nature portfolio

Peer Review File

Host macrophages/monocytes promote malaria transmission by modulating mosquito microbiota via SR-A-mediated phagocytosis

Corresponding Author: Professor Wenyue Xu

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

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
In this manuscript, He et al report a very interesting study showing that mouse monocytes and/or macrophages enhance transmission of murine malaria via phagocytosing bacteria of the mosquito microbiota. Overall, the study is well-conducted and the main conclusions would definitely be worth a publication in Nature Communications. I have however a few comments to be fully convinced by this study.

Major comments:
The main point is that some experiments have been performed only twice, I think that a third replicate in all the experiments presented in the main figures would be required for publication in Nature communications, especially that the overall numbers are sometimes reasonable but low. Also, it would be interesting to see the n of each replicate below charts instead of the total amount of mosquitoes.

Secondly, I wonder if the authors have pointed survival of their mosquitoes at least until dissection time. Microbiota proliferation after the blood meal has been found to affect mosquito lifespan (Gendrin et al, 2015) and I imagine that a reduction in phagocytosis, besides reducing vector competence, may as well reduce mosquito survival. Whether log-rank or a simple one-time point observation at the time of dissection, such survival data would make the study more convincing.

Considering stats, I would like to see GLMMs used instead of Mann Whitney U test, to take into account random effects of replicates. When the authors make a point on the non significance (after antibiotic treatment), it would like to see the actual p-value rather than a "ns". This "ns" may be close to significance, but only 2 replicates have been done to test this point so a third one can clarify this.

The manuscript requires extensive English and latin proofreading, checks on the use of italics. Besides mistakes the main text, I spotted some in titles or axis titles in Fig 1B,D, 2A, S2B,D,G; S3A,C,E.

Minor points:
Line 255 "in the control bacteria" please clarify this sentence.
S4E: are both pictures two representative pictures in each condition? Don't the authors have a larger sample size to show?
Line 509 : are there differences between batches in the % of not fully engorged mosquitoes?
In my opinion, at least some items of figure 3 should be used in a main figure, because it makes an important point (maybe removing some data from figure 1 e.g. choose one parasite for main and one for supplements).

In sum, I am excited by this study, but I know that I have been disappointed by very beautiful stories that have not been confirmed. I am hesitating between this excitement and a lack of trust, and I hope that revision will help me decide what I think about it...

Reviewer #2

(Remarks to the Author)

The manuscript by He et al examines the effects of mammalian immune cells on infection outcomes in the mosquito vector using rodent models of malaria infection. The study is simple, yet elegant, in its design and is clear in its presentation that macrophage/monocyte populations in the mosquito blood bolus influence the bacteria in the mosquito gut to influence malaria infection outcomes. The results are therefore very interesting and provide new insight into a previously unexplored aspect of malaria transmission. My comments are very minor and only look to increase the clarity of the presented study.

My comments are as follows:
-Please edit the abstract to improve readability and clarity, particularly for lines 16-24.
-There are several instances where more detailed editing is required that includes missing words, changes to improve clarity, and multiple times where Anopheles should be italicized.
-Line 73-74: Please revise for clarity.

Reviewer #3

(Remarks to the Author)
This manuscript by He et al. reports a novel and mechanistically detailed finding: that macrophages and monocytes taken up during mosquito blood feeding facilitate Plasmodium transmission by suppressing gut microbiota through SR-A-mediated phagocytosis. The authors demonstrate that this immunological interaction promotes ookinete development and enhances transmission for rodent malaria parasites. They show that this process undermines the efficacy of transmission-blocking antibodies (anti-Pfs25) against a P. berghei line expressing P. falciparum Pfs25, an effect that can be rescued by macrophage/monocyte depletion or SR-A neutralization. This represents an important contribution to our understanding of host-vector-parasite interactions.

The work is technically sound, conceptually novel, and of high potential interest to the malaria transmission, immunology, and vector biology communities. It is supported by a comprehensive series of in vivo experiments and microbial/molecular analyses in rodent and humanised models. However, several scientific, mechanistic, and editorial issues require attention. These range from clarifications of the main claims and better contextualisation, to refinements in data interpretation, presentation, terminology, and writing.

Major comments
• The authors repeatedly state that macrophages and monocytes “promote transmission.” This should be clarified throughout as an indirect effect, mediated via suppression of anti-Plasmodium bacteria, rather than any direct facilitation of parasite development.
• The role of SR-A in bacterial phagocytosis is convincingly demonstrated, but it is not clear whether this is the sole receptor involved. The possible contributions of other receptors (e.g., CD36, DC-SIGN, MR) are mentioned in the discussion but should be more explicitly addressed or acknowledged as a limitation.
• The finding that SR-A blockade restores the full efficacy of anti-Pfs25 antibodies is compelling, but the presentation could be improved. For example, clarifying that these are parallel and synergistic mechanisms (i.e., microbiota-mediated blocking and antibody-mediated blocking), rather than interacting ones. Also, include time-course data to address how long SR-A remains blocked after antibody injection and how long macrophages/monocytes persist in the mosquito midgut.
• The human data (addition of monocytes in SMFA) is an important proof of concept but limited to a single experimental system. It would strengthen the manuscript to show whether similar monocyte expansions are observed in naturally infected individuals or gametocyte carriers.
• The translational implications of this work, particularly for transmission-blocking vaccines or antibody combinations, deserve stronger emphasis in both the abstract and discussion. Currently, relevant statements in the abstract are unclear or overly compressed.
• The roles of E. anophelis, S. sonnei, K. oxytoca, and S. marcescens are central to the proposed mechanism. However, their relative contributions to parasite suppression are not disentangled. Do they act independently, additively, or synergistically? This should be discussed more clearly.
• While the manuscript shows that gametocytemia and ookinete development are unaffected by clodronate liposome (CL) treatment, this is presented too late. These controls should be moved earlier in the Results and shown in main figures to reassure the reader that effects on transmission are not due to altered gametocyte production.
• Similarly, depletion of neutrophils and NK/NKT cells is shown to have no effect on transmission, but the results and conclusions (lines 120-129) should be supported by replication and earlier gametocyte data.
• The effect of CL treatment on systemic cytokine responses (e.g., IL-10, IFN-γ), which may influence parasite development or gametocyte activation, is not addressed. Even if not measured, this should be acknowledged as a limitation.
• The claim that “macrophages and monocytes actively phagocytose microbiota during early blood digestion, while ookinetes opsonised by anti-Pfs25 antibodies might evade phagocytosis through Fc receptors” is intriguing but speculative. Further discussion or qualification is needed.
• More broadly, the relevance to natural P. falciparum transmission remains indirect. Additional data or discussion is needed to support extrapolation from the rodent model to the human system.
• Lines 117-118 and 125-129: The authors should show that CL treatment does not affect gametocyte levels at these stages, not only later in the manuscript.
• Lines 120-124: Are the results in the figure from a single replicate? Three replicates are referenced but what is shown in the figure can’t be from all.
• Line 221-223: Were these experiments (with and without antibiotics) performed in parallel? There appears to be a baseline difference, which should be clarified, replicated, and discussed.
• Lines 252-257: Please indicate the number of replicates and mosquito batches tested. Are the results consistent?

Minor comments
• The title could be rephrased to reflect the indirect nature of the effect. For example: “Host monocytes/macrophages promote malaria transmission by modulating mosquito microbiota via SR-A-mediated phagocytosis.”
• Avoid phrases like “enhance” or “promote transmission” unless immediately followed by the mechanistic qualifier (e.g., “by reducing gut bacteria”).
• Statistical significance symbols should be consistently defined in all figure legends.
• There is no need to explain species name abbreviations; this is a standard convention.
• Line 22 (abstract): The phrase “depletion of SR-A” is misleading. The authors refer to SR-A blockade. Please rephrase.
• Abstract: The term “immune evasion strategy” is vague. Spell out the mechanism: that the parasite co-opts host immune cells to eliminate transmission-blocking bacteria.
• Lines 54-56: Of the three references cited, only the last one supports modulation of the peritrophic matrix. Please correct.
• Lines 67-68: This sentence suggests that NK/NKT cells were previously implicated in transmission. If so, cite appropriate references. If not, rephrase for clarity.
• Lines 74-76: The start of the Results section is abrupt. Consider rewriting or referencing appropriately.
• Line 143-146: The mention of the P. falciparum SMFA appears abruptly. Better contextualisation is needed.
• Line 148: The term “anti-malarial bacteria” may be confusing. Consider “bacteria with transmission-blocking activity” or similar.
• Line 309 and onward: A microscopy image showing intracellular phagocytosed bacteria would strengthen the evidence.
• Line 335 and throughout: Replace “ug” with the correct symbol for micrograms (µg).

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
I congratulate the authors for taking all my comments into account, I am more convinced about the manuscript as it stands now. I agree with them that the differences observed after the third replicate are not contradicting the main message, and think that the confirmation of the other results after proper analyses strengthens the story.
Happy to see that the survival of mosquitoes was affected as I suspected.
Beautiful work, contratulations...

Reviewer #3

(Remarks to the Author)
The manuscript has improved in several important respects. However, a number of issues remain that should be addressed before further consideration.

The addition of a third independent replicate across the main figures is welcome and was necessary. The re-analysis using GLMMs is more appropriate and statistically sound. However, it is important to note that the inclusion of the third replicate altered several previously reported findings (e.g., immune cell frequencies in Fig. 1 and infection prevalence in Fig. 2). While the authors report these changes transparently in the rebuttal, the fact that conclusions shifted with the addition of one replicate indicates that the original dataset was underpowered and that some earlier conclusions were not robust.

At present, I have no independent means of verifying the robustness of the revised dataset beyond trusting the statistical analyses, which appear appropriately conducted. Nonetheless, it would strengthen the manuscript if the authors explicitly acknowledge in the Results section that certain immune cell changes did not remain significant after full replication.

Despite this concern, it is my view that the study remains mechanistically interesting and conceptually novel, and the additional replication and statistical re-analysis substantially strengthen the scientific core of the work.

The clarification that macrophages/monocytes influence transmission indirectly via modulation of the mosquito microbiota improves conceptual clarity. The addition of time-course data, SR-A blocking duration experiments, and bacterial colonisation experiments strengthens the mechanistic argument. However, causal phrasing still occasionally overreaches and should be moderated to avoid implying direct pro-parasitic effects where the mechanism is indirect.

There are instances where the rebuttal states that certain corrections were made, but the revised manuscript does not fully reflect these claims. For example:
- The authors state that unnecessary species name explanations were removed, yet explanatory phrasing remains in places.
- Several stylistic and grammatical issues persist.
- The description of the GLMM model structure remains insufficiently detailed in the Methods section.

In several sections, portions of reviewer comments appear to have been incorporated into the manuscript with minimal or no modification, at times retaining phrasing that reads as external critique rather than as integrated narrative.

While it is entirely appropriate to respond to reviewer suggestions, revised text should be fully integrated into the authors’ voice and the structure of the manuscript. Direct or near-direct reproduction of reviewer phrasing without careful adaptation

may give the impression of reactive editing rather than coherent revision.

The manuscript would benefit from careful stylistic harmonisation to ensure that the text reads as a unified scientific narrative rather than a patchwork of responses.

Finally, the writing style in parts of the revised manuscript suggests the possible use of generative AI tools for drafting or rephrasing. The use of such tools is increasingly common and not inherently problematic; however, best practice and many journal policies require transparent disclosure if generative AI has been used in manuscript preparation. If AI-assisted drafting or rewriting was used, this should be clearly stated in the appropriate section in accordance with journal policy.

Dear editor and reviewers,

Thank you for the opportunity to revise our manuscript and for your instructive comments, which will significantly enhance its quality. We have addressed each of the reviewers' points individually. We hope that you will be satisfied with our responses.

Response to reviewer 1' comments:

Reviewer #1 (Remarks to the Author):

In this manuscript, He et al report a very interesting study showing that mouse monocytes and/or macrophages enhance transmission of murine malaria via phagocytosing bacteria of the mosquito microbiota. Overall, the study is well-conducted and the main conclusions would definitely be worth a publication in Nature Communications. I have however a few comments to be fully convinced by this study.

Response: Thanks a lot for your appreciation.

Major comments:

The main point is that some experiments have been performed only twice, I think that a third replicate in all the experiments presented in the main figures would be required for publication in Nature communications, especially that the overall numbers are sometimes reasonable but low. Also, it would be interesting to see the n of each replicate below charts instead of the total amount of mosquitoes.

Response: We have now conducted a third round of experiments, compiling data from three independent studies to reinforce the findings presented in the main figures and supporting information. The number of replicates is indicated in the pie chart and figure legends. In response to your suggestion, we re-analyzed the pooled data using Generalized Linear Mixed Models (GLMMs). Most of our results align closely with those reported in the preliminary manuscript, with a few notable exceptions detailed in Figures 1e, 1g, 1h, and 2l.

In our previous Figure 1e, we reported a significant increase in the frequency of DCs in the peripheral blood of mice infected with *P. yoelii* or *P. berghei*. However, our recent data show that only *P. yoelii* infection significantly increased DC frequency. In Figure 1g, we initially indicated a significant increase in CD4+ T cell frequency in the peripheral blood of mice infected with either strain. Yet, our pooled data analysis did not demonstrate a significant difference in CD4+ T cell frequency between the two infections. Furthermore, in our earlier Figure 1h, we observed no significant reduction in CD8+ T cell frequency following *P. berghei* infection. In contrast, our current data reveal that *P. berghei* infection significantly decreases CD8+ T cell frequency in the peripheral blood. We hypothesize that the notable increase in innate immune cell frequency may contribute to the observed reduction in CD8+ T cells following malaria infection. Since we did not examine the roles of CD4+ and CD8+ T cells, or DCs, in malaria transmission, this discrepancy does not alter our conclusions. Figures 1e, 1g, and 1h, along with the relevant context, have been updated accordingly (lines 85-94).

In our previous Figure 2l, we stated that 100% of control mice were infected with *P. berghei* after mosquito bites. However, during the third replication, two control mice were not infected, likely due to low sporozoite production in the mosquitoes' salivary glands. After pooling data from all three replicates, we found that only 90% of control mice were infected with *P. berghei* post-bite (lines 155). This finding has been updated in the new Figure 2l. Please refer to the revised

manuscript for additional details.

Secondly, I wonder if the authors have pointed survival of their mosquitoes at least until dissection time. Microbiota proliferation after the blood meal has been found to affect mosquito lifespan (Gendrin et al, 2015) and I imagine that a reduction in phagocytosis, besides reducing vector competence, may as well reduce mosquito survival. Whether log-rank or a simple one-time point observation at the time of dissection, such survival data would make the study more convincing.

Response: Thanks a lot for your suggestion. Now, the effect of CL treatment on the survival of mosquitoes has been observed. As you expected, mosquito fed on CL-treated mice has a lower survival rate compared with mosquito fed on control mice, and the data of which has been presented as Fig3 d. The relevant context has been updated accordingly (lines220-224).

Considering stats, I would like to see GLMMs used instead of Mann Whitney U test, to take into account random effects of replicates. When the authors make a point on the non significance (after antibiotic treatment), it would like to see the actual p-value rather than a "ns". This "ns" may be close to significance, but only 2 replicates have been done to test this point so a third one can clarify this.

Response: Thanks a lot for your comments. We have re-analyze our data by GLMMs analysis as the data of mosquito oocysts often follows a Poisson or negative binomial distribution, and zero-inflation was also taken into consideration. Now, the actual p-value rather than ns have been added. Please refer to the revised manuscript. We are sincerely appreciating for your professional advice. All figure legends and method has been revised. Please refers to our updated manuscript.

The manuscript requires extensive English and latin proofreading, checks on the use of italics. Besides mistakes the main text, I spotted some in titles or axis titles in Fig 1B, D, 2A, S2B,D,G; S3A,C,E.

Response: We have carefully read thoroughly our manuscript, and tried our best to correct spelling and grammar errors. In addition, we have also corrected the mistakes in the titles or axis titles in Fig 1B, D, 2A, S2B, D, G; S3A, C, E, as you suggested. Please refer to the revised manuscript.

Minor points:

Line 255 "in the control bacteria" please clarify this sentence.

Response: Thanks. "in the control bacteria" has been revised as "for other bacteria".

S4E: are both pictures two representative pictures in each condition? Don't the authors have a larger sample size to show?

Response: Yes. Both pictures are two representative pictures in each condition, now we have added up to four representative pictures of midguts colonized with *eGFP-E. anophelis* from three independent experiments (line 950). Please refer to the revised manuscript.

Line 509: are there differences between batches in the % of not fully engorged mosquitoes?

Response: We did not find the significant difference between batches in the % of not fully engorged mosquitoes.

In my opinion, at least some items of figure 3 should be used in a main figure, because it makes an important point (maybe removing some data from figure 1 e.g. choose one parasite for main and one for supplements).

Response: Thanks a lot for your suggestion. We have re-organized our manuscript, the influence of CL, anti-Ly6G, and anti-NK1.1 have been displayed before we mention the influence of their roles in mosquito stage transmission (lines 117-128).

In sum, I am excited by this study, but I know that I have been disappointed by very beautiful stories that have not been confirmed. I am hesitating between this excitement and a lack of trust, and I hope that revision will help me decide what I think about it...

Response: We understand what you worried about. Now, we have added a third repeat for the experiments presented in the main figures. As a result, our repeated data supports our conclusion in this manuscript. Hopefully, you will be satisfied with our revision.

Response to reviewer 2' comments:

Reviewer #2 (Remarks to the Author):

The manuscript by He et al examines the effects of mammalian immune cells on infection outcomes in the mosquito vector using rodent models of malaria infection. The study is simple, yet elegant, in its design and is clear in its presentation that macrophage/monocyte populations in the mosquito blood bolus influence the bacteria in the mosquito gut to influence malaria infection outcomes. The results are therefore very interesting and provide new insight into a previously unexplored aspect of malaria transmission. My comments are very minor and only look to increase the clarity of the presented study.

Response: Thanks a lot for your appreciation.

My comments are as follows:

-Please edit the abstract to improve readability and clarity, particularly for lines 16-24.

Response: Thanks for your comments. Now, the abstract has been modified to improve readability and clarity (lines 15-27).

-There are several instances where more detailed editing is required that includes missing words, changes to improve clarity, and multiple times where Anopheles should be italicized.

Response: We have carefully read thoroughly our manuscript, and tried our best to correct spelling and grammar errors. The word of "Anopheles" has been checked and italicized.

-Line 73-74: Please revise for clarity.

Response: This sentence has been revised as "Mosquitoes acquire malaria parasites by biting infected hosts. During these bites, immune cells from the host's peripheral blood, along with the parasites, are ingested into the mosquito's midgut. However, it remains unclear whether these ingested immune cells influence the development of the parasites within the mosquito." (lines 73-76). Hopefully, it has been clear now.

Response to reviewer 3' comments:

Reviewer #3 (Remarks to the Author):

This manuscript by He et al. reports a novel and mechanistically detailed finding: that macrophages and monocytes taken up during mosquito blood feeding facilitate Plasmodium transmission by suppressing gut microbiota through SR-A-mediated phagocytosis. The authors demonstrate that this immunological interaction promotes ookinete development and enhances transmission for rodent malaria parasites. They show that this process undermines the efficacy of transmission-blocking antibodies (anti-Pfs25) against a P. berghei line expressing P. falciparum Pfs25, an effect that can be rescued by macrophage/monocyte depletion or SR-A neutralization. This represents an

important contribution to our understanding of host-vector-parasite interactions.

Response: Thanks a lot for your appreciation of our study.

The work is technically sound, conceptually novel, and of high potential interest to the malaria transmission, immunology, and vector biology communities. It is supported by a comprehensive series of in vivo experiments and microbial/molecular analyses in rodent and humanised models. However, several scientific, mechanistic, and editorial issues require attention. These range from clarifications of the main claims and better contextualisation, to refinements in data interpretation, presentation, terminology, and writing.

Response: Thanks a lot for your comments. We have thoroughly read our manuscript, and especially pay attention on the scientific, mechanistic, and editorial issues as you mentioned. Now, we have been tried our best to make our data interpretation and main claims clearer, and improve our writing. Please refer to our revised manuscript.

Major comments

• The authors repeatedly state that macrophages and monocytes "promote transmission." This should be clarified throughout as an indirect effect, mediated via suppression of anti-Plasmodium bacteria, rather than any direct facilitation of parasite development.

Response: The indirect effect of macrophages and monocytes on malaria transmission has been clarified thoroughly for our revised manuscript.

• The role of SR-A in bacterial phagocytosis is convincingly demonstrated, but it is not clear whether this is the sole receptor involved. The possible contributions of other receptors (e.g., CD36, DC-SIGN, MR) are mentioned in the discussion but should be more explicitly addressed or acknowledged as a limitation.

Response: Thanks a lot for your suggestion. The possible contributions of other receptors (e.g., CD36, DC-SIGN, MR) has been more explicitly addressed or acknowledged as a limitation (lines 514-518).

• The finding that SR-A blockade restores the full efficacy of anti-Pfs25 antibodies is compelling, but the presentation could be improved. For example, clarifying that these are parallel and synergistic mechanisms (i.e., microbiota-mediated blocking and antibody-mediated blocking), rather than interacting ones. Also, include time-course data to address how long SR-A remains blocked after antibody injection and how long macrophages/monocytes persist in the mosquito midgut.

Response: As you suggested, we have presented the data on microbiota-mediated blocking and antibody-mediated blocking in relation to TRA and TRB percentages to clarify their relationship. The new data indicate that these two mechanisms are not synergistic.

Regarding the persistence of macrophages and monocytes in the mosquito midgut, we used flow cytometry (FACS) to analyze their frequency at 1-, 3-, 12-, and 24-hours post-feeding on infected mice. Our results show that macrophages and monocytes ingested by mosquitoes can survive for at least 3 hours, but their numbers dramatically decrease at 12 hours post-blood meal, becoming difficult to detect by 24 hours. This data is presented in Figure 5c, and the corresponding context has been updated accordingly (lines 346-350).

In response to your suggestion, we also investigated the longevity of the SR-A blocking effect. We found that neutralizing SR-A 1 day before infection (5 days before the blood meal) did not affect the number of oocysts in the mosquito midgut but did reduce oocyst prevalence. Additionally, injecting anti-SR-A 1 day post-infection (3 days before the blood meal) resulted in a reduction in

transmission comparable to that observed in the CL-treated group. This indicates that neutralization of SR-A by antibody can effectively reduce malaria transmission for at least 3 days. This data is presented in Figure 5h, and the corresponding context has been updated accordingly (lines 368-374).

• The human data (addition of monocytes in SMFA) is an important proof of concept but limited to a single experimental system. It would strengthen the manuscript to show whether similar monocyte expansions are observed in naturally infected individuals or gametocyte carriers.

Response: Literatures about the expansions of monocytes in naturally infected individuals or gametocyte carriers have been cited and discussed (lines 156-157).

• The translational implications of this work, particularly for transmission-blocking vaccines or antibody combinations, deserve stronger emphasis in both the abstract and discussion. Currently, relevant statements in the abstract are unclear or overly compressed.

Response: Thanks for your suggestion. Both abstract and discussion have been revised to emphasize the translational implications of this work (lines 24-29, and lines 531-539).

• The roles of E. anophelis, S. sonnei, K. oxytoca, and S. marcescens are central to the proposed mechanism. However, their relative contributions to parasite suppression are not disentangled. Do they act independently, additively, or synergistically? This should be discussed more clearly.

Response: Thanks for your suggestion. The independent, additive, or synergistical effect of *E. anophelis, S. sonnei, K. oxytoca* on malaria transmission has been performed. As a result, we did not observe a superimposed effect when fed mosquito with multiple bacteria compared with solely colonization, indicating there is no synergistical effect of *E. anophelis, S. sonnei, K. oxytoca* on malaria transmission. This data has been presented as Figure 4h, and the relevant context has been updated accordingly (lines 284-289).

• While the manuscript shows that gametocytemia and ookinete development are unaffected by clodronate liposome (CL) treatment, this is presented too late. These controls should be moved earlier in the Results and shown in main figures to reassure the reader that effects on transmission are not due to altered gametocyte production.

Response: As you suggested, the result of CL treatment on gametocytemia and ookinete development has been moved to the S Figure 2, and mentioned before their effects on transmission. Please refer to our revised manuscript.

• Similarly, depletion of neutrophils and NK/NKT cells is shown to have no effect on transmission, but the results and conclusions (lines 120-129) should be supported by replication and earlier gametocyte data.

Response: Thanks. The depletion of neutrophils and NK/NKT cells on transmission has been repeated for three times and their effect on gametocytemia has been included (S Fig 2a, b).

• The effect of CL treatment on systemic cytokine responses (e.g., IL-10, IFN-γ), which may influence parasite development or gametocyte activation, is not addressed. Even if not measured, this should be acknowledged as a limitation.

Response: Thanks. The effect of CL treatment on systemic cytokine responses (e.g., IL-10, IFN-γ), which may influence parasite development or gametocyte activation, has been addressed in the discussion (lines 510-518).

• The claim that "macrophages and monocytes actively phagocytose microbiota during early blood digestion, while ookinetes opsonised by anti-Pfs25 antibodies might evade phagocytosis

through Fc receptors" is intriguing but speculative. Further discussion or qualification is needed.

Response: Thanks. Further discussion has been added (lines 497-501).

• More broadly, the relevance to natural P. falciparum transmission remains indirect. Additional data or discussion is needed to support extrapolation from the rodent model to the human system.

Response: Thanks. As we did not provide the direct evidence that the neutralization of SR-A to reverse the enhanced effect of human monocytes on *P. falciparum* transmission, we have added a sentence of "However, the direct evidence whether the neutralization of SR-A could reverse the promoted effect of human monocytes on P. falciparum transmission is still required for the extrapolation from our findings in rodent model to human" in discussion to show the gap of extrapolation from the rodent model to the human system (lines 487-489).

• Lines 117-118 and 125-129: The authors should show that CL treatment does not affect gametocyte levels at these stages, not only later in the manuscript.

Response: The result that CL treatment does not affect gametocyte levels has been moved forward as you suggested.

• Lines 120-124: Are the results in the figure from a single replicate? Three replicates are referenced but what is shown in the figure can't be from all.

Response: Sorry for that, we only showed a single replicate in previously manuscript. Now, we have re-analyzed the pooled data from three independent experiments, and showed the consistent results. Please refers to our revised manuscript.

• Line 221-223: Were these experiments (with and without antibiotics) performed in parallel? There appears to be a baseline difference, which should be clarified, replicated, and discussed.

Response: Yes, those experiments were performed in parallel, and replicated. There are two control, one control is treated with antibiotics, and another is not. After clearance of microbiota by antibiotics treatment, the oocysts number was much higher than that of the control mosquitoes not treated with antibiotics, which is consistent with previous study (doi:10.1371/journal.ppat.1000423). Also, we have discussed in our updated manuscript (lines 228-231).

• Lines 252-257: Please indicate the number of replicates and mosquito batches tested. Are the results consistent?

Response: Thanks for your comments. Now, we have updated our figure legend, number of each experiment has been indicated, and the results were consistent. Twenty mosquito midguts were pooled as one biological replicate, and three biological replicates were performed. Three independent experiments were performed for each experiment and the data were pooled. Please refers to our revised manuscript.

Minor comments

• The title could be rephrased to reflect the indirect nature of the effect. For example: "Host monocytes/macrophages promote malaria transmission by modulating mosquito microbiota via SR-A-mediated phagocytosis."

Response: The title has been revised as you suggested (lines 1-2).

• Avoid phrases like "enhance" or "promote transmission" unless immediately followed by the mechanistic qualifier (e.g., "by reducing gut bacteria").

Response: It has been revised as your suggestion.

• Statistical significance symbols should be consistently defined in all figure legends.

Response: We have re-done all the statistical analyze, now the actual p-value has been added in

each figure, and the sentence of "*P*<0.05 was considered as statistically significant" was consistently added in all figure legends.

- There is no need to explain species name abbreviations; this is a standard convention.

Response: Thanks for your suggestion. Now, the name abbreviations have been removed.

- Line 22 (abstract): The phrase "depletion of SR-A" is misleading. The authors refer to SR-A blockade. Please rephrase.

Response: Yes, the phrase of "depletion of SR-A" has been revised as SR-A neutralization in the abstract (line 22).

- Abstract: The term "immune evasion strategy" is vague. Spell out the mechanism: that the parasite co-opts host immune cells to eliminate transmission-blocking bacteria.

Response: Thanks for your suggestion. The term "immune evasion strategy" has been specified as your suggestion (line26).

- Lines 54-56: Of the three references cited, only the last one supports modulation of the peritrophic matrix. Please correct.

Response: Yes. You are right. There is only one reference supporting modulation of the peritrophic matrix, the other two references are supporting the modulatory role of microbiota in the mosquito immune responses. To make it clear, the references have been separately cited to support the modulation of PM or immune response by microbiota, respectively (line 56).

- Lines 67-68: This sentence suggests that NK/NKT cells were previously implicated in transmission. If so, cite appropriate references. If not, rephrase for clarity.

Response: The sentence seems to be confusing, misleading the known role of NK/NKT cells in transmission. Now, it has been removed for clarity (lines 66-69).

- Lines 74-76: The start of the Results section is abrupt. Consider rewriting or referencing appropriately.

Response: Now, the start of the Results section has been modified (lines 73-76).

- Line 143-146: The mention of the P. falciparum SMFA appears abruptly. Better contextualisation is needed.

Response: The mention of the *P. falciparum* SMFA has been modified (lines 156-160).

- Line 148: The term "anti-malarial bacteria" may be confusing. Consider "bacteria with transmission-blocking activity" or similar.

Response: The term "anti-malarial bacteria" has been modified as "bacteria with transmission-blocking activity". We have cheeked thought out our manuscript, and all the "anti-malarial bacteria" has been re-phased with "bacteria with transmission-blocking activity" or similar as you suggested (line 260, line 306, and lines 533-534).

- Line 309 and onward: A microscopy image showing intracellular phagocytosed bacteria would strengthen the evidence.

Response: The microscopy image showing intracellular phagocytosed bacteria now has been included in Fig 5b of the revised manuscript (line 378).

- Line 335 and throughout: Replace "ug" with the correct symbol for micrograms (μ g).

Response: Thanks for your careful review, "ug" has been replaced with "μg" (line 364).

Dear editor and reviewer,

Thank you very much for your valuable comments, which have significantly enhanced our manuscript. We have thoroughly reviewed both the previous rebuttal and the main article file, removing statements made by the reviewers and rephrasing relevant sections in our own words. Furthermore, we have acknowledged the use of AI in the Acknowledgments section of the manuscript. All revisions have been highlighted in yellow. Below, you will find our point-by-point response to Reviewer 3's comments. We hope our responses meet your expectations.

Response to Reviewer #3:

The manuscript has improved in several important respects. However, a number of issues remain that should be addressed before further consideration.

Response: Yes. Now, we have fixed these issues.

The addition of a third independent replicate across the main figures is welcome and was necessary. The re-analysis using GLMMs is more appropriate and statistically sound. However, it is important to note that the inclusion of the third replicate altered several previously reported findings (e.g., immune cell frequencies in Fig. 1 and infection prevalence in Fig. 2). While the authors report these changes transparently in the rebuttal, the fact that conclusions shifted with the addition of one replicate indicates that the original dataset was underpowered and that some earlier conclusions were not robust.

Response: Yes. This result is also out of our anticipation, and this also reminds us to repeat the important experiments for three times in our future study.

At present, I have no independent means of verifying the robustness of the revised dataset beyond trusting the statistical analyses, which appear appropriately conducted. Nonetheless, it would strengthen the manuscript if the authors explicitly acknowledge in the Results section that certain immune cell changes did not remain significant after full replication.

Response: Thanks. It has been explained as you suggestion (line 85-87).

Despite this concern, it is my view that the study remains mechanistically interesting and conceptually novel, and the additional replication and statistical re-analysis substantially strengthen the scientific core of the work.

Response: Thanks a lot for your appreciation of the statistical re-analysis.

The clarification that macrophages/monocytes influence transmission indirectly via modulation of the mosquito microbiota improves conceptual clarity. The addition of time-course data, SR-A blocking duration experiments, and bacterial colonisation experiments strengthens the mechanistic argument. However, causal phrasing still occasionally overreaches and should be moderated to avoid implying direct pro-parasitic effects where the mechanism is indirect.

Response: Thanks. Now, we have rephrased some sentences to avoid implying direct pro-parasitic effects where the mechanism is indirect. Please see line 17, 22, 66,180, 279-280, 291-292, 301, and 315-316.

There are instances where the rebuttal states that certain corrections were made, but the revised manuscript does not fully reflect these claims. For example:

- The authors state that unnecessary species name explanations were removed, yet explanatory phrasing remains in places.

Response: We have checked the manuscript thoroughly, and removed all explanatory phrasing for

species. Please see line 78, 141, 189, 216, 239, 383, 385 and 398.

- Several stylistic and grammatical issues persist.

Response: Thanks. We have read the manuscript carefully and corrected stylistic and grammatical issues as possible as we can. Please refers to the highlighted words or sentences.

- The description of the GLMM model structure remains insufficiently detailed in the Methods section.

Response: Now, the detailed description of the GLMM model structure has been supplemented in the Methods section, lines 553-555.

In several sections, portions of reviewer comments appear to have been incorporated into the manuscript with minimal or no modification, at times retaining phrasing that reads as external critique rather than as integrated narrative.

While it is entirely appropriate to respond to reviewer suggestions, revised text should be fully integrated into the authors' voice and the structure of the manuscript. Direct or near-direct reproduction of reviewer phrasing without careful adaptation may give the impression of reactive editing rather than coherent revision.

The manuscript would benefit from careful stylistic harmonisation to ensure that the text reads as a unified scientific narrative rather than a patchwork of responses.

Response: Thanks for your comments. We have carefully checked the manuscript, especially the revised parts, and rephrased in our own words instead of statements made by reviewers and kept stylistic harmonization of the whole manuscript. Please see the line 349-351.

Finally, the writing style in parts of the revised manuscript suggests the possible use of generative AI tools for drafting or rephrasing. The use of such tools is increasingly common and not inherently problematic; however, best practice and many journal policies require transparent disclosure if generative AI has been used in manuscript preparation. If AI-assisted drafting or rewriting was used, this should be clearly stated in the appropriate section in accordance with journal policy.

Response: Thanks for the reminding. We did use the AI to help us to rephase our manuscript, which has been stated in the acknowledgement section (line 726-727).