 tumors is not due to a lack of immunological memory specific to 4T1.

Therefore, rechallenging cured mice from MC38 with Pan02 in the background of C57BL/6 mice or rechallenging cured mice from CT26 with a Balb/c derived cell line that originally responds to SAM-FC(+) may provide stronger evidence.

Author's response: According to the reviewer's suggestion, we performed a tumor rechallege experiment in MC38 tumor-eradicated C57BL/6 mice. As MC38 and B16F10 tumor cells were originated from C57BL/6 mice, the MC38 tumor-eradicated mice were simultaneously rechallenged with both of these cells. Secondary tumors appeared only in B16F10 but not in MC38 transplants, indicating that the immunological memory of the tumor-eradicated mice after SAM-FC(+) treatment was tumor cell specific. We have added the new data (MC38 tumor-eradicated mice) to Supplementary Fig. 8 together with the data of original Supplementary Fig. 8c, d (CT26 tumor-eradicated mice) to the revised manuscript. We changed the sentences "Moreover, the established long-term memory … implanted into the same mouse strain (Supplementary Fig. 9a, b) to "Similar results were obtained using MC38 tumor-eradicated mice, in which only B16F10 cell-derived tumors but not MC38 cell-derived tumors grew (Supplementary Fig. 8c, d)" in the revised manuscript (page 16, lines 504-506).

7. In most figure legend, the authors only claimed sample size, didn't mention whether it is a representative experiment from at least two independent experiments or merged results from different batches of experiments. Fig2.b/c/d/e/f/g/i/j/k have a sample size of 5, Fig2h has a sample size of 3; Fig3b/c/d has a sample size of 3~4; Fig.3f/g/h has a sample size of 3~4; Fig.3i-j has a sample size of 5; all figures in Fig.4 have a sample size of 3; most figures in Fig.5 also have sample size of 3~5 ; Fig.6a/b/c have a sample size of 3 and Fig.6d-e have sample size of 5.

For all mentioned figures, it seems that the authors only performed the experiments once with a small sample size, please justify this issue and provide repeated experiment results if they really did

Author's responses: The experiments in **Figs. 1, 2, 3, and 5** were conducted independently two or three times but the data were merged to facilitate data arrangement in the original manuscript.

So, we have added the number of independent experiments to the figure legends of the revised manuscript. The experiments in **Fig. 4 and 6** were performed once to support our hypothesis that SAM-FC is effective for the treatment of solid tumors and metastasis in multiple tumor models. We did not repeat experiments when the results were significant even with small sample sizes (3-5 mice per group). Also, we made mention as "Experiment was done once" if an experiment had been performed once.

Minor:

Q1. *supplementary figure 6o-r, intratumoral cytokine quantification, the unit should be pg/gram, not pg/ml.*

Author's response: We have corrected this mistake in Supplementary Fig 6o-r in the revised manuscript.

Q2. *in the line of 289-290: " This suggests that an increase in M2-to-M1 polarization (Fig. 3d, Supplementary Fig. 9b), is sufficient for the SAM-FC-mediated inhibition of tumor". Neither Fig3d or Supplementary Fig.9b was referenced to M2-to-M1 polarization. It should be type?*

Author's response: We removed the citations of Fig. 3d and Supplementary Fig. 9b from the sentence of the original manuscript and re-wrote the sentence as follows "Together, these results indicate that FlaB-secreting bacteria induce functional M2-to-M1 macrophage polarization, which is essential for the inhibition of metastasis by phagocytosis" in revised manuscript (page 11, lines 334-336).

Q3. the legend of Fig3e-f about MC38 model are not clear enough:

"e Experimental scheme relating to generation of the C57BL/6 mice MC38 liver metastatic model. MC38 cells were administered to mice by ***subcutaneously implantation (5 × 10^6 cells) (SC), or simultaneously via both the subcutaneous (5 × 10^6 cells) and intrasplenic (1 × 10^6 cells) routes (SC + liver).*** *f Average growth curves of subcutaneous tumors. SC + liver refers to mice with both MC38 subcutaneous and liver tumors. h Metastatic scores of intrasplenic tumors in MC38 dual tumor-bearing mice. Metastatic nodules on the liver surface on Day 25 were counted. n = 3–4;"*

Since authors used sc or sc+liver, we assumed there should be sc results and sc+liver results separately, but it seems that Fig3.g-h are results from sc+liver model. Supplementary 9f labels better and easy to read.

Author's response: We used SC + liver tumor mice in Fig. 3 and Supplementary Fig. 10e-m. So, the statement was incorrect. We have removed the statement and moved "subcutaneously implantation (5×10^6 cells) (SC), or" from Fig. 3e, f in the original manuscript to Fig. 3h, i in the revised manuscript.

Q4. *M1 markers here used is CD80, not the same as in Fig2.b and Fig.3c. Is there any consideration?*

Author's response: We only used CD86 as a maker of M1-like macrophages. We have fixed the error in the relevant figures of the revised manuscript (Fig. 3d, e in revised manuscript).

Q5. *there seems to be a type in Supplementary 10e, the second row of samples should be SAM-FC(-), rather than SAM-FC(+)?*

Author's response: We corrected this error in the revised manuscript.

Q6. *Supplementary Figure 8a is the only direct data in this paper shows that the anti-tumor immunity induced by SAM-FC(+) is T cell mediated, it might be included in Figure 2.*

Author's response: We moved Supplementary Figure 8a, b to Fig. 2l, m in the revised manuscript.

Q7. *There seems to be a type in the legend of Supplementary 9c, which obviously are C57BL/6 mice, not "(c) Individual images of lung metastasis in 4T1-Luc2 tumor-bearing BALB/c athymic nu-/nu- mice (n = 5)."*

Author's response: We fixed the legends of Supplementary Fig. 9b and c "(b) Bioluminescence images ..... the metastatic tumors (n= 3-4)." to "**e** *In vivo* bioluminescent imaging of SC + liver MC38-Luc mice (*n* = 3). Left, images; right, quantification of bioluminescent signals in the lungs.". This data were moved to Supplementary Fig 10e in the revised manuscript.

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

The authors established multi-drug loaded Salmonella delivery system based on their inducible bidirectional Tet system for reprogramming the tumor immune microenvironment by robust ICD and macrophage-activating capacity. Localized secretion of ClyA from SAM-FC induced immunogenic cancer cell death and promoted release of tumor-specific antigens and damage-associated molecular patterns, which establish long-term antitumor memory. Localized secretion of FlaB promoted phenotypic and functional remodeling of intratumoral macrophages that then markedly inhibited tumor metastasis in mice bearing tumors of mouse and human origin. But the results do not explain the necessity of combing ClyA and FlaB. Results demonstrated that the SAM-FC induced functional reprogramming of tumor immune microenvironment by activating both the innate and adaptive arms of the immune system. However, a few questions should be carefully solved before further consideration for publishing.

Q1. *For the cancer metastasis inhibition evaluation via immunotherapy, immunocompetent BALB/c and immunocompromised BALB/c athymic nu−/nu− mice were chosen as animal model, why not choose the C57BL/6 mouse?*

Author's response: In the original version (Fig. 3e-h and Supplementary Fig. 9c-f), we already presented experimental cases of liver metastasis in C57BL/6 mice bearing MC38 subcutaneous tumor and liver metastasis (SC + liver) (Fig. 3h-k and Supplementary Fig. 10a-i; page 12, lines 354-384 in the revised manuscript).

Q2. *As shown in Fig 4b, the expression of leukocytes (CD45+) in both SAM-FC(-) and SAM-FC(+) treated groups are significantly increased compared to PBS group. But it shows that relevant difference in SAM-FC(-) group (△=4.9%) is more obvious than SAM-FC(+) group (△=4%), does it means that SAM itself will contribute the process of inhibition of cancer metastasis, not just ClyA and FlaB?*

Author's response: The percentages of leukocytes ($CD45^+$) in the PBS, SAM-FC (-), and SAM-FC (+) groups were 6.6%, 11.5%, and 15.5%, respectively. Therefore, the percentages of leukocytes in the SAM-FC(-) and SAM-FC(+) groups were 4.4% and 8.9%, respectively, higher than that of the PBS group. Bare bacteria [SAM-FC(-)] can induce leukocyte infiltration into the metastatic livers of SC + liver mice, and anti-tumor payloads enhance immune cell infiltration. We changed the sentence in original manuscript "Compared with the other treatment…….in the liver significantly (Fig. 4b and Supplementary Fig. 10m)" to "The percentages of leukocytes in the livers of PBS, SAM-FC(-), and SAM-FC(+) groups were 6.6%, 11.5%, and 15.5%, respectively (Fig. 4b, Supplementary Fig. 10m), suggesting that ClyA and FlaB secreted by SAM-FC(+) were more effective in promoting leukocyte infiltration in metastatic lesions than bare bacteria [SAM-FC(-)]." in the revised manuscript (Page 12, lines 365-369).

Q3. *In section 'SAM-FC enhances the HMGB1-mediated cross-presentation of TSAs', result data demonstrated that SAM-FC(+) could promote the activation of DCs, CD4+ T cells, CD8+ T cells, and Th1 response synchronously via HMGB1. However, whether, which one (APC process, or T cell activation process) is core process should be further explored.*

Author's response: In response to the reviewer, we examined the potential role of HMGB1 in DC maturation and T cell activation with or without DC involvement using *ex vivo* splenocytes. We observed that TLR4, the HMGB1 receptor, is expressed not only in DCs[54] but also in splenic T cells (Supplementary Fig. 11c). This suggests that HMGB1 not only induces maturation of $CD11c^+$ DCs (increased levels of $CD86^+$, $MHCII^{Hi}$, and $CCR7^+$) but also directly stimulates $CD3^+$ T cells (increased expression of $TCR\beta^+$, Ki-67^+, and $CD25^+$) by binding to TLR4, even in the absence of DCs (Supplementary Fig. 11d-i). Notably, T cell activation markers were higher in samples of rHMGB1 + $CD3^+$ T cells than in samples of rHMGB1 + $CD3^+$ T cells + $CD11c^+$ DCs. However, without an antigen-presenting process, these T cells lack anti-tumor capabilities. By contrast, HMGB1-stimulation of DCs, which increases antigen-presentation to T cells, results in the formation of T cells with effective anti-tumor responses, highlighting the dual role of HMGB1 in immune cell activation and responses. We have added this new description to the revised manuscript as "Using splenocytes and bone marrow (BM) derived DCs, … These

findings highlight the dual role of HMGB1 in immune cell activation and responses[52-54]" (page 13, lines 388 - 401). New citations (52–54) have been added to these sentences and the corresponding references have been added in the reference list of the revised manuscript (page 31, lines 982-987).

Q4. *For bilateral tumor model by unilateral intratumoral injection, although increased frequency of proliferating CD4+ and CD8+ T cells were observed in SAM-FC(+) group, but the bioluminescent signals of SAM-FC(+) were observed in both treated and untreated tumors after intratumoral injection, which means that the terminal tumor inhibition was contributed by SAM-FC(+), not immunotherapy, please carefully clarify the contribution.*

Author's response: As you pointed out, the migration of intratumorally injected SAM-FC to distal tumors was verified in the present study using bioluminescent SAM (chromosomally integrated lux operon, Fig. 6a). Additionally, expression of the payload in bilateral tumors was presented in Fig 6b. We speculate that bacterial colonization of distal tumors induces an antitumor immune response, similar to that observed in the primarily injected proximal tumors. Indeed, SAM-FC(+) exhibited superior efficacy and elicited a more robust immune response against both the locally injected tumor and the distal tumor than SAM-FC(-). These effects can be attributed not only to the intrinsic effects of the bacteria itself (SAM) but also to the enhanced immune responses induced by functional ClyA and FlaB expression, which we have shown has additional tumor-suppressive effects.

Q5. *Besides bilateral tumor model, pls employing the unilateral tumor model for SAM-FC(+) treatment, after the primary tumor was eliminated, then inoculated the distant tumor model for ICD-mediated immunotherapy evaluation.*

Author's response: Following to reviewer's suggestion, we evaluated ICD-mediated immunotherapy induced by SAM-FC(+). We observed complete eradication of CT26 tumors by day 12 following SAM-FC(+) treatment. The mice were then immediately rechallenged with CT26 cells on the opposite flank on the following day (day13), resulting in successful tumor rejection by day 17 or day 19. These findings suggest that antitumor immunological memory was

established at an early stage after SAM-FC(+) treatment, similar to what was observed in the rechallenge study (Fig. 1e, i in revised manuscript). The results are presented as follows.

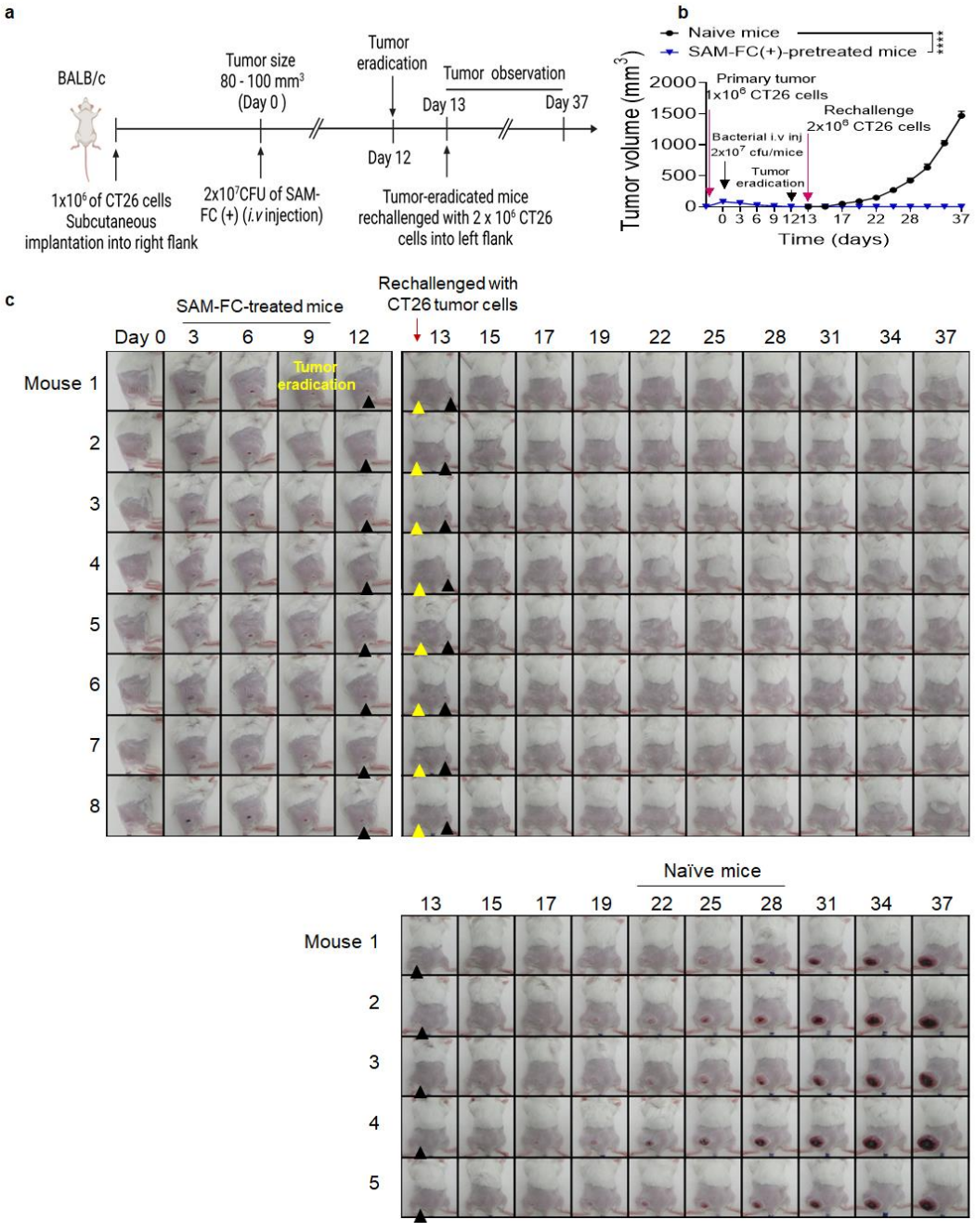

Figure R: Tumor rechallenge of at the early stage after CT26 primary tumor eradication by SAM-FC(+). a Experimental scheme. Briefly, CT26 cells (1×10^6) were subcutaneous implanted into the right hind flank of BALB/c mice (black triangles). When tumors reached approximately 100 mm^3 (day 0), the mice were intravenous injected with SAM-FC (2×10^7 cfu). Bacteria-treated mice were then given Doxy (orally, 1.7 mg/kg) every day starting on day 3. +, with Doxy. On the next day that tumors were eradicated (day 13), the mice were rechallenged with the same tumor cells (2×10^6) into the left flank (yellow triangles) ($n = 8$). Experiments were done twice independently ($n = 4$ a time). Naïve

age-matched mice (n = 5) were implanted with the same tumor cells (1×10^6) and used as tumor challenge controls. **b** Average growth curve of primary and rechallenged tumors. ****P < 0.0001, two-way ANOVA with Tukey's multiple comparisons test. All data are shown as the mean ± s.e.m. **c** All images of of the mice (upper panel). Naïve mice challenged with CT26 tumors as control (bottom panel).

Reviewer #4 (Remarks to the Author), with expertise in Bacteria, immunology:

In their manuscript, Nguyen et al describe the use of an attenuated Salmonella Typhimurium strain that has been equipped with inducible bacterial genes to target transplantable murine tumors. The therapeutic genes are encoding cytolysin A and the flagellar protein FlaB. They provide extensive data on the characterization and in vitro as well as in vivo function. Although, this work is in general interesting to my mind the novelty of the findings is rather limited. Cytolysin A and FlaB expressed by tumor targeting bacteria has been described before. The combined expression shows only additive effects. The effects on the immune cells from various tissues are due the particular bacterial protein, has been by enlarge described before, and therefore were expectable. Together with some inconsistencies within the experimental strategy. I find this manuscript not acceptable and more prone for publication in a specialized journal. There are some major and some minor point. I did nt separate them. In detail:

Author's response: Thank you very much for this insightful comment. As you rightly pointed out, ClyA and FlaB as therapeutic payloads were described in our previous studies, and there have been reports of Salmonella-induced anti-cancer immunity. However, what was particularly novel in the present study was that ClyA and FlaB synergistically activated both innate and adaptive immunity, resulting in the effective eradication of primary and metastatic cancer lesions. Notably, we rigorously investigated the type of immune response produced by SAM-FC and demonstrated that it induces an anti-cancer immune response through TSA presentation along with DAMP production, particularly that of HMGB1. Therefore, we believe that treatment with both ClyA and FlaB together could represent a valuable and effective form of bacterial cancer therapy.

Q1. *Title: multi-drug loaded is an overstatement. Only two are described beside the luciferase.*

Author's response: We changed the title "Reprogramming the tumor immune microenvironment using engineered multiple-drug loaded *Salmonella*" in original manuscript to

"Reprogramming the tumor immune microenvironment using engineered dual-drug loaded *Salmonella"* in the revised manuscript.

Q2. *Introduction: it would be helpful to shortly introduce the TAAs and TSAs in general and in particular.*

Author's response: We inserted the following sentences about TAAs and TSAs: "Tumor antigens can be divided into two major classes, tumor-associated antigens (TAAs) and tumor-specific antigens (TSAs) … TSAs elicit strong tumor-specific immune responses in the absence of T cell-mediated central tolerance" into the revised manuscript (Page 4, line 103-108). Also, we added citations to new references (5) and added the references to the reference list in the revised manuscript (Page 29, lines 877-878).

Q3. *Line 129: it is not entirely true that bacteria alone, i.e. without recombinant anti-tumor compound did not lead to an entire eradication in preclinical models especially of CT 26. Should be corrected.*

Author's response: According to reviewer's suggestion, we changed this statement to: "However, such bacteria have shown inconsistent anti-tumor efficacy in different preclinical and clinical tumor models[21-23, 28-32]." in revised manuscript (page 5, lines 134-135).

Q4. *Results: It would be helpful if the components of the plasmids would be named before the abbreviation is used.*

Author's response: We changed the sentences "Plasmids expressing payloads such as ClyA, FlaB, and *Renilla* luciferase variant 8 (Rluc8) were then constructed based on pJH18 and then transformed into SAM bacteria." to "Plasmids expressing payloads such as ClyA (C), FlaB (F), and *Renilla* luciferase variant 8 (Rluc8; R) were then constructed based on pJH18 and then transformed into SAM" in the revised manuscript (page 6, lines 164-166).

Q5. *Line 170-171 is misleading should be corrected.*

Author's response: We corrected this as "ClyA and FlaB secreted from the transformed SAM exerted hemolytic effects and induced TLR5 simulation, respectively, only in the presence of Doxy (denoted by a "+")" in the revised manuscript (page 6, lines 174-175).

Q6. *"Bom" as a component of the plasmids should be introduced here and the function explained as it appears to be an important function of the plasmid.*

Author's response: We have inserted information about "Bom" gene as "probably because of *bom* gene activity, which prevents the division of plasmid-free bacteria" into the revised manuscript (page 7, lines 211-212).

Q7. *Line 197: anti-tumor payload is meant. Should be corrected.*

Author's response: We changed 'tumor payload(s)' to 'anti-tumor payload(s)' in the revised manuscript (page 7, 204; page 14, line 441; page 15, lines 456, line 468; page 17, line 540)

Q8. *My impression is that the experiments were carried out only once. Experiment should be reproduced. Of course, I can see that quite a large number of mice was included in most of the experiments and two or three tumor models were employed.*

Author's responses: The experiments in **Figs. 1, 2, 3, and 5** were conducted independently two or three times but the data were merged to facilitate data arrangement in the original manuscript. So, we have added the number of independent experiments to the figure legends of the revised manuscript. The experiments in **Fig. 4 and 6** were performed once to support our hypothesis that SAM-FC is effective for the treatment of solid tumors and metastasis in multiple tumor models. We did not repeat experiments when the results were significant even with small sample sizes (3-5 mice per group). Also, we made mention as "Experiment was done once" if an experiment had been performed once.

Q9. *Line 205 and followed: the results of the control of SAM-E should be mentioned here. It is only found in the figure.*

Author's response: We changed the sentence "In CT26 tumor-bearing mice, the best therapeutic effect was observed following treatment with SAM-FC(+) bacteria." to "...although treatment with plasmid free bacteria (SAM-E) also suppressed the tumors" in the revised manuscript (page

7, lines 216-217). Also, we inserted the sentence "SAM-E significantly suppressed tumor growth but did not eradicate the tumors." in the revised manuscript (page 8, lines 233-234).

Q10. *Line 253:tetramers against a peptide from MuLV gp70 was used as TAA. No references is given. Why was peripheral blood analyzed and not TILs and draining LN cells and spleen as in the other cases. The flow cytometry data should be shown because the tetramer staine shown on suppl. Fig. 10i is not convincing.*

Author's response: Following to reviewer's suggestion, we added citations to new references in the revised manuscript (Ref 41, 42, 43 in page 9, lines 268) describing the detection of TAA-specific T cells using the use of H-2L^{d}MuLV gp70 peptide (SPSYVYHQF) tetramer-bound CD8^{+} T cells (Tet^{+} CD8^{+}) and added the new references to the reference list in the revised manuscript (page 30, lines 956-962).

In the original manuscript, tumor-specific T cells, identified using a MuLV gp70 tetramer peptide, were detected in the peripheral blood of mice treated with ClyA-secreting bacteria [SAM-FC(+) and SAM-CR(-)]. We conducted these experiments again in TdLNs and tumors from mice treated SAMs, but the results were the same. We have added the new data to Fig. 2h and Supplementary Fig. 7d-f in the revised manuscript. Also, we changed the sentence "These T cell responses were specific for TAAs, as indicated by the increase in the number of H-2L^{d}MuLV gp70 peptide (SPSYVYHQF) tetramer-bound CD8^{+} T cells (Tet^{+} CD8^{+}) in the peripheral blood of CT26-bearing mice after treatment with SAM-FC(+) or SAM-CR(+)" to "These T cell responses were specific for TAAs, as indicated by the increase in the number of H-2LdMuLV gp70 peptide (SPSYVYHQF) tetramer-bound CD8^{+} T cells (Tet^{+} CD8^{+}) in the peripheral blood, tumor-draining lymph nodes (TdLNs), and tumors of CT26-bearing mice after treatment with SAM-FC(+) or SAM-CR(+)$^{41-43}$" in the revised manuscript (Page 9, lines 264-268).

Q11. *Line 270 and follow: the T cells in the draining LN were only characterized be general surface molecules. It is therefore not clear whether they were tumor specific or possibly specific for Salmonella. Why were the TILs not analyzed.*

Author's response: This reviewer' question was addressed in our reply to Q10 (tumor-specific T cells were detected in tumor tissues, Fig. 2h, Supplementary Fig. 7f in revised manuscript).

Q12. *The metastasis part is to my mind the weakest part. Why was 4T1 not applied orthotopic. It is usually injected into the fat pad of female mice. The findings can simply be explained by assuming that the reduction of the cell number is sufficient to delay metastasis formation. More characterizations have to be done to allow this statement. For instance, is there any influence on the premetastatic niche etc.*

Author's response: We assessed the efficacy of SAM-FC in inhibiting lung metastasis in BALB/c mice inoculated with 4T1 tumor cells into the mammary fat pad (orthotopic 4T1 tumor and metastasis). Although empty bacteria (SAM-E) and ClyA-secreting SAM-CR(+) reduced lung metastasis more than the PBS control, FlaB-secreting bacteria [SAM-FC(+) or SAM-FR(+)] were more effective in inhibiting metastasis. We have added the following sentences "FlaB-secreting bacteria were superior to ClyA-secreting bacteria in inhibiting 4T1 lung metastasis. The mice treated with SAM-FC(+) and SAM-FR(+) exhibited the strongest inhibition in 4T1 lung metastasis. Treatment with SAM-E or SAM-CR(+) was more effective in reducing lung metastasis than treatment with PBS (Fig. 3c, and Supplementary Fig. 9a). These findings suggest that FlaB-secreting bacteria are more potent in inhibiting metastasis than ClyA-secreting bacteria." to the revised manuscript (page 10, lines 303-309).

We also observed changes in immune cells in lung metastasis. The ratio of M1($F4/80^+CD86^+$)/M2($F4/80^+CD206^+$) macrophages was similarly increased by SAM-FC(+) and SAM-FR(+). These results indicate that macrophage polarization toward the M1 phenotype was more effectively induced by FlaB-secreting bacteria than by empty bacteria or ClyA-secreting bacteria. Additionally, a significant increase in the NK cell population ($CD3^-$ $CD49^+$) was observed in lungs following treatment with FlaB-secreting bacteria. The new data has been added to Fig. 3a, d-e (for macrophages) and Supplementary Fig. 9h, j (for NKs) in the revised manuscript. We have added the following sentences: "We assessed whether the anti-metastasis effect of FlaB-secreting bacteria was mediated by functional macrophage polarization … which might synergistically promote anti-metastatic outcomes (Supplementary Fig. 9h, j)" to the revised manuscript (page 10, lines 310-317; page 11, lines 318-321). Citations to new references

(46 - 49) have been added and the new references have been included in the reference list (page 31, lines 967-976).

Also, we performed macrophage neutralization with αCSF-1R antibody in the same mice innoculated with 4T1 tumor cells. The SAM-FC(+) plus αCSF-1R group showed a significant decrease of the M1/M2 ratio in lung metastasis compared with the SAM-FC(+) group alone. αCSF-1R increased metastasis of the control mouse (PBS), but SAM-FC(+) reversed the αCSF-1R effect, indicating that macrophage neutralization abolished the suppression of metastasis by SAM-FC(+). The relevant data has been added to Fig. 3a-e and Supplementary Fig. 9e-g. The sentences "To further explore the role of macrophages in the inhibition of 4T1 metastasis, we neutralized macrophages using the specific antibody αCSF-1R… suppressed primary tumor growth and metastasis in the SAM-FC(+)-treated group (Fig. 3a-c, Supplementary Fig. 9e, f)" have been added to the revised manuscript (Page 11 line 324-329).

Finally, we assessed a role of macrophages in the suppression of metastasis in BALB/c athymic nu^-/nu^- mice (nude mice) treated with SAM-FC. In addition to macrophage analysis of primary tumor and metastasis of nude mice bearing subcutaneous 4T1-FLuc2 tumors reported in the original manuscript (Supplementary Fig. 9b), we analyzed macrophages in the lung metastasis of nude mice innoculated with human MDA-MB-231-FLuc-GFP tumor cells into the mammary fat pad. After bacterial treatment, SAM-FC(-) was more effective in suppressing lung metastasis than PBS but was less effective than SAM-FC(+), indicating that other immune cells, not T cells, were required for the inhibition of metastasis by SAM-FC. We also conducted a phagocytosis assay using macrophages from lung metastasis of the same mice. The highest phagocytic activity was observed in mice treated with SAM-FC(+), followed by those treated with SAM-FC(-) and PBS. The data have been added as Fig. 3f, g. The sentences "Notably, SAM-FC(+) was able to significantly reduce metastasis in immunodeficient BALB/c athymic nu^-/nu^- mice bearing human MDA-MB-231-FLuc-GFP or 4T1-FLuc2 tumors... which is essential for the inhibition of metastasis by phagocytosis." have been added to the revised manuscript (page 11, lines 329-336).

Q13. *Why was a solid sc tumor implanted besides the intrasplenic injection of MC38.*

Author's response: In the experiment in Fig. 3e, we implanted MC38 in the right flank (below the intrasplenic injection site), whereas we implanted MC38-Fluc on the left flank (opposite site of the intrasplenic injection site), which made it easy to image both primary and liver metastasis under the cooled-CCD camera.

Q14. *Why was the liver not analyzed for colonization by bacteria. The fast growing tumor colonies in the liver should already display a size that allows colonization by bacteria at the time of bacterial injection.*

Author's responses: We additionally evaluated the number of viable bacteria numbers in livers from mice bearing only the subcutaneous MC38 tumor (SC) and mice bearing both the subcutaneous MC38 tumor and its liver metastasis (SC + liver tumors) after SAM-FC treatment. In the mice bearing the SC tumor, bacterial counts in the liver gradually decreased until day 5 after bacterial injection, despite the high number of bacteria detected on day 1. However, in the mice bearing SC + liver tumors, the bacterial numbers in the liver significantly increased until day 5 (Supplementary Fig. 10a-d in revised manuscript). These results indicated that SAM-FC specifically targets and proliferates in metastatic liver tissue after intravenous injection. We have added the data to Supplementary Fig. 10a-d in the revised manuscript. We also inserted the sentence "After intravenous injection of SAM-FC, …. thereby triggering immune cell responses." in the revised manuscript (page 12, line 360-364).

Q15. *Line 314: what does mean "liver directed SAM-FC treatment?*

Author's response: We changed "liver-directed SAM-FC treatment" to "SAM-FC(+) treatment" in the revised manuscript (page 12, line 379).

Q16. *Line 377: similar experiments with similar results have been carried out and published several years ago. They should be quoted.*

Author's response: We quoted reference 27 and referred to it in the revised manuscript (page 15, line 465).

Q17. *Line 447: "to the best of our knowledge…." Is an overstatement. The parts of the system*

have been described before and the dual expression has been published by the authors. Please remove.

Author's response: In the revised manuscript, we removed "to the best of our knowledge" in the revised manuscript (page16, line 491).

Q18. *The authors show extensive data of imaging. The panels are so small that nothing can be recognized especially when using hard copies. Similarly, nothing can be recognized on the micrographs. It is also missing how many field of view were analyzed.*

Author's response: The manuscript contains very large amounts of data, which make it difficult to include all of the data in the figures without compromising figure quality. We did our best to increase the quality of the figures in the revised manuscript. If the paper is accepted for publication, we will discuss this issue with the journal office.

Q19. *Legend Suppl. Fig 8b. the description is confusing. Eradicated might have to be added.*

Author's response: We changed the legend of original Supplementary Fig. 8b "mice without primary CT26 tumors" to "CT26 tumor-eradicated mice" and moved the data together with the legends (original Supplementary Fig. 8a, b) to Fig. 8a, b in the revised manuscript.

Q20. *Fig. 1: An effect by the bacteria carrying empty vector is seen. –why due the mice not survive longer.*

Author's response: Despite SAM-E showing initial tumor suppression, all mice were euthanized when the tumor volume exceeded 1,000 mm^3 around 25-30 days after bacterial injection.

Q21. *Suppl. Fig. 2c: the +/- of SAM-FC is mixed up.*

Author's response: We corrected this mistake in Supplementary Fig. 2e in the revised manuscript.

REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

The revised manuscript is recommended for publication.

Reviewer #2 (Remarks to the Author):

I am pleased with the authors' responses and the revisions made to the manuscript.

Reviewer #5 (Remarks to the Author):

I am satisfied with the answers to the majority of the questions. But I keep my criticism of the limited novelty. Some new data are not convincing either.

In terms of novelty, the authors stated that “what was particularly novel in the present study was that ClyA and FlaB synergistically activated both innate and adaptive immunity”. However, a number of important pieces of data have demonstrated that single drug (SAM-CR/SAM-FR) are equally potent as dual drugs (SAM-FC) in inducing anti-tumor responses. These examples include T cell memory (Figs. 2j,k), T cell activation (Fig. S6g), and animal survival (Figs. 1d, 1g, 1h). There was no significant difference between the single and dual drug combinations, according to the present statistics. Additionally, the synergistic index was not calculated. Dual-drug might have additive rather than synergistic effects. These data collectively do not support the statement.

Regarding the responses to Q10 and Q11 under Reviewer 4, the key data added to Fig. 2h seem dubious to me. First, comparing SAM-CR group versus SAM-FC group for Tet+CD8+ T cells in tumors yields a P value of 0.0058. In the scatter plots, however, the percentages of this population are of very similar levels in both groups. How was this statistical analysis performed? FAC scatter plots and original data are required. Secondly, the percentage of Tet+ in CD8+ T cells is around 0.05% in SAM-FC-treated tumors, which is extremely low. At this level, antigen-specific T cells could hardly produce a noticeable tumor-killing effect.

Reviewer #1 (Remarks to the Author):

The revised manuscript is recommended for publication.

Reviewer #2 (Remarks to the Author):

I am pleased with the authors' responses and the revisions made to the manuscript.

We thank and appreciate the time and effort reviewer #1 and reviewer #2 have invested in reviewing our work.

Reviewer #5 (Remarks to the Author):

I am satisfied with the answers to the majority of the questions. But I keep my criticism of the limited novelty. Some new data are not convincing either. In terms of novelty, the authors stated that "what was particularly novel in the present study was that ClyA and FlaB synergistically activated both innate and adaptive immunity". However, a number of important pieces of data have demonstrated that single drug (SAM-CR/SAM-FR) are equally potent as dual drugs (SAM-FC) in inducing anti-tumor responses. These examples include T cell memory (Figs. 2j,k), T cell activation (Fig. S6g), and animal survival (Figs. 1d, 1g, 1h). There was no significant difference between the single and dual drug combinations, according to the present statistics.

Author's response:

Comparison with SAM-CR: Although there was no significant difference in memory T cell (Figs. 2j, k) and T cell activation (Supplementary Fig. 6g) levels, mice treated with SAM-FC(+) exhibited an increased tumor-suppressive population of M1 ($F4/80^+CD86^+$) and $GzmB^+CD8^+$ T cells, as well as decreased population of immunosuppressive M2 macrophages, when compared to those treated with SAM-CR(+) (Fig. 2b, c, g). This shift towards a tumor-suppressive immune profile is consistent with the improved therapeutic outcomes observed with SAM-FC(+) treatment over SAM-CR(+) (Fig. 1c, g). Furthermore, the suppression and eradication of tumors were significantly more pronounced in mice treated with SAM-FC(+) than in those receiving SAM-CR(+) in both CT26 tumor-bearing BALB/c mice and MC38 tumor-bearing C57BL/6 mice (Fig. 1c, d, g, h) [SAM-FC(+) 75% (24/32) *versus* SAM-CR(+) 57.14% (12/21) in tumor eradication of CT26 tumor-bearing BALB/c mice; SAM-FC(+) 80% (12/15) *versus* SAM-CR(+) 50% (5/10) in tumor eradication of the MC38 tumor-

bearing C57BL/6].

Comparison with SAM-FR: SAM-FC significantly enhanced the activation of DCs, effector $CD4^+$ T cells, $GzmB^+PD\text{-}1^+CD8^+$ T cells, Tet^+CD8^+ T cells, and memory T cells, as well as decreased Treg cell population (Fig 2d-k). This immunological shift also corresponds to improved therapeutic effects and the establishment of effective long-term anti-tumor memory in the treatment with SAM-FC(+) compared to SAM-FR(+) (Fig. 1c-e). Although SAM-FC(+) did not show significant tumor suppression compared to SAM-FR(+) in MC38 tumor-bearing C57BL/6 mouse model (Fig. 1g, h), it exhibited a significant difference in tumor suppression and prolonged mouse survival in the CT26 tumor-bearing BALB/c mouse model (Fig. 1c, d). Consistent with this, potent long-term anti-tumor memory was more firmly established in SAM-FC(+) treated mice than SAM-FR(+) treated mice in both CT26 and MC38 tumor-bearing mice model, even though SAM-FC(+) and SAM-FR(+) similarly suppressed tumor growth and prolonged mouse survival in MC38 tumor-bearing C57BL/6 mouse model (Fig. 1g, h).

Additionally, SAM-CR(+) and SAM-FR(+) demonstrated a preference for tumor suppression in specific cancer models: CT26 tumors in BALB/c mice and MC38 tumors in C57BL/6 mice, respectively. However, SAM-FC(+) exhibited the strongest therapeutic effects independently of cancer types (CT26 and MC38) and host genetic background (BALB/c and C57BL/6). It suggests that SAM-FC exhibits the broad-spectrum efficacy in cancer treatment compared to SAM-CR or SAM-FR.

Additionally, the synergistic index was not calculated. Dual-drug might have additive rather than synergistic effects.

In response to the reviewer's comment, we measured the Coefficient of Drug Interaction (CDI) to analyze whether tumor suppression by the dual-drug effect was additive or synergistic, as previously described [Lu, L. *et al*. Synergistic cytotoxicity of ampelopsin sodium and carboplatin in human non-small cell lung cancer cell line SPC-A1 by G 1 cell cycle arrested. *Chinese journal of integrative medicine* **23**, 125-131 (2017)]. The CDI was calculated using the equation [$CDI = AB/(A \times B)$], where 'A' and 'B' represent the effects of single drugs, and 'AB' represents the effect of the dual-drug combination. CDI values less than, equal to, or greater than 1 indicate that dual-drugs effects are synergistic, additive, or antagonistic, respectively. A CDI value below 1 indicates a synergistic interaction between the drugs.

The data were obtained from the average tumor volumes for each group on day 15 (for the CT26 tumor model) or day 12 (for the MC38 tumor model), as shown in Figure 1c and g of the revised

manuscript. The ratios of average tumor volumes for the SAM-FC(+), SAM-FR(+), and SAM-CR(+) groups relative to the control group (SAM-E) were designated as 'AB', 'A', and 'B', respectively. The results demonstrated that ClyA and FlaB in SAM bacteria synergistically affect each other in tumor suppression. This synergy was evident as the CDI values for the CT26 and MC38 tumor models were 0.33 and 0.88, respectively, as shown in Supplementary Table 3 of the revised manuscript.

The new data has been added as Supplementary Table 3 to the revised manuscript. We have modified the sentence from "These results strongly suggest that SAM engineered to express ClyA and FlaB could have a therapeutic anti-cancer vaccination effect" to "These results strongly suggest that SAM engineered to express both ClyA and FlaB could synergistically enhance a therapeutic anti-cancer effect (Supplementary Table 3)" in revised manuscript. Additionally, we added the following description "Synergism evaluation of SAM-FC….. The ratios of average tumor volumes in the SAM-FC(+), SAM-FR(+), and SAM-CR(+) groups relative to the control group (SAM-E) were designated as 'AB', 'A', and 'B', respectively." to the revised manuscript (page 8, line 226 – 228 and page 26, line 806 – 816, respectively). We also have added the reference to the reference list of the revised manuscript (Page 30, line 1000 – 1002).

Supplementary Table 3: CDI values of SAM-FC(+) treatment in CT26 and MC38 tumor models

Average tumor volume (mm^3)	Control (SAM-E)	A [SAM-FR(+)]	B [SAM-CR(+)]	AB [SAM-FC(+)]	CDI	Interpretation
CT26 tumor model (day 15)	555.50	383.05	224.05	51.58	0.33	Synergism
MC38 tumor model (day 12)	476.33	63	521.60	60.87	0.88	Synergism

CDI, Coefficient of Drug Interaction; CDI = {(AB/control)/[(A/control) x (B/control)]}

Regarding the responses to Q10 and Q11 under Reviewer 4, the key data added to Fig. 2h seem dubious to me. First, comparing SAM-CR group versus SAM-FC group for Tet+CD8+ T cells in tumors yields a P value of 0.0058. In the scatter plots, however, the percentages of this population are of very similar levels in both groups. How was this statistical analysis performed? FAC scatter plots and original data are required. Secondly, the percentage of Tet+ in CD8+ T cells is around 0.05% in SAM-FC-treated tumors, which is extremely low. At this level, antigen-specific T cells could hardly produce a noticeable tumor-killing effect.

Author's response:

We have corrected this mistake in Fig. 2h, right. in the revised manuscript.

The FAC scatter plots and original data are shown in Supplementary Figure 7f. For analysis of Tet^+ in $CD8^+$ T cells, we isolated tumors on day 9 after SAM injection. At that time, tumors had significantly shrunk and tended to be completely eradicated on day 12 or day 15, suggesting high population of Tet^+ in $CD8^+$ T could be observed on day 5-8 and gradually decreased by day 9. This is because, after killing the tumor cells, they tend to migrate to lymphoid organs such as the spleen and lymph nodes (Fig. 2h, left and middle) to differentiate into central memory T cells (Fig. 2j and k). In response to the reviewer's suggestion, we plan to conduct analyses at earlier time points (day 5 to 8 after SAM injection) in future studies to verify the elevated presence of Tet^+ $CD8^+$ T cells within tumors."