

Methylene Blue Treatment of Fatal Cerebral Malaria and Identification of Potential Blood Biomarkers

Corresponding Author: Dr Benoît Malleret

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)

This manuscript by Malleret and colleagues uses the Rhesus *P. coatneyi* model of cerebral malaria in humans to show that methylene blue can be used as a potential therapeutic for cerebral malaria. In particular this has been shown using mouse models and this study, in part, aimed to demonstrate applicability to humans by repeating this observation in non-human primates. The study relies heavily on transcriptomics and the main findings were that 1) methylene blue treatment led to resolution of the neuroinflammatory transcriptome upon disease resolution, particularly in the brain stem and 2) comparison of the transcriptomic data set to that of PBMCs from *P. coatneyi* animals and human PBMCs in *P. falciparum* infections identified 9 DEGs that could potentially be used as biomarkers for CM. The methods are all fairly standard.

In general, the data is presented well with easy to follow figures. The manuscript in general is a valuable addition to the transcriptomics available for malaria and for that reason I am supportive of the study. That being said the authors can improve some aspects of the manuscript to increase the utility of the data presented. One issue with the study design is that there not naïve a group of Rhesus where the effect of methylene blue treatment alone can be parsed out. Is there a naïve / methylene blue dataset available from the literature that could be discussed (any animal)?

Most of my other comments relate to the introduction and discussion. There is no explanation or discussion of methylene blue – what is it and how does it work? Similarly there are references to various pathways / immune cells that the transcriptomics may be relevant to – but no real discussion of how these cells may be important. The literature is poorly cited in this respect (studies are listed in Table 1 for the 9 biomarkers only but they are not properly integrated in the manuscript and not discussed as a group of molecules, rather these are treated almost like independent molecules - they are likely linked). This lack of information limits the utility of the data for the reader. From a discussion perspective why would methylene blue be able to treat cerebral malaria? There is no discussion of how cerebral malaria occurs (mechanisms as per current knowledge) and where methylene blue fits into this pathway.

In the introduction, is there edema or hemorrhage in the brain from *coatneyi* infection? This review (<https://journals.sagepub.com/doi/full/10.1177/0300985815583098>) suggests that there is, and if so it should probably listed as well.

Figure 2: Why was the liver not different? This seems unlikely given what is known about malaria. Arguably the kidney should also show differences. Which areas of the kidney / liver were taken as tissue – there is nothing specified. Can the authors discuss some hypotheses about why this treatment appears to specifically target transcriptomics in the brainstem? CM rarely occurs in with damage in the brain / CNS alone. It is not clear why lung was not also included.

It was good that the authors have made PCAs for each brain region. However I think the claim “In the brain tissues, the gene expressions of the Pcoat+ MB– samples were distinct, whereas those of the Pcoat+ MB+ and Pcoat– MB– samples were indistinguishable from each other, and this is especially apparent in the brainstem (Fig. 2B).” is too strong. Instead of saying that this is especially apparent in the brainstem, I would say that it’s only apparent in the brainstem.

I would change the title of this section to either say “Brainstem genes that were modulated by infection and treatment are central to the Ingenuity Pathway Analysis neuroinflammation signaling pathway.” or “Brainstem genes that were modulated by infection and treatment are central to neuroinflammation signaling pathways.” As it reads now it suggests to me that the

“Neuroinflammation signaling pathway” is some specific pathway defined by convention independently of IPA.

I'm assuming IPA is “Ingenuity pathway Analysis” but I don't think it's ever explicitly stated

Reviewer #2

(Remarks to the Author)

The study conducted by Hang & Leong et al. describes the transcriptional profiles of diverse tissues of splenectomized *M. mulatta* infected with *P. coatneyi* and after treatment with methylene blue (MB). They demonstrate that irrespective of the dose, MB treatment reduced most of the parasitemia and prevented severe malaria in 67% of animals by day 12. Infection induced differential expression mainly in different regions of the brain, in particular, the brainstem and genes modulated by the infection were also affected by MB treatment. Additional analysis attempts to demonstrate a 9-gene signature as a potential biomarker for cerebral malaria (CM). It represents an interesting model to study CM and evaluate therapeutic efficacy. However, there are several major issues that need attention.

Major points

- Although MB treatment was successful in reducing parasitemia and preventing more severe disease in most animals, previous study demonstrates that all animals treated only with MB presented recrudescence, and treatment rescued most but not all animals from fatal outcomes (Ref 63). Although the models and treatment regimens are different it should be considered whether the MB treatment works by clearing parasites, which in turn modulates the inflammatory response or whether MB also displays direct immunomodulatory activity.
- The only parameters used to demonstrate that MB treatment was successful was parasitemia and brain gene expression. The authors describe clinical data very succinctly in the Supplementary Data 1. The data should be demonstrated and analyzed, if possible, from each animal, including white blood cell counts, RBC counts, temperature, which symptoms and signs of malaria and more specifically of CM were recorded for each animal and whether those clinical alterations could be reversed by MB treatment.
- The findings that the transcriptional regulation in the brain is the most affected during infection is important. However, there are no other types of data corroborating their CM model. They conclude that neutrophils might underlie the neuroinflammation in their model, but the results are not conclusive. I suggest cellular deconvolution analysis of their RNAseq data to predict immune and brain cell frequencies using widely known computational tools such as CYBERSORT. Also explore key specific genes whose expression is modulated by treatment at the single gene level.
- Histopathological analysis of the brain regions would certainly improve the understanding of whether parasite sequestration and/or inflammatory infiltration might explain the differences in the number of DEGs in different brain regions. Analysis of other organs can also explain why there are no expressive differences in gene expression.
- Validation of some key proteins suggested by the transcriptional data would strength and confirm some conclusions. It could be immunohistochemistry, ELISA or any other method that corroborating for example, increased neuroinflammation and or other significant pathways.

- The analyses related to the identification of biomarkers for CM consist of overlapping different DEG lists from 3 datasets that consist in a total of 38 samples. This experimental setting would be ideal for identifying prognostic blood biomarkers of CM. Did the authors collect venous blood from animals before infection and during the course of infection and treatment? Because, this could reveal genes predicting CM before it is apparent, which would be prognostic and not diagnostic. Here, the signature is diagnostic, because the changes in the gene expression occur when the individuals already experience acute symptoms and possibly signs of CM. Although the expression of 9-gene signature is conserved among the evaluated datasets, other cohorts also need to be used for validation including GSE117613, GSE1124, GSE72058, GSE33811, E-MTAB-6413. Model performance should be tested using ROC curve AUCs reported for each cohort. If the signature is a biomarker for CM, it should also discriminate between uncomplicated and other severe phenotypes of malaria, such as severe anemia. How does ROC AUCs from the 9-gene signature in this study compares to other previous CM gene signatures?

- The results do not support the conclusion that this study elucidates the role of neutrophils and neuroinflammation in CM pathogenesis. Further study about the efficacy of MB treatment is needed and the identified biomarkers need to be tested in a larger set of samples and independent cohorts.

Minor points

- It would be more informative to demonstrate the hierarchical clustering of samples by genes in a heatmap instead of JSD distances. Because, as expected, samples cluster mostly by organ and clustering by infection or treatment is not evident.
- The association of the 9 gene signature with neutrophils would benefit from a statistical enrichment approach to understand whether they are indeed enriched only in neutrophil transcriptomes or they might also be expressed by other leukocytes and cells.

Reviewer #3

(Remarks to the Author)

The manuscript by Hang et al addresses the need of additional therapies and diagnostic markers for cerebral malaria (CM), an important concern of higher mortality in African children and a prolonged neurological sequelae in survivors. With parenteral administration of artesunate being the only option, the authors have shown the potential of Methylene Blue (MB) for CM treatment. The earlier studies on MB have only addressed its transmission-blocking and antimalarial effects, except for one study in rodent parasite model on preventing experimental cerebral malaria. The present study has utilized *P. coatneyi*-infected rhesus macaques as non-human primate model to study the potential of MB in CM treatment. The authors have performed detailed transcriptomics studies for the brainstem, thalamus and cerebellum of the *P. coatneyi*-infected rhesus macaques (in addition to heart, liver and kidney) and shown that MB treatment can reverse the gene expression changes in the brainstem of the infected animals. The authors have compared the RNA-Seq data of the present study with two of the published datasets representing the blood samples of *P. coatneyi*-infected macaques with acute infections and *P. falciparum*-infected humans with CM. This in turn has led to an important finding of identifying a set of 9 potential biomarkers with many of them associated with neutrophil function and CM. In particular, this is of clinical relevance with the existing limitations in accurate CM diagnosis by neuroimaging and fundoscopy, and distinguishing it from other non-CM encephalopathies. Although the findings are interesting, there are several concerns that need to be addressed in detail.

Major Comments:

- 1) While the utilization of *P. coatneyi*-infected rhesus macaques as a CM model is commendable, the studies could have been designed slightly better. For example, the manuscript lacks the histological evidences for various brain parts to correlate the MB treatment and gene-expression findings with cerebral pathologies, leaky vasculature, parasite sequestration, leukocyte infiltration etc. With only a small amount of tissue required for transcriptomics studies, the rest of it could have been utilized for such data to strengthen the findings. It is also not clear why other brain parts like basal ganglia and corpus collosum are not examined in the present study since they also contribute to the neurological complications in CM.
- 2) The reason behind the incomplete clearance of terminal parasitemia and treatment failure in *P. coatneyi*-infected animals treated with higher doses of 10 mg/kg and 17 mg/kg of MB is not clear. Two third of animals exhibit decreased mentation, epistaxis, anorexia etc. Does it indicate any MB toxicity especially when there is a blood-brain barrier breach in CM? The 100% success rate of 6 mg/kg could have been verified with another cohort to ensure that the findings are consistent.
- 3) A cohort of uninfected macaques treated with MB would have served as an additional control, especially to understand the gene-expression changes (if any) in brain because of MB treatment since MB is brain permeable. Such data might have also given some idea for why the number of DEGs observed for Pcoat+MB+ vs Pcoat+MB- is twice than that of Pcoat+MB- vs Pcoat-MB-.
- 4) The ability of MB to reverse the gene expression changes in the brainstem of *P. coatneyi*-infected animals to the extent of uninfected animals is interesting. Nevertheless, there are chances of irreversible/prolonged/direct/indirect consequences of neurological complications that might still persist and lead to adverse outcome. This could be a reason for the treatment failure observed despite a significant reduction in the terminal parasitemia and reversal of gene expression changes in 10 mg/kg and 17 mg/kg groups.
- 5) The authors have compared the transcriptomics dataset of the present study with the published datasets available for the blood samples of acute *P. coatneyi* infections and human CM patients to narrow down their findings and identify potential blood biomarkers for CM diagnosis in humans. Although the findings are interesting, the comparison with just six human CM blood samples from a single study of Thiam et al., is inadequate to propose them as biomarkers. The authors should attempt to include more number of CM samples.
- 6) It is also important to confirm the specificity of these biomarkers for CM by analyzing the non-cerebral severe malaria samples. There is also a small cohort of non-cerebral severe malaria samples in Thiam et al study itself. Is it omitted for any technical/experimental reason? Equally important is to confirm the results with the samples from other non-malarial encephalopathies.

Minor Comments

- 1) It has been reported that the knobs present in the *P. coatneyi*-infected RBCs of splenectomized animals are incompletely formed in comparison to animals with intact spleen. It is not clear why the present study is carried out in splenectomized rhesus macaques. There should have been at least a discussion on this aspect providing clarity.
- 2) In the supplementary figure 1, Pcoat+MB- data is missing for the liver principal component analysis.
- 3) Although the authors have proposed the feasibility of performing flow cytometry and antibody staining, these are difficult in clinical set up especially in the context of diagnostic window in CM. The more realistic option would be a real-time PCR or ELISA analyses.
- 4) The fold-changes observed for the expression of some of the markers (for example IL1RN and S100A8 of human samples provided in Supplementary data 3.5) may not meet the diagnostic criteria.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

This manuscript details the transcriptomic changes in several organs with reduced *P. coatneyi* parasitemia from methylene blue administration. The authors have attempted to address most of my comments. Whilst this has improved clarity in a few areas there are still some issues that could not / have not / could not been resolved.

1. No naive / methylene blue data set so we don't know what methylene blue does to transcripts (if anything) to separate if there are any effects on the tissue from methylene blue administration vs reducing parasitemia.
2. there is still no validation of transcripts
3. Regarding the liver signature, the lack of infected / MB- samples for this means it is impossible to say anything about the transcriptomic impacts when infection are lessened with administration of methylene blue. It is not clear why these are not included (Supplementary Fig1) when they are in the other panels. This point should at least be stated more clearly that there may be a difference but it is not possible to say without this comparative group.

With regards to the rebuttal:

"Thank you for this comment. We would like to clarify that our study uses a blood-stage malaria challenge rather than a sporozoite challenge. As a result, the liver stage of malaria, which is typically associated with significant pathology in the liver, does not occur in our model. This explains why we did not observe the expected transcriptomic changes in the liver, as the liver involvement seen in other malaria models is absent here."

I did just want to correct the authors on this point. The liver stage of malaria is clinically silent with minimal inflammation. On the other hand blood stages are cleared by the liver and sequester in the liver (to a greater or lesser extent depending on model) creating an inflammatory response in this organ and liver damage (as the authors mention in the rebuttal letter). I would expect to see some differences given the lower parasitemia levels.

Given these weaknesses I am still somewhat supportive of the publication of this data as this manuscript provides a good resource for the CM community.

Reviewer #2

(Remarks to the Author)

My concerns were addressed, but a minor suggestion is to add representative H&E images of brain sections from Pcoat+ MB- and Pcoat+ MB+, so one can visualize the effect of the treatment in the tissue. I would include this image as Figure 1c, because it would be as important as the reversal in peripheral parasitemia.

Reviewer #3

(Remarks to the Author)

In the revised version of the manuscript, Hang et al. have attempted to address the concerns that were raised earlier by comparing their RNA-Seq data with the additional datasets that are available for uncomplicated and severe (non-cerebral and cerebral) malaria. They have also included West Nile Virus dataset for non-malarial encephalopathy. These efforts have strengthened the RNA-Seq data that suggest the potential of the nine candidate genes as biomarkers for cerebral malaria. However, there are other aspects that need to be addressed.

1) As it was mentioned earlier, the data presented on methylene blue (MB) for cerebral malaria (CM) treatment is not convincing. The treatment failure observed for *P. coatneyi*-infected animals administered with higher doses (10 mg/kg and 17 mg/kg) of MB is debatable although the treatment initiation at 15% (instead of 10% for 6mg/kg) has been proposed as a reason for this outcome. The symptoms at euthanasia overlap with CM and they occur despite the better clearance of the parasites. In the responses given, the authors emphasize that they do not intend to use MB in clinical settings. However, in the discussion section of the manuscript, MB treatment has been mentioned as a therapeutic option for CM and alternative to artemisinin-based therapies. The authors should tone down such claims.

2) In the Supplementary Table 1, the authors should include the details for untreated *P.coatneyi*-infected animals, and the parasitemia details at the time of MB treatment initiation for all the groups. The time point of euthanasia varies between 9-14 days for the different groups and therefore, the red arrow provided for the experimental endpoint in Figure 1 has to be corrected.

3) The histopathological images for the brain and other organ samples of the *P. coatneyi*-infected animals with and without MB treatment have to be included in the manuscript along with the details provided in the Supplementary Table 2. The experimental details to be included in the Methods section.

4) Given the importance of spleen in the immune responses of blood-stage infections and the disease severity including CM, it would be appropriate if the authors discuss the potential differences / miss outs that may arise in the splenectomised P. coatneyi model. Especially, in the context of other immune cells, and the comparison of data from splenectomised animals with spleen-intact human samples.

5) With the commercial antibodies available for the identified biomarkers, the authors could have attempted to validate their findings at protein levels for at least a couple of biomarkers. This would have strengthened their potential use in CM diagnosis through antibody staining and flow cytometry.

Version 2:

Reviewer comments:

Reviewer #3

(Remarks to the Author)

The authors have attempted to address my comments in the revised manuscript. The following points shall be taken care.

1) It would have been better if the representative H&E images are provided for brainstem, cerebellum and thalamus for all the three doses tested instead of providing different brain regions for different doses. Or else, the brainstem images shall be provided for all the three doses.

2) From the H&E images, there appears to be only a partial reversal of pathology in the brain irrespective of the doses tested. Nevertheless, the DGE pattern of the brainstem shows complete reversal similar to that of naïve rhesus macaques. The authors may comment on this finding.

3) The scale bar is missing for the H&E sections. Just by looking at the images, the magnifications seem to be different and this shall be verified.

4) The rationale behind initiating the methylene blue treatment at higher blood parasitemia levels of 30-40% for 10 and 17 mg/kg doses is not clear.

5) The authors could have attempted to confirm the findings at protein levels for one or two candidates, if not with the blood samples at least with the brainstem sections/samples. The commercial antibodies generally get examined for cross-reactivity with the species that are commonly used in research. These antibodies may cross-react with rhesus macaque proteins as well. Although the authors highlight the difficulties in non-human primate research, I feel that the sample collections, preservations etc., could have been planned well.

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REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

This manuscript by Malleret and colleagues uses the Rhesus *P. coatneyi* model of cerebral malaria in humans to show that methylene blue can be used as a potential therapeutic for cerebral malaria. In particular this has been shown using mouse models and this study, in part, aimed to demonstrate applicability to humans by repeating this observation in non-human primates. The study relies heavily on transcriptomics and the main findings were that 1) methylene blue treatment led to resolution of the neuroinflammatory transcriptome upon disease resolution, particularly in the brain stem and 2) comparison of the transcriptomic data set to that of PBMCs from *P. coatneyi* animals and human PBMCs in *P. falciparum* infections identified 9 DEGs that could potentially be used as biomarkers for CM. The methods are all fairly standard.

In general, the data is presented well with easy to follow figures. The manuscript in general is a valuable addition to the transcriptomics available for malaria and for that reason I am supportive of the study. That being said the authors can improve some aspects of the manuscript to increase the utility of the data presented. One issue with the study design is that there not naïve a group of Rhesus where the effect of methylene blue treatment alone can be parsed out. **Is there a naïve / methylene blue dataset available from the literature that could be discussed (any animal)?**

Thank you for your positive feedback and supportive comments. We acknowledge the importance of including a naïve group treated with MB to clearly distinguish the specific effects of MB treatment. Unfortunately, to the best of our knowledge, there is currently no publicly available transcriptomic dataset for naïve animal treated solely with MB (including rhesus macaques or other animal models). The absence of such data in the literature highlights the need for further studies that focus specifically on the standalone effects of MB, which would provide a clearer understanding of its impact on various biological systems. However, in our study, we observed a minimal difference of only 2 DEGs between the $P_{coat}^- MB^-$ (naïve-untreated) group and the $P_{coat}^+ MB^+$ (infected-treated) group (**Fig. 3a**). This minimal difference may suggest a limited effect of MB on the animals and study outcomes.

Most of my other comments relate to the **introduction and discussion**. There is no explanation or discussion of methylene blue – what is it and how does it work? Similarly there are references to various pathways / immune cells that the transcriptomics may be relevant to – but no real discussion of how these cells may be important. The literature is poorly cited in this respect (studies are listed in Table 1 for the 9 biomarkers only but they are not properly integrated in the manuscript and not discussed as a group of molecules, rather these are treated almost like independent molecules - they are likely linked). This lack of information limits the utility of the data for the reader. From a discussion perspective why would methylene blue be able to treat cerebral malaria? There is no discussion of how cerebral malaria occurs (mechanisms as per current knowledge) and where methylene blue fits into this pathway.

Thank you for your detailed feedback. We appreciate your insights into the areas where additional context was needed. In response, we have expanded both the Introduction and Discussion sections to include a more thorough explanation of the mechanisms underlying CM and MB. Please refer to Line 58 and Line 400 for the updated content.

Besides, we have included an analysis to assess the association of the 9 gene signature with the immune population in **Fig. 8b**. The results indicate that seven of the genes are predominantly expressed in neutrophils, while NFE2 is highly expressed in neutrophils, though not predominantly. MAG, on the other hand, is highly expressed in plasmacytoid dendritic cells. These findings support the role of neutrophils in CM pathogenesis, and we have discussed the association at Line 363.

In the **introduction**, is there edema or hemorrhage in the brain from *coatneyi* infection? This review (<https://journals.sagepub.com/doi/full/10.1177/0300985815583098>) suggests that there is, and if so it should probably listed as well.

Thank you for this valuable suggestion. Indeed, there is evidence of edema and hemorrhage in the brain associated with *P. coatneyi* infection, which we have observed in our histopathological analysis and included in **Supplementary Table 2**. These features highlight *P. coatneyi*'s suitability as a model for studying CM, as such neuropathological changes are characteristic of severe malaria pathology, including blood-brain barrier disruption and associated complications.

Figure 2: Why was the liver not different? This seems unlikely given what is known about malaria. Arguably the kidney should also show differences. Which areas of the kidney / liver were taken as tissue – there is nothing specified. Can the authors discuss some hypotheses about why this treatment appears to specifically target transcriptomics in the brainstem? CM rarely occurs in with damage in the brain / CNS alone. It is not clear why lung was not also included.

Thank you for this comment. We would like to clarify that our study uses a blood-stage malaria challenge rather than a sporozoite challenge. As a result, the liver stage of malaria, which is typically associated with significant pathology in the liver, does not occur in our model. This explains why we did not observe the expected transcriptomic changes in the liver, as the liver involvement seen in other malaria models is absent here.

While CM can contribute to liver and kidney pathology, it primarily affects the brain, where microvascular obstruction, inflammation, and blood-brain barrier disruption lead to the most severe pathology. This focus on the CNS, particularly the brainstem, aligns with the predominant transcriptomic changes and pathology observed in our CM model.

Both kidneys and liver were extensively sampled post-euthanasia, with up to 10 samples taken from each organ, targeting lesion margins, lesion centers, and adjacent normal tissue. For kidneys, samples included both cortical and medullary regions, with tissue slices kept below 1 cm thick to ensure optimal formalin penetration. Despite this thorough sampling, no significant transcriptomic changes were detected in either the liver or kidneys, which suggests their relatively minor role in CM pathology.

In the lungs, although *P. coatneyi*-infected macaques displayed sequestration of infected erythrocytes within pulmonary microvessels, histopathological signs of injury were minimal. There was mild alveolar septal expansion and occasional interstitial pneumonitis, but no significant pulmonary edema, inflammatory destruction, or hyaline membrane formation, which are indicative of diffuse alveolar damage or ARDS. Given this limited involvement, our primary focus remained on the brain, renal and hepatic pathology, where acute injury was more prominent.

It was good that the authors have made PCAs for each brain region. However I think the claim “In the brain tissues, the gene expressions of the Pcoat+ MB– samples were distinct, whereas those of the Pcoat+ MB+ and Pcoat– MB– samples were indistinguishable from each other, and this is especially apparent in the brainstem (Fig. 2b).” is too strong. Instead of saying that this is especially apparent in the brainstem, I would say that it’s only apparent in the brainstem.

Thank you for your thoughtful feedback. We agree that the statement could be made more precise, we have revised the sentence as follows at Line 159:

“In the brain tissues, the gene expressions of the Pcoat+ MB– samples were distinct, whereas those of the Pcoat+ MB+ and Pcoat– MB– samples were indistinguishable from each other, and this is only apparent in the brainstem (**Fig. 2b**).”

I would change the title of this section to either say “Brainstem genes that were modulated by infection and treatment are central to the Ingenuity Pathway Analysis neuroinflammation signaling pathway.” or “Brainstem genes that were modulated by infection and treatment are central to neuroinflammation signaling pathways.” As it reads now it suggests to me that the “Neuroinflammation signaling pathway” is some specific pathway defined by convention independently of IPA.

Thank you for your suggestions regarding the section title. We agree that the current wording could imply that the “neuroinflammation signaling pathway” refers to a specific, universally recognized pathway, rather than an analysis outcome specifically derived from IPA. We have revised the section title to “Brainstem genes that were modulated by infection and treatment are central to neuroinflammation signaling pathways”.

I’m assuming IPA is “Ingenuity pathway Analysis” but I don’t think it’s ever explicitly stated.

Thank you for point this out. You are correct. We have updated the manuscript to clarify this on Line 198:

To explore the biological relevance of the 574 brainstem genes modulated by infection and reversed by MB treatment, we analyzed those genes with Ingenuity pathway analysis (IPA) and found that granulocyte adhesion and diapedesis, as well as IL-10 signaling were the two most significantly affected pathways, although their direction of regulation could not be predicted (**Fig. 4a**).

Reviewer #2 (Remarks to the Author):

The study conducted by Hang & Leong et al. describes the transcriptional profiles of diverse tissues of splenectomized *M. mulatta* infected with *P. coatneyi* and after treatment with methylene blue (MB). They demonstrate that irrespective of the dose, MB treatment reduced most of the parasitemia and prevented severe malaria in 67% of animals by day 12. Infection induced differential expression mainly in different regions of the brain, in particular, the brainstem and genes modulated by the infection were also affected by MB treatment. Additional analysis attempts to demonstrate a 9-gene signature as a potential biomarker for cerebral malaria (CM). It represents an interesting model to study CM and evaluate therapeutic efficacy. However, there are several major issues that need attention.

Major points

- Although MB treatment was successful in reducing parasitemia and preventing more severe disease in most animals, previous study demonstrates that all animals treated only with MB presented recrudescence, and treatment rescued most but not all animals from fatal outcomes (Ref 63). Although the models and treatment regimens are different it should be considered whether the MB treatment works by clearing parasites, which in turn modulates the inflammatory response or whether MB also displays direct immunomodulatory activity.

We appreciate your insightful comment and understand your concerns. Our future work will focus on elucidating the mechanisms underlying the therapeutic effects of MB treatment. To achieve this, we plan to conduct a series of studies that include immune profiling of treated animals to assess changes in cytokine levels and immune cell populations. This approach will help determine whether MB's primary role is in clearing parasites, or if it also has direct immunomodulatory effects. Both *in vivo* and *in vitro* experiments could be employed to dissect the pathways involved in immune response modulation, providing a comprehensive understanding of how MB influences disease outcomes in malaria.

- The only parameters used to demonstrate that MB treatment was successful was parasitemia and brain gene expression. The authors describe clinical data very succinctly in the Supplementary Table 1. The data should be demonstrated and analyzed, if possible, from each animal, including white blood cell counts, RBC counts, temperature, which symptoms and signs of malaria and more specifically of CM were recorded for each animal and whether those clinical alterations could be reversed by MB treatment.

Thank you for your valuable feedback. We have addressed your concern by providing detailed clinical data in **Supplementary Table 1**, which includes information on white blood cell counts, RBC counts, temperature, and the symptoms of malaria, including those specifically related to CM, for each animal.

- The findings that the transcriptional regulation in the brain is the most affected during infection is important. However, there are no other types of data corroborating their CM model. They conclude that neutrophils might underlie the neuroinflammation in their model, but the results are not conclusive. I suggest cellular deconvolution analysis of their RNAseq data to predict immune and brain cell frequencies using widely known

computational tools such as CYBERSORT. Also explore key specific genes whose expression is modulated by treatment at the single gene level.

Thank you for your suggestion. We appreciate your recommendation to include additional data to further substantiate our CM model. In response, we conducted a CIBERSORT analysis and found that neutrophils represent a higher percentage in the untreated samples compared to both uninfected and treated samples (**Supplementary Fig. 8a**). Furthermore, we explored the expression of the nine signature biomarkers across various immune cell subsets using data from the Human Protein Atlas. This allowed us to assess the expression patterns of these biomarkers in different immune cell types, providing a clearer understanding of their potential roles in the immune response to CM (**Supplementary Fig. 8b**). Our results showed that, with the exception of MAG and NFE2, these biomarkers were predominantly expressed in neutrophils. This finding further supports the potential role of neutrophils in neuroinflammation during CM, aligning with our hypothesis.

Additionally, we have included the top 50 key specific genes whose expression is modulated by treatment at single-gene level (**Supplementary Fig. 3c**). Notably, 15 genes from this list were highly expressed in the neutrophils: LILRA3, LILRA5, CD177, CSF3R, CEBPB, FCAR, IL1RN, LCN2, MMP9, S100A8, S100A9, MMP9, SECTM1, SOCS3, SPI1. We believe these additions will strengthen our findings and offer a more comprehensive understanding of the role of neutrophils and transcriptional changes in the brain during CM and MB treatment.

- Histopathological analysis of the brain regions would certainly improve the understanding of whether parasite sequestration and/or inflammatory infiltration might explain the differences in the number of DEGs in different brain regions. Analysis of other organs can also explain why there are no expressive differences in gene expression.

Thank you for your insightful comment. We agree that histopathological analysis of different brain regions could provide valuable context for understanding the differences in the number of DEGs and whether parasite sequestration and/or inflammatory infiltration play a role. This point was also highlighted by the reviewer #1. In response, we have included a comparative histopathological analysis in **Supplementary Table 2**, which highlights the observed changes in brain regions and other organs, providing further insights into the potential mechanisms underlying the gene expression differences.

- Validation of some key proteins suggested by the transcriptional data would strengthen and confirm some conclusions. It could be immunohistochemistry, ELISA or any other method that corroborating for example, increased neuroinflammation and or other significant pathways.

Thank you for your valuable suggestion. We agree that validating key proteins suggested by the transcriptional data would provide additional strength and confirmation to our conclusions, particularly regarding the increased neuroinflammation and other significant pathways identified in our study. While we were not able to incorporate this validation within the current study, we recognize the importance of such experiments.

As part of our future research, we plan to validate these findings using malaria-infected human patient cohort. Specifically, we intend to employ techniques such as immunohistochemistry, Luminex, and potentially other complementary methods to confirm the expression of key proteins and further explore their role in neuroinflammation and other relevant pathways in CM. However, we would like to note that a Luminex kit capable of targeting all the identified CM biomarkers across both human and macaque samples is currently unavailable, which limited our ability to include this approach in the present study.

We appreciate your thoughtful suggestion, and we are committed to incorporating these validations in our ongoing and future studies to enhance the robustness of our conclusions.

- The analyses related to the identification of biomarkers for CM consist of overlapping different DEG lists from 3 datasets that consist in a total of 38 samples. This experimental setting would be ideal for identifying prognostic blood biomarkers of CM. Did the authors collect venous blood from animals before infection and during the course of infection and treatment? Because, this could reveal genes predicting CM before it is apparent, which would be prognostic and not diagnostic. Here, the signature is diagnostic, because the changes in the gene expression occur when the individuals already experience acute symptoms and possibly signs of CM. Although the expression of 9-gene signature is conserved among the evaluated datasets, other cohorts also need to be used for validation including GSE117613, GSE1124, GSE72058, GSE33811, E-MTAB-6413. Model performance should be tested using ROC curve AUCs reported for each cohort. If the signature is a biomarker for CM, it should also discriminate between uncomplicated and other severe phenotypes of malaria, such as severe anemia. How does ROC AUCs from the 9-gene signature in this study compares to other previous CM gene signatures?

We appreciate your insightful suggestion regarding the potential for identifying prognostic biomarkers for CM. This indeed would be a valuable extension of the current work. In our study, we did collect venous blood samples from the animals at multiple time points, both before and during the infection and treatment phases. However, the onset of CM in this model can be remarkably rapid, typically manifesting within a very narrow time window after the onset of a yet to be determined threshold parasitaemia. This often occurs within 7 to 9 days post-infection, with seemingly low-risk animals (even with low peripheral parasitemia) slipping into a coma within an hour or two. This rapid progression makes it challenging to capture early changes that might serve as true prognostic indicators of CM before clinical symptoms emerge. Our current study focused primarily on diagnostic biomarkers for CM. While our findings are diagnostic in nature, we agree that future studies would benefit from exploring earlier time points to assess the prognostic potential of these biomarkers. We see significant value in further developing this model to enhance its utility for studying prognostic markers, which could ultimately improve early detection and intervention strategies for CM.

Additionally, we have already included the datasets you mentioned, such as GSE117613, GSE1124, GSE72058, GSE33811, and E-MTAB-6413, and the results show that the 9 gene signature can successfully discriminate CM from other severe malaria phenotypes, including severe anemia. In fact, the ROC AUC for our 9 gene

signature classifies CM with 100% accuracy in our RNA-seq brainstem samples (**Fig. 7b**), 95% accuracy in public RNA-seq datasets (**Supplementary Fig. 5b**), and 80% accuracy in public microarray dataset (**Supplementary Fig. 5e**). To compare our model's performance, we also tested the public CM gene signature BCL2L13, NFIX, S100P, S100A8, RETN, PGLYRP1, BIRC5, IL1R2, MCOLN1, MED25, FUCA1, LZTFL1, SLC25A38, RPA1, RPIA, ACVR1, ATP6V0E2, SPSB3, and PIGQ (Silva et al., 2024), and found it was able to distinguish 92% of the groups (**Fig. 7a**). This comparison highlights the strong performance of our 9-gene signature in distinguishing CM cases from other malaria phenotypes. We believe these results demonstrate that our 9-gene signature holds significant promise as a robust biomarker for CM, capable of distinguishing CM from other malaria phenotypes with high accuracy across different datasets.

- The results do not support the conclusion that this study elucidates the role of neutrophils and neuroinflammation in CM pathogenesis. Further study about the efficacy of MB treatment is needed and the identified biomarkers need to be tested in a larger set of samples and independent cohorts.

Thank you for your valuable feedback. We understand your concern regarding the need for further evidence to support the conclusion about neutrophils and neuroinflammation in CM pathogenesis. To address this, we have shown that neutrophils show a higher abundance in the brainstem in our analysis (**Fig. 8a**), and the 9 biomarkers identified in our study are predominantly expressed in neutrophils (**Fig. 8b**), suggesting neutrophils' potential involvement in the immune response during CM. Further analysis revealed that many of the top 50 genes modulated by MB treatment are highly expressed in neutrophils (**Supplementary Fig. 2c**). Among these, we identified several genes, including LILRA3, LILRA5, CD177, CSF3R, CEBPB, FCAR, IL1RN, LCN2, MMP9, S100A8, S100A9, MMP9, SECTM1, SOCS3, and SPI1, which are closely linked to neutrophil function. While these findings support the idea of neutrophils playing a key role in neuroinflammation, we acknowledge that additional studies, particularly focusing on functional validation, would strengthen our understanding of their role in CM pathogenesis.

Regarding the efficacy of MB treatment, while our study primarily focuses on identifying biomarkers, future research could include functional assays to further evaluate MB's impact on neutrophil activation and neuroinflammation. This would involve assessing neutrophil responses, inflammatory markers, and the progression of CM in treated versus untreated animals. These steps would help establish a more direct link between neutrophil activity, neuroinflammation, and MB treatment efficacy in CM.

As for the 9 biomarkers, we have tested them in a larger set of samples and independent cohorts. The results are presented in **Fig. 7** and **Supplementary Fig. 5**, where we demonstrate that our biomarkers can accurately distinguish CM from other severe malaria phenotypes in both our RNA-seq brainstem samples and several public datasets. This validation across different cohorts further supports the reliability and potential clinical relevance of these biomarkers in CM diagnosis.

Minor points

- It would be more informative to demonstrate the hierarchical clustering of samples by genes in a heatmap instead of JSD distances. Because, as expected, samples cluster mostly by organ and clustering by infection or treatment is not evident.

Thank you for your suggestion. We agree that a hierarchical clustering heatmap would be more informative. We have updated the analysis to include this visualization (**Fig. 2a**), which provide a clearer view of sample relationships based on gene expression, especially in the context of infection or treatment.

- The association of the 9 gene signature with neutrophils would benefit from a statistical enrichment approach to understand whether they are indeed enriched only in neutrophil transcriptomes or they might also be expressed by other leukocytes and cells.

Thank you for your valuable suggestion. We have included the relevant data in **Fig. 8b**, where we assess the association of the 9 gene signature with the immune population. The results show that seven of the genes are predominantly expressed in neutrophils, while NFE2 is highly expressed in neutrophils but not predominantly. MAG, on the other hand, is highly expressed in plasmacytoid dendritic cells. This finding further supports the role of neutrophils in CM pathogenesis. We believe this addition addresses your concern and provides a more comprehensive understanding of the gene signature.

Reviewer #3 (Remarks to the Author):

The manuscript by Hang et al addresses the need of additional therapies and diagnostic markers for cerebral malaria (CM), an important concern of higher mortality in African children and a prolonged neurological sequelae in survivors. With parenteral administration of artesunate being the only option, the authors have shown the potential of Methylene Blue (MB) for CM treatment. The earlier studies on MB have only addressed its transmission-blocking and antimalarial effects, except for one study in rodent parasite model on preventing experimental cerebral malaria. The present study has utilized *P. coatneyi*-infected rhesus macaques as non-human primate model to study the potential of MB in CM treatment. The authors have performed detailed transcriptomics studies for the brainstem, thalamus and cerebellum of the *P. coatneyi*-infected rhesus macaques (in addition to heart, liver and kidney) and shown that MB treatment can reverse the gene expression changes in the brainstem of the infected animals. The authors have compared the RNA-Seq data of the present study with two of the published datasets representing the blood samples of *P. coatneyi*-infected macaques with acute infections and *P. falciparum*-infected humans with CM. This in turn has led to an important finding of identifying a set of 9 potential biomarkers with many of them associated with neutrophil function and CM. In particular, this is of clinical relevance with the existing limitations in accurate CM diagnosis by neuroimaging and fundoscopy, and distinguishing it from other non-CM encephalopathies. Although the findings are interesting, there are several concerns that need to be addressed in detail.

Major Comments:

- 1) While the utilization of *P. coatneyi*-infected rhesus macaques as a CM model is commendable, the studies could have been designed slightly better. For example, the manuscript lacks the histological evidences for various brain parts to correlate the MB treatment and gene-expression findings with cerebral pathologies, leaky vasculature, parasite sequestration, leukocyte infiltration etc. With only a small amount of tissue required for transcriptomics studies, the rest of it could have been utilized for such data to strengthen the findings. It is also not clear why other brain parts like basal ganglia and corpus collosum are not examined in the present study since they also contribute to the neurological complications in CM.

We appreciate the reviewer's insightful comment regarding the inclusion of additional brain regions, such as the basal ganglia and corpus callosum, in our study on CM. These regions are indeed crucial for understanding the full spectrum of neurological complications associated with CM. In this study, we focused primarily on brain regions most commonly associated with severe CM outcomes, specifically the cerebral cortex and hippocampus. These areas were selected based on their well-documented involvement in the neuropathological changes associated with CM, particularly in relation to parasite sequestration, which is a hallmark of this model. Given the study's scope and resource constraints, we prioritized these regions to ensure a thorough and focused analysis.

Additionally, the collection of material for histopathological analysis did not provide the necessary anatomical resolution to specifically identify the basal ganglia or corpus callosum. The study utilized a wide range of retrospective archival material from previous work by authors such as Aikawa, but unfortunately, this material lacked the detailed anatomical information needed to pinpoint the origin of the tissue within the cerebrum. Consequently, we were only able to perform higher-level anatomical

analysis. Nevertheless, our histopathological findings align with recent studies by Lombardini et al. (2022) on *P. coatneyi* histopathology, which have been incorporated into the manuscript at Line 132, Line 340 and **Supplementary Table 2**. This reference further supports the conclusions of our study and provides valuable context for understanding CM-related pathologies. Future studies would benefit from a more meticulous examination of the microanatomical components of the brain to provide a more comprehensive understanding of CM-related pathologies.

- 2) The reason behind the incomplete clearance of terminal parasitemia and treatment failure in *P. coatneyi*-infected animals treated with higher doses of 10 mg/kg and 17 mg/kg of MB is not clear. Two third of animals exhibit decreased mentation, epistaxis, anorexia etc. Does it indicate any MB toxicity especially when there is a blood-brain barrier breach in CM? The 100% success rate of 6 mg/kg could have been verified with another cohort to ensure that the findings are consistent.

Thank you for your insightful comment. We acknowledge that challenges regarding the incomplete clearance of parasitemia in animals treated with higher doses (10 mg/kg and 17 mg/kg) of MB. It's important to note that these higher doses did clear parasitemia at a faster rate compared to the lower dose (6 mg/kg). However, the treatment were initiated at a higher parasitemia threshold (15%), as compared to 6 mg/kg (10%). Given how quickly *P. coatneyi* parasitemia escalates, it becomes extremely challenging to monitor and treat at the exact optimal time, which may contribute to treatment failure.

Regarding the clinical signs of decreased mentation, epistaxis, and anorexia, while these could raise concerns about MB toxicity, they are also consistent with symptoms of CM, especially when the blood-brain barrier is compromised. We cannot rule out toxicity, but these symptoms might be more directly linked to the severity of the disease itself. We have included the symptoms of CM, as well as the symptoms at euthanasia in **Supplementary Table 1**.

Lastly, we agree that verifying the 100% success rate of the 6 mg/kg dose with another cohort would be ideal to further confirm the consistency of the findings, and we will consider this in future studies.

- 3) A cohort of uninfected macaques treated with MB would have served as an additional control, especially to understand the gene-expression changes (if any) in brain because of MB treatment since MB is brain permeable. Such data might have also given some idea for why the number of DEGs observed for Pcoat+MB+ vs Pcoat+MB- is twice than that of Pcoat+MB- vs Pcoat-MB-.

We acknowledge that a cohort of uninfected macaques treated with MB would have provided valuable insights, especially regarding gene expression changes in the brain due to MB treatment. However, due to strict limitations set by the IACUC on the number of animals used, our study was limited to infected macaques only. Nevertheless, we did observe only 2 DEGs between the Pcoat-MB- and Pcoat+MB+ groups (**Fig. 3a**), suggesting that the effect of MB on the outcome is likely minimal, despite its brain permeability.

- 4) The ability of MB to reverse the gene expression changes in the brainstem of *P. coatneyi*-infected animals to the extent of uninfected animals is interesting.

Nevertheless, there are chances of irreversible/prolonged/direct/indirect consequences of neurological complications that might still persist and lead to adverse outcome. This could be a reason for the treatment failure observed despite a significant reduction in the terminal parasitemia and reversal of gene expression changes in 10 mg/kg and 17 mg/kg groups.

Thank you for highlighting this important consideration. The treatments for the 10 mg/kg and 17 mg/kg groups were initiated at a higher parasitemia threshold (15%) compared to the 6 mg/kg group (10%). Given the rapid progression of *P. coatneyi* infection, achieving precise timing for treatment initiation poses a significant challenge, which may contribute to treatment failure. This delay might allow for neurological complications to persist, despite reductions in parasitemia and some reversal of gene expression changes.

Now that we know CM typically develops between Day 7 and 9 post-infection, we can adjust the timing of MB treatment to effectively target CM at the optimal stage, ensuring timely administration. This will enable us to study the progression of CM and its neurological impact more effectively in the animal model. However, it is important to emphasize that we do not intend to use MB in clinical settings. Our primary objective is to establish a robust CM animal model for identifying potential drug targets. Additionally, we plan to monitor the long-term neurological effects of CM in the animals to assess any persistent or irreversible consequences, which will deepen our understanding of the disease and aid in the development of future therapeutic strategies.

- 5) The authors have compared the transcriptomics dataset of the present study with the published datasets available for the blood samples of acute *P. coatneyi* infections and human CM patients to narrow down their findings and identify potential blood biomarkers for CM diagnosis in humans. Although the findings are interesting, the comparison with just six human CM blood samples from a single study of Thiam et al., is inadequate to propose them as biomarkers. The authors should attempt to include more number of CM samples.

Thank you for your feedback. We appreciate your concern regarding the limited number of human CM samples in the study by Thiam et al. In response, we have now included 5 additional datasets (GSE1124, GSE33811, GSE72058, GSE116306 and GSE117613) to strengthen our analysis. The updated results, which further support the alignment of our findings, can be found in **Fig. 7c** and **Supplementary Fig. 5**.

- 6) It is also important to confirm the specificity of these biomarkers for CM by analyzing the non-cerebral severe malaria samples. There is also a small cohort of non-cerebral severe malaria samples in Thiam et al study itself. Is it omitted for any technical/experimental reason? Equally important is to confirm the results with the samples from other non-malarial encephalopathies.

Thank you for your valuable comment. We agree that confirming the specificity of the biomarkers for CM is essential. In response, we have compared our findings with non-CM cases, including non-cerebral severe malaria and mild cases as controls, as well as with samples from a non-malarial encephalopathy, specifically, the West Nile Virus infection. These results, which can be found in **Supplementary Fig. 5 and 6**, show that the identified biomarkers remain specific to CM, even when compared with this broader

context. We believe these findings further support the potential of these biomarkers as specific indicators for CM diagnosis.

Minor Comments:

- 1) It has been reported that the knobs present in the *P. coatneyi*-infected RBCs of splenectomized animals are incompletely formed in comparison to animals with intact spleen. It is not clear why the present study is carried out in splenectomized rhesus macaques. There should have been at least a discussion on this aspect providing clarity.

Spleen intact rhesus macaques do not reliably achieve a consistent or severe enough degree of parasitemia so as to be able to assess therapeutic treatment protocols. Furthermore, as part of the refinement of the protocols comprising this study, it was noted that parasitemia spikes would occur at differing, and unpredictable rates and result in death of animals. Splenectomy, combined with hourly parasitemia assessment permitted predictable mapping of parasitemia, and the ability to achieve parasitemia levels consistent with severe disease. Anecdotal historical evidence from spleen intact donor animals at AFRIMS suggests that parasitemia may even be cleared in immunocompetent animals.

At the stage of merozoite development within schizonts, the malaria pigment crystals begin to coalesce, forming larger irregular granules and the parasitized erythrocyte enlarges and is eventually obscured by the schizont (Coatney, 1971). During this stage, parasitized erythrocytes can still be identified in peripheral blood, but their numbers are significantly decreased in spleen-intact animals. Schizont rupture and release of merozoites occurs with relative synchrony and results in the periodicity of parasitemia eliciting the pyrogenic response in the host which parallels the severity of histopathological lesions. In splenectomized monkeys, these late stages of the parasite are also found in peripheral blood, along with the ring forms.

In our previous study, "Ultrastructural Characterization of Host-Parasite Interactions of *Plasmodium coatneyi* in Rhesus Macaques" (Lombardini et al., Parasitology, February 2022, Vol. 149, Issue 2, pp. 161-170), we observed the formation of knob-like structures on the surface of *P. coatneyi*-infected red blood cells sequestered in the brain. These structures bear a resemblance to those found in *P. falciparum*, offering insights into the ultrastructural similarities between the two *Plasmodium* species in splenectomized rhesus macaques.

We have added a discussion on this aspect at Line 337 in the revised manuscript to provide further clarity on the rationale for using splenectomized rhesus macaques in our study.

- 2) In the supplementary figure 1, Pcoat+MB- data is missing for the liver principal component analysis.

Thank you for highlighting this point. The liver samples for Pcoat+MB- did not pass the quality threshold for RNA sequencing. Therefore they are not included in the analysis.

- 3) Although the authors have proposed the feasibility of performing flow cytometry and antibody staining, these are difficult in clinical set up especially in the context of

diagnostic window in CM. The more realistic option would be a real-time PCR or ELISA analyses.

Thank you for your suggestion. We agree that antibody staining and flow cytometry can present logistical challenges in a clinical setting. However, they offer the advantage of providing rapid results, which is crucial given the very fast progression of CM. While real-time PCR or ELISA would offer more practical and accessible diagnostic alternatives, yet they are more time-consuming and may not be able to provide the quick results needed in such rapidly evolving cases. Therefore, we believe that flow cytometry, despite its challenges, may still be an important tool in critical, time-sensitive situations.

- 4) The fold-changes observed for the expression of some of the markers (for example IL1RN and S100A8 of human samples provided in Supplementary Table 3.5) may not meet the diagnostic criteria.

Thank you for your comment. We acknowledge that the fold-changes observed for some markers, such as IL1RN and S100A8 in the human samples (revised to **Supplementary Table 4.5**), may not meet the diagnostic criteria. We have validated these markers in larger, independent cohorts and publicly available datasets. Both IL1RN and S100A8 demonstrated the ability to distinguish CM from both mild and severe malaria cases, suggesting their potential utility in diagnosing CM (**Supplementary Fig. 5**).

REVISION 2

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

This manuscript details the transcriptomic changes in several organs with reduced *P. coatneyi* parasitemia from methylene blue administration. The authors have attempted to address most of my comments. Whilst this has improved clarity in a few areas there are still some issues that could not / have not / could not been resolved.

1. No naive / methylene blue data set so we don't know what methylene blue does to transcripts (if anything) to separate if there are any effects on the tissue from methylene blue administration vs reducing parasitemia.

The Methylene Blue study conducted at AFRIMS was done under the strict review of the AFRIMS Institutional Animal Care and Use Committee (IACUC) and under the Department of Defense's Animal Care and Use Review Office (ACURO), which provides oversight of the implementation of US Army Medical Research and Development Command, U.S. Army, and Department of Defense (DoD) policies regarding use of animals in research, development, testing, evaluation, and training. All DoD policies governing animal use are in compliance with the United States FDA and USDA guidelines and mandates as to use of non-human primates. Furthermore, AFRIMS complies with all laws, and regulations of the Kingdom of Thailand regarding appropriate use of non-human primates in medical research. The regulatory agencies have adopted the concept of the 3Rs (Replacement, Reduction, Refinement) in the Animal Welfare Act (AWA). The AWA and its regulations require researchers to consider alternatives to procedures that may cause more than momentary pain or distress to animals, and to avoid unnecessarily duplicating previous experiments. This is especially true in mammals such as non-human primates. As such, the Methylene Blue study depended on the maximum number of animals needed to demonstrate efficacy in treating the *Plasmodium coatneyi* parasitemia, depending on archival material for the histopathology of untreated animals. There was no ethical reason or logistical possibility of sacrificing animals for the sole purpose of demonstrating histopathological effect of methylene blue in uninfected animals.

2. there is still no validation of transcripts

We appreciate the reviewer's suggestion to validate our findings at the protein level. We have investigated the availability of commercial antibodies for the identified biomarkers. While antibodies for flow cytometry exist for all markers except CHIT1, only CD177 shows cross-reactivity with rhesus macaque. Unfortunately, we no longer have blood cells from our samples, so it is not feasible to perform flow cytometry at this stage. Additionally, there are currently no available Luminex assays that cross react with rhesus macaque proteins, which prevent us from performing this analysis with plasma samples from our cohort. To address this, we are exploring the development of an in-house Luminex kit compatible with rhesus biomarkers for our next study, although the feasibility of this effort remains uncertain. We also plan to validate the protein expression of these markers in future studies using sample from human malaria patients.

3. Regarding the liver signature, the lack of infected / MB- samples for this means it is impossible to say anything about the transcriptomic impacts when infection are lessened with administration of methylene blue. It is not clear why these are not included (Supplementary Fig1) when they are in the other panels. This point should at least be stated more clearly that there may be a difference but it is not possible to say without this comparative group.

Thank you for pointing this out. The liver Pcoat⁺ MB⁺ dataset is unfortunately missing due to the quality control criteria for RNA sequencing not being met. As a result, we were unable to include these samples in the analysis. While our study primarily focuses on the brain due to its direct relevance to cerebral malaria, we certainly do not overlook the pathophysiological impact of *P. coatneyi* infection on the liver in this context. We acknowledge that the absence of this comparative group limits our ability to draw conclusions about the transcriptomic impacts of methylene blue on liver infection, and we have made this point clearer in the revised manuscript at **Line 170** in the Result section.

With regards to the rebuttal:

"Thank you for this comment. We would like to clarify that our study uses a blood-stage malaria challenge rather than a sporozoite challenge. As a result, the liver stage of malaria, which is typically associated with significant pathology in the liver, does not occur in our model. This

explains why we did not observe the expected transcriptomic changes in the liver, as the liver involvement seen in other malaria models is absent here."

I did just want to correct the authors on this point. The liver stage of malaria is clinically silent with minimal inflammation. On the other hand blood stages are cleared by the liver and sequester in the liver (to a greater or lesser extent depending on model) creating an inflammatory response in this organ and liver damage (as the authors mention in the rebuttal letter). I would expect to see some differences given the lower parasitemia levels.

Thank you for your insightful comment and for bringing this to our attention. We apologize for the misunderstanding in our previous rebuttal. You are absolutely correct that while the liver stage of malaria is clinically silent, the liver does play a significant role in clearance of infected red blood cells. As shown in the liver H&E staining (**Supplementary Figure 2**), we detected the presence of hemozoin in the liver prior to MB treatment, which supports the notion of physiopathology induced by the presence of hemozoin. Following MB treatment, there was a reduction in hemozoin accumulation, although it was not entirely cleared. This suggests that while MB treatment reduces the impact of *P. coatneyi* infection on the liver, it may not fully eliminate the inflammatory response or liver damage associated with this process.

Given these weaknesses I am still somewhat supportive of the publication of this data as this manuscript provides a good resource for the CM community.

Reviewer #2 (Remarks to the Author):

My concerns were addressed, but a minor suggestion is to add representative H&E images of brain sections from Pcoat⁺ MB⁻ and Pcoat⁺ MB⁺, so one can visualize the effect of the treatment in the tissue. I would include this image as Figure 1c, because it would be as important as the reversal in peripheral parasitemia.

Thank you for your suggestion. We appreciate your recommendation to include representative H&E images of brain sections from both Pcoat⁺ MB⁻ and Pcoat⁺ MB⁺ groups. We agree that these images would provide valuable visual insight into the tissue-level effects of the treatment and could help highlight the changes associated with MB treatment in the brain.

Rather than placing these images in **Figure 1c**, we have included them in a newly added **Figure 3** to better align with the structure of our findings. In **Figure 2**, we show that among the organs analyzed, only the brain sections display a distinct transcriptomic signature differentiating untreated animals from both naïve and MB-treated animals, suggesting that the brain is uniquely modulated by treatment. Therefore, we chose to separate the brain histopathology (**Figure 3**) from that of other organs (**Supplementary Figure 2**) and present it in a dedicated figure that follows directly from our gene expression analysis. This allows us to highlight the pathological impact of MB treatment specifically in the brain and supports our molecular findings with histological evidence.

Additionally, to represent the full range of treatment doses used in our study, we selected H&E sections from different brain regions corresponding to each dose: 10 mg/kg for the cerebrum, 6 mg/kg for the cerebellum, and 17 mg/kg for the brainstem. Each of these regions consistently shows a reduction in pathology upon treatment, and together they provide a full comprehensive view of the histological impact across the dosing spectrum. We believe this representative approach effectively conveys the therapeutic effect. We appreciate your suggestion, which has helped improve the clarity and narrative flow of the manuscript.

Reviewer #3 (Remarks to the Author):

In the revised version of the manuscript, Hang et al. have attempted to address the concerns that were raised earlier by comparing their RNA-Seq data with the additional datasets that are available for uncomplicated and severe (non-cerebral and cerebral) malaria. They have also included West Nile Virus dataset for non-malarial encephalopathy. These efforts have strengthened the RNA-Seq data that suggest the potential of the nine candidate genes as biomarkers for cerebral malaria. However, there are other aspects that need to be addressed.

1) As it was mentioned earlier, the data presented on methylene blue (MB) for cerebral malaria (CM) treatment is not convincing. The treatment failure observed for *P. coatneyi*-infected animals administered with higher doses (10 mg/kg and 17 mg/kg) of MB is debatable although the treatment initiation at 15% (instead of 10% for 6mg/kg) has been proposed as a reason for this outcome. The symptoms at euthanasia overlap with CM and they occur despite the better clearance of the parasites. In the responses given, the authors emphasize that they do not intend to use MB in clinical settings. However, in the discussion section of the manuscript, MB treatment has been mentioned as a therapeutic option for CM and alternative to artemisinin-based therapies. The authors should tone down such claims.

Thank you for your thoughtful comment. We agree with your assessment and have revised the discussion section to tone down the claims regarding MB as a therapeutic option for cerebral malaria (**Line 456**). The revised text now emphasizes the need for further investigation and acknowledges the limitations observed in our study, including the treatment failures at higher doses and the persistence of CM-like symptoms despite parasite clearance, which may be related to delayed treatment initiation or other host factors. We no longer present MB as a definitive alternative to artemisinin-based therapies, but rather as a compound with potential that warrants additional preclinical evaluation.

2) In the Supplementary Table 1, the authors should include the details for untreated *P.coatneyi*-infected animals, and the parasitemia details at the time of MB treatment initiation for all the groups. The time point of euthanasia varies between 9-14 days for the different groups and therefore, the red arrow provided for the experimental endpoint in Figure 1 has to be corrected.

Thank you for pointing this out. We have updated **Supplementary Table 1** to include the details for the untreated *P. coatneyi*-infected animals, as well as the parasitemia levels at the time of MB treatment initiation for all relevant groups. Upon reviewing the data, we realized there was an error in the parasitemia values reported in our previous response. We sincerely apologize for this mistake and regret any confusion it may have caused. After rechecking the data, we found that the 6 mg/kg group was treated with MB at approximately 17% parasitemia, while the higher-dose groups (10 mg/kg and 17 mg/kg) were initiated at approximately 37% parasitemia. We truly appreciate your understanding and continued constructive feedback.

Additionally, we have corrected the arrows indicating the experimental endpoint in **Figure 1b** to accurately reflect the variation in euthanasia time points (ranging from Day 9 to 14). These changes have been incorporated into the revised manuscript and supplementary materials.

3) The histopathological images for the **brain and other organ** samples of the *P. coatneyi*-infected animals with and without MB treatment have to be included in the manuscript along with the details provided in the Supplementary Table 2. The experimental details to be included in the Methods section.

Thank you for your valuable feedback. This comment was also raised by Reviewer 1. We agree that including the histopathological images for the brain and other organ samples from the *P. coatneyi*-infected animals with and without MB treatment will strengthen the manuscript. We have included these images in **Figure 3** (brain sections) and **Supplementary Figure 2** (other organs), and provided the details of these histopathological analyses in the Result and Methods section at **Line 175** and **Line 524** respectively.

To illustrate the effects of MB treatment across the range of doses used in our study, we have selected H&E sections from different brain regions that correspond to specific treatment doses: 10 mg/kg for the cerebrum, 6 mg/kg for the cerebellum, and 17 mg/kg for the brainstem. These images collectively demonstrate a consistent reduction in pathological features following treatment. We believe the selected images provide a representative and informative overview of the therapeutic impact.

4) Given the importance of spleen in the immune responses of blood-stage infections and the disease severity including CM, it would be appropriate if the authors discuss the potential

differences / miss outs that may arise in the splenectomised *P. coatneyi* model. Especially, in the context of other immune cells, and the comparison of data from splenectomised animals with spleen-intact human samples.

Thank you for this important observation. We fully acknowledge the limitations introduced by the use of splenectomised *P. coatneyi*-infected animals in our model. As suggested, we have added a discussion in the revised manuscript at **Line 386**, addressing the potential immunological differences and limitations this may introduce, particularly in the context of immune cell dynamics and when comparing to spleen-intact human cases. We now highlight that the absence of the spleen may alter monocyte and T cell responses, and that caution should be exercised when extrapolating our findings to natural human infections. We appreciate your feedback in helping us clarify this important aspect of the study.

5) With the commercial antibodies available for the identified biomarkers, the authors could have attempted to validate their findings at protein levels for at least a couple of biomarkers. This would have strengthened their potential use in CM diagnosis through antibody staining and flow cytometry.

Thank you for this valuable suggestion. We note that this point was also raised by Reviewer 1, and we appreciate the opportunity to clarify. We agree that protein-level validation would strengthen the diagnostic relevance of the identified biomarkers. We have examined the availability of commercial antibodies and found that while most biomarkers (except CHIT1) have antibodies suitable for flow cytometry, but only CD177 exhibits confirmed cross-reactivity with rhesus macaque. Unfortunately, we no longer have blood cells from our cohort, so flow cytometric validation is not currently feasible. Additionally, commercially available Luminex assays do not cross react with rhesus proteins, preventing us from using frozen plasma samples for protein quantification. We are actively exploring the development of an in-house Luminex assay tailored to rhesus biomarkers, although the feasibility and timeline for this remain uncertain. Importantly, we plan to validate the protein expression of these candidate biomarkers in future studies using samples from human malaria patients to support their potential clinical application.

REVISION 3

REVIEWER COMMENTS

Reviewer #3 (Remarks to the Author):

- 1) It would have been better if the representative H&E images are provided for brainstem, cerebellum and thalamus for all the three doses tested instead of providing different brain regions for different doses. Or else, the brainstem images shall be provided for all the three doses.

Thank you for the suggestion. We have now included representative H&E images of brainstem, cerebrum and cerebellum for all three doses tested to allow better comparison across treatment groups (**Figure 3**). Please note that the cerebrum images do not specifically highlight the thalamus, which is not readily visualized in standard H&E sections without deeper dissection. This has been clarified in the Methods section at **Line 561**.

- 2) From the H&E images, there appears to be only a partial reversal of pathology in the brain irrespective of the doses tested. Nevertheless, the DGE pattern of the brainstem shows complete reversal similar to that of naïve rhesus macaques. The authors may comment on this finding.

Thank you for this important observation. We agree with the reviewer's point, and we have added a corresponding explanation to the Discussion section at **Line 399**. Briefly, certain pathological changes, particularly in the brain, are likely to be irreversible or exhibit a degree of chronicity. Neurons do not regenerate, so once degeneration or necrosis occurs, there is permanent loss of the original tissue. It is also important to keep in mind that histopathological assessment offers only a snapshot in time, capturing a static moment within what is often a dynamic, ongoing process.

Moreover, the H&E photomicrographs represent an extremely small fraction of the overall tissue, typically just an 8 to 10 μm section. As such, they may not fully reflect the broader biological or molecular changes occurring within the entire organ. Therefore, one should be cautious when directly correlating localized microscopic findings with global molecular readouts such as gene expression patterns. That said, the observation of complete reversal in the DGE profile of the brainstem, despite only partial histological recovery, may suggest functional compensation. While structural damage might persist, the brain is known to exhibit a degree of physiological redundancy and plasticity. In response to injury, alternative neural pathways and synaptic connections may develop to restore some level of function, even if the original architecture cannot be fully regenerated.

- 3) The scale bar is missing for the H&E sections. Just by looking at the images, the magnifications seem to be different and this shall be verified.

Thank you for pointing this out. We have now included scale bars in all H&E sections to ensure accurate representation of magnification. The magnifications have been verified and are consistent with the stated imaging parameters (**Figure 3** and **Sup. Figure 2**).

- 4) The rationale behind initiating the methylene blue treatment at higher blood parasitemia levels of 30-40% for 10 and 17 mg/kg doses is not clear.

Thank you for the comment. The higher parasitemia levels (30-40%) at the time of MB treatment initiation in the 10 and 17 mg/kg groups were unintentional and resulted from the rapid progression of parasitemia. Due to the swift onset of infection, despite close monitoring, the window to initiate treatment at the desired threshold was sometimes missed. This made it difficult to match treatment initiation precisely across groups. We acknowledge that starting treatment at higher parasitemia likely contributed to the reduced efficacy observed with the higher doses. To clarify this, we have now included the parasitemia levels at the time of MB administration at **Line 127**, and the rationale for the treatment timing at **Line 485**.

- 5) The authors could have attempted to confirm the findings at protein levels for one or two candidates, if not with the blood samples at least with the brainstem sections/samples. The commercial antibodies generally get examined for cross-reactivity with the species that are commonly used in research. These antibodies may cross-react with rhesus macaque proteins as well. Although the authors highlight the difficulties in non-human primate research, I feel that the sample collections, preservations etc., could have been planned well.

We thank the reviewer for the thoughtful follow-up comment and agree that protein-level validation would enhance our findings. However, due to the retrospective nature of the study, tissue sample preservation was optimized for transcriptomic and histopathological analysis, and not compatible with reliable protein detection.

We only identified CD177 antibody with confirmed cross-reactivity to rhesus macaque and recognize its potential for future validation. In parallel, we are actively exploring the development of an in-house Luminex assay tailored to rhesus biomarkers, which may enable protein quantification from stored plasma samples. However, the feasibility and timeline for this remain uncertain.

We appreciate the reviewer's point regarding study planning. Moving forward, we are implementing more comprehensive sample preservation strategies – including those suitable for protein analyses – in ongoing and future NHP studies. We hope the reviewer will appreciate the logistical and ethical constraints of NHP research, and agree that our current findings, together with planned validation efforts, offer valuable insights into the biology and diagnostic potential of cerebral malaria.