Author’s response to reviews

Title: Modulation of innate immune responses at birth by prenatal malaria exposure and association with malaria risk during the first year of life

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Response to Reviewer’s comments
We thank the Reviewers for carefully evaluating our manuscript and for their constructive criticism. Please see below our point-by-point response to reviewer’s comments.

Reviewer #1:

This work is quite impressive, and stands out by including large enough numbers of subjects to make the results significant. However, some of the groups including acute PM and non-exposed are small in comparison with others. What strikes me as a bit odd is that the group "Past PM vs non exposed" stands out by showing significant differences for all cytokines, while the results for other groups are more varied. Could this be a result of the differences in numbers of voters in each group? You may want to comment more on the effect on the various numbers of individuals included in the analyses and the consequences for the significance on the results.

RESPONSE: We agree with reviewer #1 that this is one of the limitations of our study. We were already mentioning in the discussion, but now, as suggested by reviewers #2 and #3, we are presenting the limitations of this study in a separate section (Lines 778-784) in which we commented on why we obtained these small numbers in certain PME groups and its potential consequences for the significance on the results.

Another factor to consider that is not mentioned clearly is the effect on IPT and any treatment during pregnancy for the outcome. It may or may nor influence the results, but I think it may be worth mentioning how many were in the CSST/IPT arm and how many in the IPT only arm. Also, how many were actually treated for malaria during pregnancy? One more point is any practical implications on the findings here in relation to the recent paper in Journal of Infectious Diseases describing the impact on screening and treatment.

RESPONSE: We thank reviewer #1 for these suggestions and have added the number of women in each arm in Table 1. In addition, we mention the proportion of women who received at least 2 SP doses and the proportion of those who received at least 1 AL treatment. We have also added in Figure 1 the information of study arm in each PME group.

This study is complementary to the findings reported in the recent JID paper as it provides details on how PME impacts the risk of malaria in infants through the modulation of the fetal immune system. Given that past placental infections, which potentially occur early during pregnancy, have a profound effect on the fetal immune system, the practical implication here could be that a strategy based on screening and treatment should be implemented as early as possible during the first trimester to improve the long term benefit in infants. This has been added to the manuscript’s discussion (Lines 786-799).
Reviewer #2:

In this paper by Natama et al, the authors describe an ambitious analysis of cord blood innate cell responsivity to TLR stimulation among a birth cohort of children whose mothers had evidence of malaria in pregnancy. The authors had two overarching questions: 1) What impact do different manifestations of malaria in pregnancy have on both spontaneous and TLR-stimulated cytokine production? 2) Is spontaneous or TLR-stimulated cytokine production at birth associated with protection from malaria in infancy? The authors found that spontaneous cytokine, chemokine, and growth factor production were all significantly lower in samples with evidence of PME than in those that were unexposed, but, following TLR7/8 stimulation, samples with evidence of resolved placental malaria (pigment only) were "hyperresponsive" in comparison to samples without evidence of prenatal exposure. Furthermore, certain responses (both spontaneous and following TLR stimulation) were associated with differential malaria risk in infancy. Strengths of the manuscript include large numbers of samples studied (n=313), use of placental histopathology and longitudinal data in pregnancy to detail prenatal malaria exposure, ability to evaluate longitudinal associations with clinical malaria in infancy, and appropriate statistical analyses (with correction for numerous comparisons) employed.

Although the authors have described several interesting associations, I have several suggestions that would greatly enhance interpretability of the manuscript:

1) Was this cohort made up from both arms from the parent COSMIC study? Given the differences in follow-up between the treatment arms, how often was parasitemia assessed by RDT during pregnancy? Were these similar/different in parasite prevalence/treatment between the various PME arms? This should be reported and added to Table 1.

RESPONSE: We thank reviewer #2 for these suggestions, some of which suggestions are indeed similar to the ones made by the reviewer #1 in relation to number of women in each arm from the parent COSMIC study and anti-malarial drugs received (See additional data in Table 1). Also, in Figure 1, details regarding maternal infections in each COSMIC study arm are shown. In addition, we clarified the point around how often parasitemia was assessed by RDT in the CSST/IPTp-SP Arm during pregnancy as we were already mentioning that in the methods section, and treatment received for clinical malaria cases in both arm (Lines 190-196). Regarding the point around similarity/difference in parasite prevalence/treatment between the various PME arms, there was no difference in parasite prevalence across PME groups since all the mother-child pairs were selected based on documented malaria infection during pregnancy. This is the reason why we do have higher number of participants from the CSST/IPTp-SP arm (65.2%) as...
there was an active detection of malaria infection during pregnancy in this group. Also the non-exposed group was selected from the CSST/IPTp-SP arm. For AL treatment, the differences between PME groups are now shown in Figure 1 legend.

2) Figure 1 would greatly benefit from additional details regarding subject selection. Furthermore, there should be some mention as to how representative this subset is to the overall cohort (i.e., were associations between PME and subsequent child malaria risk also found in the subset of children where TLR responses were not measured?).

RESPONSE: We have modified Figure 1 accordingly to take into account this suggestion. As you can see in the Figure 1, we now provide new details regarding the selection process. Based on available data at delivery about peripheral infections during pregnancy as detected by RDT and/or LM, cord blood samples were stimulated. Then the history of malaria infection during pregnancy was subsequently confirmed by qPCR and placental infections by histology. Finally, infants were categorized as follows: 7 in acute PM group (CSST/IPTp-SP=3; IPTp-SP=4), 38 in chronic PM (CSST/IPTp-SP=24; IPTp-SP=14), 185 in past PM (CSST/IPTp-SP=117; IPTp-SP=68), 61 in the exposed/no PM group (CSST/IPTp-SP=38; IPTp-SP=23). The non-exposed group was composed by 22 infants from only mothers allocated to CSST/IPTp-SP.

How this subset is representative to the overall cohort could be found in the paper that we have published recently in which we showed associations between PME and subsequent risk of malaria during the first year of life in the overall cohort (Natama et al., JID 2018:217, 1967-76). Indeed we have shown that both peripheral infections during pregnancy and PM (mostly past PM) were significantly associated with malaria risk during the first year of life in the overall cohort. Therefore, we do have evidence that this subset is representative of the overall cohort.

3) One major concern is the choice of the control group (Prenatal malaria exposure unexposed, n=22, or only 7% of the overall cohort) and whether this group is truly "unexposed."

RESPONSE: We explain below what did we assess to ascertain the group was truly unexposed.

a. How was this group selected? How representative is this unexposed group in comparison to the unexposed group with the parent COSMIC cohort, since only a subset of the overall cohort was studied?

RESPONSE: A statement has been added in the malaria detection and definition section to clarify that (Lines 281-284). Actually, the control group was composed of pregnant women only recruited among the CSST/IPTp-SP intervention arm as in this arm there was active malaria
detection. All these women had negative RDT/LM results during pregnancy through monthly screening and ANC visits and subsequently confirmed by qPCR while placenta histology showed no evidence of placental infection including past PM (resolved PM with presence of malaria pigments).

In addition, in figure 1, details have been provided to show that initially 60 women (based on RDT and LM results available at delivery) were in this group but, after the correction of malaria status by qPCR and placental histology the number was reduced to 22.

b. Although this control group is utilized to report associations between various PME groups, given its small size (and the small size observed in the some of the other groups) lack of significant associations between groups may simply be a reflection of inadequate power. This should be discussed.

RESPONSE: This point is now discussed in the section related to the study limitations (Lines 778-784).

c. Did the authors perform statistical analyses to evaluate whether there were differences in cytokine production between PME groups that were exposed? This was not reported. Relatedly: in Figure S3, several of the features appear to have a trend towards decreasing production among unstimulated samples with increasing "severity" of PME. Did the authors assess for trend across these categories?

RESPONSE: We thank the reviewer #2 for pointing out these missing informations. We have now reported in the results section our findings related to: (i) the comparison of cytokine production between PME groups in cord blood and (ii) the trend analysis that allows in fact to demonstrate a trend towards decreasing production among un-stimulated samples with increasing "severity" of PME for some biomarkers (lines 351-356).

4) Relatedly: categories of acute, chronic, past placental malaria. Both acute and chronic PM have evidence of active placental infection - did authors evaluate whether combining these 2 groups increased statistical power to observe for differences in TLR responses?

RESPONSE: The reason why we analysed these two groups separately was that in previous studies, the impact on immune response was different for these two groups. Therefore, given that only 7 infants were born from mothers with acute PM, we have analysed the effect of these two categories separately, which gives the opportunity to observe (i) the effect of chronic PM in a
none-negligible group (n=38) of individuals and (iii) the trend in infants born to mothers with acute PM.

5) Regarding the category of exposed/no PM. Did these women have clinical malaria, asymptomatic parasitemia, or both during pregnancy? Please provide additional details.

RESPONSE: We clarified that point indicating that women in the exposed/no PM group experienced either clinical episode (N=6) or asymptomatic infection (N=55) during the course of their pregnancy (Line 316-317).

6) The authors make no mention in the results as to which cells may be producing these cytokines, nor to whether differential admixture of cell types in the PME categories may be explanatory for any differences observed. Did the authors measure cellular frequencies? Would also expand the discussion of this (lines 378-380, 404-408)

RESPONSE: We now expanded the discussion around cells population across PME categories as potential explanation of the observed differences (Lines 440-456 and lines 458-467). Unfortunately, cellular frequencies in cord blood were not measured in this study. The reviewer #3 also raised this concern. Therefore, we have modified the discussion accordingly to include this as one of the limitations for this study (Lines 778-784).

7) Re: Prospective protection: figure one shows that children born to mothers with placental malaria have a lower risk of malaria during the first 6 months. That is in contrast to what has been reported in several epidemiologic studies. Why do the authors postulate this reduced risk? Does their data help to explain this observation? Authors should address this in the discussion

RESPONSE: We agree with reviewer #2 that these data were not discussed. The potential explanation of these observations is now addressed in the discussion (Lines 688-695). We mention that both maternal antibodies and the strong effect of seasonality in Nanoro, which may make dynamics different from other sites, could explain why children from mothers with placental malaria have a lower risk during the first months of life. Of note, we are currently under discussions to address the issue of maternal antibodies in future studies.

a. Relatedly - authors only discuss time to clinical malaria. Did they observe any different associations between analytes measured and repeated malaria in infancy (incidence), parasite prevalence in infancy, or severity of symptoms if infected? Did the authors observe
associations with risk of non-malarial febrile illness given evidence of hyper-responsivity to TLR7/8 stimulation?

RESPONSES: As part of the present study, the focus in our pre-defined analytical plan was put on time-to-event analysis. Therefore, recurrent events analysis was not performed. In our experience with other studies in the same area, we do not find different outputs of time to first vs multiple events. Given that we generated a lot of data, we did not consider appropriate to add more endpoints (e.g. parasite prevalence) and thus complexity to the study. In addition, too many exploratory analyses would be more like a fishing expedition.

In this study, few cases of severe malaria occurred in the total cohort (1.5% of total clinical cases (Natama et al., Mal. J. 2018, 17:163)), thus, we did not have enough statistical power to fit any robust/reliable model for the exploration of such association.

Regarding the association with risk of non-malarial febrile illness, separate work is on-going to investigate the effect on different non-malarial fevers including diagnosis of viruses, which will allow us to make a more accurate analysis of this association. And since this was not the aim of the study, these results will be reported in a separate paper.

8) Overall the discussion section was fairly difficult to follow. The first paragraph didn't clearly summarize the study's main findings and this could be useful to setup the rest of the discussion. Furthermore, although the question of how prenatal malaria exposure may shape innate immune responses is interesting, I find the more critical question is how innate immune responsivity at birth may influence prospective risk. I would elevate this in the discussion. That paragraph should also be reorganized to discuss cytokines/chemokines/growth factors associated with protection followed by those associated with risk, since that may make the section easier to follow.

RESPONSE: We have modified the first paragraph accordingly to clearly summarize the study main findings (Lines 410-438). In addition, we have re-organized the discussion section to highlight better how innate immune responses at birth influence malaria risk in infancy as suggested (Lines 685-784).

9) The paper lacks a limitation section in the discussion. Some of the points addressed above could be included.

RESPONSE: We have introduced a limitation section in the discussion (Lines 778-784), which includes in fact some of the points addressed above.
Reviewer #3:

This study aims to examine the impact of malaria in pregnancy on the innate immune response in whole cord blood in responses to different TLR agonists. Key findings show that certain TLR agonists are more likely to induced cytokine/chemokine responses than others, that newborns of mothers with chronic placental are more likely to have TLR-induced cytokine responses, and spontaneous cytokine production is reduced in cord blood of newborns exposed to malaria during pregnancy compared to those not exposed. In addition there is some association between innate immune response to certain cytokines/chemokine with either increased or decreased risk of malaria infection in the newborns over a year of follow-up. Similar studies have been performed in the past, but there are unique characteristics of this study, including better definition of the types of material exposure based on recent or past placental malaria, as determined by placental histology.

There are several concerns with this study;

General comments:

1. There could be better definition of malaria exposure during pregnancy of the mothers of the different groups. Samples for study were selected as part of larger study examining different treatment regimens during pregnancy. For example how often were mothers examined for presence of malaria? How often were they infected? When were mothers enrolled and how often did they receive malaria chemoprophylaxis during pregnancy? These are important variables that could affect exposure maternal and thus fetal exposure. Having this information is an asset the study.

RESPONSE: We thank reviewer #3 for these suggestions. Some of these suggestions are indeed similar to the ones made by reviewers #1 and #2 in relation to number of women in each arm from the parent COSMIC study and anti-malarial drugs received for both malaria prevention and treatment (See additional data in Table 1). In table 1, we also added the mean gestational age at enrollment of pregnant women. In addition, more details regarding the information on how often were mothers examined for presence of malaria have been added in the methods section (Lines 190-196).

2. Cytokine responses were performed with whole blood. However there is no reported measurement of WBC populations and lymphocyte subsets in cord blood at delivery. There is evidence that prenatal malaria exposure can alter myeloid subsets abundance could account for the differences observed to TLR agonist. If this information is not available, it should be stated as such and potential limitations in the interpretation of their data.
RESPONSE: We agree with the reviewer #3 that this is one of the limitations for this study, and this is now stated in the limitation section (Lines 778-784).

3. A challenge in evaluating malaria risk in the first year of life is exposure. Obviously mothers with malaria exposure during pregnancy are more likely to live in an environment with higher malaria exposures, which will impact their infants. This has always been a challenge in interpreting the impact of prenatal malaria exposure of on subsequent malaria during infancy. For example their observation that offspring of PM+ mothers was decreased in the first 6 months would be a results of passive transfer of protective maternal antibodies and then increased risk from 6 to 12 months from increase malaria exposure. These factors can easily obscure any prenatal immune response. How exposure controlled for at all?

RESPONSE: We thank the reviewer #3 for this comment. Actually, the study was conducted in a rural area with similar malaria transmission intensity in the health district. The major characteristic of this study area is that malaria transmission is highly seasonal (Natama et al, Mal. J. 2018, 17:163). Therefore to account for difference in the risk of infections between infants, birth season was included in the multivariable models using an interaction term with the timing of clinical malaria. Moreover, PME, which influences level of maternal antibodies, was also included in the multivariate models to control for the effect of in-utero exposure to malaria parasites and/or antigens (Lines 390-399).

Also, as suggested by the reviewer #2, we are addressing now in the discussion the observation that PM has a lower risk of malaria during the first 6 months in contrast to what has been reported in several epidemiologic studies (Lines 688-695). Indeed, maternal antibodies could be one of the factors that could help to explain why children from mothers with placental malaria have a lower risk during the first months of life and we are planning to address in the near future this issue in our cohort.

4. We usually do not think of innate immune response having memory. So how does malaria exposure earlier in pregnancy, influence innate immune responses observed at delivery? Impact more rapid maturation of innate immune response? There is some evidence for memory in NK cells, but there is also controversy whether NK cells express much TLR 7/8. There should be some discussion about the mechanisms of how prenatal malaria exposure in utero affects innate immune responses.

RESPONSE: The increasing evidence of a memory innate immune response has recently led to a paradigm shift and we think it is now well accepted that the innate system has adaptive characteristics. Innate stimulations can lead to sensitization following pathogen exposure and this has been termed trained innate immunity, and it seems to be developed by epigenetic
reprogramming. This possible mechanism is now discussed in Lines 458-467 of the Discussion. In addition, we already explained in the discussion that malaria pigment in the placenta was associated with the maturation of cord blood myeloid and plasmocytoid DCs in previous studies to show that the differential admixture of cell types across PME categories may be explanatory of the observed differences in cytokine production in the present study (Lines 469-522).

Specific comments:

1. Overall the paper was difficult to follow, lots of data. Admittedly parsing out all the cytokine/chemokine data is challenging. The abstract need significant revision. Key points to do not come out well.

RESPONSE: We thank reviewer #3 for this suggestion and have revised the abstract accordingly (Lines 58-87).

2. The discussion is too long and not well focused on mechanisms as discussed above and limitations of the study.

RESPONSE: We have now added some discussion on mechanisms as suggested and included a limitation section.

3. Figure 2 showing principal components are not well described and does not help much in interpreting the data. If the PC analysis was able to group cytokine/chemokine responses in an unbiased fashion and the PC could be used a predicting variables rather an individual cytokine/chemokines, then this would be useful in the analysis. Otherwise Supp Fig 5 would be most helpful in understanding the immune responses.

RESPONSE: Actually we have evaluated innate immune responses in 2 steps:

- The first step was to assess the variation of cytokine production across the selected TLR agonists for which we used the PCA to visualize the variance of cytokine responses between subjects and stimuli and also by using boxplots (Additional file 1, including Fig. S1 and S2). That is the reason why the PCA was not performed using predicting variables.

- The second step for understanding the innate immune responses was the assessment of cytokine changes across PME groups, which is described in the results section “PME and cytokine responses at birth” and illustrated with boxplots in the additional file 2 (including Figs S3-S6).
4. **Unclear how much Figs 4 and 5 contribute to results in an impactful way.**

RESPONSE: As mentioned in the manuscript, the figs 4 and 5 show the risk of malaria in infants by birth weight and birth season, respectively. In this study, LBW appeared to be a significant risk factor for clinical malaria during the first year of life while the birth season allows to account for differences in malaria exposure between infants as malaria transmission in the study area is highly seasonal (Natama et al, Mal. J. 2018, 17:163). Therefore, figs 4 and 5 have been used to show how these covariates influence the risk of malaria during the first year of life.