nature portfolio

Peer Review File

Probiotics functionalized with a gallium-polyphenol network modulate the intratumor microbiota and promote anti-tumor immune responses in pancreatic cancer

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author): with expertise in biomaterials, cancer, bacteria

The inefficiency of immunotherapy in pancreatic cancer is attributed to an imbalance in the intratumor microbiome. This study presents a novel approach utilizing a layer-by-layer self-assembly technique to fabricate Lactobacillus rhamnosus GG probiotics equipped with a gallium polyphenol network (LGG@Ga-poly). By disrupting the iron-dependent metabolic functionality, LGG@Ga-poly selectively eradicates detrimental Proteobacteria and diminishes the concentration of lipopolysaccharides. Moreover, the reconfiguration of the intratumor microbiome triggers the activation of tumoral Toll-like receptors, resulting in a decrease in immunosuppressive PD-L1 and IL-1β expression by tumor cells, a reduction in immunotolerant myeloid populations, and an enhancement in the infiltration of cytotoxic T lymphocytes within tumors. The administration of LGG@Ga-poly significantly delays the development of pancreatic ductal adenocarcinoma in a preventive context, inhibits the growth of established tumors and lung metastasis, and improves the effectiveness of immune checkpoint blockade immunotherapy. The following are specific comments for consideration.

1# After screening various microbes in Fig 3b, the lack of clear indication regarding the specific bacteria used in Fig 3c is perplexing

2# In Fig 4g and Fig 4h, the application of LGG@Ga-poly resulted in a reduction in the abundance of Proteobacteria, consequently leading to Lactobacillus winning the competition for niche occupancy. However, it remains unclear why Lactobacillus is unaffected by LGG@Ga-poly. Are there any plausible explanations for this phenomenon?

3# Many results were illustrated by immunohistochemistry staining, such as Fig 5e and Fig 5k. However, the inclusion of quantification of the immunohistochemistry data would enhance the persuasiveness of the findings.

4# In Supplementary Fig. 12, bacterial colony, excessive concentration of bacterial colonies will affect the accuracy of counting. It is advisable to moderately adjust the dilution ratio to mitigate this effect.

5# In Supplementary Fig. 25, H&E images of lungs exhibits some differences among different groups. What is the reason for this phenomenon?

Reviewer #2 (Remarks to the Author): with expertise in pancreatic cancer, microbiota

Pancreatic cancer is a difficult cancer to detect and has low survival rates. In recent years, the number of patients with pancreatic cancer has increased significantly. Fortunately, through the study of the microbiome, we have been able to understand the important correlation between microbes in the tumor microenvironment and pancreatic cancer. In recent years, many studies have also demonstrated the effectiveness of using probiotics as direct treatment or in combination with chemotherapy or immunotherapy. The authors demonstrated that LPS released by intestinal or intratumor bacteria leads to tumor cell expression of PD-L1, MDSC proliferation, and conversion of M1 macrophages into M2 macrophages through the TLR pathway. In order to treat pancreatic cancer, the authors used probiotic LGG as a carrier to carry Ga3+ (LGG@Ga-polymer) into pancreatic tumors to compete with Fe3+, thereby limiting the survival of pathogenic microorganisms in the tumor. This also leads to a decrease in LPS levels within the tumor, and the tumor-infiltrating immune cell population tends to be anti-tumor, ultimately leading to cancer cell growth arrest and cell death, including apoptosis and necrosis. These antitumor effects are associated with a range of immune-related responses, including innate and adaptive immunity. The authors also demonstrated the safety of LGG@Ga-polymer.

Overall, this research is very novel, the experimental results are comprehensive, and it is very positive for the treatment of pancreatic cancer. However, there are still several opinions and questions as follows

1. The source of the anti-PD-L1 antibody is unknown.

2. Figure 4a indicated that most of the Cy5-labeled LGG were located in the liver but not the tumor mass, however, the photoacoustic signal of Cy5-labeled LGG@Ga-poly more specifically detected at the tumor but not the liver. I am curious why LGG@Ga-poly specifically senses and targets pancreatic cancer. Although the authors believe that the anaerobic condition of the tumor microenvironment attracts LGG@Ga-poly. Is there any evidence to confirm it? In addition, how does the LGG@Ga-poly get into tumors? Can only surviving LGG@Ga-poly enter the tumor? On the other hand, can LGG still grow and reproduce after entering the tumor? And does LGG@Ga-poly exist extracellularly or

intracellularly in tumor tissue? If it is inside a cell, what type(s) of cells are the host cells? What is the penetration mechanism of LGG@Ga-poly?

3. Various bacteria such as Porphyromonas gingivalis and Fusobacterium nucleatum are considered risk factors for pancreatic cancer and are able to enter cells. Does Ga3+ also need to enter cells and then inhibit the growth of Fe3+-dependent bacteria?

4. Because pancreatic cancer is difficult to detect, long-term prevention is needed. But from a preventive perspective, will long-term use of LGG@Ga-poly affect physiological phenomena, especially the immune system, before tumors occur? If the answer is yes, can you anticipate what might happen?

5. The PANC-02 is a mouse pancreatic ductal adenocarcinoma, not cells at the precancer stage. For prevention, is the LSL-KrasG12D-Pdx-1-Cre transgenic mouse model better than the orthotopic mouse model?

6. It will be interesting to observe whether residual tumor cells in LGG@Ga-poly-treated PANC02 orthotopic mice are more resistant to a second treatment with LGG@Ga-poly.

7. Is the percentage of cancer stem cells in the tumor mass of LGG@Ga-poly-treated mice higher than that of PBS-treated mice?

Reviewer #3 (Remarks to the Author): with expertise in intratumor microbiota, pancreatic cancer, immunology

Zi-Yi Han, et al., reported that lactobacillus with gallium-polyphenol can enhance immunotherapy in PDA. The work is interesting but has many weakness which need to be addressed. Orthotropic model is very aggressive and it is suggested to show similar finding in slow progressive KC model or many other PDA model. Authors need to verify using multiple cell lines to show the efficacy of probiotics. FISH staining has non-significant staining. The analysis and data presented on microbiome is very preliminary and may not support role of probiotics encased in gallium-polyphenol can Enhancement of

immunotherapy in pancreatic cancer through intratumor microbiota modulation.

Other comments:

Why there was no bacteria in subcutaneous tumors?

Figure 1b and 2b is very intense FISH suggesting a background non-significant hybridization. It is difficult to determine the presence of bacteria in tissue without full scan.

Proteobacteria is also a normal flora however why authors did not see any Proteobacteria in control?

Only polymyxin B (PmB) treatment improve the efficacy of α-PD-L1 treatment need to be verified in another slow progression animal model. The provided data is not enough to make this assumptions.

There are many bacteria produce LPS authors need to clarify what is the meaning of microbial LPS.

Why Ga is used other ions have similar activity like Mg and Ca. There is no significant difference between Ga and these ions.

Antibacterial activity of Ga3+ with polyphenols is not clear. Graph plotted is not correct and confusing. How many bacteria were used.

What is the role of siderophores.

Line 184. Do not expand every time the names of bacteria.

Figure 3j should be removed doesn't provide any information.

SFig 15 there is very much variability in bacterial richness and abundance. It shows that not firmicutes is increased but it is also desulfobacteria in two animals.

Legends of figures are incomplete. Need to be modified even in supplement figs.

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

The work of Han et al shows interesting data in a murine model of PDAC on the use of a gallium-polyphenol conjugayed Lactobacillus rhanmnosus GG, and may be potentially groundbreaking if holds true in the human context, especially if combined with immunotherapy and chemotherapy. It is of particular interest the tumor selectivity of the LGG@Ga-poly and its capability to eliminate intratumoral bacteria and to potentially reactivate the immune system.

Authors perform a series of experiments to ensure that the compound is safe, to

demonstrate in vivo its bioavalabilty and biodistribution. Authors demonstrate in vitro its functional capability to eliminate different pathobionts and its functional resilience in hostile environments. Moreover, authors evaluated the functional efficacy of the compound in experimental tumor settings, both in preventive and therapeutic administrations. In both cases the compound demonstrates efficacy in reducing the tumor burden. Authors then provide some mechanistic insights to understand how proteobacteria-derived LPS may be responsible for the reduced efficacy of ICB in this context. Authors conclude that the elimination of intratumor microbiota impedes the activation of tumoral Toll-like receptors, reducing immunosuppressive PD-L1 and IL-1β expressed by tumor cells. From the immunological point of view this elimination diminishes the differentiation of immunotolerant myeloid populations and improves the infiltration of cytotoxic T lymphocytes.

The study is surely interesting and in general well-conceived and designed, and it can provide important insights to ameliorate PDAC antitumor responses. However, some weaknesses limit the full appreciation of the work, especially those regarding the immunological part. All the data of the immune infiltrate and its functional responses are surely less solid than the rest of the work and in my opinion would require an extensive revision.

Specifically:

1. There are no plots of the immune infiltrate, no gating strategy, not a single description of how the cell population have been defined based on the expression markers (Figure 6A-G) and in general primary data to appreciate the quality of the data as well as the phenotypical and functional modifications of the immune system pre-post treatments.

The only plots available are those for CD4/CD8 in 7G, and authors then show only the cumulative graph for CD8

2. The plots in supplementary 19B, it is not clear why CD4+ cells are not present. Was it a technical problem?

3. The plots in supplementary 20 are not understandable. How authors chose where to put the bar of positivity?

4. The ELISA of Figure 6 i-l, especially those of IL6 and IL12 seem below the limit of detection. It is unclear which is the assay used and which is the limit of detection.

6. Beside that, one major issue of the immunological part is that all the experiments

addressing the function of the IS show variations in frequency rather than in function. This is true for in vivo experiments where authors report variations in the intratumoral frequency of CD8/ Macrophages/MDSC as well as in vitro experiments of polarization. In both cases, it is not shown whether the treatment with the compound directly influences (and how, by which mechanisms) the cytotoxic function of CTLs, or if the MDSC or M2 macrophages truly become conditioned and influence in an immunosuppressive fashion the activities of other immune cells.
Moreover, in many experiments authors evaluate the presence of CD3/ CD4 cells but never identify them as Treg or effectors (not even based on surface markers or transcription factors, or cytokine produced) and are not shown their functional activities.

Minor: it is never mentioned how many replicates of the experiment have been performed (it seems only once, with 3-5 mice/group).

Fig2i: how immune populations have been defined? Why only three mice when in the experiments there are at least 5? Has it been repeated?

Fig8: what about Macrophages and MDSC? Is the TME altered as in the setting without chemo?
Fig8: is the microbiome composition affected? Chemotherapy generally impacts on the composition and function of the gut microbiome and would be interesting to evaluate if LGG@Ga-poly also contributes to microbiome stabilization.

Response to comments

We are very grateful for the support and suggestions on our manuscript. Our manuscript has been carefully revised according to these valuable comments. Major changes have been highlighted in red. The point-by-point response to the comments is listed as follows:

Reviewer #1: with expertise in biomaterials, cancer, bacteria

The inefficiency of immunotherapy in pancreatic cancer is attributed to an imbalance in the intratumor microbiome. This study presents a novel approach utilizing a layer-by-layer self-assembly technique to fabricate Lactobacillus rhamnosus GG probiotics equipped with a gallium polyphenol network (LGG@Ga-poly). By disrupting the iron-dependent metabolic functionality, LGG@Ga-poly selectively eradicates detrimental Proteobacteria and diminishes the concentration of lipopolysaccharides. Moreover, the reconfiguration of the intratumor microbiome triggers the activation of tumoral Toll-like receptors, resulting in a decrease in immunosuppressive PD-L1 and IL-1β expression by tumor cells, a reduction in immunotolerant myeloid populations, and an enhancement in the infiltration of cytotoxic T lymphocytes within tumors. The administration of LGG@Ga-poly significantly delays the development of pancreatic ductal adenocarcinoma in a preventive context, inhibits the growth of established tumors and lung metastasis, and improves the effectiveness of immune checkpoint blockade immunotherapy. The following are specific comments for consideration.

Reply: We sincerely appreciate the insightful comments and suggestions. The specific modifications were as follows:

1# After screening various microbes in Fig 3b, the lack of clear indication regarding the specific bacteria used in Fig 3c is perplexing

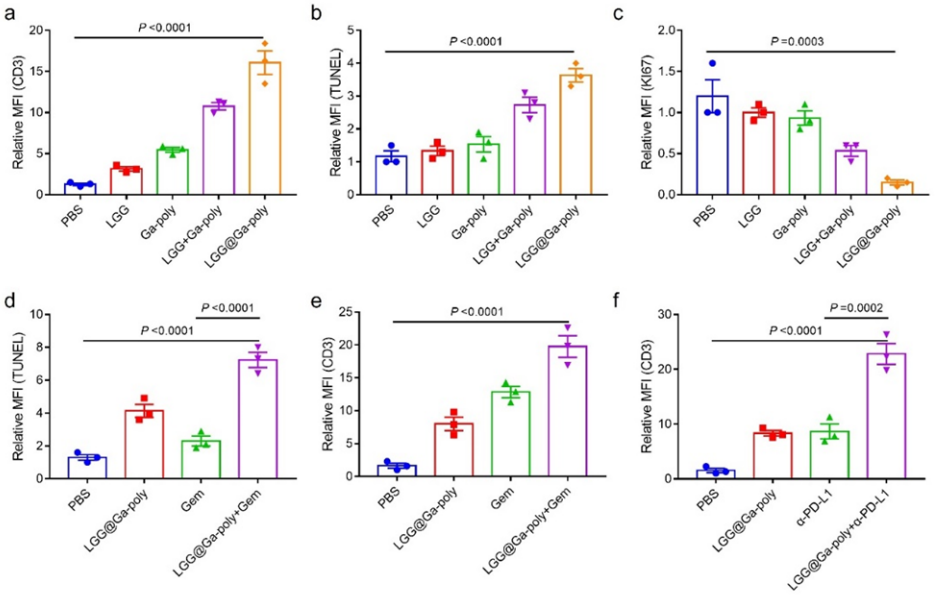
Reply: Sorry for making this mistake. The bacteria used in Fig 3c was *E. coli*. The related illustration was added into the main text and legend.

2# In Fig 4g and Fig 4h, the application of LGG@Ga-poly resulted in a reduction in the abundance of Proteobacteria, consequently leading to Lactobacillus winning the competition for niche occupancy. However, it remains unclear why Lactobacillus is unaffected by LGG@Ga-poly. Are there any plausible explanations for this phenomenon?

Reply: Sorry for confusing you. LGG@Ga-poly led to a decreased abundance of *Proteobacteria*, which subsequently allowed *Lactobacillus* to dominate the niche. Ga^{3+} acts as a mimic of iron and eliminates bacteria by inhibiting their iron metabolism. Importantly, the growth of *Lactobacillus* is not solely dependent on iron, which allows LGG@Ga-poly to selectively target and eradicate harmful *Proteobacteria* without significantly affecting *Lactobacillus*.

3# Many results were illustrated by immunohistochemistry staining, such as Fig 5e and Fig 5k. However, the inclusion of quantification of the immunohistochemistry data would enhance the persuasiveness of the findings.

Reply: Thanks for your kind suggestion. To enhance the persuasiveness of our findings, we have included quantitative data in the immunohistochemistry sections (Figs 5e, 5k, 8d, 8e, and 8k, as shown in Supplementary Fig. 25 in the revised version).



Supplementary Fig. 25 The quantification of the immunofluorescence staining data for *Figure 5e* (a), *5k* (b, c), *8d* (d), *8e* (e), *8k* (f). n = 3 samples. Data are means ± sem.

4# In Supplementary Fig. 12, bacterial colony, excessive concentration of bacterial colonies will affect the accuracy of counting. It is advisable to moderately adjust the dilution ratio to mitigate this effect.

Reply: Thanks for your kind suggestion. We have moderately adjusted the dilution ratio and assured that bacterial colonies were uniformly distributed (as shown in Supplementary Fig. 10d in the revised version).

5# In Supplementary Fig. 25, H&E images of lungs exhibits some differences among different groups. What is the reason for this phenomenon?

Reply: There may have been an issue with the initial staining. To minimize any doubts, we repeated the H&E staining of major organs. The repeated results confirmed that the treatment with the material had no significant adverse effects on lung tissues (as shown in Supplementary Fig. 23a in the revised version).

Reviewer #2: with expertise in pancreatic cancer, microbiota

Pancreatic cancer is a difficult cancer to detect and has low survival rates. In recent years, the number of patients with pancreatic cancer has increased significantly. Fortunately, through the study of the microbiome, we have been able to understand the

important correlation between microbes in the tumor microenvironment and pancreatic cancer. In recent years, many studies have also demonstrated the effectiveness of using probiotics as direct treatment or in combination with chemotherapy or immunotherapy. The authors demonstrated that LPS released by intestinal or intratumor bacteria leads to tumor cell expression of PD-L1, MDSC proliferation, and conversion of M1 macrophages into M2 macrophages through the TLR pathway. In order to treat pancreatic cancer, the authors used probiotic LGG as a carrier to carry Ga^{3+} (LGG@Ga-poly) into pancreatic tumors to compete with Fe^{3+}, thereby limiting the survival of pathogenic microorganisms in the tumor. This also leads to a decrease in LPS levels within the tumor, and the tumor-infiltrating immune cell population tends to be anti-tumor, ultimately leading to cancer cell growth arrest and cell death, including apoptosis and necrosis. These antitumor effects are associated with a range of immune-related responses, including innate and adaptive immunity. The authors also demonstrated the safety of LGG@Ga-poly.

Overall, this research is very novel, the experimental results are comprehensive, and it is very positive for the treatment of pancreatic cancer. However, there are still several opinions and questions as follows

Reply: Thanks for the insightful comments and suggestions. The specific modifications were as follows:

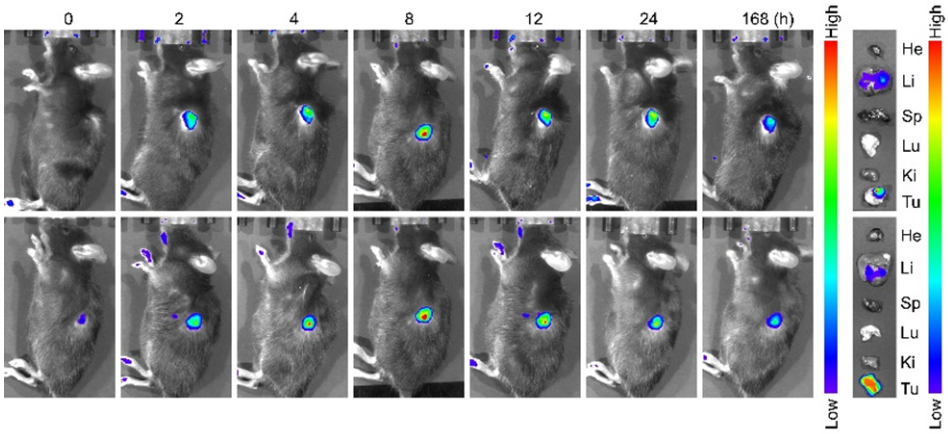
1. The source of the anti-PD-L1 antibody is unknown.

Reply: Sorry for making this mistake. The source of the anti-PD-L1 antibody was indicated in the Supporting Information. The source of the antibodies used for FCM was also added.

2. Figure 4a indicated that most of the Cy5-labeled LGG were located in the liver but not the tumor mass, however, the photoacoustic signal of Cy5-labeled LGG@Ga-poly more specifically detected at the tumor but not the liver. I am curious why LGG@Ga-poly specifically senses and targets pancreatic cancer. Although the authors believe that the anaerobic condition of the tumor microenvironment attracts LGG@Ga-poly. Is there any evidence to confirm it? In addition, how does the LGG@Ga-poly get into tumors? Can only surviving LGG@Ga-poly enter the tumor? On the other hand, can LGG still grow and reproduce after entering the tumor? And does LGG@Ga-poly exist extracellularly or intracellularly in tumor tissue? If it is inside a cell, what type(s) of cells are the host cells? What is the penetration mechanism of LGG@Ga-poly?

Reply: In order to more objectively characterize the distribution of the materials in the mice after oral administration, we repeated the experiment and found that some bacteria or materials indeed enter the liver (as shown in Supplementary Fig. 12a in the revised version). The part of LGG and material enriched in the liver may be attributed to the leakage of intestine, and therefore they enter the systemic circulation. From the quantitative result, LGG@Ga-poly mainly accumulated in the tumor (Fig. 4b). To compare the penetrating ability of free Cy5 and LGG@Cy5 into tumors, we only

detected the signal in the tumor and confirmed that compared to the free Cy5, LGG@Cy5 can penetrate the tumor tissues, thereby possessing superior signal. The PAI signal in the liver was not monitored.



Supplementary Fig. 12 a, In vivo and ex vivo fluorescence imaging of orthotopic PDAC mice after oral administration of Cy5.5 labeled LGG or LGG@Ga-poly (A parallel repeat trial). He-heart, Li-liver, Sp-spleen, Lu-lung, Ki-kidney, Tu-tumor. The MFI in the tumor was higher than that in the liver.

The specific tumor-targeting capability of LGG@Ga-poly is attributed to the anaerobic nature of LGG, which allows it to specifically target hypoxic tumor tissues. Additionally, the nutrient-rich and immunosuppressive environment of these tissues further attracts bacterial accumulation. This phenomenon of bacterial tumor-targeting has been confirmed by multiple reports (Nat. Nanotechnol. 11, 941-947 (2016); Adv. Mater. 33, e2100241 (2021); Science, 382, 211-218 (2023); Chem. Soc. Rev. 15, 12576-12615 (2021)).

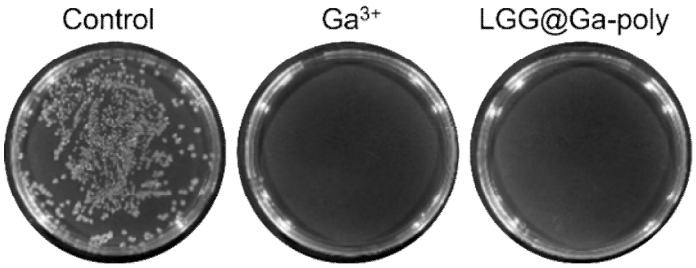
As previously found, LGG@Ga-poly reaches tumors via the gut-pancreas axis after translocation (Cancer Discov. 8, 403-416 (2018); Nano Lett. 9, 8608-8617 (2022)). Protected by chitosan, the majority of LGG@Ga-poly survives passage through the gastrointestinal tract and subsequently enters the tumors. Once within the tumors, these LGG can continue to grow and reproduce, thereby engaging in ecological niche competition (Adv. Func. Mater. 33, 2302728 (2023); Nat. Commun. 13, 7903 (2022)).

LGG@Ga-poly predominantly exists extracellularly within tumors, though a small fraction is internalized by cells, primarily tumor cells, macrophages, and dendritic cells (Science, 368, 973-980 (2020)). Recent studies have shown that facultative anaerobic bacterial strains can spontaneously invade hypoxic tumor cells (Nat. Biotechnol. (2023). https://doi.org/10.1038/s41587-023-01957-8). Certainly, the specific mechanisms underlying these interactions require further investigation.

3. Various bacteria such as Porphyromonas gingivalis and Fusobacterium nucleatum are considered risk factors for pancreatic cancer and are able to enter cells. Does Ga^{3+}

also need to enter cells and then inhibit the growth of Fe^{3+}-dependent bacteria?

Reply: For most of bacteria, Ga^{3+} as an iron mimic killed bacteria directly via inhibition of bacterial iron metabolism. For intracellular bacteria, the antimicrobial action of Ga^{3+} relies on its uptake by the bacteria, which allows it to disrupt iron metabolism and induce bacterial death. Therefore, Ga^{3+} must enter the cells to exert its lethal effects. To demonstrate this, we developed a tumor cell model infected with *Fusobacterium nucleatum*. After these cells were treated with Ga^{3+} and LGG@Ga-poly, we detected significant levels of Ga^{3+} inside the cells and confirmed that the *F. nucleatum* was successfully eradicated.



Supplementary Fig. 10 e, In a *F. nucleatum* - infected cell model, the bacterial colonies of *F. nucleatum* on agar plates after different treatments were shown.

4. Because pancreatic cancer is difficult to detect, long-term prevention is needed. But from a preventive perspective, will long-term use of LGG@Ga-poly affect physiological phenomena, especially the immune system, before tumors occur? If the answer is yes, can you anticipate what might happen?

Reply: All components of the formulation, including LGG, Ga^{3+}, and chitosan, are generally regarded as safe (GRAS), underscoring the biosafety of LGG@Ga-poly for use as a healthcare product without adversely affecting physiological functions. Extensive research on microbiota-immune interactions suggests that long-term oral administration of LGG@Ga-poly may positively influence the immune system or, at the very least, not compromise it. To investigate this further, we administered LGG@Ga-poly orally to healthy mice for three months and observed no significant changes in immune-related indicators (as shown in Supplementary Fig. 15b in the revised version).

5. The PANC-02 is a mouse pancreatic ductal adenocarcinoma, not cells at the precancer stage. For prevention, is the LSL-KrasG12D-Pdx-1-Cre transgenic mouse model better than the orthotopic mouse model?

Reply: Thanks for your kind suggestion. The KPC mouse model, as a genetic mouse model, accurately replicates many features of human pancreatic cancer, making it an exceptionally relevant model for this disease. Following the suggestion, we established a KPC transgenic mouse model to assess the prevention effects of LGG@Ga-poly. As depicted in Supplementary Fig 15a, LGG@Ga-poly demonstrated the superior tumor prevention efficiency.

6. It will be interesting to observe whether residual tumor cells in LGG@Ga-poly-treated PANC02 orthotopic mice are more resistant to a second treatment with LGG@Ga-poly.

Reply: Thanks for your kind suggestion. The material developed in this study inhibits tumors by activating anti-tumor immunity, achieved through the regulation of the microbial community within the tumor. Unlike conventional treatments that directly target tumor cells, this indirect method of treatment reduces the likelihood of tumor cells developing resistance.

7. Is the percentage of cancer stem cells in the tumor mass of LGG@Ga-poly-treated mice higher than that of PBS-treated mice?

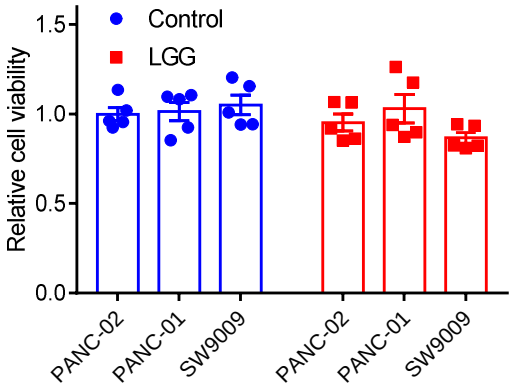
Reply: Thanks for your kind suggestion. As suggested, we examined cancer stem cells across different treatment groups and observed that the material treatments did not alter the proportion of cancer stem cells. Notably, the percentage of cancer stem cells in the tumor mass of the LGG@Ga-poly-treated group was comparable to that in the PBS-treated mice (as shown in Supplementary Figs. 17c-e in the revised version).

Reviewer #3: with expertise in intratumor microbiota, pancreatic cancer, immunology

Zi-Yi Han, et al., reported that lactobacillus with gallium-polyphenol can enhance immunotherapy in PDA. The work is interesting but has many weakness which need to be addressed. Orthotropic model is very aggressive and it is suggested to show similar finding in slow progressive KC model or many other PDA model. Authors need to verify using multiple cell lines to show the efficacy of probiotics. FISH staining has non-significant staining. The analysis and data presented on microbiome is very preliminary and may not support role of probiotics encased in gallium-polyphenol can Enhancement of immunotherapy in pancreatic cancer through intratumor microbiota modulation.

Reply: Thanks for your kind comments. As suggested, we employed a slow progressive KPC mouse model to evaluate the therapeutic effects. In this model, we also showed that LGG@Ga-poly could significantly prevent tumors in a prophylactic regimen (as shown in Supplementary Fig. 15a in the revised version) and inhibit tumor growth in a therapeutic setting (as shown in Supplementary Fig. 18 in the revised version). These results demonstrate that LGG@Ga-poly possessed potential for PDAC treatment.

Our strategy aims to inhibit PDAC progression by modulating the intratumor microbiota, rather than directly targeting tumor cells. Accordingly, we primarily assessed the effects in vivo to provide more robust support for our findings. In line with our approach, our in vitro cell experiments revealed that the probiotics did not exert direct cytotoxic effects on various pancreatic cell lines.



The cytotoxic effects of LGG probiotics on different lines of pancreatic cancer cells.

To further substantiate our conclusions, we followed your recommendations for microbiota modulation data presentation, including comprehensive FISH staining for bacterial identification. The microbiota analysis via 16S gene sequencing demonstrated that LGG@Ga-poly treatment increased the abundance of *Lactobacillus* and decreased that of LPS-producing *Proteobacteria* within the tumors. This approach is consistent with established methods in pancreatic cancer microbiota research. These results support that LGG@Ga-poly could enhance PDAC immunotherapy via modulation of intratumor microbiota.

Other comments:

Why there was no bacteria in subcutaneous tumors?

Reply: Sorry for making the mistake. The subcutaneous tumors harbor bacteria, but very little compared to the orthotopic pancreatic tumors. Research indicates that many tumors, including some epidermal tumors, are colonized by bacteria. Our study involving subcutaneous tumors in mice, which were artificially transplanted, showed very little bacterial colonization. This observation was confirmed by the FISH staining probing the intratumor bacteria.

Figure 1b and 2b is very intense FISH suggesting a background non-significant hybridization. It is difficult to determine the presence of bacteria in tissue without full scan.

Reply: Thanks for your kind suggestion. The full scan and locally amplified scan of the FISH staining, which probed for bacteria, was provided to confirm their presence in pancreatic tumors. This finding is consistent with previous research.

Proteobacteria is also a normal flora however why authors did not see any Proteobacteria in control?

Reply: *Proteobacteria*, a component of the normal flora, commonly colonize many tissues, including the gut. However, in the healthy pancreas, *Proteobacteria* are rarely present. This observation aligns with numerous reports in the literature (Cancer Discov.

8, 403-416 (2018)).

Only polymyxin B (PmB) treatment improve the efficacy of PD-L1 treatment need to be verified in another slow progression animal model. The provided data is not enough to make this assumptions.

Reply: As suggested, we conducted experiments using a slow progression KPC mouse model and confirmed that PmB treatment enhances the efficacy of PD-L1 antibody therapy (as shown in Supplementary Fig. 3a in the revised version).

There are many bacteria produce LPS, authors need to clarify what is the meaning of microbial LPS.

Reply: LPS, produced by bacteria, can activate Toll-like receptors within tumors. This activation increases the expression of immunosuppressive molecules like PD-L1 and IL-1β in tumor cells and immunotolerant myeloid populations. It also reduces the infiltration of cytotoxic T lymphocytes (CTLs) into the tumor. Consequently, bacterial LPS in pancreatic tumors contributes to local tumor immunosuppression (Figs 1i, 6, 7).

Why Ga is used other ions have similar activity like Mg and Ca. There is no significant difference between Ga and these ions.

Reply: Ga^{3+} selectively kills bacteria by inhibiting their iron-dependent respiration. Unlike many bacteria, the proliferation of the probiotic LGG is not dependent on iron metabolism, making it resistant to the bactericidal effects of Ga^{3+}. Consequently, when LGG@Ga-poly reaches the tumor, it eradicates LPS-producing bacteria without harming LGG, thereby enhancing the competitive advantage of LGG and further suppressing *Proteobacteria*. In contrast, other metal ions such as Mg^{2+} and Ca^{2+} lack this selectivity, killing bacteria indiscriminately, including LGG, and therefore do not effectively regulate the microbial community in pancreatic cancer.

Antibacterial activity of Ga^{3+} with polyphenols is not clear. Graph plotted is not correct and confusing. How many bacteria were used.

Reply: Ga^{3+}, acting as an iron ion analogue, antagonizes the uptake of iron by bacteria via their iron carriers (siderophores), which are polyphenolic structures. Therefore, the self-assembly of Ga^{3+} with polyphenols can increase the uptake of Ga^{3+} by bacteria, enhancing its antibacterial efficacy. In our experiments, when bacterial growth reached a plateau, Ga^{3+}, as well as combinations of Ga^{3+} with different polyphenols, were added, followed by plating. As expected, the self-assembled Ga^{3+} and polyphenol complexes exhibited superior antibacterial effects (fewer bacterial colonies) compared to free Ga^{3+}. Detailed experimental procedures are provided in the Supporting Information (Antibacterial evaluation of Ga^{3+}).

What is the role of siderophores.

Reply: Siderophores are the iron carriers of bacteria, responsible for iron uptake. Ga^{3+},

acting as an analogue of the iron ion, can enter bacteria via these siderophores, disrupting iron metabolism and effectively killing the bacteria. Siderophores are polyphenolic structures, and therefore, the self-assembly of Ga^{3+} with polyphenols can increase the uptake of Ga^{3+} by bacteria, thereby enhancing its antibacterial efficacy.

Line 184. Do not expand every time the names of bacteria.
Reply: Thank you for your valuable advice. We have carefully revised the manuscript as suggested, using full names for bacteria upon their first mention and abbreviations thereafter.

Figure 3j should be removed does not provide any information.
Reply: Thanks for your kind advise. Figure 3j has been removed from the manuscript.

SFig 15 there is very much variability in bacterial richness and abundance. It shows that not firmicutes is increased but it is also desulfobacteria in two animals.
Reply: Sorry for confusing you. The abundance of *Actinobacteriota* and *Firmicutes*, indicated by red color, increased, whereas that of *Desulfobacteria*, shown in orange, did not. *Actinobacteriota* is commonly found in the healthy gut, where it is a typical colonizer (as shown in Supplementary Fig. 13 in the revised version).

Legends of figures are incomplete. Need to be modified even in supplement figs.
Reply: Thanks for your kind advise. We have carefully completed and revised the legends for both the main text figures and the supplementary figures as suggested.

Reviewer #4: with expertise in cancer immunology, microbiome
The work of Han et al shows interesting data in a murine model of PDAC on the use of a gallium-polyphenol conjugayed Lactobacillus rhanmnosus GG and may be potentially groundbreaking if holds true in the human context, especially if combined with immunotherapy and chemotherapy. It is of particular interest the tumor selectivity of the LGG@Ga-poly and its capability to eliminate intratumoral bacteria and to potentially reactivate the immune system.
Authors perform a series of experiments to ensure that the compound is safe, to demonstrate in vivo its bioavalabilty and biodistribution. Authors demonstrate in vitro its functional capability to eliminate different pathobionts and its functional resilience in hostile environments. Moreover, authors evaluated the functional efficacy of the compound in experimental tumor settings, both in preventive and therapeutic administrations. In both cases the compound demonstrates efficacy in reducing the tumor burden. Authors then provide some mechanistic insights to understand how proteobacteria-derived LPS may be responsible for the reduced efficacy of ICB in this context. Authors conclude that the elimination of intratumor microbiota impedes the activation of tumoral Toll-like receptors, reducing immunosuppressive PD-L1 and IL-

1β expressed by tumor cells. From the immunological point of view this elimination diminishes the differentiation of immunotolerant myeloid populations and improves the infiltration of cytotoxic T lymphocytes.

The study is surely interesting and in general well-conceived and designed, and it can provide important insights to ameliorate PDAC antitumor responses. However, some weaknesses limit the full appreciation of the work, especially those regarding the immunological part. All the data of the immune infiltrate and its functional responses are surely less solid than the rest of the work and in my opinion would require an extensive revision.

Reply: Thank you for acknowledging the innovation and thoroughness of our work. Regarding the immunological aspects of the article, we have extensively revised this section based on your expert feedback. These revisions aim to more accurately depict the immune activation following treatment with the material.

Specifically:

1. There are no plots of the immune infiltrate, no gating strategy, not a single description of how the cell population have been defined based on the expression markers (Figure 6A-G) and in general primary data to appreciate the quality of the data as well as the phenotypical and functional modifications of the immune system pre-post treatments. The only plots available are those for CD4/CD8 in 7G, and authors then show only the cumulative graph for CD8

Reply: Sorry for making this mistake. As suggested, we have included plots detailing the immune infiltrate and our gating strategy (as shown in Supplementary Fig. 26 in the revised version). Additionally, we have expanded our analysis to include the functional characteristics of immune cells, specifically IFNγ^+ CD8$^+$ T cells and Granzyme B$^+$ CD8$^+$ T cells. The plots for each immune cell type are provided as in Figure 6 and Supplementary Figure S19 in the revised version.

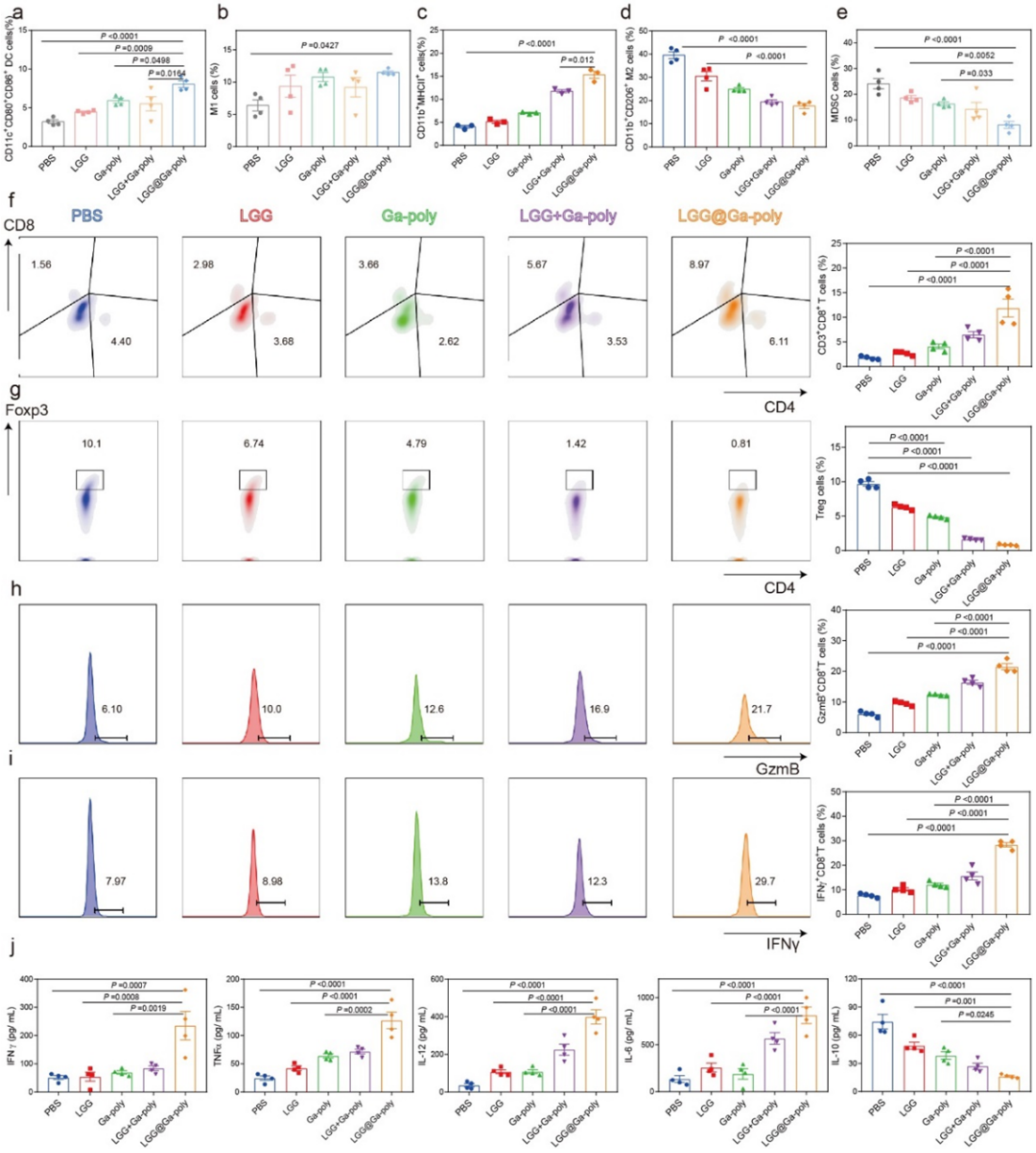


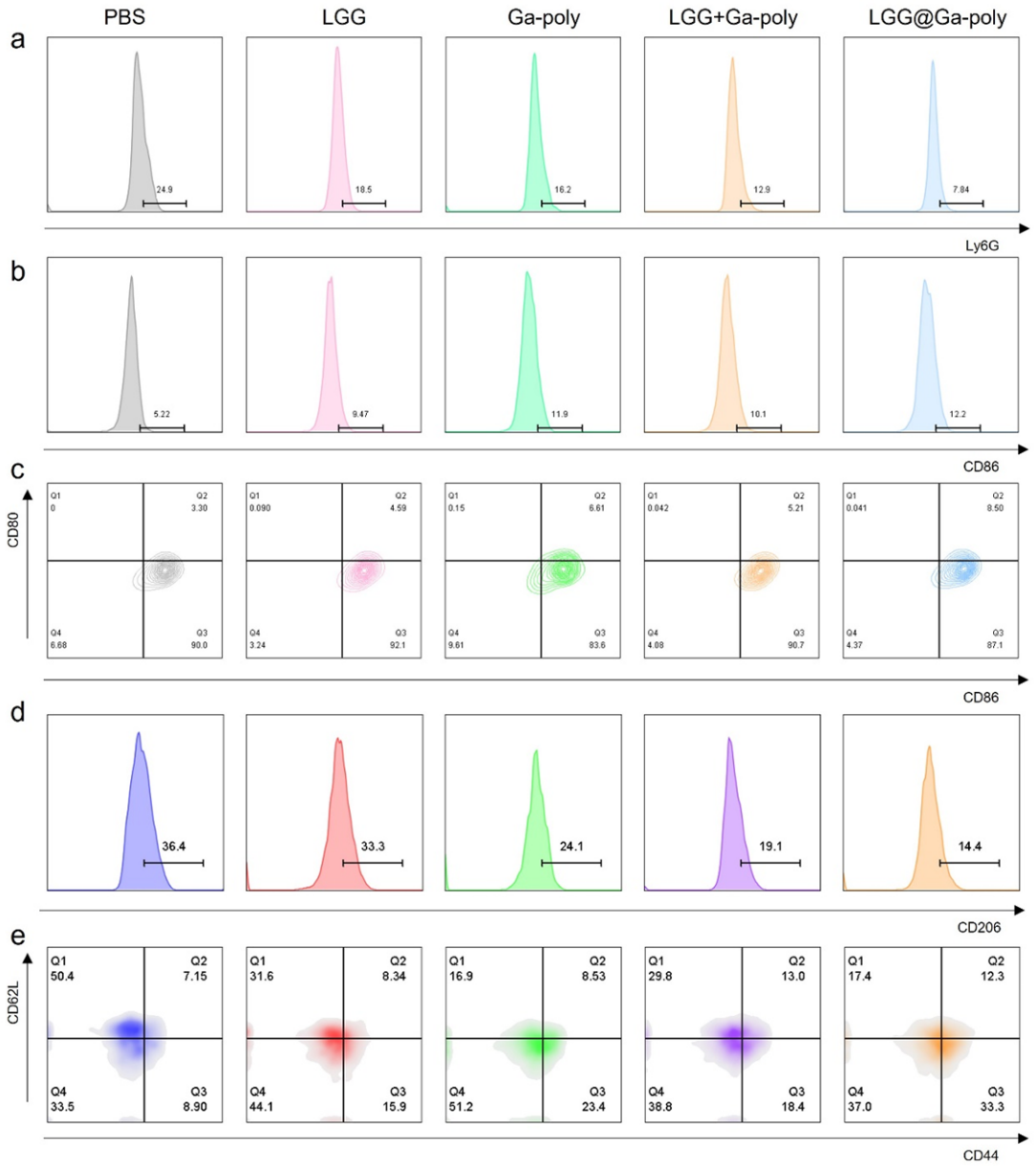
Fig. 6. LGG@Ga-poly treatment induced an immune-active PDA TME.

2. The plots in supplementary 19B, it is not clear why CD4+ cells are not present. Was it a technical problem?

Reply: Sorry for making this mistake. It may have been a technical issue; we have repeated the experiment and confirmed the presence of $CD4^+$ cells (as shown in Fig. 6f in the revised version).

3. The plots in supplementary 20 are not understandable. How authors chose where to put the bar of positivity?

Reply: Sorry for confusing you. To better illustrate our choice, we have provided the gating strategy in the figures to clearly show where the positivity threshold was set (as shown in Supplementary Fig. 26 in the revised version). The FCM images were provided in the Supplementary Fig. 19.



Supplementary Fig. 19 FCM images of $CD11b^+Ly6G^+$ MDSC cells **a**, $CD11b^+CD86^+$ M1 cells **b** within tumors after different treatments; $CD11c^+CD80^+CD86^+$ DC cells **c** within lymph nodes; $CD11b^+CD206^+$ M2 cells **d** within tumors and $CD3^+CD8^+CD44^+$ $CD62L^-$ T_{EM} cells **e** within spleens after different treatments.

4. The ELISA of Figure 6 i-l, especially those of IL6 and IL12 seem below the limit of detection. It is unclear which is the assay used and which is the limit of detection.
Reply: We utilized the ELISA test method, and initially encountered potential issues with the kit. After repeating the experiment, we observed an increase in IL6 and IL12 levels following material treatment. The concentrations measured were consistent with those reported in previous literature (as shown in Fig. 6j in the revised version).

5. Beside that, one major issue of the immunological part is that all the experiments addressing the function of the IS show variations in frequency rather than in function.

This is true for in vivo experiments where authors report variations in the intratumoral frequency of CD8/ Macrophages/MDSC as well as in vitro experiments of polarization. In both cases, it is not shown whether the treatment with the compound directly influences (and how, by which mechanisms) the cytotoxic function of CTLs, or if the MDSC or M2 macrophages truly become conditioned and influence in an immunosuppressive fashion the activities of other immune cells.

Reply: Thanks for your kind suggestion. We expanded our analysis to include the functional characteristics of immune cells, specifically IFNγ^{+}CD8^{+}T cells and GranzymeB^{+}CD8^{+}T cells. As depicted in Figs. 6h, i, LGG@Ga-poly treatment significantly enhanced the cytotoxic function of CTLs. This suggests that LGG@Ga-poly can counteract the immunosuppressive tumor microenvironment by decreasing populations of MDSCs, M2 macrophages, and Tregs, and boosting CTL infiltration and function.

6. Moreover, in many experiments authors evaluate the presence of CD3/ CD4 cells but never identify them as Treg or effectors (not even based on surface markers or transcription factors, or cytokine produced) and are not shown their functional activities.

Reply: Thanks for your kind suggestion. To assess whether CD3/CD4 cells function as regulatory T cells (Tregs) or effector cells, we analyzed these cells using specific surface markers. We found that treatment with LGG@Ga-poly significantly reduced the proportion of Tregs (as shown in Fig. 6g in the revised version).

Minor: it is never mentioned how many replicates of the experiment have been performed (it seems only once, with 3-5 mice/group).

Reply: Sorry for making this mistake. As suggested, we provided the replicated of the experiments, and added replicates of each experiment in the legend.

Fig2i: how immune populations have been defined? Why only three mice when in the experiments there are at least 5? Has it been repeated?

Reply: Sorry for confusing you. We monitored tumor growth by recording the tumor weight of each mouse, with five mice in total per group. To define the immune populations after different treatments, we randomly selected three to four mice from each group for treatment and subsequent FCM analysis.

Fig8: what about Macrophages and MDSC? Is the TME altered as in the setting without chemo?

Reply: As shown in Figs. 6b, d, e in the revised version, LGG@Ga-poly treatment could increase the proportion of M1 macrophages while reducing the MDSC and M2 macrophage population. Chemo could induce the immune cell death of tumor cells, releasing antigens, and evoking antitumor immunity. Therefore, combining LGG@Ga-poly treatment with chemotherapy could reverse immunosuppressive TME and resulted in an enhanced anti-tumor immune response.

Fig8: is the microbiome composition affected? Chemotherapy generally impacts on the composition and function of the gut microbiome and would be interesting to evaluate if LGG@Ga-poly also contributes to microbiome stabilization.

Reply: Thanks for your kind suggestion. As shown in the Supplementary Fig 23c in the revised version, the relative level of probiotic *Butyricicoccus* in the gut microbiome following LGG@Ga-poly plus chemo treatment remained unchanged compared to the PBS control. This suggests that LGG@Ga-poly contributes to the stabilization of the gut microbiome.

REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

The authors figured out all of my concerns.

Reviewer #2 (Remarks to the Author):

In response to the reviewers' comments, the authors performed several experiments to demonstrate the value of their work. These additional results please me. Even without further research addressing other issues, the fact that they provide appropriate and sufficient references is enough to convince me. The authors give an excellent example of responses to the reviewer's questions, making this article more valuable for future clinical applications.

However, there is only one question that needs to be clarified. As shown in Supplementary Figure 5, why did LGG grow so slowly, even without adding Ga3+? What kind of broth did the author use? Wasn't this MRS broth?

Reviewer #3 (Remarks to the Author):

Authors responded to many of the concerns but still lack solid data on immune response and microbiome. I will suggest authors to please read question and concerns once more and reply in details.

Also in the light of new paper

Annals of Surgery ():10.1097/SLA.0000000000006299, April 16, 2024. | DOI: 10.1097/SLA.0000000000006299

Suggesting that there is no role of microbiome in PDAC.

Please add text why your results are significant.

Reviewer #4 (Remarks to the Author):

Authors replied satisfactorily to all my queries. manuscript greatly improved. I have nothing else to add.

A point-by-point response to the reviewers' comments

We very much appreciate the reviewers, who give us valuable comments to improve the paper. The manuscript was revised accordingly, and the answers to comments were listed below.

Reviewer #1 (Remarks to the Author):

The authors figured out all of my concerns.

Reply: We greatly appreciate the reviewer's recognition of our revised manuscript.

Reviewer #2 (Remarks to the Author):

In response to the reviewers' comments, the authors performed several experiments to demonstrate the value of their work. These additional results please me. Even without further research addressing other issues, the fact that they provide appropriate and sufficient references is enough to convince me. The authors give an excellent example of responses to the reviewer's questions, making this article more valuable for future clinical applications.

However, there is only one question that needs to be clarified. As shown in Supplementary Figure 5, why did LGG grow so slowly, even without adding Ga^{3+}? What kind of broth did the author use? Wasn't this MRS broth?

Reply: We are immensely grateful to the reviewer for your invaluable assistance in enhancing the quality of our manuscript and for recognizing the improvements in the revised article.

We apologize for any confusion caused by the data presented in Supplementary Figure 5. In this experiment, we utilized M9 medium, which contains fewer nutrients compared to MRS Medium, resulting in slower bacterial growth. Our results demonstrated that Ga^{3+} exerted significant inhibitory effects on *E. coli*, but only minimal effects on LGG. We have updated the manuscript to clarify this distinction. Thank you for bringing this to our attention.

Reviewer #3 (Remarks to the Author):

Authors responded to many of the concerns but still lack solid data on immune response and microbiome. I will suggest authors to please read question and concerns once more and reply in details.

Also in the light of new paper: Annals of Surgery:10.1097/SLA.0000000000006299, April 16, 2024. | DOI: 10.1097/SLA.0000000000006299

Suggesting that there is no role of microbiome in PDAC.

Please add text why your results are significant.

Reply: We extend our heartfelt thanks once again to the reviewer for your insightful comments and recognition of our manuscript. The data concerning microbiota and

immunity robustly support the core premise of our research: that LGG@Ga-poly effectively enhances antitumor immunity by modulating intratumor microbiota.

Regarding the literature you provided, although it suggests that microbiota may not play a significant role in PDAC, a substantial body of evidence (*Science* **357**, 1156-1160 (2017); *Cancer Discov.* **8**, 403-416 (2018); *Cell* **178**, 795-806 (2019); *Cell Death Dis.* **12**, 1033 (2021); *Nature* **615**, 168-174 (2023)), supports a strong link between intratumoral flora and both the progression and treatment of PDAC. Our experimental results demonstrate that our material specifically enhances PDAC immunotherapy by targeting intratumoral flora. Moreover, our findings provide a microbiota-targeted strategy applicable to various tumors colonized by microbial communities, as supported by additional studies (*Science* **368**, 973-980 (2020); *Cell* **185**, 1356-1372 (2022); *Nat. Microbiol.* **9**, 1467-1482 (2024); *Nat. Commun.* **15**, 4194 (2024)). The relevant content has been incorporated into the Discussion section of the manuscript to further elaborate on these findings.

Reviewer #4 (Remarks to the Author):

Authors replied satisfactorily to all my queries. manuscript greatly improved. I have nothing else to add.

Reply: We thank the reviewer again for the comments.