Response letter

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Dear Editors,
We would firstly like to thank the reviewers and editors for donating time and efforts to our manuscript entitled “Fecal microbiota transplantation alleviates experimental colitis through the Toll-like receptor 4 signaling pathway”.
We greatly appreciate the valuable insights and constructive feedback provided by the reviewers, which have significantly enhanced the coherence and rationality of our study. Our manuscript has been thoroughly revised as recommended. Below, we present our point-by-point responses addressing each of the reviewers' comments.
We shall look forward to hearing from you at your earliest convenience.

Best Regards.
Yours sincerely,

Xiao-Zhong Yang
The Affiliated Huaian No.1 People’s Hospital of Nanjing Medical University

Responses to the comments of Reviewer #1:
I would like to thank the authors for their important work.
Comments:
Title and abstract:
• What “DSS-induced mice” stands for? (is it Dextran sodium sulfate?)
Response: Thanks for your careful review. In the context of this manuscript, DSS stands for Dextran sodium sulfate. We have modified the abbreviation in the manuscript.

• TLR4 is only mentioned as abb in the title > please mention in full
Response: Sorry for our unclear description. TLR4 means Toll-like receptor 4. We have modified the title and stated this point more clearly in the manuscript.

Methods:
• It is not clear why the TLR-4 knock-out mice still have colitis, their coltitis might resolve by time from itself due to absent TLR-4? • and if the microbiota of FMT were not affected in this group due to lack of inflammation in the first place, could the authors delineate these two points?
Response: Thank you for your insightful comments regarding our manuscript.

We would like to address these points as follows: For one thing, it is indeed possible that TLR4-knockout (KO) mice exhibit increased tolerance to dextran sodium sulfate (DSS) treatment due to the lack of TLR4-mediated inflammatory responses. However, this does not imply the absence of colitis. In comparison to wild-type (WT) mice, KO mice tend to have lower disease activity index (DAI) scores. Nonetheless, when compared to normal KO mice, their intestinal condition is still compromised, as evidenced by softer stool consistency, positive fecal occult blood tests, and histopathological indications of inflammatory cell infiltration. For another, it is important to note that DSS-induced colitis is a transient inflammation model. Once the administration of DSS is discontinued, the inflammation associated with colitis tends to gradually subside and the intestinal tissue begins to undergo repair processes. As mentioned before, it is indeed observed that the intestines of mice exhibit a certain level of inflammation following DSS treatment. According to our study, we found that Fecal Microbiota Transplantation (FMT) has altered the microbiota composition in KO mice. Our research findings primarily emphasize that FMT did not yield favorable therapeutic effects in KO mice.

• As the authors explained "We further investigated whether the protection against DSS-induced colitis was attributed to TLR4 knockout or microbiota re-composition. We detected the gut microbiota of WT and KO mice in the basal and DSS-treated states.">> but the effect on colitis in the knockout mice may take time , and early state may not be an enough indicator, while the treated state (could be ignored) as it could be resolved by time without treatment, could you elaborate more on this point?

Response: Thank you for your feedback on our manuscript. In this study, we used the same approach as the WT group to better present our results, which allowed us to assess the impact of TLR4 gene deletion on colitis and reduce the variability. We acknowledge that colitis is a complex disease process that may take longer to fully develop and resolve. We greatly appreciate the importance of long-term observations and in-depth analysis as suggested by you. In the future study, we will continue to conduct long-term monitoring and further analysis to gain a better understanding of the impact of TLR4 gene deletion on colitis and its underlying mechanisms.

Results:

• Well presented. I suggest adding a figure of the proposed pathway and its link to the specific microbiota re-balance as mentioned in the conclusion.

Response: Thank you for your comment. We appreciate your suggestion regarding including additional results. We believe that the currently presented figures provide a comprehensive representation of our study findings. Really appreciate for your suggestion.

Discussion:

• Please add this reference to your discussion: Guo, J., Liao, M., & Wang, J. (2021). TLR4 signaling in the development of colitis-associated cancer and its possible interplay with microRNA-155. Cell communication and signaling : CCS, 19(1), 90. https://doi.org/10.1186/s12964-021-00771-6

Response: Many thanks for your intelligent suggestion. We have included the reference you mentioned in our revised manuscript.

• The authors wrote "However, in this study, FMT did not exert effect on colonic inflammation in TLR4-KO mice. It is intriguing to detect that the abundance of Akkermansia, which had dominated in TLR4-KO mice, significantly decreased after FMT.">> could this be due to the reason I mentioned before in the methods section, kindly elaborate?

Response: Thank you for the comment and interesting suggestions on research questions. According to our previous study, we observed that in WT mice where *Lactobacillus* was the dominant genus, FMT effectively alleviated DSS-induced colitis by increasing the relative abundance of *Lactobacillus*. However, in KO mice with *Akkermansia* as the dominant genus, although FMT changed the relative abundance of gut microbiota (including an increase in the relative abundance of *Lactobacillus*), it did not ameliorate colitis in the TLR4-/- mice. That is to say, it is possible that the altered immune status and inflammation in KO mice could have shaped their gut microbiota, leading to the enrichment of specific microbial species such as *Akkermansia*. However, the introduction of donor microbiota through FMT might have disrupted this dominance and triggered changes in the overall microbiota composition. Therefore, the efficacy of FMT may require the involvement of the TLR4 signaling pathway. In general, the interaction between the host immune system, gut microbiota, and the effectiveness of FMT is complex and multifaceted. In this study, we found that the initial microbiota composition of KO mice could impact their response to FMT. We appreciate your insightful question, and we have provided a thorough explanation in our revised manuscript to address your inquiry.

Conclusions: needs modification. References: needs modification.

Response: Thanks for your suggestion. We have modified the conclusions and references. We appreciate your valuable input, which has contributed to improving the overall quality of our paper.

Responses to the comments of Reviewer #2:

This is an interesting study, finding the role of TLR4 in FMT inhibiting colitis. The results are good enough to support this conclusion. However, some issues need to be solved as follows:

1. What is the relationship between AKK and TLR4?

Response: Greatly appreciate for your positive comments of our work and detailed suggestions to improve the quality of our manuscript. Studies have suggested a potential link between *Akkermansia* and TLR4 signaling. *Akkermansia* has been reported to promote the integrity of the intestinal barrier and regulate immune homeostasis, potentially by interacting with TLR4. However, the precise mechanisms and interactions between *Akkermansia* and TLR4 are still not fully understood and require further investigation. Many thanks.

2. Where is the results of the compare of WT groups? There should be 6 groups compared together: WT (Water), KO (Water), WT (DSS+Water), KO (DSS+Water),WT (DSS+FMT), KO (DSS+FMT).

Response: Thanks for your careful review. Really appreciate for your suggestion. Comparisons between other groups can be found in our published paper "Wen X, Wang HG, Zhang MN, Zhang MH, Wang H, Yang XZ. Fecal microbiota transplantation ameliorates experimental colitis via gut microbiota and T-cell modulation. World J Gastroenterol 2021;27(21):2834-2849[PMID: 34135557 DOI: 10.3748/wjg.v27.i21.2834]". In this manuscript, we focus on the changes of DSS-induced colitis in KO mice and the relationship between FMT and TLR4-related gut microbiota and genes.

3. What's Aqp4, Clca4a, Dpm3, Fau, Mcrip1, Meis3, Nupr1l, Pank3, Rps13 ? What is the connection between them and TLR4?

Response: Truly thanks for your constructive comments. These DEGs were the top 9 genes related to *Akkermansia*. Aqp4 stands for Aquaporin 4, which is a transmembrane protein involved in regulating the transport and balance of water across cell membranes. It plays a crucial role in water metabolism, cerebrospinal fluid circulation, and neuronal signaling. Clca4a represents Chloride Channel Accessory 4a, a protein involved in regulating the activity of chloride ion channels. However, there are few studies on it. The remaining genes are primarily associated with cell cycle regulation, transcriptional control, apoptosis, and stress responses. These functions highlight its potential role in modulating various cellular activities. Their functions align with the main processes identified in the GO analysis, indicating their involvement in crucial biological pathways. While some of the mentioned genes may have roles in immune regulation, inflammation, or cellular processes that could intersect with TLR4 signaling, their specific relationships with TLR4 are not extensively characterized and require further research for a comprehensive understanding. Once again, we sincerely appreciate your valuable input, which has helped us

provide a more comprehensive explanation of these genes and their relevance to our study.