External Reviewer Assessment

Project: [Redacted] | Field: Immunology / Microbiome | Reviewer: [Anonymous] | Time: 6+ hours

How would you evaluate the novelty of this ongoing study? Please comment on its originality and contribution to the field.

As the authors state in the introduction, the role of the gut microbiome in influencing the onset of allergic inflammation has already been described previously and therefore does not constitute the primary novelty of this study. The authors themselves have previously reported that supplementation with Microorganism X alleviates allergic reactions in mice. And in the same study they also noted that Disease A patients show reduced levels of Microorganism X. Therefore, some reviewers may perceive certain aspects of the current work as confirmatory or derivative rather than field-defining. However, in my view, in the present study the authors describe a much more specific and mechanistically detailed pathway, and for this reason, I believe the study nevertheless provides substantial novelty. The link between Lipid Mediator L and asthma is also not entirely novel. For example, [Prior Study]. 2013 Dec 5;6(1):61–65.) reported that specific lipid species are elevated in the lungs of asthmatic subjects. In my view, however, this prior work supports the relevance of the current study rather than detracting from its novelty. Overall, I think the authors present an impressive body of experimental evidence that together describes an entire pathway, starting from a specific gut microorganism, the mechanism by which this microorganism affects Lipid L levels, the effect of Lipid L on mast cells, and the identification of the receptor on mast cells that mediates the Lipid L effect. The comprehensive description of this causal chain, linking the gut microbiome to host immune phenotypes, in my view, substantially enhances the novelty of the study. An additional aspect that further strengthens the originality of this work is the demonstration that plasma Lipid L levels can stratify Disease A patients into high and low-risk groups for relapse, suggesting that Lipid L may have value as a biomarker.

What are the primary strengths and weaknesses of the study? Please specify any aspects that stand out positively or areas needing improvement.

I think this is an ambitious and potentially high-impact study. A major strength is the breadth of data and analyses presented. The study brings together human patient data, metagenomics, multiple in vivo disease models, and in vitro experiments. The ongoing study is developing and will, in its final form, provide support for a coherent causal chain linking a specific gut microorganism to allergic disease phenotypes. One area I noticed requires improvement is the refinement of some claims and figure titles. In several places, certain statements could be perceived as overclaiming, as I will detail in my point-by-point assessment. In addition, there are several analyses that would benefit from more explicit statistical testing, and I have also outlined those cases in my detailed point-by-point assessment. I believe that with relatively few targeted refinements of the analysis, and some additional experiments that demonstrate causality in vivo (some of which the authors already plan), the manuscript could be substantially strengthened.

Based on its novelty and potential impact, do you believe this study is a suitable candidate for publication in a top-tier journal? Why or why not?

I do think the study is a suitable candidate for publication in a top-tier journal. What I find compelling is the breadth of experiments presented, the novel mechanistic insights provided, and the clinical relevance for Disease A and asthma. I have detailed below what I think will be central for the authors to focus on to further strengthen the manuscript.

What specific steps or enhancements would you recommend to significantly improve the quality of the manuscript, bringing it to the standards expected of leading journals?

Before I go through the figures in detail, here are the major experiments/adjustments that I believe would most substantially strengthen the manuscript:

1) The experiments already planned by the authors evaluating the effect of Lipid L on (i) mast cell activation/vascular permeability and (ii) the Disease B model in the context of Receptor R knockout in mast cells.

2) Using the same genetic approach to test whether the protective effect of Microorganism X in the Disease A model (In Vivo Assay/vascular permeability) and/or in the Disease B model is reduced or lost when Receptor R is knocked out in mast cells.

3) Demonstrating that the Enzyme E gene in Microorganism X is required for the beneficial effects of this microorganism. As I understand, genetic manipulation of this microorganism might be challenging; an activity-guided biochemical fractionation approach (as outlined in my comment to Figure 1, point 9) may be the most practical alternative.

4) Increasing the rigor of the statistical analysis by explicitly testing the significance of the relevant interaction terms (as outlined below in the detailed comments).

I have organized my detailed comments by Figure.

Figure 1

I have the impression that the current title of Figure 1 may be perceived as overclaiming. I understand that the In Vivo Assay assay strongly implicates mast cells, but I wonder whether, without actually demonstrating that perturbation of mast cells eliminates the effect, one can claim that this is a mast cell-dependent effect. Maybe a safer version would be to say: Microorganism X inhibits IgE/antigen-induced vascular permeability ….

At the same time, this might be an opportunity to further increase the already commendable rigor in this manuscript. Since the authors already plan experiments with Receptor R-flox animals crossed to a mast cell Cre line, they could test whether the protective effect of Microorganism X in vivo is mediated through the Receptor R/mast cell axis. I think demonstrating that the beneficial effects of Microorganism X observed in the Disease A model (In Vivo Assay/vascular permeability) and the Disease B model are dependent on Receptor R in mast cells would be a very strong addition.

Figure 1A. Y-axis indicates “Abundance” - I think the axis label would benefit from indicating the unit of what was measured.

Figure 1A and 1B show the different abundance of Microorganism X between HC and Disease A (A) and HC and asthma (B), but different analyses were used. I would suggest showing these comparisons in the same way. Both types of analyses could be performed for both comparisons. Alternatively, is there a specific reason why these comparisons are shown using different approaches? The reason I suggest a more systematic presentation is that reviewers may otherwise become suspicious and wonder whether the data were displayed differently because the alternative analyses did not yield a significant result.

Figure 1H. It is difficult to see what the p-values are based on that color scale. It might help to actually show -log10 p values.

Figure 1I. For many lipid species, the data is shown twice. I think that is okay, but maybe it should be briefly acknowledged that the two terms contain a lot of overlapping lipid species.

Figure 1K. Glycerophospholipid metabolism in MX. The figure panel with this title is potentially misleading. I am uncertain what was measured in this panel. If it was mapping reads to Glycerophospholipid metabolism genes of MX, my impression would be that it does not add significant value over what was already shown in panel A, namely the reduced abundance of Microorganism X in the Disease A group. Based on the title, I would expect to gain insight into the Glycerophospholipid metabolism in MX, for example, different metabolic activity of that pathway, but this is likely information that cannot be derived from the data shown in the panel.

Figure 1L. I would add the names of the enzymes to the schematic instead of the numbers. The numbers alone tell the reader not much. I think it is better if the reader can get as much information from the figure panels without having to consult the legend.

Figure 1O. I believe the data should be interpreted as total lipid mediator-processing capacity. I am not sure if this assay can unambiguously attribute the activity to one specific enzyme, as implicitly indicated by the title of the panel: Enzyme E activity.

I think an opportunity to strengthen the mechanistic rigor of the study would be to demonstrate that the observed lipid mediator-processing activity of Microorganism X is mediated via Enzyme E. This would really help complete the full pathway description from the bacterial gene to the host phenotype. Ideally, this would be demonstrated by genetic manipulation of Enzyme E in Microorganism X showing loss (and rescue) of lipid mediator-processing activity, though I understand that this might be impractical. If this is not feasible, a strong alternative would be an activity-guided fractionation strategy, in which lipid mediator-processing activity is tracked across biochemical fractions. The active fraction could be analyzed by proteomics. This could demonstrate that activity co-fractionates with Enzyme E and would substantially strengthen the claim that Enzyme E is the causal factor in Microorganism X.

Figure 2

Based on panel 2A alone, I believe the claim that “Microorganism X alleviates skin inflammation by downregulating Lipid L” is too strong. The part I am not convinced by is that it is doing so by downregulating Lipid L. The drop of OD610 in the MX-LPC_PCA condition compared to LPC_PCA is clear. But a very similar drop is also seen in the absence of Lipid L when comparing RHO_PCA to In Vivo Assay. Since that reduction is seen in both the absence and presence of Lipid L, it seems independent of Lipid L. To evaluate whether MX reduces vascular permeability by reducing Lipid L, I would test whether MX specifically reduces the Lipid L-induced increase in permeability. This can be tested by comparing the difference MX-LPC_PCA minus MX-In Vivo Assay to the difference LPC_PCA minus In Vivo Assay. If MX-LPC_PCA minus MX-In Vivo Assay is significantly smaller than LPC_PCA minus In Vivo Assay, this would indicate a clear interaction between the effects of MX and Lipid L and support an Lipid L-mediated mechanism. A more formal description of this type of analysis (two-way ANOVA / linear model with an interaction term) is provided below in my comments to Figure 4 and Figure 8, and the same statistical framework would be appropriate here.

Figure 1B and 1C. I would suggest analyzing the data in Figures 1B and 1C in the same way as described above for Figure 1A.

Figure 2G. I think the authors demonstrate well that the addition of Microorganism X reduces systemic plasma Lipid L levels under both In Vivo Assay and Lipid L-In Vivo Assay conditions. However, the relative increase in plasma Lipid L upon Lipid L challenge appears to be very similar whether Microorganism X was added or not. To me, this suggests that Microorganism X influences baseline (endogenous) Lipid L levels, but it is unclear whether it influences the added Lipid L. An effect on the added Lipid L would, in my view, be necessary to support the claim that the Microorganism X effect on vascular permeability is mediated via Lipid L in this experimental setup. Since this does not appear to be the case, I would conclude that the evidence remains weak. While Lipid L levels are reduced following Microorganism X addition, this effect

is currently correlative with the reduced vascular permeability observed in the In Vivo Assay assay. Strong causal evidence that the phenotype is mediated via Lipid L is, in my view, still missing.

To demonstrate that the reduced vascular permeability in the In Vivo Assay is mediated by Microorganism X reducing Lipid L, I can think of two indirect approaches that would support this claim. First, if it is feasible to interfere with Enzyme E function in Microorganism X (genetic knockout?, CRISPRi/downregulation?), showing that the mutant strain loses the ability to reduce In Vivo Assay/dye leakage would provide strong evidence that Enzyme E is necessary for the effect. Second, blocking the relevant Lipid L sensing/signaling pathway in the host and showing that this abrogates the effect of MX would support the idea that the phenotype depends on Lipid L signaling. I believe the experiment described in my comments to Figure 1, using the Receptor R-flox animals crossed to a mast cell Cre line, would address this point.

Figure 4

In my view, the current figure title for Figure 4 ("…alleviates Disease B model …") is somewhat imprecise. Rather than stating that the model itself is alleviated, I think it would be clearer to specify what was alleviated in the model. For example: "Microorganism X attenuates airway inflammation in an Disease B model."

If the RHOxLPC interaction is statistically supported (please see comments below), the wording "by lowering Lipid L levels" could be added: "Microorganism X attenuates airway inflammation in an Disease B model by lowering Lipid L levels."

In Figure 4, the authors provide strong evidence that Microorganism X alleviates many symptoms in the Disease B model. Whether these effects are mediated by reducing the levels of Lipid L, I think, requires additional analysis of the data. I would suggest keeping the bar graph illustration and analysis as is, but add an additional analysis that more specifically addresses the question of whether there is an interaction between MX and Lipid L.

The experiment includes four conditions: Allergen alone, LPC_OVA, MX-Allergen, and MX-LPC_OVA (I focus here on what I believe are the most important conditions in this experiment). The conditions differ by (i) the presence or absence of Microorganism X and (ii) the presence or absence of Lipid L. This is a classical 2 by 2 (2x2) experimental design. Therefore, I would suggest analyzing these data using a two-way ANOVA (which is equivalent to fitting a linear model), with main effects for Microorganism X and Lipid L and an interaction term between the two.

Formally: y = b0 + b1(MX) + b2(Lipid L) + b3(MX x Lipid L) + epsilon,

where y is the measured outcome (for example, cell counts in fluid sample). The key parameter is the MX x Lipid L interaction term, which tests whether the effect of Lipid L differs depending on whether Microorganism X is present.

This analysis evaluates whether the increase caused by Lipid L (LPC_OVA minus Allergen) is smaller when Microorganism X is present (MX-LPC_OVA minus MX-Allergen). If the interaction between MX and Lipid L is statistically significant, this indicates a reduced Lipid L effect in the presence of Microorganism X, which would support an Lipid L-mediated component of the protective effect.

Figure 5

Figure 5B : A small detail, but I would recommend showing the conditions in the same order in the bar graphs. The Lipid L and IgE+DNP+Lipid L categories are flipped in Cell Type 3 compared to the other Cell Type 1 and Cell Type 2.

The data shown in Figure 5B support the conclusion that Lipid L can induce mast cell degranulation. However, the

claim in the figure legend ("(B) Lipid L promotes the release of IgE/DNP mediated β - hexosaminidase in Cell Type 1, Cell Type 2, and Cell Type 3."), is only partially supported in my opinion. The extent to which Lipid L promotes IgE/DNP-mediated β - hexosaminidase release seems to be cell-type dependent. In Cell Type 1 the combined IgE+DNP+Lipid L condition does not exceed the release rate seen in response to Lipid L alone. In Cell Type 2 the release rate is significantly higher in the IgE+DNP condition than in the combined IgE+DNP+Lipid L condition. Does this mean Lipid L actually inhibits the release? In Cell Type 3, the combined stimulus (IgE+DNP+Lipid L) exceeds both the IgE+DNP stimulus and the Lipid L stimulus. Thus, the Cell Type 3 data support that Lipid L potentiates IgE-mediated degranulation. The broader claim that Lipid L promotes the release in all the mast cell types tested seems not well supported.

The observations may point to a certain level of tissue specificity, which is actually intriguing. And it may be biologically meaningful, as I would imagine lung mast cells are the relevant effector population in the Disease B model. The authors may wish to highlight this point as these in vitro observations fit well with the in vivo asthma data.

Figure 5F. To support the claim made in the figure legend ("Lipid L promotes Growth Factor induced Cell Type 1 (top) and Cell Type 2 (bottom) migration."), the authors should evaluate whether the cell count is significantly higher in the Growth Factor+Lipid L condition compared to the Growth Factor alone condition.

Figure 6

Figure 6A. The illustration of these data is not very clear to me. I assume the same UMAP is shown for HC and Disease A side by side, but it appears that there are almost no cells in the HC group. In this situation, I would suggest showing three UMAPs side by side: first, a combined UMAP including all cells from both groups; and then UMAPs where the cells are split by group. In addition, because the number of cells is so low in the HC group, it would be helpful to draw dashed lines around the clusters in the combined UMAP and then show these same boundaries in the same positions on the UMAPs of the individual groups. This would make it easier to see that clusters present in the Disease A condition are largely absent in the HC condition.

Figures 6B and 6C. The difference in expression level between the responders and non-responders should be evaluated statistically. In 6B, it is not clear under which condition the expression level is higher. Receptor R seems to have been detected in a larger fraction of the cells in the non-responders, but the expression in the cells of the responders seems to be higher.

Figure 6D : I suggest labeling the panel so that the reader can immediately see which UMAP corresponds to the non-asthma condition and which one to the asthma condition.

Figure 7

Figure 7 is strong. I have no specific comments for improvement.

Figure 8

I think the results shown in Figure 8B are already strong. The authors may wish to formally test whether Receptor R knockdown attenuates Lipid L-induced b-hex release, analyzing the experiment as a 2x2 design (sh-mock vs sh-Receptor R; plus/minus Lipid L) as indicated below:

Run a two-way ANOVA / linear model with an interaction term:

y = b0 + b1(KD) + b2(Lipid L) + b3(KD x Lipid L) + epsilon,

where KD is 1 for the sh-Receptor R condition and 0 for the sh-Mock condition, and Lipid L is 1 for the Lipid L condition and 0 for the control condition without Lipid L.

Test and report whether the coefficient b3 of the KDxLPC interaction term is statistically significant. This directly tests whether the Lipid L-induced increase is significantly reduced upon Receptor R knockdown.

The same statistical test as described above for Figure 8B should be applied to the data presented in Figures 8D, E, and F.

Figure 9

Figure 9D : It is somewhat unclear to me how patients were stratified into a low and high-risk group. I assume this was done based on plasma Lipid L levels, and the numbers indicated suggest a median split. The authors could explicitly indicate how the patients were stratified into groups.

Are there particular experimental approaches or methodologies that require refinement or additional validation?

Besides the statistical analyses outlined above, what comes to mind is the presentation of the single-cell genomics data in Figure 6. I am not sure whether these datasets were generated by the authors or whether they are analyses of publicly available data. If the authors generated these datasets themselves, they represent a highly valuable resource. Currently, the manuscript presents only the downstream analyses without clearly introducing the datasets themselves. If these data were generated as part of this study, I would strongly recommend highlighting this more explicitly. Introducing each dataset with a schematic overview indicating the number of patients, the tissue and cell types analyzed, and the single-cell or spatial methodology used would help emphasize the value of these datasets and ensure that the authors' contribution in generating these resources is appropriately recognized. Also, my general impression from the single-cell data shown in Figure 6A-D is that, since we do not yet have the manuscript text, it is unclear what conclusions the authors intend to draw from these data. In their current form, the data appear to be presented more qualitatively than quantitatively. For drawing solid conclusions, a more quantitative statistical analysis would be required. It is possible that the statistical power is limited due to the relatively small number of individuals analyzed. If no statistically significant differences are observed, I think it is acceptable to present the data in a more qualitative manner. However, the authors would need to clearly state that the data are suggestive and indicate a trend rather than definitive differences, and that these findings would need to be corroborated in a larger cohort. Given that the authors focus on a specific target gene, validation in a larger sample size could also be achieved using a more cost-effective approach than single-cell genomics.

What is your assessment of the translational value of this study? How effectively do you think the findings could be applied in a clinical or real-world setting?

Overall, I would assess the translational value of this study as high. As mentioned above, one aspect that seems clearly translational is the demonstrated ability to stratify Disease A patients based on plasma Lipid L levels. Since the authors show that relapse risk is associated with plasma Lipid L levels and that therapeutic efficacy is also linked to this measure, I think they have already demonstrated a meaningful degree of clinical relevance, and translation into a clinical or real-world setting does not seem far-fetched. What would be important to further bridge the gap to clinical application is validation of these findings in a much larger cohort, although this clearly goes beyond the scope of the current study.

A second layer of translational value lies in the mechanistic insights presented, which directly point toward potential interventional strategies. One straightforward approach that comes to mind is intervention at the level of the gut microbiome, but strategies targeting the lipid mediator itself or the receptor on mast cells could also be envisioned. While such approaches would clearly require careful development and evaluation, I see substantial value in this study laying the conceptual foundation for future interventional strategies that could ultimately have significant clinical relevance and real-world impact.

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